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CRISPR–Cas12a with split crRNA for the direct and sensitive detection of microRNA†

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microRNAs (miRNAs) have been identified as potential biomarkers. Despite the prevalence of quantitative PCR in the field of miRNA detection, this technology is encumbered by the complexity of its methodology. This study presents a novel CRISPR/Cas12a-based method for the direct and sensitive detection of miRNA-21 using split crRNA. The system comprises Cas12a protein, crRNA-handle, and activator DNA complementary to the target miRNA. In the presence of the target miRNA, it binds to the activator DNA, forming a duplex. The formed duplex, in conjunction with the crRNA-handle, activates Cas12a's trans-cleavage activity. This leads to cleavage of a fluorescent reporter, generating an enhanced signal. The method enables direct RNA detection without reverse transcription or sample amplification, offering simplicity and efficiency. This method demonstrates high sensitivity with a minimum detectable limit of 5 pM. Furthermore, the method's specificity is substantiated by its capacity to discern point mutations in miRNA. This system has been shown to quantitatively analyse miRNA-21 levels present within serum, as evidenced by the recovery experiment. Therefore, the method's simplicity, stability, and cost-effectiveness render it a powerful tool for nucleic acid detection, with potential for clinical applications.

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Introduction

microRNAs (miRNAs) are critical regulators of gene expression, and their dysregulation is implicated in numerous human diseases.[1,2] The development of simple, robust, and reliable miRNA detection methods is of great significance in the study of the biological function of miRNAs, molecular diagnostics, treatment of diseases, and targeted drugs.[3] Over the past decades, quantitative polymerase chain reaction (qPCR) has been considered as the gold standard technique for the detection of miRNAs.[4] However, qPCR-based diagnostic methods require expensive instruments and time-consuming procedures.[5–7]

The utilization of nucleic acid detection, facilitated by CRISPR technology, offers a rapid, sensitive, and deployable approach for the identification of miRNAs.[8–12] Although studies have shown that CRISPR–Cas13a enables direct detection of miRNAs,[13–16] compared to Cas12a that uses ssDNA probes, Cas13a uses RNA reporter probes that are more expensive and easily degraded, resulting in a higher risk of false positive results and higher detection costs.[17–19] Among the discovered CRISPR–Cas enzymes, CRISPR–Cas12a was developed for the ultrasensitive detection of DNA and RNA.[20–23] In contrast to the direct detection of DNA, RNA substrates must first be converted into DNA through reverse transcription before they can be recognised by Cas12a.[24–27] However, there remain challenges that limit its practical applications, such as the need for pre-amplification, resulting in extended experiment times and a significant increase in the risk of aerosol contamination.[28–32] Therefore, there is an urgent need to develop diagnostic tools based on Cas12a for the direct and sensitive detection of microRNA. Recent research has revealed that split crRNA has the capacity to activate the trans-cleavage activity of Cas12a in the presence of target DNA.[23,33] Consequently, researchers have developed innovative sensing methods for highly sensitive detection of RNA.[34]

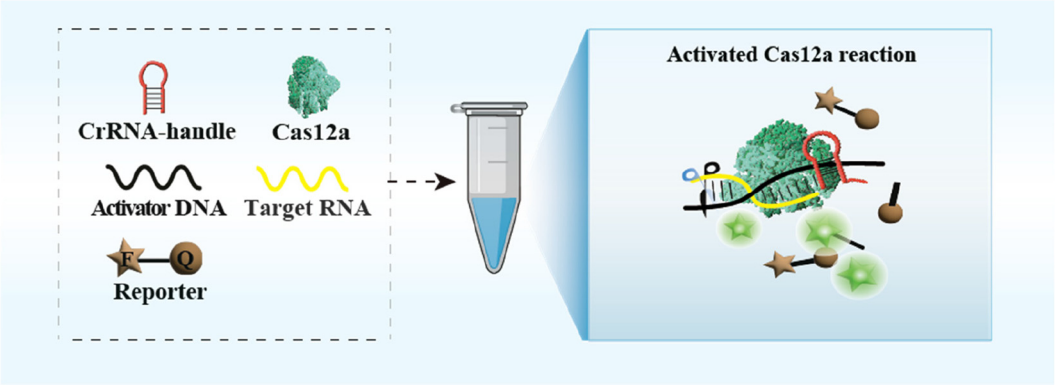
Inspired by the preceding works, a novel method was developed in this study, utilizing CRISPR/Cas12a with split crRNA for the direct and sensitive detection of microRNA. The reaction principle of this method is illustrated in Scheme 1. The system consists exclusively of Cas12a protein, crRNA-handle (scaffold RNA), and activator DNA (containing 20 nt deoxyribonucleotides complementary to the target microRNA). In the

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Scheme 1 Experimental principle of CRISPR–Cas12a with split crRNA.

presence of target miRNA, it hybridizes with the activator DNA, forming a hybrid duplex. The duplex then synergizes with the crRNA-handle to activate Cas12a's trans-cleavage activity. Consequently, the activated Cas12a persists in cleaving the reporter (ssDNA labelled with FAM and BHQ1 at its ends), thereby generating a substantially enhanced fluorescence signal. The developed method enables direct detection of RNA molecules without the necessity for reverse transcription and sample amplification, thus demonstrating a high level of simplicity. Consequently, this method based on CRISPR/Cas12a with split crRNA provides a simple sensing platform for the detection of miRNAs.

Experimental section

Reagents and materials

EnGen LbaCas12a (Cpf1) and 10× 2.1 buffer were procured from New England BioLabs. DEPC-treated water and 30% acrylamide were obtained from Sangong Biotech (Shanghai, China). All DNA and RNA oligonucleotides were synthesized and modified by Sangong Biotech (Tables S1 and S2†). The 20 bp DNA ladder and 6× DNA gel loading dye were purchased from Takara. TEMED was supplied by Diamond. 4S GelRed, EDTA, and Tris were procured from BBI. The serum used in the study was obtained from healthy individuals at Chongqing Baoshihua Hospital. All DNA and RNA oligonucleotides were dissolved in DEPC (RNase-free) water and stored at −20 °C for subsequent experiments. Fluorescence measurements were performed using an F-4700 spectrophotometer (Hitachi, Japan). The excitation and emission wavelengths of FAM were 490 nm and 518 nm, respectively. This study was approved by the Ethics Committee of Chongqing Medical University (Approval no. 2024035). The ethics committee granted a waiver of informed consent as the samples were anonymized and the study posed minimal risk to participants.

Polyacrylamide gel electrophoresis

The process of polyacrylamide gel electrophoresis was performed using a standard protocol. In brief, 10 μL of the reaction products was premixed with 2 μL of 6× DNA gel loading dye and then loaded onto a premade 12% polyacrylamide gel. Electrophoresis was then carried out for 35 minutes at a constant voltage of 110 volts. 2.5 μL of 20 bp DNA ladder was used for molecular weight reference. Following staining with GelRed, the gel images were then visualised by means of a Tanon image system.

CRISPR–Cas12a with split crRNA for the detection of miRNA

The concentrations of the target miRNA, CRISPR–Cas12a, crRNA-handle, and DNA activators were optimized through a series of trials, in which the components were assembled in 2.1 buffer and DEPC-treated H_2O. Following this, the mixture was incubated at 37 °C for 10 minutes. Subsequently, the mixtures were added to a 100 μL reaction volume containing 1 μM reporter. The reactions were then conducted at 37 °C for 150 minutes, after which the fluorescence changes at 518 nm were recorded. It should be noted that the usage of each component can be adjusted according to the experimental consumption. The final concentrations of Cas12a, crRNA-handle, and target ssDNA activators employed in all the assays were 250 nM, 500 nM, and 20 nM, respectively.

Results and discussion

Feasibility analysis

As demonstrated in Fig. 1A, electrophoresis illustrates the fragments produced by CRISPR–Cas12a cleavage following activation. As shown in Fig. 1B, the red curve verifies that the pronounced fluorescence was exclusively produced when the Cas12a enzyme, the crRNA-handle, the target microRNA, and the activator ssDNA were all present. Conversely, the absence of either the target molecule or the crRNA-handle resulted in the absence of collateral cleavage activity and a negligible level of fluorescence. These results demonstrate that the Cas12a enzyme, the crRNA-handle, the target miRNA, and the activator ssDNA are all necessary to rebuild the Cas12a RNP with full trans-cleavage activity.

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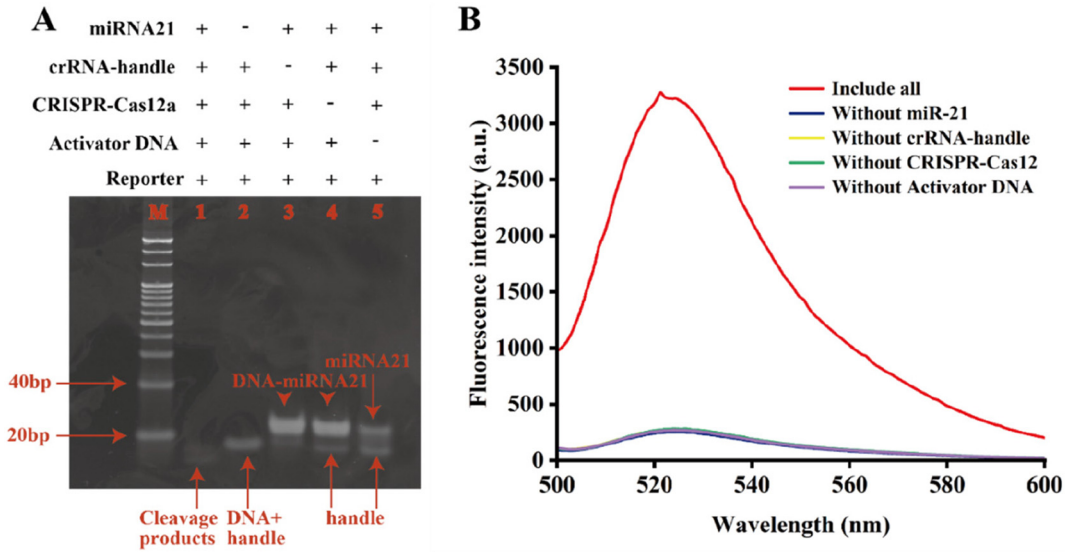


Fig. 1 Feasibility analysis of fluorescence sensing. (A) Polyacrylamide gel electrophoresis analysis. The concentrations of miRNA-21, activator DNA, and reporter were 1 μM. The concentrations of crRNA-handle and CRISPR–Cas12a were 500 nM. (B) Fluorescence spectroscopy analysis; the concentration of miRNA-21 was 500 pM.

Optimization of experimental conditions

Four key parameters were subjected to optimization, including the reaction time, the concentration of crRNA-handle, the concentration of activator DNA, and the concentration of CRISPR–Cas12a. The experimental design utilized elevated the concentrations of CRISPR–Cas12a and crRNA-handle to enhance the sensitivity of the assay. It is evident from the data presented in Fig. 2A, B, and D that as the reaction time increases, the concentrations of both the crRNA-handle and CRISPR–Cas12a also increase and the fluorescence signal becomes stronger. Following a comprehensive analysis of the experimental data, we concluded that the optimal concentrations for the experimental system are a final concentration of 500 nM for the crRNA-handle and a final concentration of 250 nM for CRISPR–Cas12a, with a reaction time of 150 minutes. It is acknowledged that excessive activator DNA has the capacity to influence the efficiency of CRISPR–Cas12a trans-cleavage reporter, thereby affecting the strength of the fluorescence signal. To this end, we utilized varying concentrations (10 nM–700 nM) of activator DNA to ascertain the optimal reaction conditions. As demonstrated in Fig. 2C, the results show that the concentration of activator DNA has a significant effect on the Δfluorescence intensity, which increases to a peak before gradually decreasing. Consequently, 20 nM was determined to be the optimal activator DNA concentration for the assay. In summary, when the concentration of crRNA-handle is 500 nM, the concentration of CRISPR–Cas12a is 250 nM, the concentration of activator DNA is 20 nM, and the reaction time is 150 minutes; the fluorescence biosensing method has good detection performance.

Performance analysis

In order to evaluate the sensitivity of the sensor, the target microRNA-21 was measured at varying concentrations under optimal conditions. As demonstrated in Fig. 3A, with an increase in target concentration, the fluorescence signal value also increases. The concentration of the target (miRNA-21) ranges from 50 fM to 750 pM, and the logarithmic concentration of the target follows an "S-shaped" curve with the fluorescence signal value. As shown in Fig. 3B, when the concentration of the target molecule is in the range of 5 pM–750 pM, a strong linear relationship exists between the fluorescence intensity and the target concentration. The regression equation for this relationship is $F = 7.15C + 467.04$ ($R^2 = 0.9804$). In this equation, F represents the fluorescence signal value at a wavelength of 520 nm and C represents the concentration of the target. The minimum detectable limit of the system is 5 pM.

Selectivity analysis

In order to evaluate the selectivity of the assay, several types of miRNAs were tested and compared (miRNA-1: miRNA-21 with single base mutations, miRNA-2: miRNA-21 with 2 base mutations, miRNA-3: miRNA-21 with 3 base mutations, miRNA-4: miRNA-21 with 4 base mutations, miR-106a, and miR-20a) with the miRNA-21 target (Fig. 4). In the presence of miRNA-21, a strong fluorescence signal can be observed. However, it is evident that the experimental system's ability to distinguish single base mutations is still insufficient. With the exception of miRNA-1, the enhancement of the fluorescence signal was not observed for any of the non-targeted miRNAs, thereby indicating a high degree of specificity for this biosensing method.

Recovery experiments

The interference of complex sample matrices is an important limiting factor for the clinical application of biosensing strat-

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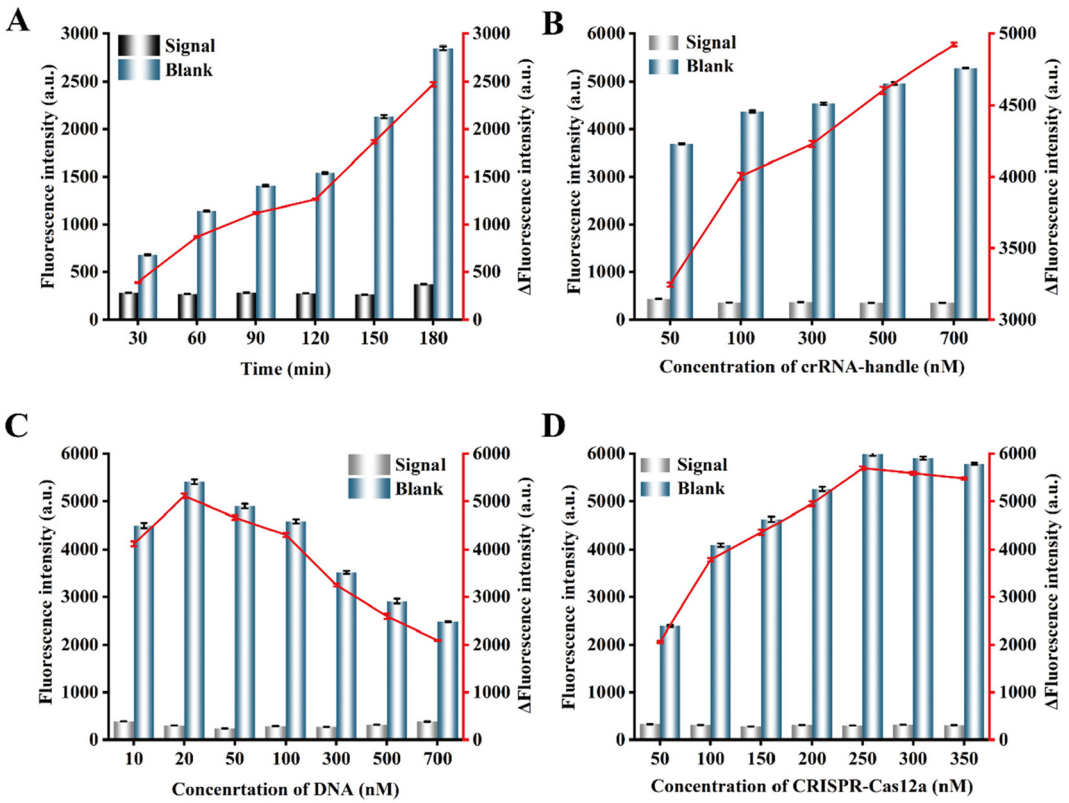


Fig. 2 Optimization of detection conditions: (A) reaction time, (B) the concentration of crRNA-handle, (C) the concentration of activator DNA, and (D) the concentration of CRISPR–Cas12a.

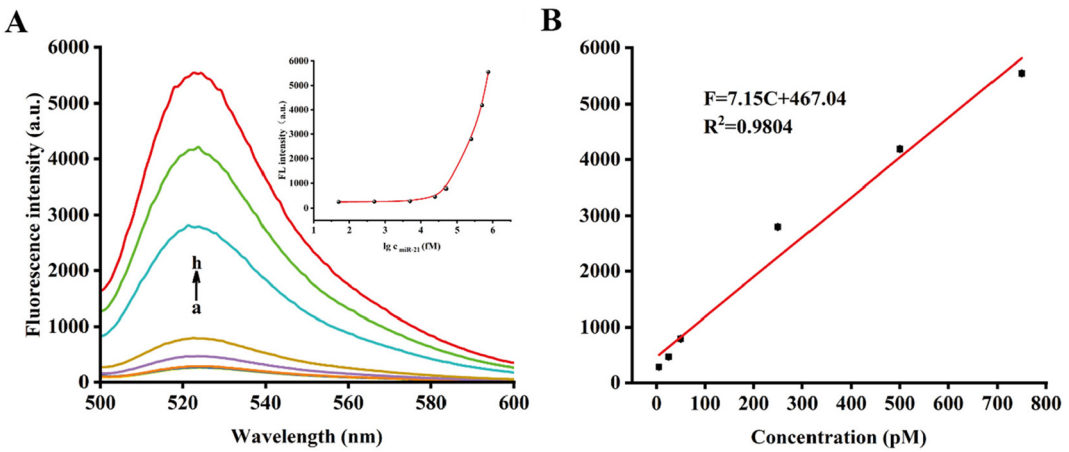


Fig. 3 Fluorescence spectral response (A) and calibration curves (B) for series concentrations (50 fM, 500 fM, 5 pM, 25 pM, 50 pM, 250 pM, 500 pM, and 750 pM: from a to h) of miRNA-21.

egies. In order to verify the feasibility of this biosensing strategy in clinical sample detection, four different concentrations of miRNA-21 were added to diluted 10% normal human serum in order to simulate the actual samples. As demonstrated in Table S3,† the detection outcomes for the four distinct concentrations of miRNAs in the serum matrix demonstrate substantial consistency with the actual added values, exhibiting recovery rates ranging from 96.33% to 117.60%, along with a standard deviation (RSD) ranging from 1.8% to 6.2%. The aforementioned results indicate that the fluorescence biosensing strategy based on CRISPR–Cas12a with split crRNA has certain resistance to complex matrix interference, and the results are stable, with the potential for high-sensitivity detection of miRNA-21 in clinical samples.

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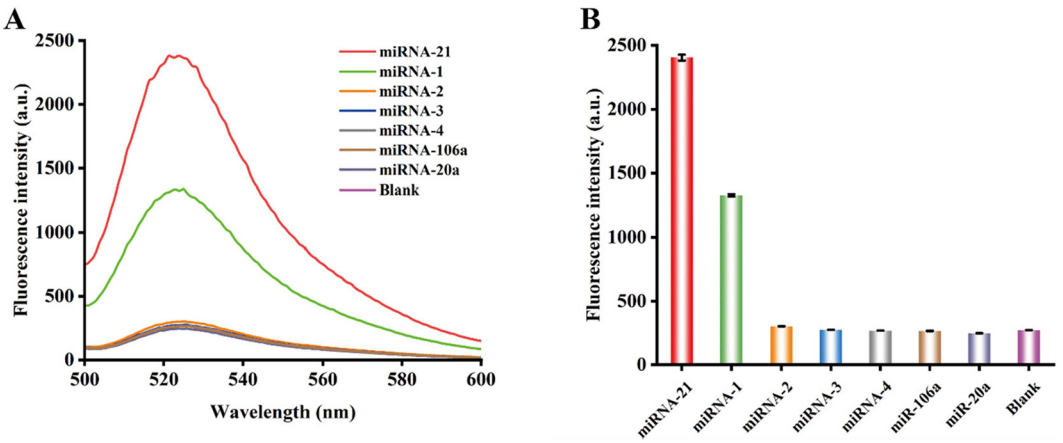


Fig. 4 (A) Fluorescence spectra and (B) fluorescence peak intensities in response to various miRNAs. The concentrations of miRNA-21, miRNA-1, miRNA-2, miRNA-3, miRNA-4, miR-106a, and miR-20a were 250 pM.

Conclusion

In order to take advantage of RNA molecules that are complementary to a given sequence as part of a CRISPR–Cas system, we have devised a fluorescence biosensing strategy based on CRISPR–Cas12a with split crRNA. This strategy has been shown to achieve highly sensitive and selective detection of miRNA without the need for reverse transcription and pre-amplification. The practical potential of this biosensor in cancer diagnosis and prognosis is promising.

Despite the promising advantages of the split crRNA-based CRISPR–Cas12a fluorescence biosensing strategy for miRNA detection, several limitations and challenges remain to be addressed. First, while the method demonstrates high sensitivity and selectivity, its linear range and detection limit may still fall short for certain clinical applications, particularly when detecting ultra-low abundance miRNAs in complex biological matrices. Second, the performance of the biosensor in real clinical samples, such as blood, serum, or tissue extracts, needs further validation to ensure robustness against interfering substances and variability in sample composition. Additionally, the current reliance on recovery experiments, although useful for preliminary validation, does not fully capture the complexity of clinical scenarios, where factors such as sample heterogeneity, degradation, and the presence of inhibitors can significantly impact detection accuracy. Therefore, future studies should prioritize testing in diverse clinical samples to evaluate the practical applicability.

Future research should focus on addressing these challenges to enhance the robustness and practicality of the biosensor. Integrating the split crRNA strategy with other isothermal amplification techniques, such as catalytic hairpin assembly or rolling circle amplification, could further improve sensitivity and expand the linear detection range. Additionally, exploring the adaptability of this approach to other CRISPR–Cas systems, such as Cas13 or Cas14, could broaden its application to detect a wider array of nucleic acid targets, including DNA and RNA viruses. Optimizing crRNA design and Cas12a enzyme activity could also help reduce off-target effects and improve detection efficiency. Finally, large-scale clinical validation studies are essential to demonstrate the biosensor's reliability and accuracy in real-world diagnostic scenarios, particularly for cancer diagnosis and prognosis. By addressing these limitations and exploring these future directions, the split crRNA-based biosensing strategy could evolve into a transformative tool for molecular diagnostics, with significant potential for commercialization and clinical implementation.

In summary, the fluorescence biosensing strategy has the following advantages: (1) no amplification or reverse transcription is required, allowing for direct identification and simple operation. (2) Only one enzyme, Cas12a, is involved, ensuring high stability and relatively low experimental costs. Furthermore, the simple "split-crRNA-activator" strategy of this work can be expanded to other CRISPR–Cas enzymes for developing innovative molecular diagnostics. Therefore, this work presents a powerful nucleic acid detection approach with the potential for commercial use in clinical applications.

Data availability

Data will be made available on request.

Conflicts of interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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