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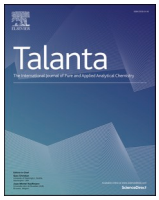

Bis-enzyme cascade CRISPR-Cas12a platform for miRNA detection

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ABSTRACT

MicroRNA (miRNA) play a vital role in the pathological development of many diseases. It is considered to be the diagnosis and potential biomarkers of prognosis. Herein, we proposed **B**is-enzyme cascade **P**latform by combining **T**7 RNA polymerase and CRISPR-**C**as12a (BPTC) for a miRNA detection. In the proposed BPTC, the RNA to DNA conversion ability of phi29 amplification and *trans*-cleavage of CRISPR-Cas12a are combined. The target miRNA can be amplified after binding to the recognizer ssDNA, and then transcribed the CRISPR-derived RNA (crRNA) by T7 RNA polymerase. The produced crRNA can thereby be assembled by CRISPR-Cas12a and recognized with its target dsDNA, thus triggered its *trans*-cleavage towards surrounding fluorescent reporters, labeled with a fluorophore and a corresponding quenching group. Based on the bis-enzyme cascade system, the biosensor shows highly sensitivity and excellent specificity. Moreover, this study provided a novel all-in-one detect strategy for miRNA and may open a new idea for the design of CRISR-Cas-based miRNA biosensing platforms.

1. Introduction

MicroRNA (miRNA), an endogenous, small (approximately 19–24 nucleotides) and non-coding functional RNA, plays a vital role in regulating gene expression, epigenetic mechanisms and cell cycle regulation [1–3]. Moreover, miRNAs are key factors in regulation of tumor and the abnormal expression of miRNA is related to occurrence of tumor [4,5]. Thus, it can be applied as tumor diagnostic biomarkers and various methods for miRNAs detection have been explored such as RT-qPCR, exponential amplification reaction (EXPAR), duplex-specific nuclease signal amplification (DSNSA), hybridization chain reaction (HCR), catalyzed hair-pin amplification (CHA) and rolling circle amplification (RCA) [6–9].

Recently, clustered regularly interspaced short palindromic repeats and associated proteins (CRISPR-Cas) has been widely used for nucleic acid testing since the complementarity-dependent of programmable guide RNA (crRNA) for targeting nucleic acids offers excellent specificity of CRISPR-Cas system [10–13]. CRISPR-Cas12a, it can not only cleave both double-stranded DNA (dsDNA) and single-stranded DNA (ssDNA) based on its *cis*-cleavage activity, but also has *trans*-cleavage activity to cut any other ssDNA [14–17]. Thus, it has been well applied to nucleic acid detection of pathogenetic bacteria and viruses [18–20], and can be further combined with other amplification methods or instruments to achieve ultrasensitive, portable and fast detection [21–24].

Moreover, CRISPR-Cas system can be employed as an amplified tool due to thousands of report probe, which is a nucleic acid molecule bound to a quencher molecule and a reporter fluorophore (FQ report probe), can be cleaved by single activated Cas protein [25–29]. Inspired by these characters of CRISPR-Cas, single-stranded RNA (ssRNA) was used as a target for detection of miRNA [30–32]. However, the pre-prepared crRNA and RNA FQ probe are easily degraded and requiring rigorous storage conditions, which is not conducive to long-distance transportation and long-term environmental testing outside the laboratory [33,34]. Therefore, it is urgent to develop a strategy that is without the requirement of crRNA pre-preparation and RNA FQ probe. Other than to Cas13, the Cas12a (previously called Cpf1) has also been confirmed to

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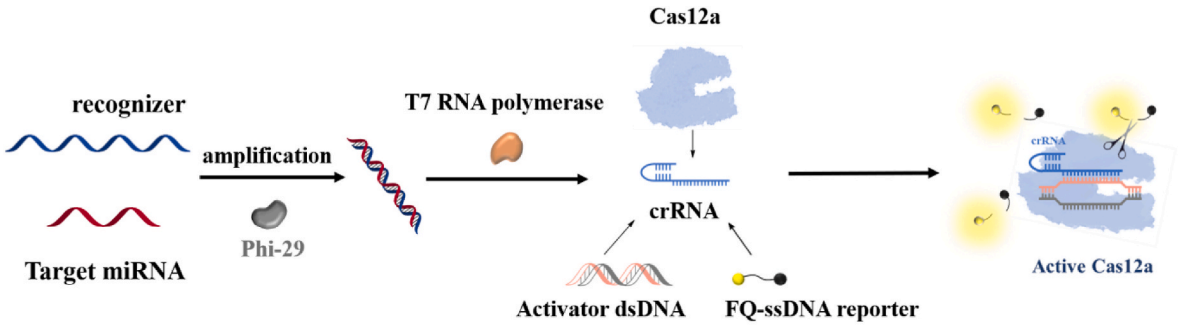


Fig. 1. The principle of Bis-enzyme Cascade Platform by combining T7 transcription and Cas12a cleavage activity. The target miRNA combines with recognizer, and then extend it with phi29 DNA polymerase. The obtained double-stranded DNA (dsDNA) products contains the crRNA template. The Cas12a enzyme first bound to the crRNA obtained by T7 transcription of the above amplified products, and then guided the system for specific recognition and binding to the target dsDNA. Thus, the cleavage activity of the CRISPR-Cas12a system was triggered to cleave the FQ reporter and fluorescent signal generated in relative fluorescence units (RFUs).

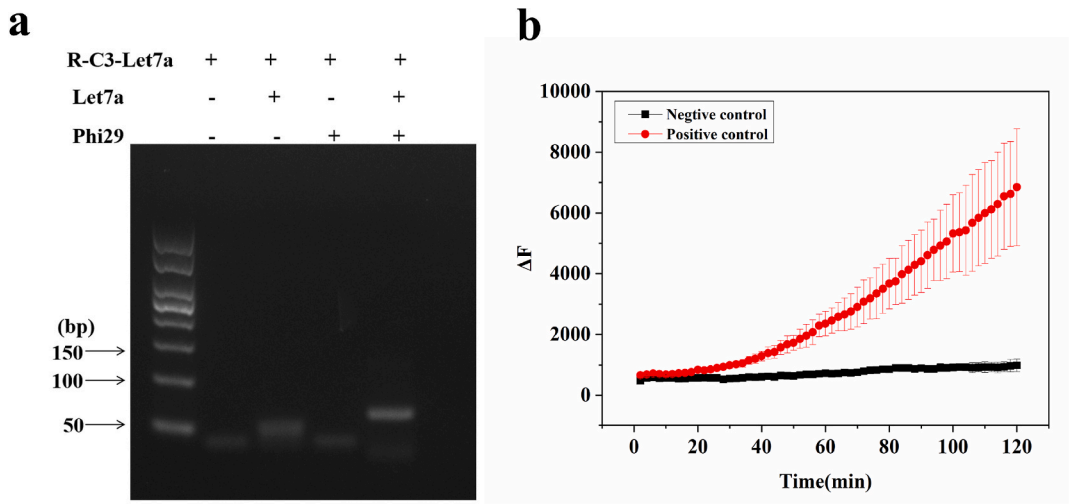


Fig. 2. Feasibility of Bi-enzyme Cascade Biosensor by combining T7 transcription and Cas12a cleavage activity. (a) Agarose gel electrophoresis analysis of the feasibility of phi29 amplification M: DNA marker. Lane 1: R–C3-Let7a. Lane 2: R–C3-Let7a + Let7a. Lane 3: R–C3-Let7a + phi29 DNA polymerase. Lane 4: R–C3-Let7a + Let7a + phi29 DNA polymerase. (b) Fluorescence measurement of the feasibility, no Let7a was used as negative control, 100 pM Let7a was added as positive control. (n = 3 technical replicates, bars represent mean ± s.d. ΔF represents background subtracted fluorescence).

possess a collateral cleavage activity [35–37]. Cas12a effectors can *trans*-cleave collateral their target ssDNA, which makes it possible to get rid of the reliance on RNA probe. It is more stable and easier to be stored using ssDNA as the probe [38,39].

Nevertheless, it is still necessary to generate a ternary complex of Cas12/crRNA/target DNA to activate the collateral cleavage of Cas12a. Thus, preparation of crRNA is a vital step [40,41]. We aim to take the construction of on-site preparation of Cas12a-activated crRNA method to detect miRNA. T7 transcription, an efficient mean of amplification, has currently been used in analytical field. There have been reports that T7 transcription amplification obtains RNA to activate Cas13a. However, the targeting RNA transcribed by T7 is unable to active Cas12a [42–44]. Therefore, we considered whether the miRNA detection method that we proposed above could be achieved by on-site preparation of crRNA combined with T7 transcription.

Herein, we aim to develop a Bis-enzyme Cascade Platform by combining T7 transcription and Cas12a cleavage activity (BPTC). The biosensor contains two stages for amplification. In detail, ssDNA recognizer can form a double-stranded DNA (dsDNA) with a T7 promoter probe in the presence of target miRNA assisted with phi29. At the first stage, one miRNA can induce thousands of crRNA produce assisted with T7 RNA polymerase. At the second stage, crRNA/Cas12a *trans*-cleaves FQ probe at a rate of approximately 250 every second which significantly enhanced the response intensity to obtain a more efficient outcome. The BPTC shows acceptable sensitivity by combining two stages for amplification. Besides, the detection strategy of "all-in-one" based on the BPTC can greatly shorten the detection time. This study can provide a simple, rapid miRNA detection methods and may open a new perspective for construct of Cas-based biosensor.

2. Material and methods

2.1. Activity verification of Cas12a and phi29 proteins

We initially purified Cas12a and phi29 proteins (the purification steps for both proteins are documented in Materials and methods in Supplementary Information), and the purification results were subsequently verified by SDS-PAGE gel electrophoresis. The results show that

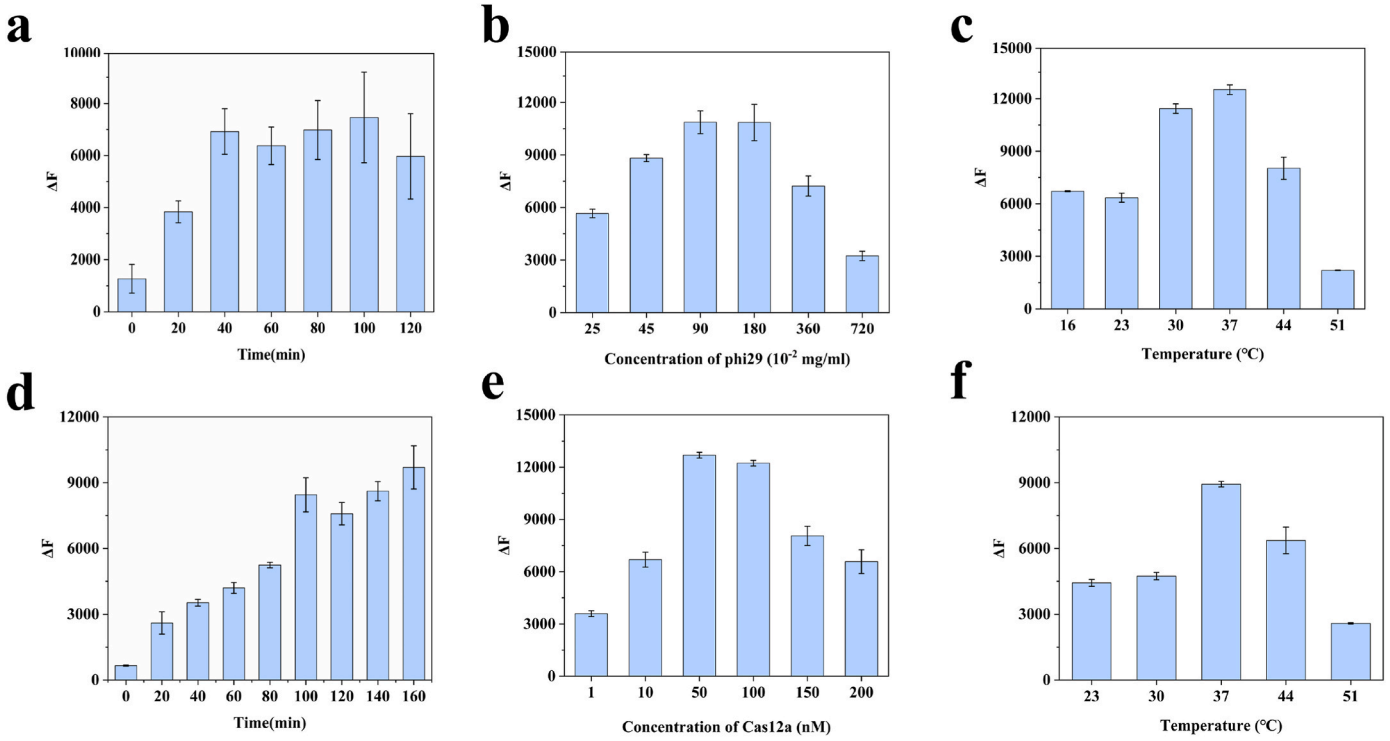


Fig. 3. (a) Fluorescence signal values detected at 120 min under different phi29 amplification time and (b) different concentration of phi29. (c) Fluorescence signal values detected at 120 min at different phi29 amplification temperatures. (d) Fluorescence signal values detected at 120 min under different T7 RNA polymerase transcription time. (e) Fluorescence signal values detected at 120 min under different concentration and (f) different reaction temperature of Cas12a. ($n = 3$ technical replicates, bars represent mean ± s.d. ΔF represents background-subtracted fluorescence).

after the final purification step, we obtained a single target protein (Fig. S1 and Fig. S2). Correspondingly, we performed activity verification on the purified two proteins respectively.

For activity verification of Cas12a, 50 nM Cas12a and 50 nM crRNA were mixed and incubated in 1 × Cas12a reaction buffer (50 mM NaCl, 10 mM Tris-HCl, 10 mM $MgCl_2$, 100 μg/ml BSA, pH 7.9) at 37 °C for 30 min, thus a binary complex of Cas12a binding to crRNA was obtained. This binary complex was then mixed with the target strand and FQ-reporter (Table S2) in 1 × Cas12a reaction buffer. The reaction was monitored using a fluorescence microplate reader (BioTek H1) at 37 °C for a maximum time of 120 min, with fluorescence measurements (λ_{ex}: 492 nm; λ_{em}: 520 nm) every 2 min. The FQ-reporter, corresponding crRNA and its target (Table S2) were ordered from Shanghai Sangon Biotechnology Co., Ltd.

Meanwhile, the activity of phi29 protein has also been verified. 150 nM circular DNA template, 150 nM primer, 10 mM dNTPs and 0.9 mg/ml phi29 protein were mixed and reacted in 1 × phi29 reaction buffer (50 mM Tris-HCl, 10 mM $MgCl_2$, 4 mM DTT, 10 mM $(NH_4)_2SO_4$, pH 7.5) for 60 min, in order to demonstrate its amplification performance better, we also used the commercially available phi29 DNA polymerase (purchased from HaiGene Biotech Co., Ltd) for comparison, and agarose gel electrophoresis was then performed to verify the amplification results.

2.2. BPTC system for miRNA detection

miRNA recognizer was artificially synthesized including the crRNA template sequence, T7 promoter and a C3 modifier at 3' ends to avoid false positives. The miRNA recognizer sequences were available in Table S1 crRNA templates were amplified from miRNA recognizer by phi29 DNA polymerase and T7 RNA polymerase. The miRNA recognizer was primarily mixed with target miRNA at 94 °C for 5 min and then 25 °C for 5 min to make them better combine. Next, mixed up the conjugate, 0.9 mg/ml phi29 DNA polymerase and 10 mM dNTP mixture in 1 × phi29 reaction buffer at 30 °C for 60 min Phi29 was then inactivated at 94 °C for 5 min. Immediately, 100 U T7 RNA polymerase (New England Biolabs) and 10 mM NTP mixture was added to the phi29 amplification product, and the crRNA was obtained by in vitro transcription at 37 °C for 2 h.

The crRNA products mentioned above (12.75 μl) were mixed with 50 nM Cas12a in 1 × Cas12a reaction buffer at 37 °C for 30 min. Then mixed the above incubation products with 100 nM target dsDNA (Table S1) and 1 μM FQ probe in 1 × Cas12a reaction buffer. The reaction (30 μl, 384-well microplate) was triggered by the cutting activity of Cas12a on the fluorescent probe and used a fluorescence microplate reader (BioTek H1) to monitor fluorescence signal at 37 °C with a maximum time of 120 min using fluorescence measurement (λ_{ex}: 492 nm; λ_{em}: 520 nm) taken every 2 min.

2.3. "All-in-one" detection

In order to achieve faster and more convenient detection, we studied the feasibility of this method to be applied in "all-in-one" detection of miRNA (Fig. 5a). We firstly prepared the assay master mixture, including 200 μM miRNA recognizer, 10 mM dNTP mixture, 10 mM NTP mixture, 0.9 mg/ml phi29 protein, 100 U T7 RNA polymerase, 50 nM Cas12a, 100 nM target dsDNA and 1 μM FQ probe in 1 × phi29 buffer and 1 × Cas12a buffer, the target miRNA was then added and mixed together. The reaction each containing 30 μl in 384-well microplate was inducted by the cleavage activity of Cas12a on the fluorescent probe and used the fluorescence plate reader (BioTek H1) to monitor fluorescence signal at 37 °C with a maximum time of 120 min using fluorescence measurement (λ_{ex}: 492 nm; λ_{em}: 520 nm) taken every 2 min.

In addition, we further explored this method for the detection of real samples. Different concentrations of Let7a were diluted in blood sample, and these different concentrations of Let7a were added to the pre-configured assay master mixture. The reaction each also containing

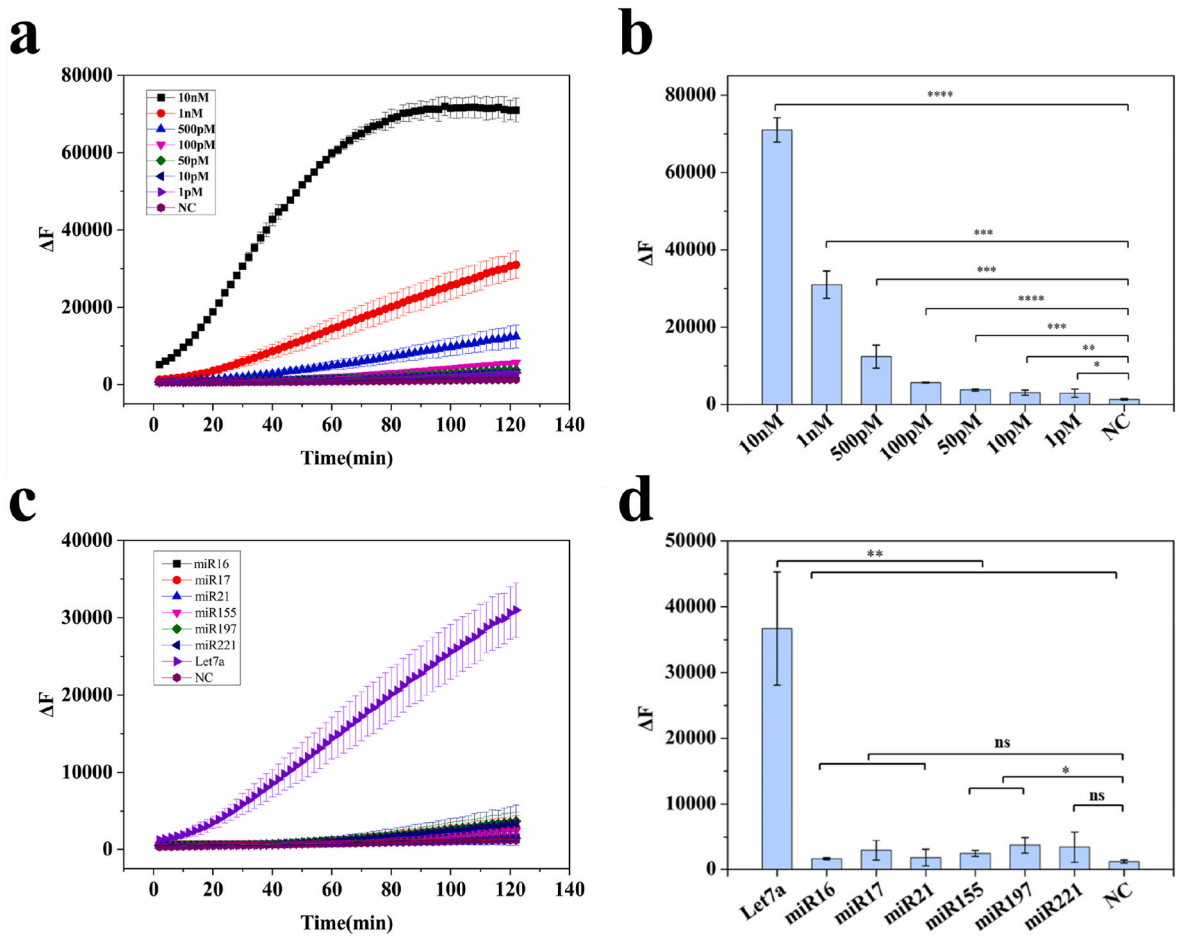


Fig. 4. The sensitivity and specificity of CRISPR-Cas12a combining phi29 detection method. (a) Fluorescence detection kinetic curve of the method in the presence of target miRNA with a series of concentrations ranging from 1 pM–10 nM (b) and the fluorescence signal value detected at 120 min. (c) Fluorescence response for differential detection of six different miRNAs (d) and the fluorescence signal value detected at 120 min ($n = 3$ technical replicates; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; bars represent mean ± s.d.; ΔF represents background subtracted fluorescence; NC represents no template control.).

30 μl in 384-well microplate was inducted by the cleavage activity of Cas12a on the fluorescent probe and used the fluorescence plate reader (BioTek H1) to monitor fluorescence signal at 37 °C with a maximum time of 120 min using fluorescence measurement (λ_{ex}: 492 nm; λ_{em}: 520 nm) taken every 2 min.

3. Results and discussion

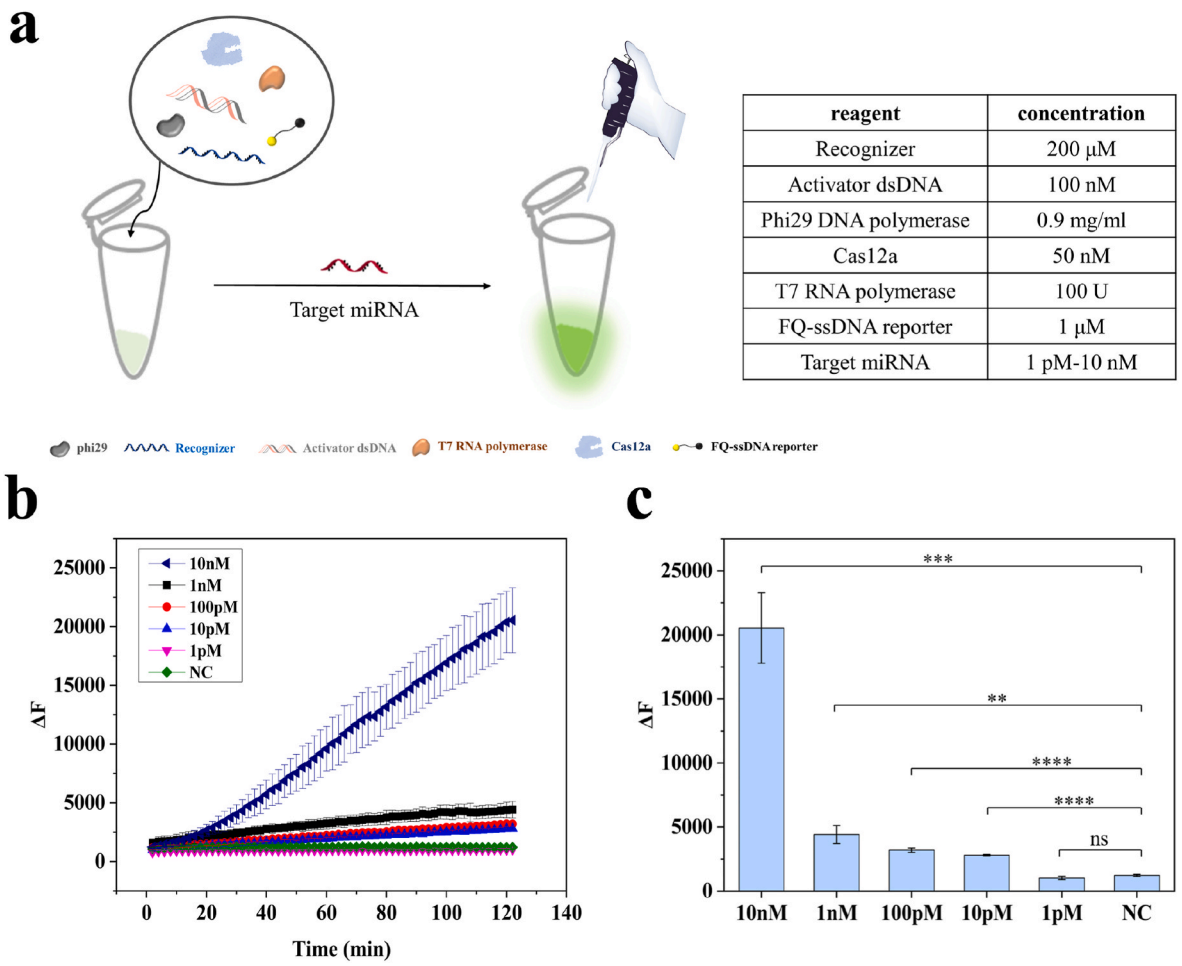
3.1. Principle of BPTC

We proposed an easy-to-use strategy to accomplish diagnostic analysis on miRNA without the complex operation based on Bis-enzyme Cascade Platform by combining T7 transcription and Cas12a cleavage activity (BPTC). Fig. 1 depicted the working-flow of the designed detection strategy of miRNA. The whole process is divided into two steps. One is an amplification process using T7 RNA polymerase assisted with phi29. The target miRNA could combine with miRNA recognizer, extend it with phi29 DNA polymerase and then transcript using T7 RNA polymerase. Consequently, the double-stranded DNA (dsDNA) products contain the crRNA template. The second critical step is the specific cleavage based on CRISPR-Cas12a system. The Cas12a enzyme first bound to the crRNA obtained from the above amplified products to complete a CRISPR-Cas12a platform, and then guided it for a specific recognition and binding to the target dsDNA. Thus, the cleavage-activity of the CRISPR-Cas12a system was initiated to cleave the FQ reporter, which could quickly produce fluorescent signal. The quantity of activated Cas12a and the amount of the target miRNA are shown by the intensity of the fluorescence signal. Considering the importance of Let7a as a marker for early diagnosis of tumors, we applied it as the target for subsequent validation.

3.2. Feasibility of BPTC

The Cas12a enzyme and phi29 DNA polymerase were considered as key factors in the proposed method. Therefore, we firstly expressed and purified the Cas12a and phi29 (Fig. S1 and Fig. S2). Then the activity of Cas12a and phi29 was verified. The result showed that fluorescence signal significantly increased in the presence of crRNA, target dsDNA and FQ reporter when the control groups remain negligible by contrast (Fig. S3). The phi29 DNA polymerase possesses proof-reading activity, strand displacement activity and the ability for generating long synthetic products, making it most suitable to effectively amplify circular DNA molecules, which is generally applied for rolling-circle amplification (RCA). Thus, the RCA test with phi29 was applied to assess its activity. Fig. S4 shows that the band of interest can be amplified, which is consistent with the amplification results of the commercially available phi29 DNA polymerase.

To assess whether the Cas12a and phi29 could be used in the construction of this platform, we firstly used phi29 to perform amplification experiments on miRNA recognizer and target miRNA complexes. As shown in Fig. 2a, in the presence of both miRNA recognizer which is C3-modified ssDNA recognizing Let7a (R–C3-Let7a) and target miRNA



reagent	concentration
Recognizer	200 μM
Activator dsDNA	100 nM
Phi29 DNA polymerase	0.9 mg/ml
Cas12a	50 nM
T7 RNA polymerase	100 U
FQ-ssDNA reporter	1 μM
Target miRNA	1 pM-10 nM

Fig. 5. The "all-in-one" strategy detect let7a in real clinic sample. (a) Schematic diagram of "all-in-one" strategy. (b) Fluorescence detection kinetic curve of "All-in one" strategy and (c) the fluorescence signal value in the presence of target miRNA with a series of concentrations ranging from 1 pM–10 nM in plasma. ($n = 3$ technical replicates; $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$; bars represent mean ± s.d.; ΔF represents background subtracted fluorescence; NC represents no template control.)

(Let7a), the longer amplification product (around 59 bp) was observed in the agarose gel (Lane 4), revealing that phi29 amplification had been successfully triggered by target miRNA. By contrast the normal amplification cannot be performed when only miRNA recognizer was present (Lane 1) and the target miRNA or phi29 DNA polymerase was absent (Lane 2–3). Afterwards the transcribed crRNA was further combined with Cas12a enzyme, the Cas12a/crRNA complex was mixed with the target dsDNA and fluorescent probe. The result was shown in Fig. 2b and Fig. S5, the fluorescence signal increases significantly when Cas12a/crRNA complex, target dsDNA and FQ probe all exist while the control groups remain negligible by contrast.

3.3. Optimization of experimental parameters

To optimize the detection rate of the proposed strategy, we first optimized the amplification time of phi29 DNA polymerase and the T7 transcription time. To assess the most appropriate amplification time, we used 20 min as a gradient to detect the fluorescence intensity after amplification from 0 min to 120 min (Fig. S6a). And the selected fluorescence signal value was detected at 120 min as comparative analysis (Fig. 3a). The result of florescent signal assay indicated that the fluorescence intensity got enhanced with the increasing amplification time, indicating that the amplification effect of phi29 DNA polymerase gradually increases from 0 min to 40 min. Furthermore, the fluorescence intensity reached the peak at 40 min and then maintained basically same after 40 min. Thus, 40 min was selected as the optimal amplification time in the following experiments. Then the same strategy was applied to detect the fluorescence intensity corresponding to the transcription time from 0 min to 160 min (Fig. S7a). As shown in Fig. 3d, we selected the fluorescence signal value detected at 120 min for further analysis. The result showed that the fluorescence signal reached its maximum value at 100 min and remained almost the same rate with the transcription increasing time. Therefore, the optimized transcription time was considered as 100 min.

In addition, the concentration and reaction temperature of each enzyme were also optimized. To evaluate the most suitable concentration of phi29, we detected the fluorescence intensity after amplification with different phi29 concentrations and selected fluorescence signal value at 120 min as comparative analysis (Fig. 3b and Fig. S6b). The results showed that with the increase of the concentration, the fluorescence signal value at 120 min raised first and then decreased and reached peak with phi29 concentration of 0.9 mg/ml. Therefore, 0.9 mg/ml was selected as the concentration of phi29 for subsequent experiments. We also evaluated the most suitable concentration of T7 RNA polymerase (Figs. S7b and c) and Cas12a, according to the result shown in Fig. 3e and Figs. S6d and 100 U and 50 nM was selected as the concentration for subsequent experiments. Moreover, the reaction temperature of phi29 and Cas12a has also been optimized, and the results of fluorescence signal showed that the optimal temperature for phi29 and Cas12a were both at 37 °C (Fig. 3c, Fig. S6c and Fig. 3f).

3.4. BPTC strategy for Let7a detection

To investigate the sensitivity of the proposed strategy, we detected the fluorescence intensity of let7a from 1 pM to 10 nM. Fig. 4a and Fig. 4b showed that the limit of detection (LOD) of target miRNA could reach to 1 pM (signal-to-noise ratio >2:1). And we also made a fitting curve for the relationship between the concentration and the fluorescence signal (Fig. S11). To assess the discriminate ability of this method in the presence of multiple miRNAs, we then distinguished the entirely matched target (Let7a) from six miRNAs (miR-16, miR-17, miR-21, miR-155, miR-197, miR-221), the sequences were available in Table S4. We discovered that compared with the other six types of miRNAs, Let7a induced a much stronger fluorescence signal (Fig. 4c and d). The result indicated that our method had an obvious specificity for identifying complementary target. Moreover, relative standard deviation (RSD) of inter-assay and intra-assay of four experimental groups were 8.12% and 3.78% respectively, denoting that the proposed strategy had an acceptable reproducibility (Fig. S8).

3.5. "All-in-one" strategy in Let7a detection and real clinic sample

We first tested the feasibility of "All-in-one" reaction, as shown in Fig. S9, this method has been proved to be feasible that the fluorescence signal increased significantly in comparison with the control group. Correspondingly, we also confirmed the sensitivity of this scheme, as illustrated in Fig. S10, the limit of detection can reach 1 pM Let7a. Moreover, to evaluate the detectability of the all-in-one strategy for real clinic sample, we spiked different concentration of Let7a in blood sample. After separated plasma from blood cells, the let7a in plasma were detected by proposed strategy. Fig. 5b and c show that the limit of detection (LOD) of "All-in-one" strategy for blood sample could reach to 10 pM for target miRNA (signal to noise ratio >2:1).

4. Conclusions

In summary, a novel miRNA detection platform based on *trans*-cleavage of CRISPR-Cas-12a and T7 transcription was exploited. By combination of the molecule amplification technique and Cas12a-actalyzed amplification, the proposed strategy showed a sensitivity of 1 pM and present accurate (99%) feature. Due to the ready-to-use feature, our method differs from most of traditional CRISPR-based sensing systems as this method is more easy-to-operated. However, despite this study optimized the detection time, step to step of diagnosis methods is still time-consuming. Thus, we also proposed the "All-in-one" strategy and made the detection faster and easier. We suppose that this exploited sensing system not only display a promising tool for disease diagnosis, but also provide a new strategy for CRISPR-based sensing detection method.

Credit author statement

Zixuan Guo: Experimental investigation, Methodology, Data curation, Writing - review & editing. Xiao Tan: Methodology, Writing - review & editing. Haoyu Yuan: Investigation, Methodology. Ling Zhang and Jiajia Wu: Formal analysis. Zhiqing Yang: Conceptualization, Methodology, Data curation, Writing - review & editing. Ke Qu: Writing - review & editing, Yi Wan: Conceptualization, Methodology, Data curation.

Declaration of competing 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.

Data availability

No data was used for the research described in the article.

Acknowledgment

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.talanta.2022.123837.

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