ACS OMEGA

Open Access

http://pubs.acs.org/journal/acsodf

Article

Exonuclease-III Assisted Signal Cycle Integrating with Self-Priming Mediated Chain Extension for Sensitive and Reliable MicroRNA Detection

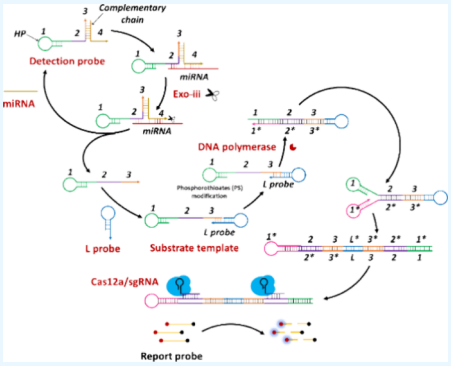
Chunmeng Li,* Xiangjian Zheng, Shangshang Xie, and Deyong Lin

Cite This: *ACS Omega* 2025, 10, 6228−6233

Read Online

ACCESS | Metrics & More | Article Recommendations | Supporting Information

ABSTRACT: MicroRNA (miRNA) is pivotal in regulating pathological progression and may serve as a significant biomarker for early diagnosis, treatment, and management strategies for atherosclerosis. This study produced a self-priming amplification-accelerated CRISPR/Cas system-based method for the sensitive and selective detection of miRNA by merging Exo-III-assisted target recycling, self-priming-mediated chain extension, and the CRISPR/Cas12a system. The sensor comprises three stages: (i) the creation of a substrate template via Exo-III mediated target recycling and DNA ligase assisted ligation; (ii) the exponential isothermal reaction facilitated by DNA polymerase for signal amplification; (iii) the *trans*-cleavage activity of CRISPR/Cas12a after recognizing the amplification product generates signals. We employed miRNA-21 as a target. The strategy enables sensitive detection of miRNA-21 without the use of primers, and the unique design of the CRISPR/sgRNA complex efficiently mitigates background signal interference. The sensor can recognize single-base mutant homologous sequences and demonstrate a steady performance in complicated biological matrices. This sensor has been effectively employed to precisely assess miRNA-21 in engineered clinical samples, showcasing its significant potential in clinical diagnostics and of atherosclerosis.



1. INTRODUCTION

MicroRNAs (miRNA), as diminutive nonmRNA molecules, have been recognized as endogenous physiological regulatory factors that can influence gene regulation during transcription in contemporary genetic research of atherosclerosis.[1−4] Aberrant miRNA expression are significant in the progression of various diseases, including the development of atherosclerosis.[5,6] A multitude of studies has demonstrated that miRNA in blood serves as a very promising atherosclerosis diagnostic marker; thus, the advancement of effective and sensitive miRNA detection strategy is crucial for early identification and the formulation of nursing strategies for atherosclerosis.[7,8] Nevertheless, owing to its inherent characteristics of diminutive size, significant sequence homology, and low blood concentration, it continues to encounter substantial technological obstacles in practical clinical advancement.

Conventional techniques for miRNA profiling including reverse transcription quantitative polymerase chain reaction (RT-qPCR),[9−12] microarrays,[13,14] and Northern blotting.[15−17] RT-qPCR is a prevalent commercial technique that offers quantitative assays with enhanced sensitivity for diagnostic purposes. However, it continues to uphold instrumental prerequisites and specialist data analysis. Furthermore, it necessitates reverse transcription operations utilizing poly-A tails or a stem-loop probe, hence augmenting the design complexity and temporal expenditures. Nucleic acid−based amplification technologies, such as hybridization chain reaction (HCR), strand displacement amplification (SDA),[18] catalytic DNAzyme-based amplification,[19] rolling circle amplification (RCA),[20−22] and loop-mediated isothermal amplification (LAMP),[23,24] have emerged as effective methods for detecting nucleic acids and non-nucleic acid targets. In comparison to RT-qPCR technology, these approaches provide the advantages of simplicity and the absence of cyclic heating; nonetheless, they still possess numerous technical deficiencies in clinical use. Loop-mediated isothermal amplification (LAMP), a recently invented isothermal signal amplification technique, has garnered significant attention for its distinctive benefits of speed and sensitivity. Nevertheless, LAMP often necessitates 4−6 primer sets, which complicates design and increases the likelihood of nonspecific amplification. Consequently, the development of innovative ways to enhance the sensitivity of the LAMP-based method and increase specificity is highly required.

Received: December 19, 2024
Revised: January 13, 2025
Accepted: January 30, 2025
Published: February 8, 2025

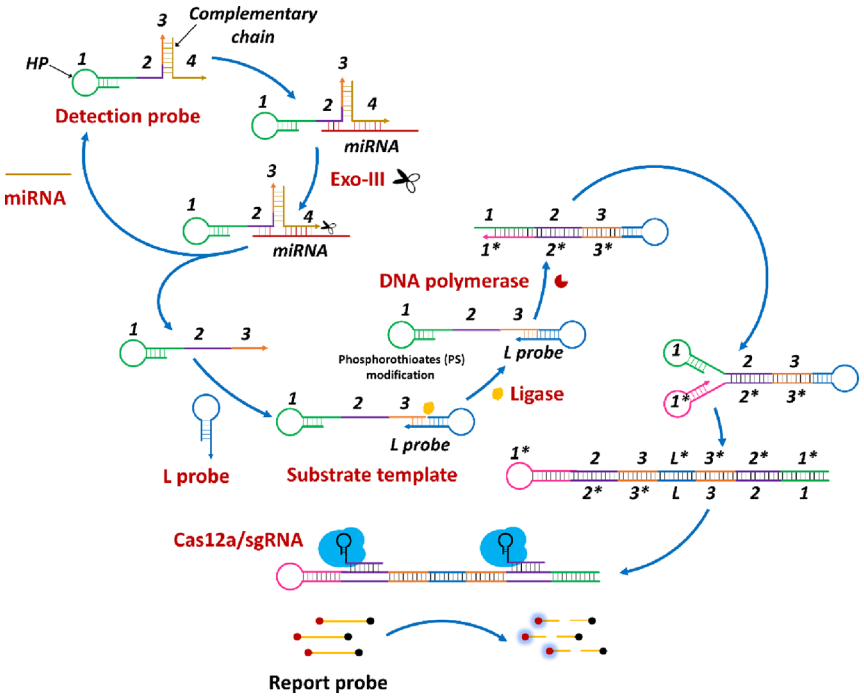


Figure 1. Principle of the proposed method for sensitive and reliable miRNA detection.

In recent years, the CRISPR (clustered regularly interspaced short palindromic repeats) system, recognized as a promising next-generation diagnostic tool, has garnered significant attention in the domain of biomarkers analysis.[25−27] Numerous CRISPR-based miRNA detection methodologies have been established by integrating diverse isothermal reactions, such as RCA and LAMP, as a preamplification step. The robust trans-cleavage activity to cut the single-stranded DNA sequences after recognizing the target double-stranded DNA sequences of the CRISPR technology significantly enhances the sensitivity and specificity of these strategies. Nonetheless, it remains incapable of eliminating numerous deficiencies in the preamplification reaction, including the intricacy and possible wrong amplification of primer design in LAMP.

In contrast to nicking endonuclease, Exonuclease-III (Exo-III) retains chain cleavage activity without necessitating a specific recognition site for target regeneration, rendering the Exo-III-assisted system a versatile and potent tool for biomarkers detection. Nonetheless, the existing Exo-III method continues to face certain intrinsic limitations. The target recognition by the Exo-III is commonly triggered by a securely structured hairpin probe mediated toehold-mediated chain displacement, necessitating a crucial yet intricate chain hybridization reaction to mediate target sensing.

We have developed a novel fluorescent miRNA detection method that integrates the Exo-III assisted target recycling, self-priming mediated chain extension, and CRISPR-Cas12a system-based fluorescence generation, leveraging the advantages of the Exo-III enzyme. In this approach, the "detection probe" is capable of specific recognition of the target miRNA and can facilitate the formation of an Exo-III-assisted signal cycle. The CRISPR-Cas12a system can precisely identify the product by detecting the high-efficiency self-priming mediated chain extension. This identification activates the trans-cleavage activity of the Cas12a enzyme, which cleaves the surrounding "report probe", resulting in the generation of fluorescence signals. Promising prospects for disease diagnosis and medical research are demonstrated by the proposed method's high sensitivity and stability for miRNA detection, which is a result of its elegant design.

2. EXPERIMENTAL SECTION

2.1. Materials and Reagents. All of the probes and miRNA sequences utilized in this study were synthesized by Sangon Biotechnology (Shanghai, China) Co., Ltd. The details of these sequences are listed in Table S1. The following items were acquired from New England Biolabs (Beijing, China): SplintR Ligase (M0375), Bst DNA polymerase, and EnGen Lba Cas12a (Cpf1). Some other reagents, including 10 mM dNTP mixture, 10× phosphate-buffered saline (PBS) buffer, and diethylpyrocarbonate (DEPC)-treated water, were acquired from Sangon Biotech (Shanghai, China).

2.2. Steps of the Biosensor for miRNA Detection. Initially, "HP" and the "complementary chain" were subjected to heating at 90 °C for 5 min and subsequently allowed to cool gradually to room temperature to yield a stable "detection probe". Subsequently, varying doses of miRNA were introduced into the tube, comprising 1 μL of a 100 nM "detection probe" and 1 μL of Exo-III. The mixture was incubated at 30 °C for 30 min and thereafter heated to 70 °C for 10 min to inactivate Exo-III. Subsequently, 1 μL of "L probe", 1 μL of PBS buffer, and 1 μL of DNA ligase (1.5 U/μL) were added. The solution was then incubated at 30 °C (30 min). Subsequently, 0.5 μL of 5 mM dNTP, 0.5 μL of 50 mM $MgSO_4$, and 1.5 μL of Bst 2.0 DNA polymerase (1.2 U/μL) were included into the solution. The enhanced reaction mixture was incubated at 30 °C for 30 min. Subsequent to the chain extension reaction, 1 μL of 2 μM sgRNA, 1 μL of 2 μM Cas12a, and 5 μL of 10 μM reporter probe were incorporated into the reaction system. Following a reaction at 30 °C for 20 min and subsequent inactivation at 70 °C for 5 min, the fluorescence intensity at 525 nm (excitation at 485 nm) was quantified by using a microplate reader.

https://doi.org/10.1021/acsomega.4c11417
ACS Omega 2025, 10, 6228−6233

3. RESULTS AND DISCUSSION

3.1. The Principle of the Proposed Method for miRNA Detection. The operational mechanism of the suggested technique is illustrated in Figure 1. This method involves a "detection probe" that incorporates a hairpin structure and a complementary sequence. In the presence of the target miRNA, it can attach to the "4" segment of the "detection probe", resulting in a blunt 3′ terminus. The Exo-III may identify and cleave the "4" segment of the "detection probe", thereby exposing the "2" and "3" segments and liberating the target miRNA from the complex. The released miRNA can bind to the "4" portion of a subsequent "detection probe," creating a signal cycle. The exposed "3" segment can serve as a binding site for the hairpin structure "L probe". Following the DNA ligase-mediated linkage of the "3" segment of the "detection probe" and the "L probe", a complete substrate template for self-priming facilitated chain extension, incorporating both the "L probe" and the "detection probe", is established. Subsequently, DNA polymerase catalyzes the self-priming of the substrate template at the 3′-terminus. Nevertheless, the phosphorothioate (PS) alteration will result in attenuation of the hydrogen connection between the hybridization chains. The 5′ end region modified with PS often separates from the complementary chain and tends to self-assemble to form a hairpin structure, thereby exposing the 3′ end in the complementary chain to mediate a next chain extension. Therefore, the self-priming amplification cycle is initiated by the DNA polymerase from the 3′-end, forming a signal cycle to produce large number of tandem double-stranded DNA products with repeated sequences in the reaction system. Subsequently, the Cas12a/sgRNA complex can specifically recognize and bind with these generated double-stranded DNA products containing pre-engineered CRISPR/Cas12a activation region, thereby activating the *trans*-cleaving activity of the Cas12a protein and producing a substantial fluorescence signal by cleaving the fluorescence reporter chain in the solution.

Assembly of the "Detection Probe" and Feasibility of the Exo-III Assisted Signal Cycle. The development of the "detection probe" is crucial for precise target identification and for facilitating subsequent signal amplification. Consequently, we initially confirmed the assembly of the "detection probe" via a fluorescence experiment. In this fluorescence test, the 3′ terminus of the "HP" is conjugated with BHQ, while the 5′ terminus of the "complementary chain" is conjugated with the FAM moiety. The mixture including equal quantities of "HP" and the "complementary chain" was heated to 90 °C for 10 min and subsequently allowed to cool gradually to room temperature to form the "detection probe". Figure 2A illustrates that the recorded fluorescence signal of the "HP" was elevated, significantly diminishing upon introduction of the "complementary chain". The alterations in the fluorescence signals indicated the successful development of the "detection probe". The target miRNA can associate with the complementary strand to provide a blunt 3′ terminus, facilitating the Exo-III-mediated target recycling process. The fluorescent moiety designated as the "detection probe" was utilized to evaluate the Exo-III facilitated target recycling process. Upon mixing the "detection probe" with the target miRNA, the resulting fluorescence intensity was comparable to that of the control group devoid of target miRNA. The introduction of Exo-III resulted in a significantly enhanced fluorescence signal,

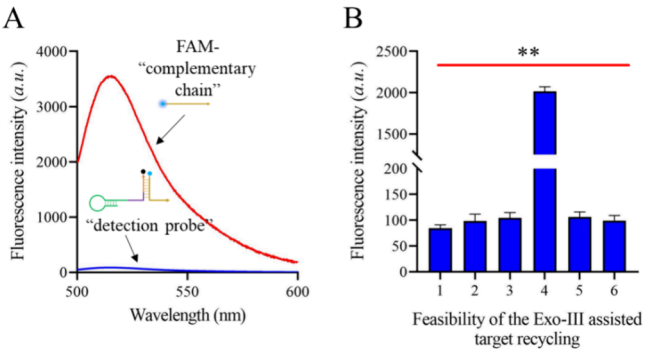


Figure 2. Construction of the "detection probe" and Exo-III assisted target recycling process. (A) Fluorescence spectrum of the FAM labeled "complementary chain" before and after assembly to the "detection probe". (B) Fluorescence intensity of the "detection probe" during the Exo-III assisted target recycling process. Column 1, "detection probe"; column 2, "detection probe"+ miRNA-21; column 3, "detection probe"+ Exo-III; column 4, "detection probe"+ Exo-III+ miRNA-21; column 5, "detection probe"+ Exo-III+ let-7a; column 6, "detection probe"+ Exo-III+ miRNA-149. **, $p < 0.05$.

suggesting that Exo-III cleaved the "complementary chain" in the presence of target miRNA, thus confirming the viability of the "detection probe" for specific target miRNA identification (Figure 2B).

3.3. Feasibility Analysis of the Method. We evaluated the feasibility of the self-priming mediated chain extension method using SYBR Green I. Specifically, SYBR Green I can produce fluorescence signals upon binding to double-stranded DNA sequences. Figure 3A illustrates that the fluorescence signal captured from the "detection probe" alone was minimal. Fluorescence signal significantly increased only in the presence of target miRNA, Exo-III, and DNA polymerase in this sensing system, indicating the production of substantial quantities of

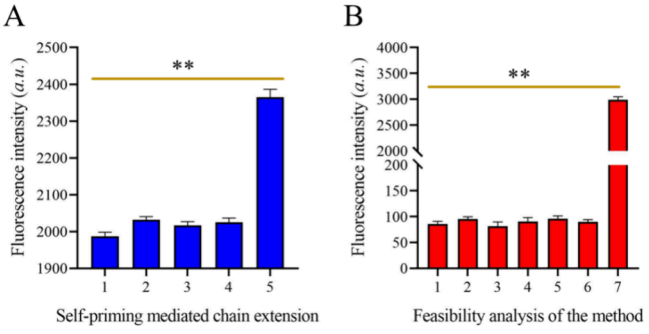


Figure 3. Feasibility analysis of the method. (A) Fluorescence intensity of SYBR Green I during the self-priming mediated chain extension process. Column 1, "detection probe"; column 2, "detection probe"+ miRNA; column 3, "detection probe"+ miRNA+ Exo-III; column 4, "detection probe"+ miRNA+ Exo-III+ "LP probe"; column 5, "detection probe"+ miRNA+ Exo-III+ "LP probe"+ DNA polymerase. (B) Fluorescence intensity of the method when essential experimental components existed or not. Column 1, "report probe"; column 2, "report probe" + "detection probe"; column 3, "report probe" + "detection probe" + miRNA; column 4, "report probe" + "detection probe" + miRNA+ Exo-III; column 5, "report probe" + "detection probe" + miRNA + Exo-III + "L probe"; column 6, "report probe" + "detection probe" + miRNA + Exo-III + "L probe" + DNA polymerase; column 7, "report probe" + "detection probe" + miRNA + Exo-III + "L probe" + DNA polymerase + Cas12a/sgRNA. **$p < 0.05$.

https://doi.org/10.1021/acsomega.4c11417
ACS Omega 2025, 10, 6228−6233

double-stranded DNA sequences during self-priming-induced chain extension.

Ultimately, we incorporated the CRISPR/Cas12a system and a fluorescent reporter probe into the self-priming mediated chain extension process to validate the viability of fluorescence detection of miRNA using this sensor. Figure 3B illustrates that only the experimental group generated substantial fluorescence signals, whereas the control group did not. The aforementioned results unequivocally demonstrate the practicality of the proposed sensor.

3.4. Optimization of Experimental Conditions. To enhance the detection capability of this sensor, we modified the experimental settings. Initially, we refined the concentration of the "detection probe". Figure 4A illustrates that a

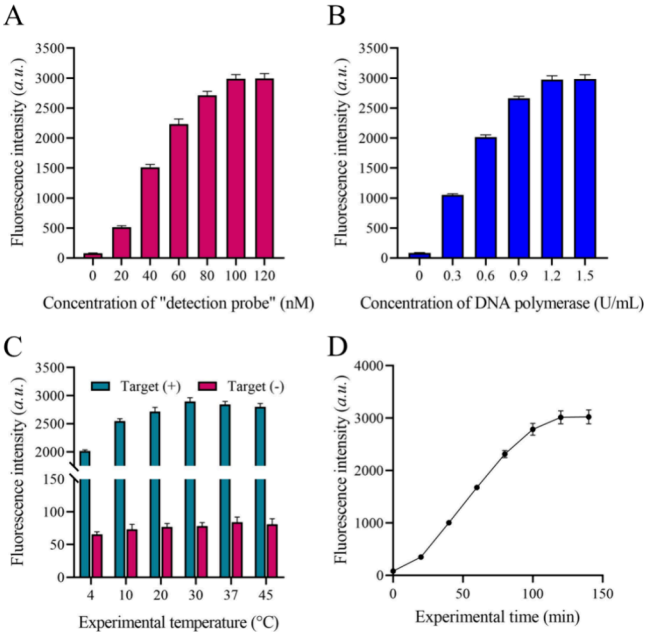


Figure 4. Optimization of experimental parameters. (A) Fluorescence intensity of the method when detecting target miRNA with different concentrations of the "detection probe" (A), different concentrations of DNA polymerase (B), different experimental temperatures (C), and various experimental times (D).

concentration of 100 nM for the "detection probe" yielded the optimal fluorescence intensity, which subsequently stabilized, indicating that 100 nM is the most effective concentration. Subsequently, we evaluated the impact of varying doses of DNA polymerase on detection efficacy; as illustrated in Figure 4B, 1.2 U/mL of Bst 2.0 DNA polymerase exhibited optimal performance, which was employed in further investigations. Additionally, we have improved the ligation reaction temperature, and the results in Figure 4C indicated that the fluorescence intensity at 30 °C surpassed that at 25 and 37 °C; therefore, we established the ligation reaction temperature at 30 °C. The optimized experimental time was determined to be 120 min according to the result in Figure 4D.

3.5. Analysis Performance of the Proposed Sensor. Following optimization of the experimental conditions, we evaluated the sensitivity of the proposed sensor. We incorporated varying quantities of miRNA-21 into the solution to assess the fluorescence intensity. Figure 5A,B demonstrates that the final signal intensity had a positive correlation with the concentration of miRNA-21, dramatically increasing as the

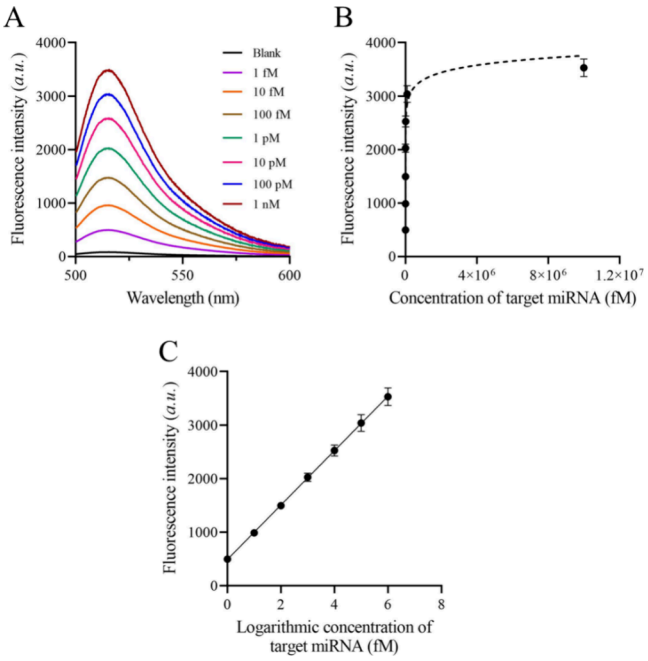


Figure 5. Sensitivity of the method for miRNA detection. (A) Fluorescence spectrum of the method when detecting different concentrations of miRNA. (B) Correlation between the peak fluorescence value and the concentration of target miRNA. (C) Linear equation of the peak fluorescence value and the logarithmic concentration of the target miRNA.

concentration of miRNA-21 climbed. Furthermore, a strong linear correlation exists between the concentration range of 1 fM to 1 nM, represented by the equation $F = 508.4 \times \lg C + 488.6$ ($R^2 = 0.9956$), with a detection limit of 0.43 fM (Figure 5C). This method's ultrasensitive detection performance far surpasses that of other previously published miRNA analysis techniques, primarily because of the multiple signal amplification effects derived from Exo-III assisted target recycling, self-priming mediated chain extension, and the CRISPR/Cas12a system.

3.6. Test of Specificity and Stability. The significant sequence homology of miRNA complicates the precise analysis. The accurate identification of miRNAs with a single-base mutation is highly significant for therapeutic application. To evaluate the selectivity of this technique, we synthesized three nonhomologous miRNAs alongside three homologous sequences as parallel controls. Figure 6A illustrates that regardless of the base mutation's location, the mutant group exhibited no substantial signal generation, with signal intensity considerably lower than that of the miRNA-21 group. Furthermore, the signals from the nonhomologous groups of let-7a, miRNA-17, and miRNA-155 closely resembled the background signal. These results unequivocally demonstrate that the approach possesses an enhanced specificity and can proficiently detect single-base alterations.

Subsequently, we quantified the signal intensity in 10% commercial serum samples with varying amounts of target miRNA to assess the stability of the proposed technique. The recovery value of the method (Figure 6B) in 10% human serum was determined to be 96.12∼103.16% with a relative standard deviation ranging from 2.23 to 4.21%, indicating that the approach exhibits robust stability and resistance to interference in complex biological matrices.

https://doi.org/10.1021/acsomega.4c11417
ACS Omega 2025, 10, 6228−6233

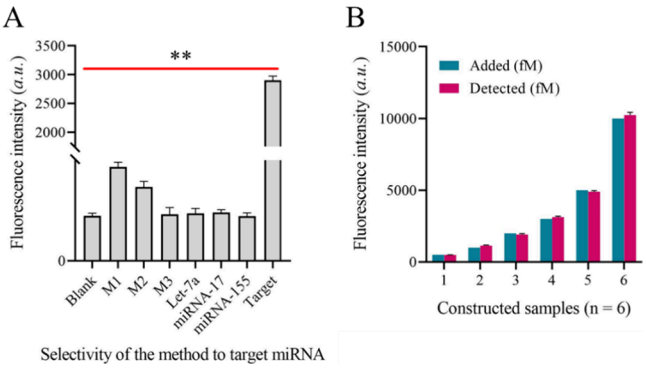


Figure 6. Specificity and stability of the method for miRNA detection. (A) Fluorescence intensity of the method when detecting different miRNA sequences. (B) Fluorescence intensity of the method when detecting six sample duplicates. $^{**}p < 0.05$.

4. CONCLUSIONS

A self-priming amplification-enhanced CRISPR sensor for sensitive miRNA measurement has been effectively established. The entire sensing mechanism involves three interconnected steps: Exo-III-assisted target recycling, self-priming-mediated chain extension, and CRISPR/Cas12a-based signal generation. In comparison to previously documented techniques, this method presents the following distinct advantages: (i) enhanced sensitivity resulting from the multiple signal amplification process, facilitating the detection of miRNA at a low limit of 0.43 fM; (ii) operational simplicity, as the design and execution are significantly streamlined without the use of primers; (iii) minimal background signal from the "reporter probe"; (iv) effective differentiation of single-base mutant homologous sequences by Exo-III and the Cas12a/sgRNA complex (Table S2). Moreover, our sensor demonstrates exceptional stability in complex materials and effectively identifies miRNA-21 in fabricated clinical samples. This conclusively demonstrates that the suggested sensor possesses significant clinical application potential and offers a novel technical concept for the advancement of miRNA point-of-care solutions.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.4c11417.

DNA and RNA sequences used in this work (Table S1); a brief comparison of the method with former ones (Table S2) (PDF)

■ AUTHOR INFORMATION

Corresponding Author

Chunmeng Li − *Department of Vascular Surgery, The Dingli Clinical Institute of Wenzhou Medical University (Wenzhou Central Hospital), Wenzhou City, Zhejiang Province 325000, China; Laboratory of Wenzhou Pan-Vascular Disease Management Center, Wenzhou City, Zhejiang Province 325000, China;* orcid.org/0009-0006-8543-4730; Email: lercermer@126.com

Authors

Xiangjian Zheng − *Department of Vascular Surgery, The Dingli Clinical Institute of Wenzhou Medical University (Wenzhou Central Hospital), Wenzhou City, Zhejiang Province 325000, China*

Shangshang Xie − *Department of Vascular Surgery, The Dingli Clinical Institute of Wenzhou Medical University (Wenzhou Central Hospital), Wenzhou City, Zhejiang Province 325000, China*

Deyong Lin − *Department of Vascular Surgery, The Dingli Clinical Institute of Wenzhou Medical University (Wenzhou Central Hospital), Wenzhou City, Zhejiang Province 325000, China*

Complete contact information is available at: https://pubs.acs.org/10.1021/acsomega.4c11417

Author Contributions

C.L.: writing-review and editing and project administration, writing-original draft, data curation, investigation, and resources. X.Z.: methodology, and formal analysis. S.X. and D.L.: data analysis. All authors adhered to the Committee on Publication Ethics guideline.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

The research is supported by the Zhejiang Provincial Health Commission Health Science and Technology Program (No. 2020KY295).

■ REFERENCES

(1) Ruggieri, F.; Jonas, K.; Ferracin, M.; Dengler, M.; Jäger, V.; Pichler, M. MicroRNAs as regulators of tumor metabolism. *Endocr.-Relat. Cancer* **2023**, *30* (8), No. e220267.

(2) Wu, Y.; Li, Q.; Zhang, R.; Dai, X.; Chen, W.; Xing, D. Circulating microRNAs: Biomarkers of disease. *Clin. Chim. Acta* **2021**, *516*, 46−54.

(3) Laggerbauer, B.; Engelhardt, S. MicroRNAs as therapeutic targets in cardiovascular disease. *J. Clin. Invest.* **2022**, *132* (11), No. e159179.

(4) Searles, C. D. MicroRNAs and Cardiovascular Disease Risk. *Curr. Cardiol. Rep.* **2024**, *26* (2), 51−60.

(5) Hajibabaie, F.; Kouhpayeh, S.; Mirian, M.; Rahimmanesh, I.; Boshtam, M.; Sadeghian, L.; Gheibi, A.; Khanahmad, H.; Shariati, L. MicroRNAs as the actors in the atherosclerosis scenario. *J. Physiol. Biochem.* **2020**, *76* (1), 1−12.

(6) Gorabi, A. M.; Ghanbari, M.; Sathyapalan, T.; Jamialahmadi, T.; Sahebkar, A. Implications of microRNAs in the Pathogenesis of Atherosclerosis and Prospects for Therapy. *Curr. Drug Targets* **2021**, *22* (15), 1738−1749.

(7) Shoeibi, S. Diagnostic and theranostic microRNAs in the pathogenesis of atherosclerosis. *Acta Physiol.* **2020**, *228* (1), No. e13353.

(8) Witarto, B. S.; Visuddho, V.; Aldian, F. M.; Atmaja, M. S. S.; Ariyanto, M. V.; Witarto, A. P.; Wungu, C. D. K.; Susilo, H.; Alsagaff, M. Y.; Rohman, M. S. Blood-based circulating microRNAs as diagnostic biomarkers for subclinical carotid atherosclerosis: A systematic review and meta-analysis with bioinformatics analysis. *Diabetes Metab. Syndr.* **2023**, *17* (10), No. 102860.

(9) Ekiz Kanik, F.; Celebi, I.; Sevenler, D.; Tanriverdi, K.; Lortlar Unlu, N.; Freedman, J. E.; Unlu, M. S. Attomolar sensitivity microRNA detection using real-time digital microarrays. *Sci. Rep.* **2022**, *12* (1), 16220.

(10) Kramer, M. F. Stem-loop RT-qPCR for miRNAs. *Curr. Protoc. Mol. Biol.* **2011**, *95*, 10.

(11) Mai, H. T.; Vanness, B. C.; Linz, T. H. Reverse transcription-free digital-quantitative-PCR for microRNA analysis. *Analyst* **2023**, *148* (13), 3019−3027.

(12) Chen, C.; Tan, R.; Wong, L.; Fekete, R.; Halsey, J. Quantitation of microRNAs by real-time RT-qPCR. *Methods Mol. Biol.* **2011**, *687*, 113−134.
(13) Kocabey, S.; Chiarelli, G.; Acuna, G. P.; Ruegg, C. Ultrasensitive and multiplexed miRNA detection system with DNA-PAINT. *Biosens. Bioelectron.* **2023**, *224*, No. 115053.
(14) Wu, D.; Hu, Y.; Tong, S.; Williams, B. R.; Smyth, G. K.; Gantier, M. P. The use of miRNA microarrays for the analysis of cancer samples with global miRNA decrease. *RNA* **2013**, *19* (7), 876−888.
(15) Alonso-Hearn, M.; Ballesteros, A.; Navarro, A.; Badia-Bringue, G.; Casais, R. Lateral-flow assays for bovine paratuberculosis diagnosis. *Front. Vet. Sci.* **2023**, *10*, No. 1257488.
(16) Esmaeilzadeh, A. A.; Yaseen, M. M.; Khudaynazarov, U.; Al-Gazally, M. E.; Catalan Opulencia, M. J.; Jalil, A. T.; Mohammed, R. N. Recent advances on the electrochemical and optical biosensing strategies for monitoring microRNA-21: a review. *Anal Methods* **2022**, *14* (44), 4449−4459.
(17) Varallyay, E.; Burgyan, J.; Havelda, Z. MicroRNA detection by northern blotting using locked nucleic acid probes. *Nat. Protoc.* **2008**, *3* (2), 190−196.
(18) Liu, Y.; Li, S.; Zhang, L.; Zhao, Q.; Li, N.; Wu, Y. Catalytic Hairpin Assembly-Assisted Rolling Circle Amplification for High-Sensitive Telomerase Activity Detection. *ACS Omega* **2020**, *5* (20), 11836−11841.
(19) Zou, L.; Wu, Q.; Zhou, Y.; Gong, X.; Liu, X.; Wang, F. A DNAzyme-powered cross-catalytic circuit for amplified intracellular imaging. *Chem. Commun. (Camb.)* **2019**, *55* (46), 6519−6522.
(20) Hui, H.; Zhao, X.; Zhang, H.; Li, X.; Fu, P.; Wu, M.; Dai, Q. Hairpin/DNA Ring Ternary Complex Initiated Rolling Circle Amplification for an Elevated Accuracy and Its Application in Analyzing Let-7a. *ACS Omega* **2023**, *8* (46), 44030−44035.
(21) Liu, X.; Zhao, X.; Zhang, J.; Wang, Y.; Ye, X. Rolling Circle Amplification Integrating with Exonuclease-III-Assisted Color Reaction for Sensitive Telomerase Activity Analysis. *ACS Omega* **2024**, *9*, 49081.
(22) Wang, R.; Zhao, X.; Chen, X.; Qiu, X.; Qing, G.; Zhang, H.; Zhang, L.; Hu, X.; He, Z.; Zhong, D.; Wang, Y.; Luo, Y. Rolling Circular Amplification (RCA)-Assisted CRISPR/Cas9 Cleavage (RACE) for Highly Specific Detection of Multiple Extracellular Vesicle MicroRNAs. *Anal. Chem.* **2020**, *92* (2), 2176−2185.
(23) Wong, Y. P.; Othman, S.; Lau, Y. L.; Radu, S.; Chee, H. Y. Loop-mediated isothermal amplification (LAMP): a versatile technique for detection of micro-organisms. *J. Appl. Microbiol.* **2018**, *124* (3), 626−643.
(24) Zhao, Y.; Chen, F.; Li, Q.; Wang, L.; Fan, C. Isothermal Amplification of Nucleic Acids. *Chem. Rev.* **2015**, *115* (22), 12491−12545.
(25) Granados-Riveron, J. T.; Aquino-Jarquin, G. CRISPR/Cas13-Based Approaches for Ultrasensitive and Specific Detection of microRNAs. *Cells* **2021**, *10* (7), 1655.
(26) Zhao, D.; Tang, J.; Tan, Q.; Xie, X.; Zhao, X.; Xing, D. CRISPR/Cas13a-triggered Cas12a biosensing method for ultra-sensitive and specific miRNA detection. *Talanta* **2023**, *260*, No. 124582.
(27) Zhao, L.; Qiu, M.; Li, X.; Yang, J.; Li, J. CRISPR-Cas13a system: A novel tool for molecular diagnostics. *Front. Microbiol.* **2022**, *13*, No. 1060947.

https://doi.org/10.1021/acsomega.4c11417
ACS Omega 2025, 10, 6228−6233