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PCDetection: PolyA-CRISPR/Cas12a-based miRNA detection without PAM restriction

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ABSTRACT

MicroRNAs (miRNAs) are small noncoding RNAs that posttranscriptionally regulate gene expression. The aberrant expression of miRNAs is related to many diseases. MiRNAs can serve as potential biomarkers for the prognosis and diagnosis of cancers and other human diseases. However, the short sequence and high sequence similarity of miRNAs impede detection. Herein, we propose a method to integrate polyA-tailing and CRISPR/Cas12a to amplify and detect all miRNAs with high specificity and sensitivity. PolyA-tailing enables efficient amplification of RNA and introduces a universal PAM sequence for Cas12a to unlock its PAM restriction. The CRISPR-Cas system guarantees the specific recognition of nucleic acid sequences with a single base mismatch. A limit of detection (LOD) as low as 50 fM was achieved. The practical application ability of polyA-CRISPR/Cas12a-based miRNA detection was validated by miRNA analyses in multiple cancer cell samples. With the increasing stability of RNA samples, low cost, excellent specificity, and sensitivity, this method demonstrates great potential to scale up to parallel diagnostic sets for miRNA-related disease.

1. Introduction

MicroRNAs (miRNAs) are small noncoding RNAs composed of approximately 22 nucleotides with important functions in the regulation of protein-coding gene expression and the pathogenesis of cancers (Bartel, 2004; Macfarlane and Murphy, 2010). Recent studies have shown that the aberrant expression of miRNAs can serve as effective biomarkers in the diagnosis, progression, and monitoring of human diseases (Calin and Croce, 2006, 2007; Garzon et al., 2010). The altered levels of extracellular circulating miRNAs in serum, plasma, saliva, and other body fluids have shown enormous potential as novel noninvasive biomarkers of disease (Ho et al., 2015; Uratani et al., 2016; Verma et al., 2015; Wang et al., 2018). Traditional techniques for miRNA detection, such as quantitative reverse transcription PCR (RT–qPCR), next-generation sequencing, in situ hybridization, and microarray-based hybridization (Baker, 2010; Dong et al., 2013; Hunt et al., 2015), require

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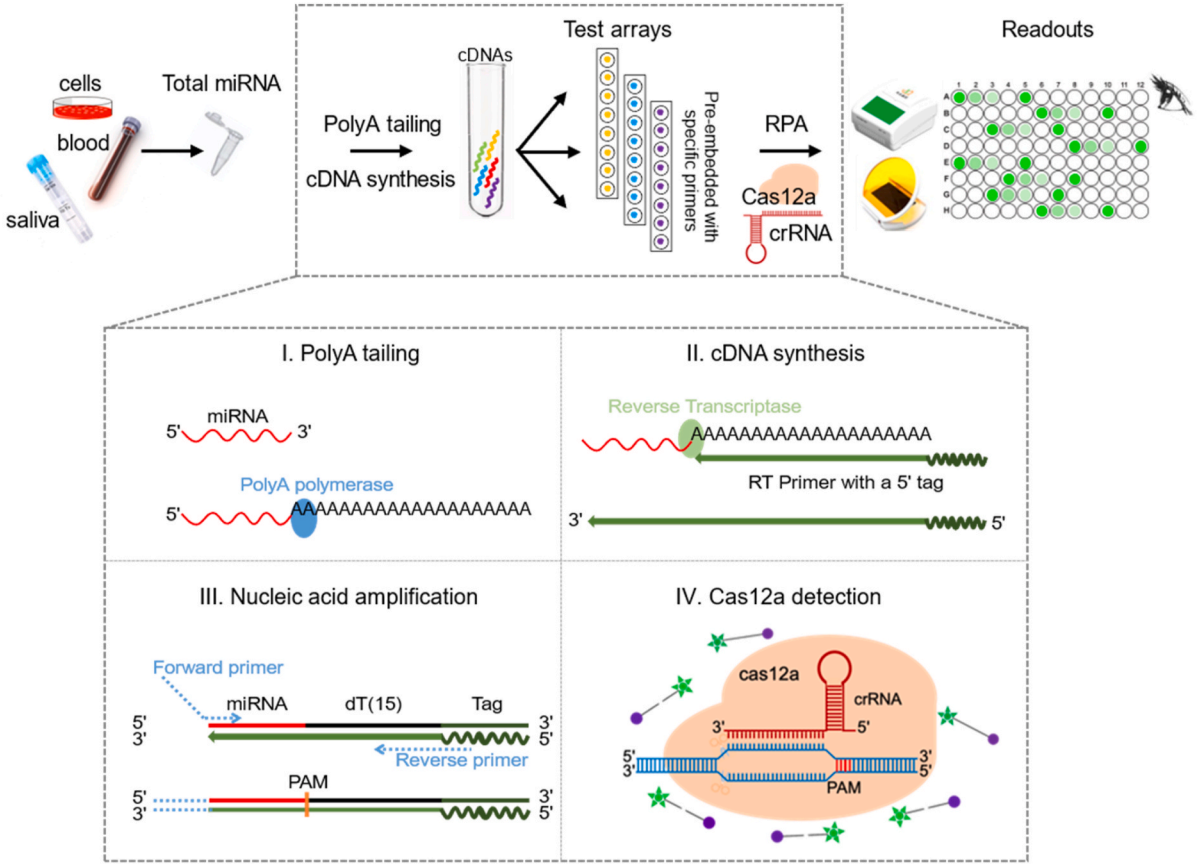
expensive equipment, complicated sample preparation, and well-trained operators, so the potential downsides limit the accessibility of these techniques.

CRISPR-Cas systems have shown great potential in gene regulation and molecular diagnosis. They are originally derived from an RNA-guided adaptive immune system against invading nucleic acid components in many bacteria and archaea (Barrangou et al., 2007; Barrangou and Marraffini, 2014; Horvath and Barrangou, 2010). The trans-cleavage activities of Cas12a, Cas13a, and Cas14a enable the development of diagnostic tools for detecting nucleic acids with high specificity, even to distinguish single-nucleotide polymorphisms (SNPs) (Chen et al., 2018; Gootenberg et al. 2017, 2018; Harrington et al., 2018; Li et al., 2018; Meng et al., 2021; Myhrvold et al., 2018). The short sequence length, high sequence similarity among family members, and sequence length variation are the main challenges for miRNA quantification (Wu et al., 2017). Cas13 has powered electrochemical sensors to detect miRNAs (Bruch et al., 2019; Liu et al., 2021; Sheng et al., 2021; Zhou et al., 2021). However, the instability of the RNA reporter in the Cas13 system is not neglectable; an alternative system is Cas12, which uses DNA sensors. Nevertheless, the Cas12 system is restricted by the available protospacer adjacent motif (PAM) in the target sequence. In this work, we propose a polyA-CRISPR/Cas12a-based miRNA detection system. PolyA-tailing unlocks PAM restriction by extending the miRNA sequence and introducing a universal PAM for Cas12a. CRISPR-Cas12a specifically recognizes the target miRNA sequence at single-base resolution. This system has improved the stability of miRNA samples, reduced the cost, and improved reproducibility. As demonstrated, polyA-CRISPR/Cas12a could enable the development of portable, accurate, and cost-effective point-of-care diagnostic systems.

2. Results

2.1. PolyA-tailing integrated CRISPR/Cas12a for miRNA detection

MicroRNAs are involved in many human diseases, including respiratory virus infection and cancer (Trobaugh and Klimstra, 2017; Zhang et al., 2014). Accurate detection and quantitation of miRNAs are critical for diagnosing and monitoring diseases (Calin and Croce, 2006). Due to the short sequence length of miRNA, which is only approximately 20 nt, the quantification of miRNAs is challenging. MiRNAs cannot use the same reverse transcription chemistry as other RNAs because miRNAs lack the unique polyA tail structure. RNA is less stable than DNA, and it is difficult to obtain the reverse transcription product from miRNA to cDNA using traditional oligo dT primers and random primers. To address this issue, we proposed the polyA-CRISPR/Cas12a miRNA detection (PCDetection) strategy (Scheme 1). We used a one-step miRNA cDNA synthesis method to add a polyA tail to the 3' end of miRNA using polyA polymerase (I) and reverse transcribed with miRNA reverse transcription (RT) primers using reverse transcriptase (II). Isothermal recombinase polymerase amplification (RPA) could be applied to amplify the nucleic acid (III). The CRISPR detection system includes the LbCas12a protein, miRNA-specific CRISPR RNAs (crRNAs), and a single-stranded DNA (ssDNA) reporter (IV). CRISPR/Cas12a recognizes the target sequence by the guide of crRNA. After activation by specific cDNA reverse transcribed from miRNA, CRISPR/Cas12a cleaves the ssDNA reporter molecule through its trans-cleavage activity, which releases the fluorophore from the ssDNA reporter.



Scheme 1. Workflow of polyA-CRISPR/Cas12a miRNA detection (PCDetection). It includes four steps, i.e., polyA-tailing (I), cDNA synthesis (II), nucleic acid amplification (III), and Cas12a enzyme-mediated detection (IV). The green fluorescence signals can be collected by a monochromator and observed by the naked eye under blue light.

2.2. Unlocking the PAM restriction for Cas12a detection

Cas12a utilizes the PAM sequence to recognize the target sequence. The PAM sequence of TTTV shows robust activity (TTTV, where V is A, C, or G) and lower but considerable activity is observed on TTTT (Kim et al., 2017; Toth et al., 2018; Yamano et al., 2017; Zetsche et al., 2015). We analyzed 2656 miRNA sequences in miRBase (the microRNA sequence database, https://www.mirbase.org/). Only 24% of miRNAs contain canonical PAM, and merely 3% could be used to design programmable crRNA (Fig. 1a). Unlocking the PAM restriction is key to the development of a universal detection system. PolyA-tailing is one solution for this challenge. A short T-rich PAM (5′-TTTN-3′) is formed by polyA-tailing, which can guide Cas protein to the miRNA.

To prove the concept of PCDetection, we randomly selected two miRNAs, i.e., miR-299 and miR-335. We successfully applied the *E. coli* polyA polymerase to extend the miRNAs with polyA tails, introducing the 5′-TTTN-3′ PAM sequences (Fig. S1). TTTT was used as PAM to design crRNA1 and crRNA4 for miR-299 and miR-335, respectively. CrRNA2, crRNA3 and crRNA5 used the TTTV sequences as PAM. MiR-299 contained a PAM sequence in its intrinsic sequence, which was used to design crRNA3. CrRNA2 (cr2) and crRNA5 (cr5) produced the best efficiency for miR-299 and miR-335, respectively (Figs. S2 and S3). From the time-course fluorescence signal, we discovered that all the miR-299-crRNAs reached saturation within 20 min (Fig. 1b). CrRNA2 and crRNA3 have similar efficiency, indicating that the crRNA targeting the polyA-tailing sequence has similar activity as the crRNA targeting the intrinsic sequence. MiR-335 can only use the PAM introduced by polyA-tailing due to the lack of T-rich PAM in its intrinsic sequence. CrRNA5 mediated a faster saturated fluorescence signal than crRNA4 (Fig. S3 and Fig. S4), which is in line with previous reports that TTTV is the optimal PAM sequence (Kim et al., 2017; Toth et al., 2018; Yamano et al., 2017; Zetsche et al., 2015). Two targets (miR-299 and miR-335) with their corresponding crRNA-mediated Cas12a detection system generated specific fluorescence signals without obvious cross-reactivity (Figs. S5 and S6). All of the above results indicate that the crRNA based on the PAM introduced by polyA can accurately recognize target miRNAs and initiate the trans-cleavage activity of Cas12a to hydrolyze the nontarget ssDNA reporter. Therefore, we used the polyA-tailing introduced TTTV sequence as the universal crRNA design for PCDetection to overcome the PAM restriction of Cas12a.

2.3. Optimization of the reaction conditions

To further improve the sensitivity of detection, we optimized miRNA amplification and poly A-tailing. RPA amplification was applied to amplify miRNA. First, we used RPA-mediated polyA-CRISPR/Cas12a to detect a series of concentrations of miR-299 and miR-335 (ranging from 4 pM to 40 nM) (Figs. S7 and S8). The results showed that the detection limit was estimated at approximately 4 pM, and the fluorescent signal of miR-335 was weaker than miR-299 due to its crRNAs limiting the activity of Cas12a. Nevertheless, the significant difference is visible by the naked eye and photographs. To increase the sensitivity of miRNA detection, the RPA amplification efficiency was improved by systematically testing 9 pairs of RPA primers (Fig. S9). As shown, the primer F3 and arbitrary R primers can yield false-positive signals. The F2 and R3 primer pair generated the highest efficiency and tolerable background value (Fig. S9 and Fig. S10). Furthermore, we analyzed the PolyA polymerase enzymatic reaction progress at six different time points (10, 20, 30, 40, 50, and 60 min) and found that 50 min was the optimal reaction time (Fig. 2a). Practically, polyA-tailing and cDNA synthesis work more efficiently by reducing the reaction volume, which improves the efficiency of polyA-tailing. The results showed a significantly stronger fluorescence signal in the optimized group than in the original group (Fig. S11).

2.4. Sensitivity and specificity analyses of PCDetection

Subsequently, we tested the sensitivity of PCDetection by analyzing different concentrations of miRNAs under the optimized conditions. As expected, the fluorescence value correlated with the amount of miRNA; moreover, in the range from 50 fM to 100 pM, the PCDetection fluorescence readout was significantly different from that of the control group (Fig. 2b). As shown in Fig. S12, all of the real-time fluorescent images conformed to the fluorescent readout corresponding to the target

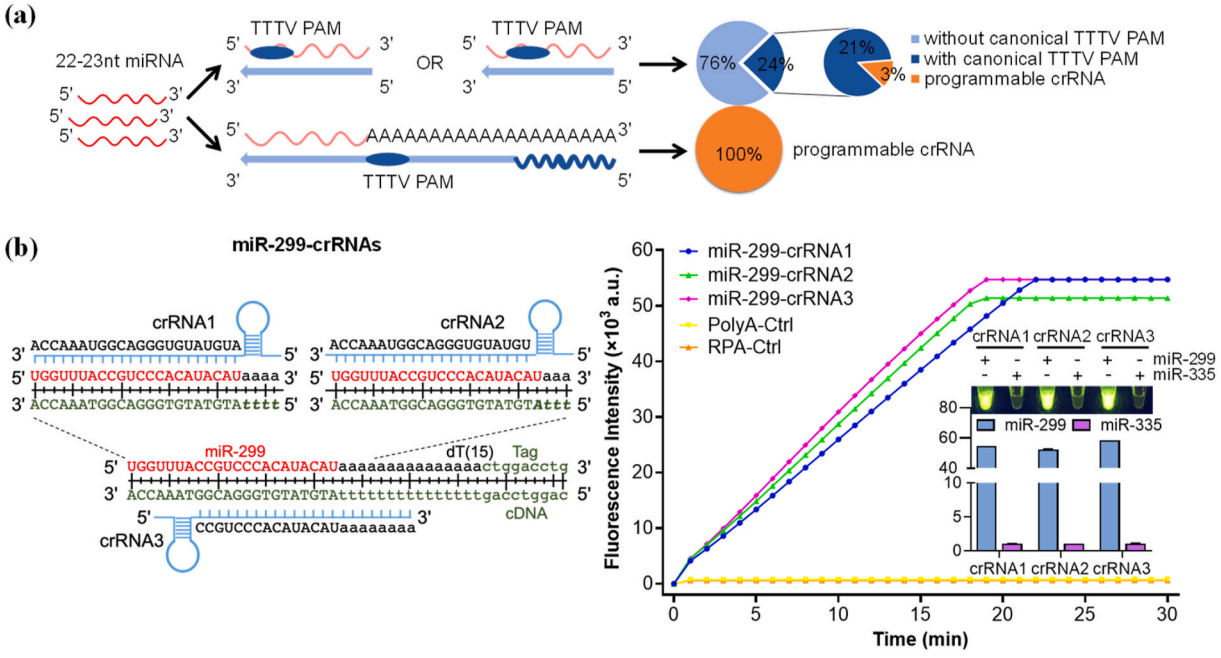


Fig. 1. Unlocking the PAM restriction of Cas12a for miRNA detection. (a) Principles for crRNA design in the polyA-CRISPR/Cas12a system. Potential PAM sequences in the known miRNA sequences were analyzed and were shown in a pie chart on the right. (b) The time-course of fluorescent intensity for miR-299 detection with miR-299-specific crRNAs, including crRNA1, crRNA2, and crRNA3. Histogram and fluorescent image depict the fluorescence intensity in 30 min.

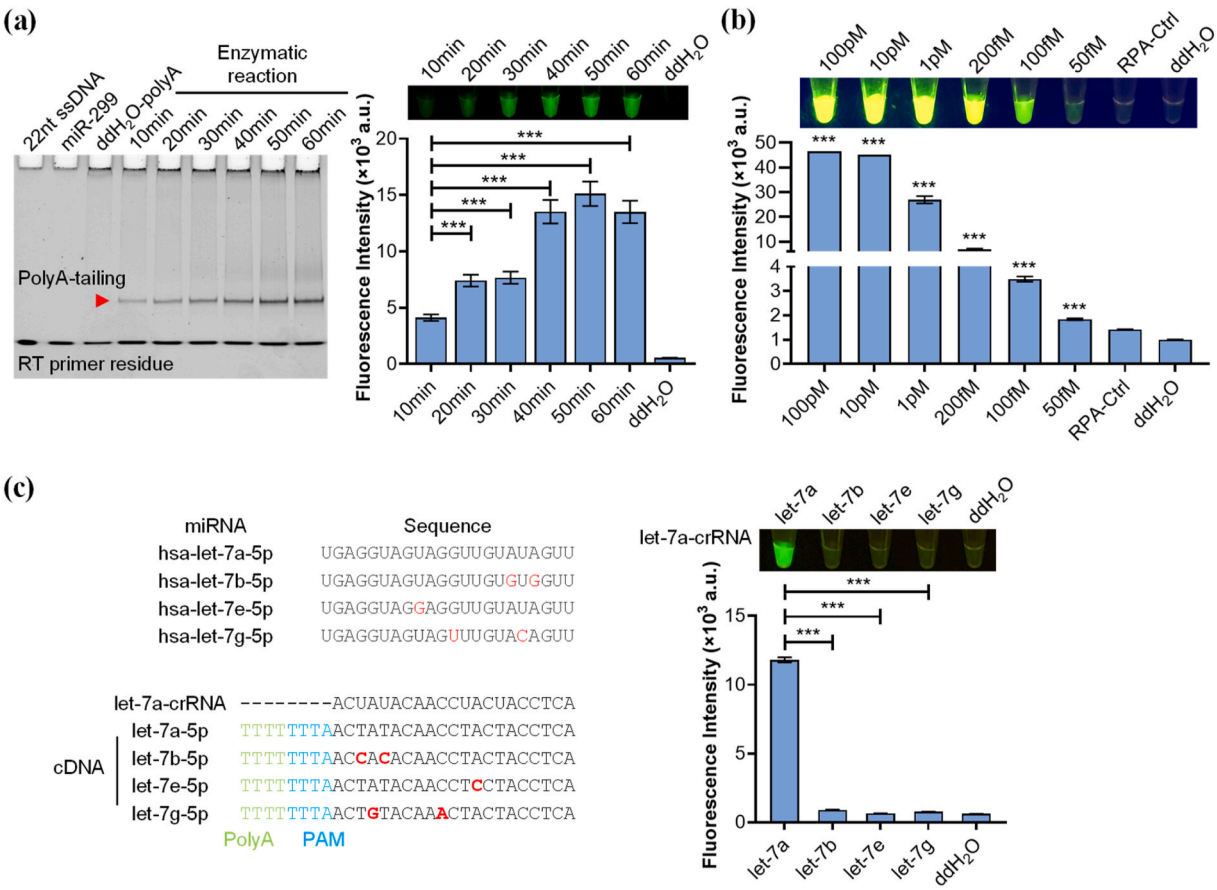


Fig. 2. Optimization of PCDetection conditions. (a) Optimization of polyA-tailing reaction. The gel electropherogram shows the time course of the enzymatic reaction of polyA polymerases, and the band shift indicates the polyA-tailing addition. The enzymatic reaction products were quantified using miR-299 PCDetection, which shows similar tendency as the electropherogram. Histogram and fluorescent image depict the fluorescence intensity in 15 min. (b) The sensitivity of miR-299 detection using PCDetection. Serially diluted synthetic miRNAs were used as detection samples. Histogram and fluorescent image depict the fluorescence intensity in 30 min. (c) Let-7a crRNA specifically activates CRISPR/Cas12a and can distinguish miRNAs with a single base mismatch. Histogram and fluorescent image depict the fluorescence intensity in 30 min.

miRNA concentration in the range from 50 fM to 100 pM. The detection limit was estimated to be as low as 50 fM for miR-299. Furthermore, a series of samples that mixed miR-299 and miR-122 at different molar ratios (1:0, 1:1, 1:10, and 1:100) were tested (Fig. S13). The lack of a significant difference in fluorescence intensity ($P > 0.05$) demonstrated that PCDetection holds potential for analyzing clinically complex samples.

The specificity of PCDetection was first evaluated by analysis of miRNAs mixtures, including miR-299, miR-335, and miR-122. The results proved that PCDetection can dramatically distinguish miR-299 from the other two miRNAs (Fig. S14). Additionally, three miRNAs (miR-299, miR-335, and miR-122) were tested individually and mixed (miR-mix). The results verified that PCDetection can specifically recognize the samples containing miR-299 within 30 min (Fig. S15). We also examined the fluorescent signal and expression level of miR-299 in the sample with the same amount of miR-299 and certain amounts of total RNA by PCDetection (Fig. S16a) and polyA-RT–qPCR (Fig. S16b). The Ct value in this experiment did not significantly differ from the Ct value in the polyA-Ctrl group (negative control). Some miRNAs have highly similar sequences, which have only one single base difference. Moreover, we tested four highly similar members from the human let-7 family: let-7a, let-7b, let-7e, and let-7g (Fig. 2c). Interestingly, ubiquitous members of the let-7 family of miRNAs were present at high concentrations. Let-7a is well studied because of its large research potential and representativeness (Gao et al., 2020). As shown in Fig. 2c, the let-7a sample produced the strongest fluorescence signal. Furthermore, we have comprehensively evaluated the specificity by detecting mimic mismatches in synthetic templates (Fig. S17) and the impact of potentially interfering substances in the serum (Fig. S18). In summary, the strong fluorescence signal and low background signal further confirmed the high specificity of the PCDetection system.

2.5. PCDetection for miRNAs in multiple cells

PCDetection was applied to detect miRNAs, i.e. miR-21 and miR-299, from different human cancer cells, including human hepatocellular carcinoma cell lines (SNU-449), human non-small-cell lung cancer cell lines (PC-9), human glioblastoma cell lines (U87MG and U251) and an immortalized line of human T lymphocyte cells (Jurkat). Human normal bronchial epidermal cells (16HBE), i.e., noncancer cells, were selected as the control sample. Briefly, cell samples were extracted, and total miRNA was isolated. The cDNA templates were generated by polyA-tailing and cDNA synthesis, amplified by RPA and finally detected by CRISPR/Cas12a. The detection can be performed in a multiwell plate or visible by the naked eye under 485 nm light (Scheme 1). As expected, the polyA-CRISPR/Cas12a fluorescent detection results indicated that miR-21 displayed remarkably high expression in U251 cells and low expression in Jurkat cells, which is consistent with the data from polyA-tailing-based RT–qPCR. All five cancer cell lines displayed varying degrees of miR-21 expression (Fig. 3a and Fig. S19). For RT–qPCR, small nuclear RNA (snRNA) U6 was used as the generic endogenous control, and the computation formula for relative expression was as follows: Fold

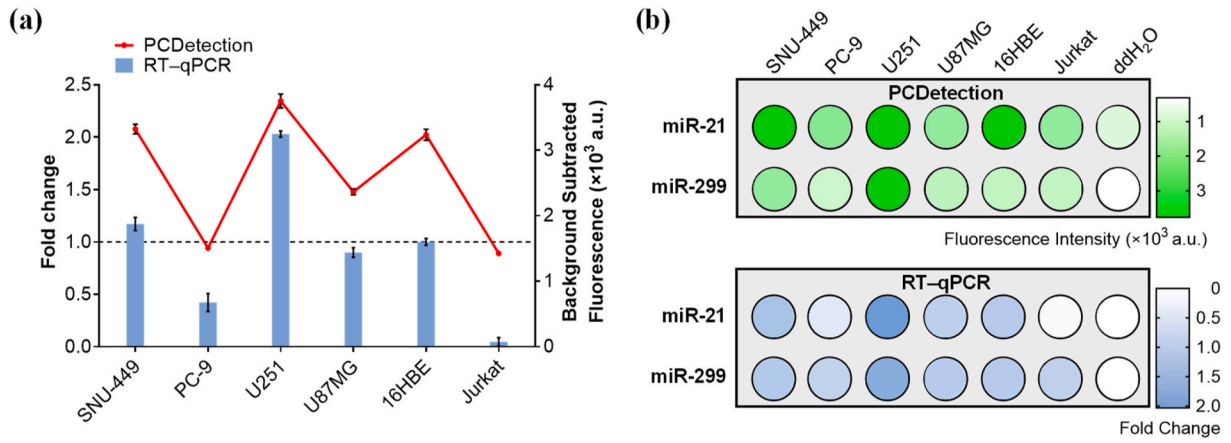


Fig. 3. PCDetection of miRNAs in cancer cells. (a) Comparison between the PCDetection and RT–qPCR for the relative expression of miR-21 in different cancer cells. (b) The PCDetection array (top panel) and RT–qPCR array (bottom panel) was used to analyze the expression of miR-21 and miR-299 in six cell samples in a parallel way.

change = $2^{-\Delta\Delta Ct}$. The Ct values and curves of polyA-tailing-based RT–qPCR were shown in Fig. S20a, Fig. S21, Fig. S23a, and Fig. S24, and the relative expression of miR-21 and miR-299 in all five cancer cell lines compared to 16HBE cells is shown in Fig. S20b and Fig. S23b. Multiwell PCDetection can simultaneously analyze multiple miRNAs in different cells (Fig. 3b, top panel), which shows similar tendency as using RT–qPCR quantification (Fig. 3b, bottom panel). It shows a similar tendency as the tube detection in Fig. S19 and Fig. S22. The results demonstrate that miR-299 is selectively expressed in U251 and SNU-449 cells. These data show the potential of PCDetection to scale up for high-throughput miRNA detection.

3. Discussion

MicroRNAs play fundamental regulatory roles in many essential cellular processes, including cellular metabolism, proliferation, and cell death, in a variety of cell types, such as stem cells, immune cells, and cancer cells (Ambros, 2003; Bartel, 2009; O'Connell et al., 2010). The aberrant expression of miRNAs correlates with the pathogenesis of cancer, cardiac diseases, immune-related diseases, and other diseases; therefore, they are potential biomarkers for disease diagnosis, prognosis, and response to treatment (Cortez et al., 2011). However, the short sequence length, high sequence similarity among family members, and sequence length variation challenge miRNA quantification (Wu et al., 2017). CRISPR-based SNP detection using crRNA specifically to recognize target sequences is promising to confront these challenges (Meng et al., 2021; Wang et al., 2021). We have summarized the CRISPR-based miRNA detection in Table S4. SplintR ligase has been applied to add the PAM sequence (Qing et al., 2021; Tian et al., 2020; Zhang et al., 2021), but the 5′-phosphorylated dC or 3′-dG will decrease the ligation efficiency (Martin et al., 1985). In catalytic hairpin assembly (CHA), miRNA can trigger the hybridization of hairpin DNAs (HPs), which contain PAM and activate Cas12a (Peng et al., 2020). Nevertheless, hybridization can be triggered by mismatched miRNAs, which interfere with specificity, especially at single-base resolution. Similar to CHA, strand displacement can also be integrated with Cas12 (Li et al., 2021), but may be disturbed by mismatched miRNAs in the biological sample. In this work, we applied polyA-tailing to unlock PAM restriction. PolyA polymerase tailed all the miRNAs and reverse amplified the DNA sequences based on the RNA sequence, which kept the original information, including mismatches, for Cas12 detection. The amplified cDNA is more stable than the RNA template that is prone to degradation in previous Cas13a-based miRNA detection strategies (Kellner et al., 2019). In addition, RPA amplifies cDNA to improve signals for sensitive detection. The platform can detect miRNAs with a detection limit of 50 fM. More importantly, the platform can be easily scaled up to analyze multiple miRNAs in various cells. The PCDetection system can be further improved by integration with electrochemical biosensors or next-generation fluorescent sensors (Bruch et al., 2019; Liu et al., 2021; Sheng et al., 2021). This system could be extended to a general detection panel to target a series of miRNA biomarkers for diagnosis in the future.

CRediT authorship contribution statement

Mingtian Zhong: Methodology, Validation, Investigation, Data curation, Writing – original draft, Visualization. **Kaizhao Chen:** Methodology, Validation, Investigation, Data curation. **Wenjun Sun:** Investigation, Data curation. **Xiangyang Li:** Validation, Investigation. **Shisheng Huang:** Validation, Investigation. **Qingzhou Meng:** Investigation, Data curation. **Bo Sun:** Writing – review & editing. **Xingxu Huang:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition. **Xinjie Wang:** Conceptualization, Supervision, Project administration, Funding acquisition. **Xiaodong Ma:** Conceptualization, Supervision, Project administration, Writing – review & editing, Funding acquisition. **Peixiang Ma:** Conceptualization, Supervision, Investigation, Project administration, Writing – original draft, Writing – review & editing, Funding acquisition.

Declaration of competing interest

The authors declare that they do not have any competing financial or personal interests which can influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bios.2022.114497.

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