Original Article

Accurate detection of lung cancer-related microRNA through CRISPR/Cas9-assisted garland rolling circle amplification

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Background: MicroRNA (miRNA) is reported to be closely related to a variety of pathophysiological processes for carcinoma and considered a potential biomarker for the diagnosis of lung cancer with brain metastasis. However, developing an accurate and sensitive miRNA detection method has proven to be a challenge. The aim of the present study was to integrate the advantages of rolling circle amplification (RCA), clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nucleases 9 (Cas9), and catalytic hairpin assembly (CHA) technologies to develop an miRNA detection method.

Methods: In the present study, we developed a novel approach for the sensitive and accurate detection of miRNA through integrating garland RCA and CRISPR/Cas9-assisted signal generation. In this method, target miRNA cyclized dumbbell padlock and triggered the RCA process to form long single-stranded DNA products with a repeated hairpin structure. Double-stranded DNA sequences (dsDNA) were formed with the addition of complementary sequences. With the assistance of the Cas9 enzyme for specific recognition and cleavage of formed dsDNA, RCA products were disassembled into hairpin probes. The generated hairpin probe could be unfolded by target miRNA to initiate the CHA process for signal generation.

Results: Through integration of the RCA and CHA processes, the method demonstrated favorable detection performance. The correlation equation between the signal and concentration of target miRNA was determined to be $Y=312.3 \times \lg C + 2108$, with a high correlation coefficient of 0.9786. The approach also exhibited high selectivity to the mismatched miRNAs.

Conclusions: Our method could be used in the screening, diagnosis, and prognosis of multiple diseases without complicated thermal cycling instrumentation.

Keywords: Garland rolling circle amplification; clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nucleases 9 (Cas9); lung cancer; microRNA (miRNA)

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Introduction

Lung cancer remains the most threatening cause of fatal malignancies, with approximately 1.6 million deaths per annum globally. Clinical success of therapies is poor due to delayed diagnosis, restricted therapeutic tools, relapse, and drug resistance. The main cause of death in lung cancer is metastasis, and the brain is one of the most common metastatic sites of lung cancer. Research shows that more than 50% of lung cancer patients will suffer from brain metastases (BM) (1,2). MicroRNA (miRNA) is a small non-coding RNA with a length of 20–24 nucleotides, and plays an essential role in inhibiting messenger RNA (mRNA) translation and promoting mRNA degradation by base pairing to complementary sites of target mRNAs. Aberrant miRNA expression is closely associated with pathophysiological processes of various carcinomas and is a promising biomarker for the early diagnosis of lung cancer (3-5). Therefore, there is an urgent need to develop an accurate miRNA detection technique for the diagnosis of lung cancer with BM.

The unique features of miRNA, such as small size, low content in tissues and cells, and similar sequences of miRNA family members in particular, increase the difficulty in establishing miRNA detection methods. The most common miRNA detection methods, including polymerase chain reaction (PCR) (6-8), northern blotting (9,10), and microarray (11), have been widely applied in clinical sample analyses. Even though these methods have been the most common and gold standard methods, they are also criticized for their own shortcomings. For example, polyacrylamide gel electrophoresis (PAGE) separation which consists of transferring membrane and multiple washing procedures is essential, whereas complex in the northern blotting. Microarray assay has a high cost of production and detection, and the complicated probe design can lead to poor specificity and sensitivity in PCR-based miRNA detection. Over the past few years, a number of methods have been proposed due to their potential in the accurate detection of miRNA (12-17). For example, Zhao *et al.* proposed an intracellular miRNA detection method through integrating upconversion nanoparticles based on light trigger and catalytic hairpin assembly (CHA), and obtained an fM level sensitivity for miRNA detection (18). Wang *et al.* established accurate miRNA detection through integrating rolling circle amplification (RCA) for signal amplification and clustered regularly interspaced short palindromic repeats (CRISPR)/CRISPR-associated nucleases 9 (Cas9) for signal generation (19,20). There is increased demand for efficient techniques and other alternative approaches with more accurate sensitivity, reasonable cost, and concise detection. Therefore, there is a need to develop a sensitive, accurate, and user-friendly method for more efficient miRNA detection. In the past few years, because of the requirements of amplification effectiveness and constant temperature operation, isothermal signal amplification techniques have been used for both *in vitro* and *in situ* miRNA detection and have been the focus of many researchers. Of these, the RCA technique has been demonstrated to be a suitable method to amplify miRNA *in vitro* and has also been applied to amplify RNA for *in situ* imaging or logic gates construction (2). According to RCA-based amplification, single-stranded nucleic acids (miRNA, short DNA) could be successfully transferred into long single-stranded DNA (ssDNA) products. However, RCA could only achieve limited amplification, and is therefore not the best choice for miRNA detection, because miRNA detection requires extremely high sensitivity.

Therefore, in the present study, we developed the CRISPR/Cas9-assisted garland RCA approach for the precise detection of miRNA. In this method, target miRNA binds with 2 terminals of dumbbell padlock and cyclizes the padlock. With the assistance of T4 DNA ligase and phi29 DNA polymerase, an ssDNA product was obtained with repeated hairpin domains. After recognition with complementary sequences, a double-stranded DNA (dsDNA) section is formed that can be identified by the CRISPR/Cas9 system. After cleavage of the dsDNA section by the Cas9 enzyme, the RCA product is dissociated into hairpin probes to trigger the CHA-based signal amplification and generation. The proposed approach is promising for miRNA detection and lung cancer diagnosis. We present the following article in accordance with the MDAR reporting checklist (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1405/rc).

Methods

Materials

All the sequences used in this research were purchased from Sangon (Shanghai, China) (Table S1). Phi29 DNA polymerase, T4 DNA ligase, dNTPs, and bovine serum albumin (20 mg/mL) used for RCA were purchased from New England Biolabs (Ipswich, MA, USA). Diethyl pyrocarbonate water was purchased from Sigma Aldrich

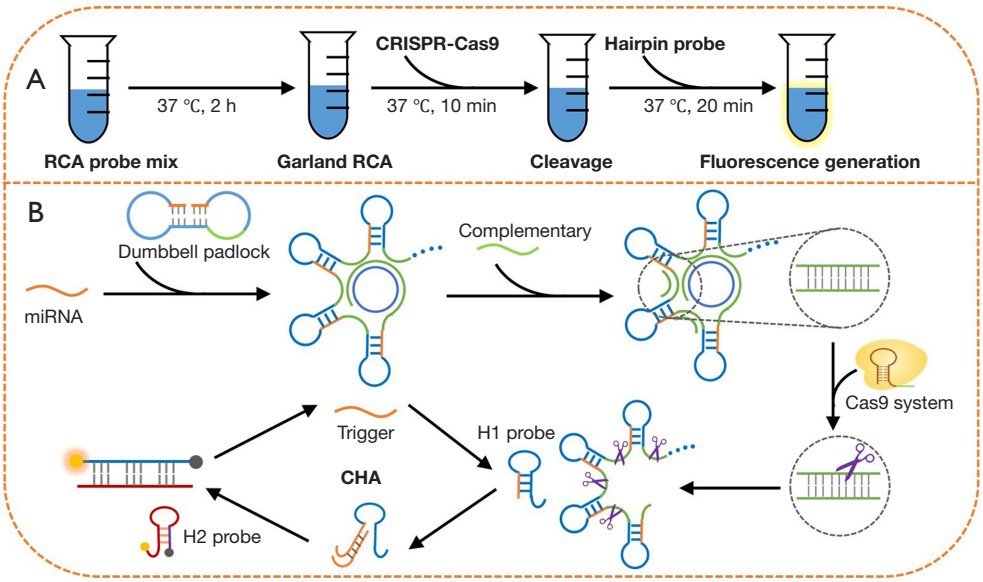

Figure 1 CRISPR/Cas9-assisted garland RCA-based miRNA detection approach. (A) Detection procedures of the method; (B) Working mechanism of the method. RCA, rolling circle amplification; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated nucleases 9; miRNA, microRNA; CHA, catalytic hairpin assembly.

(St Louis, MO, USA). The Cas9 enzyme and its CRISPR RNA (crRNA) sequences were obtained from Inovogen (Inovogen, China). Fluorescence signal was detected using a Hitachi fluorescence spectrophotometer F-4700 (Hitachi, Japan). The Mini-PROTEAN vertical electrophoresis apparatus was purchased from Bio-Rad (Bio-Rad, Hercules, CA, USA).

RCA fluorescence analysis

The RCA process was performed according to the following steps: 2 μL of the padlock template, 2 μL miRNA-21 (100 pM each), 1 μL T4 DNA ligase (2,000 U/mL), 1 μL phi29 DNA polymerase (10 U/μL), and 8 μL dNTP mixture were first added in a reaction buffer containing 50 mM Tris-HCl, 10 mM $MgCl_2$, 1 mM ATP, and 2 μL Dithiothreitol (10 mM, pH 7.5) at 25 ℃. The mixture was then incubated at 37 ℃ for 1 h, slightly vortexed, and spun down. Afterwards, the mixture was heated to 90 ℃ for 5 min and gradually cooled to room temperature. In total, 2 μL Cas9 enzyme (1 U/L) and 2 μL synthesized RNA sequences were added in the mixture and incubated at 37 ℃ for 20 min; 2 μL H2 probe (1 nM) was then added to the mixture. The fluorescence signal of the whole mixture was detected when it was incubated with the H2 probe for 20 min.

Statistical analysis

Statistical significances of all collected data were calculated by GraphPad Prism 8.0 (GraphPad Software, San Diego, CA, USA), and all data were expressed as mean ± standard deviations. Two-tailed Student's *t*-test was used to compare differences between two groups, with $P<0.01$ indicating statistical significance.

Results

Working mechanism of the proposed method

Detection procedures of the proposed method are illustrated in *Figure 1A*. In the RCA process, target miRNA could hybridize with 2 terminals of the designed dumbbell padlock to form a cyclized padlock with the assistance of the T4 DNA ligase. With the assistance of phi29 DNA polymerase for chain extension, a garland RCA product was obtained with a repeated hairpin structure section (*Figure 1B*). A dsDNA section was formed that could be recognized by the CRISPR/Cas9 system with the addition of the complementary sequences. Based on the CRISPR/Cas9-assisted specific cleavage of the formed dsDNA section, the garland RCA product was broken into hairpin structure probes (H1 probe). The generated hairpin probes could be unfolded by targeted

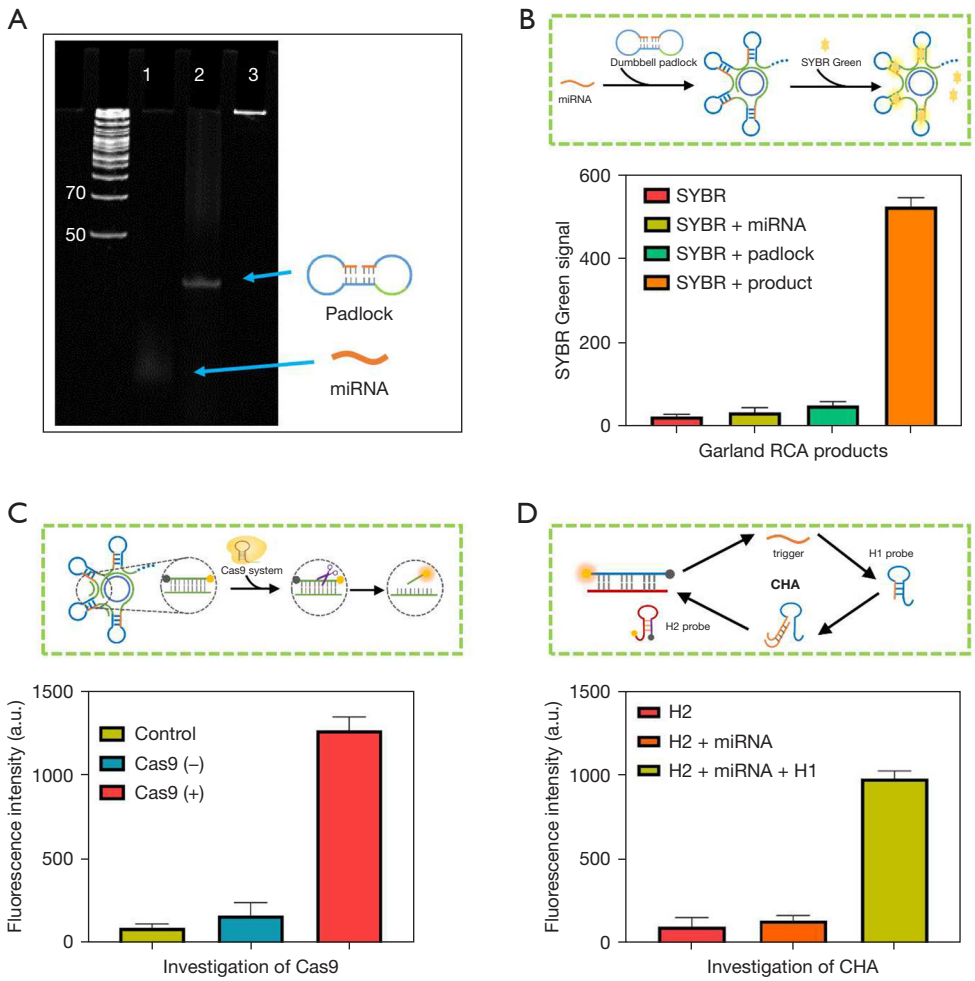

Figure 2 Feasibility of the established method. (A) Polyacrylamide gel electrophoresis result of the RCA process; (B) SYBR Green signal of the approach in the assembly of scaffold; (C) fluorescence signal of the method with and without Cas9; (D) florescence signal of the catalytic hairpin assembly process. miRNA, microRNA; RCA, rolling circle amplification; CHA, catalytic hairpin assembly; a.u., absorbance unit; Cas9, clustered regularly interspaced short palindromic repeats-associated nucleases 9.

miRNA and formed the miRNA-H1 probe complex. Fluorescence-labeled hairpin probe-2 (H2 probe) was added to hydride with the other terminal of H1 probe and replace trigger, liberated to trigger and attend next recycle which composed the CHA amplification process.

Feasibility of the method

PAGE gel electrophoresis was used to determine the feasibility of RCA in this method. As shown in *Figure 2A*, miRNA (lane 1) and padlock (lane 2) moved faster than their hybrid products, while RCA products were blocked in the loading hole, indicating the success of RCA. The formation of the hairpin structure in the RCA product was analyzed by fluorescence experiment. In the assembly of the garland RCA product, the obtained RCA products were heated to 90 ℃ for 10 min and then slowly brought to room temperature. The formation of the hairpin structure could be seen through the formation of the stem section. SYBR Green (Solarbio, Beijing, China) was used to label the stem section. As shown in *Figure 2B*, the obtained signal was much higher when the garland RCA product existed compared with only SYBR Green, indicating the success of the garland RCA product. We then investigated whether CRISPR/Cas9 could specifically recognize and cut the formed dsDNA section in garland RCA products by adding complementary

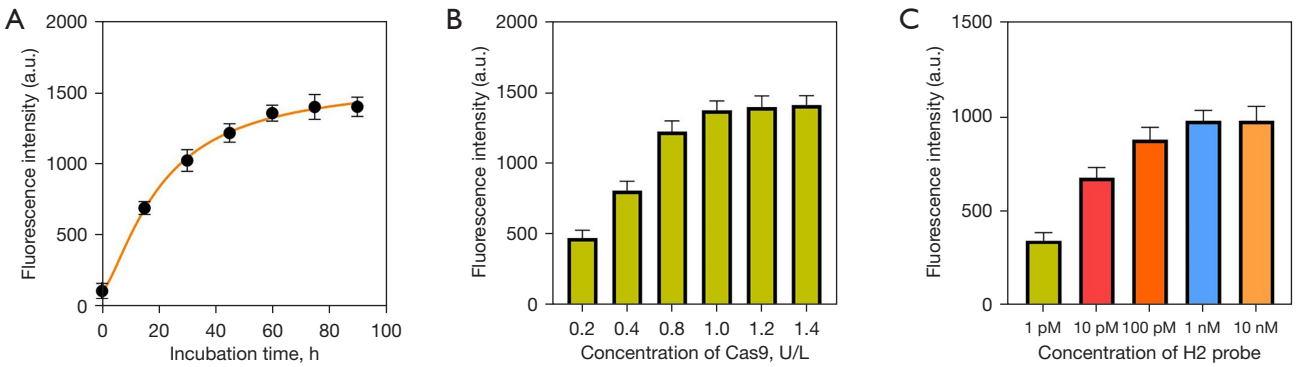

Figure 3 Optimization of the experimental conditions. (A) Fluorescence intensity of the method with different incubating time; (B) fluorescence signal of the method with different concentration of Cas9; (C) fluorescence signal of the method with different H2 probe concentrations. a.u., absorbance unit; Cas9, clustered regularly interspaced short palindromic repeats-associated nucleases 9.

sequences, which were labeled with fluorescein groups and quenched groups at both ends. The results showed that the fluorescence signal in the system was significantly enhanced in the presence of Cas9, suggesting that the complementary sequence was cut apart, leading to the re-emergence of Cy3 fluorescence (*Figure 2C*). We then studied the CHA process by synthesized H1 probe and H2 probe. As seen in *Figure 2D*, the obtained fluorescence signal of the H2 probe showed time-dependent enhancement, suggesting the feasibility of the CHA process.

Optimization of experimental conditions

We then optimized the related experimental conditions to achieve superior detection performance. Considering the importance of RCA in the overall approach, we investigated RCA amplification time, T4, and phi29 enzyme concentration. As shown in *Figure 3A*, the obtained fluorescence intensity increased with the increase of incubation time and did not increase significantly after more than 60 min of incubation. The T4 and Phi29 enzymes were finally optimized to 1 and 1.5 U/L, respectively (Figure S1). We then verified the concentration of then Cas9 enzyme. As shown in *Figure 3B*, when the concentration of Cas9 increased, the detected signal increased correspondingly, but when the concentration exceeded 1 U/L, the fluorescence signal did not change significantly. The optimized H2 concentration for miRNA detection in the CHA process was tested. As shown in *Figure 3C*, the obtained florescence intensity of the system increased when the H2 probe concentration varied from 1 pM to 1 nM, and there were no furthers increases of florescence at concentrations >1 nM.

Detection performance of the method

Under optimal experimental conditions, the sensitivity of this method was determined by detecting miRNA synthesized under different concentration gradients (10 fM to 100 pM). As shown in *Figure 4A*, the fluorescence intensity at 570 nm gradually increased with the concentration in the range of 10 fM to 100 pM. In addition, the data were fitted by exponential curve to obtain the following fluorescence concentration curve: $Y=312.3 \times lgC + 2108$ ($R^2=0.9786$, where C is the concentration of target miRNA) (*Figure 4B*). the limit of detection of the established approach was determined 3.45 fM according to the general 3σ method. These findings indicate that RCA has high detection sensitivity for the amplification of miRNA signals. The specificity of the method in 1× phosphate buffered saline buffer and clinical serum samples was evaluated. As shows in *Figure 4C*, the fluorescence intensity obtained of the target miRNA group was significantly higher than average level that obtained by other miRNA groups. The results demonstrated high selectivity of the established approach in identifying target miRNA from homologous ones.

Clinical application of the method

To investigate the potential clinical application of the constructed CRISPR/Cas9-assisted RCA system for miRNA detection, we applied this protocol to detect miRNA in serum samples from lung cancer patients with and without BM. Studies have reported that miRNA-326 is an anti-oncogene that inhibits tumor metastasis, so it is considered a potential biomarker for lung cancer detection (21). As

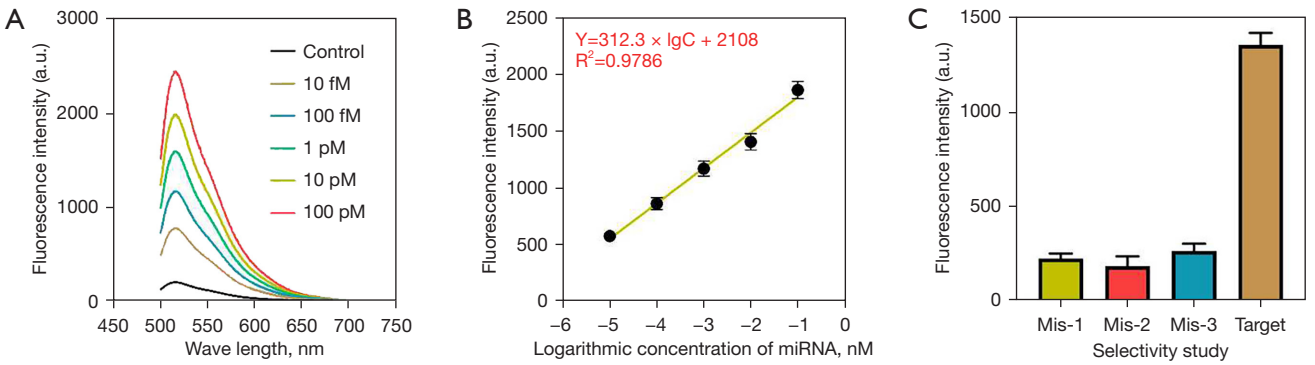

Figure 4 Detection performance of the method. (A) Fluorescence spectrum of the method when detecting different concentrations of miRNA; (B) correlation equation of the obtained fluorescence intensity and concentration; (C) fluorescence signal of the method for different miRNA detections with 1, 2, or 3 base mismatches. a.u., absorbance unit; miRNA, microRNA.

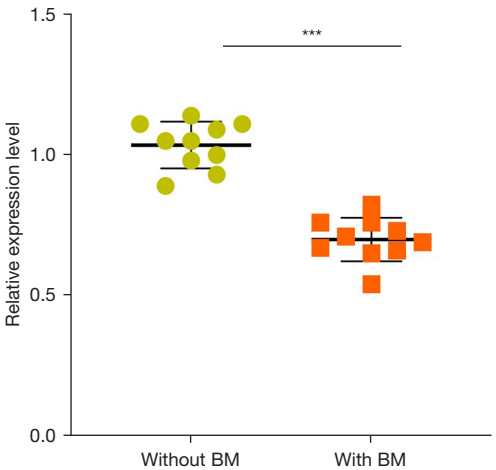

Figure 5 Expression of miR-326 in patients with lung cancer. MiR-326 in patients with lung cancer without BM was higher than that in patients with BM. Statistically significant differences are reported (***P<0.001, n=10). BM, brain metastasis.

shown in *Figure 5*, the concentration of target miRNA obtained by lung cancer patients with BM was much lower than that obtained by lung cancer patients without BM, which was consistent with previous reports (22).

Discussion

We established an accurate and sensitive miRNA detection technique through the CRISPR/Cas9-assisted integration of garland RCA and CHA. In this method, target miRNA could first be amplified into garland RCA products, which contain a complementary sequence recognizing section. With the addition of a complementary sequence, dsDNA was formed that could be recognized and cut by the Cas9 enzyme, generating a large amount of H1 probes to induce CHA-based signal amplification. Eventually, the established approach possessed a wide detection range from 10 fM to 100 pM with the LOD as low as 3.45 fM, which is comparable to most of the former established miRNA detection approaches. In addition, the method also exhibited a high selectivity to target miRNA assured by the high specificity of CRISPR-Cas9 system. Several high highlights of the approach could be summarized as: (I) the padlock sequences were designed with dumbbell structure to reduce the possible mismatch with target miRNA, improving the detection specificity; (II) RCA and CHA process were integrated in the established approach by the CRISPR-Cas9 based chain specific cleavage, endowing the method a high sensitivity; (III) CRISPR-Cas9 system specifically identify and cut the complemented section, checking the RCA products and adding the specificity of the approach. Despite all the above advantages, the requirement of labeling the FAM moiety inevitably increased the background signals. in the future, we will focus on developing the novel biosensors that can detect miRNA in a label-free way. Our proposed method for miRNA detection could be used in the screening, diagnosis of lung cancer with BM. In the future, we will focus on improving the sensitivity of our approach for clinical use.

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Footnote

Reporting Checklist: The authors have completed the MDAR reporting checklist. Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1405/rc

Data Sharing Statement: Available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1405/dss

Conflicts of Interest: All authors have completed the ICMJE uniform disclosure form (available at https://jtd.amegroups.com/article/view/10.21037/jtd-22-1405/coif). The authors have no conflicts of interest to declare.

Ethical Statement: The authors are accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Supplementary

Table S1 Nucleotide sequence

Name	Sequence (5'-3')
Padlock	p-GGG CCC TTG TAT AGC CCC CCC GGG CCC TAG TTC CTC CAG GCC CAA CTA TAC CTG GAG GAA
miRNA-326	CCU CUG GGC CCU UCC UCC AG
Complementary	CCC TAG TTC CTC CAG GCC CAA
Complementary (with fluorescence)	FAM- CCC TAG TTC CTC CAG GCC CAA-BHQ
sgRNA	CCU CUG GGC CCU UCC UCC AGU GUA GAU CAU CUU CCU CGA U
Trigger	TTC CTC CAG GTA TAG ATA TTA AAT
H2 probe	FAM-GTT GGG CCT CTG GGC CCT TAT AAT CTA CAG AGG CCC-BHQ
Mismatch 1	CCU CUG AGC CCU UCC UCC AG
Mismatch 2	CCU CUG ATC CCU UCC UCC AG
Mismatch 3	CCU CUG ATG CCU UCC UCC AG

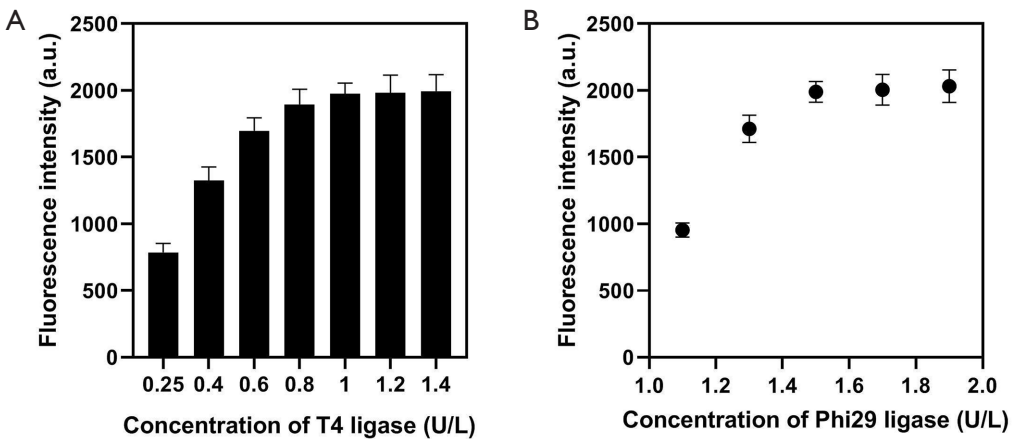

Figure S1 Optimization of enzyme concentration. (A) Fluorescence signal expression of the CRISPR/Cas9 system after adding different concentrations of T4 ligase; (B) fluorescence signal expression of the CRISPR/Cas9 system after adding different concentrations of phi29 polymerase. a.u., absorbance unit; CRISPR, clustered regularly interspaced short palindromic repeats; Cas9, CRISPR-associated nucleases 9.

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