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Hairpin/DNA Ring Ternary Complex Initiated Rolling Circle Amplification for an Elevated Accuracy and Its Application in Analyzing Let-7a

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ABSTRACT: Rolling circle amplification (RCA) is an attractive isothermal nucleic acid amplification approach and has been widely applied in constructing a variety of biosensors. However, the inevitable drawbacks of lacking enough selectivity greatly hindered further applications of RCA-based approaches. Here, we develop a novel RCA-based approach by integrating the specific target recognition capability of the hairpin/DNA ring ternary complex and multiple signal amplification and successfully applied it for let-7a detection. In this method, let-7a specifically unfolds the hairpin probe (Hp probe) in the ternary complex to induce target recycle and RCA- and DNAzyme-based signal generation. Based on this, the established approach exhibits a high selectivity to let-7a, and the response of the approach to one base pair mismatched sequences was 24.9%, indicating a significantly improved specificity. Meanwhile, the limit of detection is as low as 342 aM, which can meet the high requirement for a trace amount of miRNA detection. In all, we believe that the established approach can offer a new avenue for miRNA detection and post-tumor care.
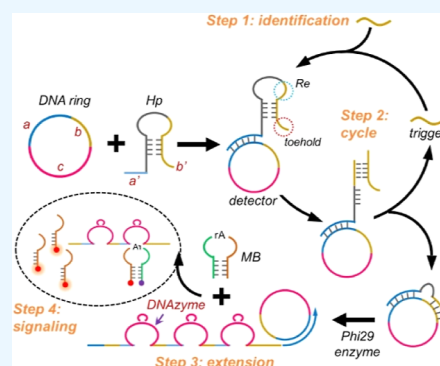
1. INTRODUCTION

Rolling circle amplification (RCA) is one of the most attractive isothermal nucleic acid amplification strategies that generate long signal strand DNA (ssDNA) sequence via a enzymatic process catalyzed by DNA polymerases (e.g., phi29 enzyme).[1−3] The RCA process is performed with a circular DNA as a template and a short ssDNA as a primer to produce ssDNA products containing repeated sections, which are complementary with circular DNA template.[4,5] Compared with other isothermal signal amplification approaches, RCA possesses the advantages of good biocompatibility and programmability, making it a popular strategy in detecting diverse biomarkers.[6] However, there are several inherent drawbacks of RCA that limited its further applications, including (i) the incomplete hybridization between primer and circular DNA is possible to initiate the RCA process thus to cause the wrong amplification, leading to a low detection specificity; (ii) the amplification efficiency of RCA is relatively low that cannot meet the high requirements of low abundant target detection.

Many efforts have been made in recent years to improve the amplification efficiency and detection accuracy of RCA-based approaches.[7−11] Considering the high specificity of the CRISPR-Cas system in even discriminating one base pair mismatch in sequences, Wang et al. developed a novel RCA-based approach, termed RACE (RCA-assisted CRISPR/Cas9 Cleavage), by integrating the CRISPR-Cas9 system and RCA techniques.[12] Despite the fact that the method showed a high

detection sensitivity and was successfully utilized in analyzing multiple miRNAs, it shows a 68% recovery in the detection Mis-1 sequence that has single-base mismatched with the target, implying the specificity of RACE needs further improvements. In addition, the CIRPSR-Cas9 system was performed following RCA in RACE, which cannot directly improve the accuracy of RCA.

Hairpin/DNA ring ternary has been exploited to improve the specificity of RCA-based approaches through inducing toehold-mediated strand displacement (TSD) in former researches. However, the specificity of TSD mainly relies on the recognition between toehold sequence and the target sequence and wrong hybridization may cause wrong amplification.[13] Therefore, it is in urgent demand to propose a novel method that integrates improved accuracy and amplification efficiency.

Lung cancer is one of the most commonly diagnosed cancers, which accounts for almost 13% of cancer diagnosis, and remains the leading cause of cancer mortality worldwide.[14−16] Precise and early diagnosis of lung cancer has attracted abundant attention due to it close relationships with

the development of personalized therapy and molecular and precise histological characterizations.[17] microRNAs (miRNAs) are a kind of small noncoding RNAs, which play a crucial role in regulating gene expression.[18] The crucial role of let-7a in mediating cell communication and influencing phenotype of recipient cells made it a promising biomarker for cancer diagnosis and guiding the post-tumor care.[19] Thus, we develop here a novel RCA-based approach with improved accuracy, and apply it for analyzing let-7a. In this method, the hairpin/ DNA ring ternary is utilized to induce specific identification of target miRNA and subsequent signal amplification. There are in total three signal amplification processes in this approach, including target recognition-triggered signal cycle, RCA, and DNAzyme-based chain cleavage, endowing the method with a high sensitivity. The high accuracy and sensitivity of the established RCA approach show a promising prospect for the early diagnosis of lung cancer and evaluation of post-tumor care.

2. EXPERIMENTAL SECTION

2.1. Reagents and Materials. The probes used in this approach are listed in Table S1. All the sequences were synthesized and purified by Shanghai Sangon Biological Engineering Technology and Services Co., Ltd. (Shanghai, China). T4 DNA Ligase, phi29 polymerase, deoxyribonucleotide triphosphates (dNTPs), and TE buffer were all obtained from Invitrogen Biotechnology Co., Ltd. (Shanghai, China). The cancer cell (adenocarcinoma epithelial BEAS-2B cell, A549 cell) was provided by the cell bank of the type culture collection of the Chinese Academy of Sciences (Shanghai, China). Commercial human serum solution was obtained from the Beyotime Biotechnology Co., Ltd. (Shanghai, China).

2.2. Assembly of a Detector. The experimental details of the perpetrating DNA ring are shown in Supporting Information. The obtained Hp probe (2 mM, 0.5 mL) was first heated to 90 °C and cooled to room temperature. Afterward, the 20 μL Hp probe was mixed with a 20 μL DNA ring for 30 min.

2.3. Fluorescence Assay to Test the Target Recognition-Based Signal Cycle. The obtained FAM labeled Hp probe was first assembled to the hairpin structure following the procedures in section 2.2. The fluorescence signal of the Hp probe before and after assembly was recorded by a Shimadzu RF-6000-PC spectrophotometer (Tokyo, Japan). 18 μL of the assembled Hp probe was mixed with 2 μL of synthesized let-7a sequences (10 fM), and the mixture was incubated at room temperature for 60 min. The fluorescence signals were recorded.

2.4. Analytical Performance of the Established Approach. To test the sensitivity of the established approach, various concentrations of let-7a sequence (2 μL) were mixed with a 2 μL detector, respectively. The mixture was then incubated at room temperature for 30 min. Afterward, 2 μL of phi29 enzyme, 5 μL of dNTPs (500 mM), and 2 μL of TE buffer were added in the mixture to perform the RCA process. After being incubated at 37 °C for 60 min, 2 μL of the MB probe (1 mM) was added in the mixture and was incubated at room temperature for 30 min. A Shimadzu RF-6000-PC spectrophotometer (Tokyo, Japan) was utilized to record the fluorescence signals. Selectivity of the approach was evaluated by detecting mismatched sequences following the former procedures.

3. RESULTS AND DISCUSSION

3.1. Working Principle of the Established Approach for miRNA Detection. The working mechanism of the established approach is shown in Figure 1. In this method, the
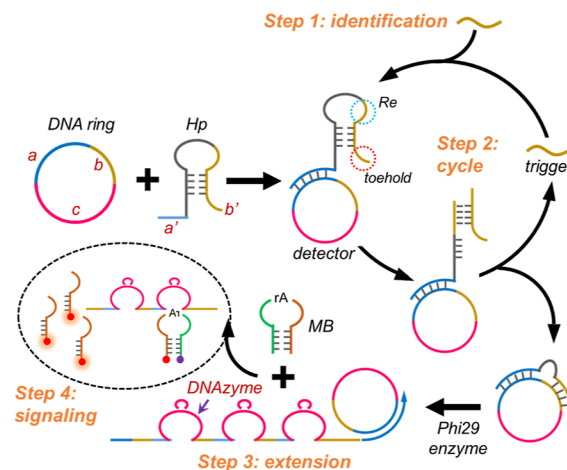

Figure 1. Working mechanism of the established approach for let-7a detection.

detector is constructed by the hybridization between the DNA ring and Hp probe. In details, the DNA ring is composed of three functional sections, **a** section that is complementary with **a**′ section in Hp probe, **b** section to induce signal cycle, and **c** section to transcribe DNAzyme sequences during the RCA process. In the presence of let-7a, it can specifically bind with the toehold sequence and gradually unfold the hairpin structure of the Hp probe, forming a let-7a-**b** duplex and exposing the Re (replace chain) section in the loop section. The exposed Re section can bind with the **b** section in the DNA ring and gradually replace let-7a. The replaced let-7a binds with a next toehold section in detector to induce signal cycles. The **b**′ section is completely hybridized with the **b** section in the DNA ring. With the **b**′ section as primer and DNA ring as template, a long ssDNA chain is obtained under the assistance of phi29 enzyme, which contains repeated transcribed DNAzyme sequences. The transcribed DNAzyme sequences can form active secondary conformation under the assistance of MB to generate a nicking site in the loops section of MB. After DNAzyme-based cleavage, the MB probe whose two terminals were labeled with the FAM signal and corresponding quenching moiety (BHQ), is unwound, leading to the recovery of FAM signal. Consequently, the recorded FAM signal is positively correlated with the amount of let-7a in the sensing system.

3.2. Feasibility of the Target Recognition-Based Signal Cycle and DNAzyme-Based Signal Generation. In this method, target miRNA binds with the toehold section in the detector to initiate signal cycles and to mediate subsequent signal amplification, which determines the feasibility of the whole approach. Therefore, we first investigated the feasibility of the target recognition-based signal cycle through a fluorescence assay, as shown in Figure 2A. The result in Figure 2B showed the FAM signal of the Hp probe at 520 nm significantly elevated when let-7a was added to the sensing system, indicating that let-7a successfully unfolded the Hp probe. By contrast, the FAM signal of the detector was much higher (approximately 2.76 times higher)
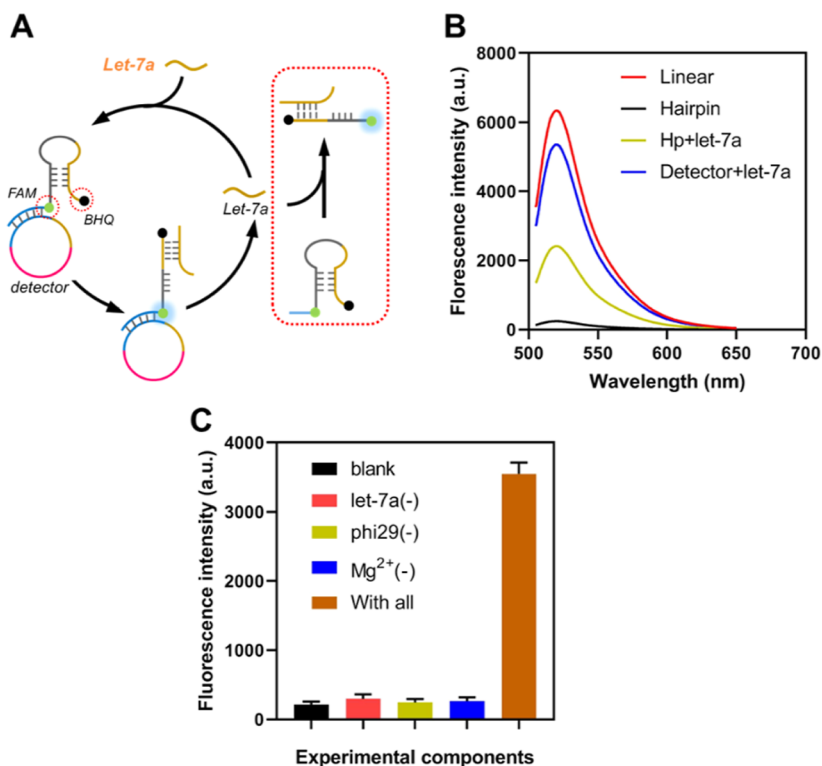
Figure 2. Feasibility of the established approach. (A) Illustration of the fluorescence assay to test the target recognition-based signal cycle. (B) Fluorescent spectrum of the FAM-labeled Hp probe when the DNA ring existed or not. The "linear" was the Hp with linear state before assembly to hairpin structure. (C) Fluorescence intensities of the approach under different experimental conditions.
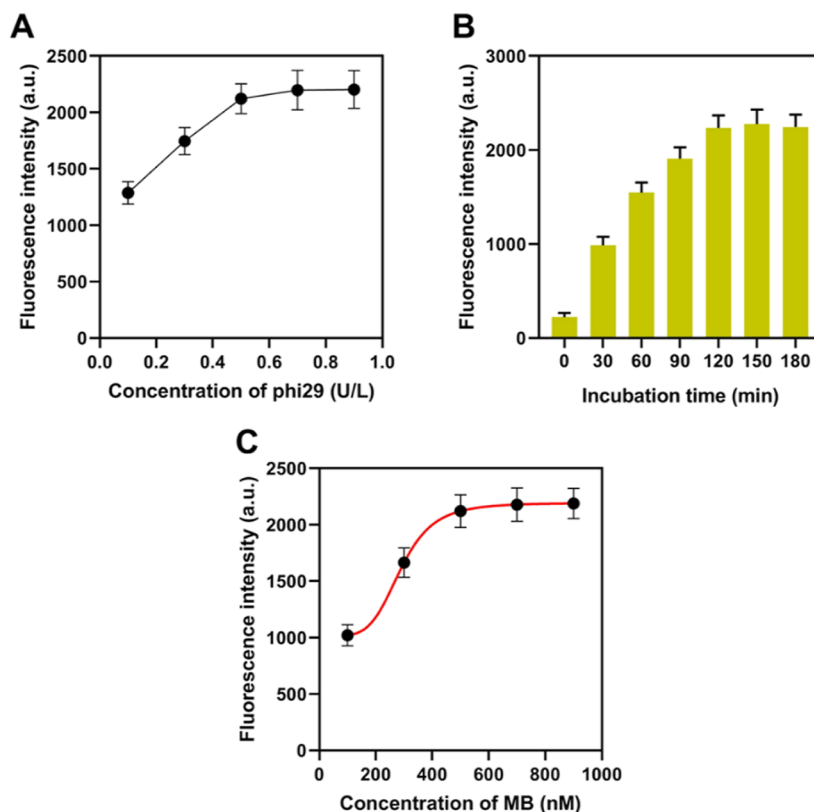
Figure 3. Optimization of experimental conditions. Fluorescence intensities of the approach with different concentrations of phi29 enzyme (A), incubation time (B), and concentration of MB probes (C).
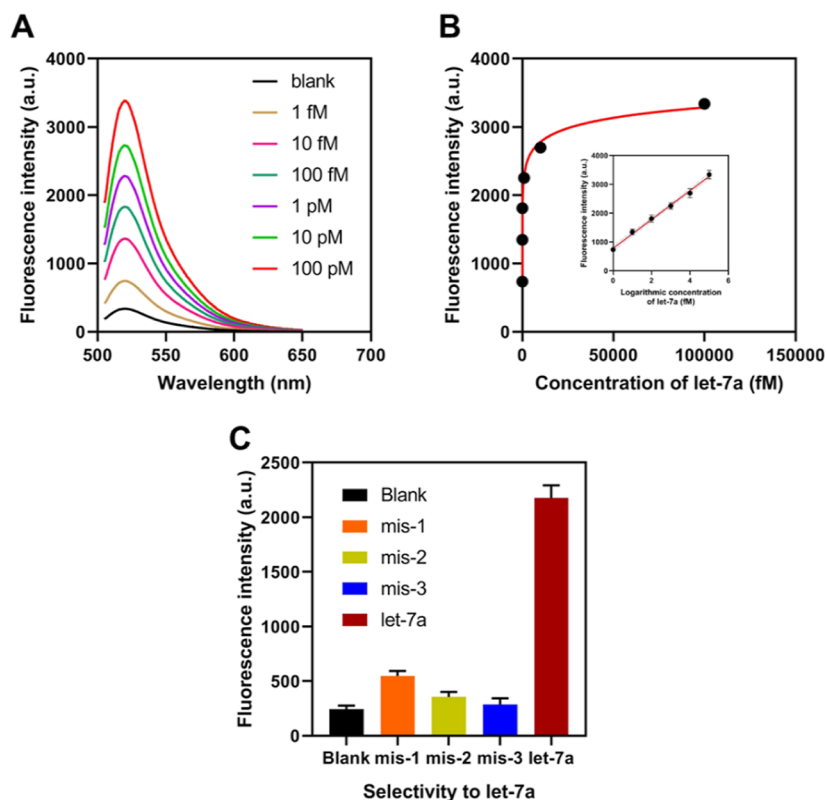
Figure 4. Analytical performance of the established approach. (A) Fluorescence spectrum of the approach when detecting different concentrations of let-7a. (B) Correlation equation between the FAM signals and the concentrations of let-7a. (C) Fluorescence intensities of the approach when detecting mismatched sequences and let-7a.

than that obtained in the Hp probe alone, implying the formation of a signal cycle. The electrophoresis result in Figure. S1 demonstrated the successful performance of the RCA process. After the RCA process, a long ssDNA chain that is composed of repeated transcribed DNAzyme sequences was produced. Therefore, the feasibility of the RCA process and cleavage activity of transcribed DNAzyme were demonstrated through utilizing RCA products to cut MB probes. The MB probe was also designed with a hairpin structure with FAM and BHQ at its two terminals. If the transcribed DNAzyme sequences can form active secondary conformation, MB probes would be cut, and the fluorescence signals of FAM could be recorded. As shown in Figure 2C, the recorded fluorescence signal of the MB probe at 520 nm was significantly enhanced compared with the control group (MB probe only), indicating the successfully assembly of active DNAzyme and cleavage of MB. Meanwhile, the recorded fluorescence signals when phi29 enzyme, Mg^{2+}, and let-7a are absent showed no differences with the control group, suggesting that the all the experimental components were essential in conducting RCA- and DNAzyme-based signal generation.

3.3. Optimization of Experimental Parameters. For a better detection performance, we optimized several experimental parameters, including the amounts of phi29 enzyme, the incubation time, and the concentration of MBs. The amount of phi29 enzyme plays a crucial role in adding dNTPs to the terminals of **b′** section and producing ssDNA chain. Thus, we first optimized the effect of amount of phi29 enzyme on the detection performance. The result in Figure 3A showed a gradually elevated fluorescence signal when the concentrations of phi29 enzyme ranged from 0.1 U/L to 0.5 U/L

phi29 enzyme, and no more increments were observed when the sensing system was incubated with more phi29 enzyme. Therefore, 0.5 U/L phi29 enzyme was selected in the following experiments. The incubation time determines the signal amplification efficiency of the established approach. Therefore, we investigated the detection performance of the established approach with different incubation time. From the result in Figure 3B, the obtained fluorescence intensity of the approach increased with the incubation time ranged from 0 to 120 min, and the no more enhancements could be observed when the sensing system was incubated with more duration. Meanwhile, the optimized concentration of MBs was 500 nM.

3.4. Analytical Performance of the Established Approach. Under the optimized experimental parameters, the detection performance of the established approach was evaluated, such as the sensitivity and selectivity. To test the sensitivity, the approach was utilized for detection of let-7a that was diluted to different concentrations. The obtained fluorescence intensities at 520 nm gradually increased when the concentrations of let-7a increased from 1 fM to 100 pM (Figure 4A), suggesting a correlation between fluorescence intensities and concentrations of let-7a. A correlation equation between the obtained fluorescence intensities and the logarithmic concentrations of let-7a was obtained as $Y = 500.0 \times \lg C + 781.1$ with the correlation coefficient of 0.9878 (Figure 4B). The limit of the detection of the approach was calculated 342 aM according to the 3σ rule. To evaluate the accuracy, the established approach was utilized to detect interferential sequences that have 1, 2, or 3 base pairs mismatched with let-7a. From the result in Figure 4C, the obtained fluorescence intensity of the approach when detecting
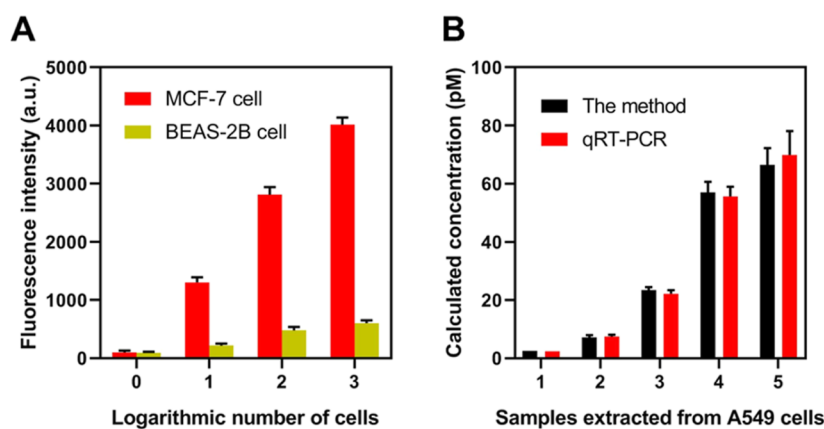
Figure 5. Clinical application potential of the established approach. (A) Fluorescence intensities of the approach when detecting miRNA extracted from MCF-7 cells and BEAS-2B cells. (B) Calculated let-7a amounts from samples extracted from A549 cells by the established approach and by qRT-PCR.

let-7a was much higher than the blank group (blank/let-7a: 14.8%). In addition, the recovery rate of the approach when detecting the mis-1, mis-2, and mis-3 sequence was 24.9, 17.6, and 15.5%. Compared with the method proposed by Wang, the method showed a greatly decreased recovery rate, indicating a high selectivity of the approach. Meanwhile, the method also exhibited a high selectivity to homologous miRNAs of let-7a, such as let-7b and let-7c (Figure. S2).

3.5. Clinical Application Potential of the Established Approach. In order to assess the feasibility of the method for detecting let-7a in actual samples, the expression levels of let-7a were examined in total RNA isolated from MCF-7 cells and human normal lung epithelial (BEAS-2B) cells. The data presented in Figure 5A demonstrates a positive correlation between the number of cancer cells and the fluorescence intensity, suggesting a corresponding increase in the concentration of let-7a. Furthermore, it is evident that the fluorescence intensity of let-7a in MCF-7 cells is significantly greater than that in BEAS-2B cells upon comparison of equal cell numbers. This result implies that MCF-7 cells possess a higher expression level of let-7a, thereby validating the utility of our approach in monitoring miRNA expression levels across various cancer cells.

Despite the fact that many miRNA detection approaches have been proposed in recent years, quantitative real-time polymerase chain reaction (qRT-PCR) remains the most widely applied one and is the gold-standard for clinical analysis of diseases. To test the clinical application potential, the established approach and qRT-PCR method were applied to detect let-7a in extracts from A549 cells. The result in Figure 5B showed the obtained fluorescence signals by the two methods elevated with the increase of the A549 cells, indicating that an increased concentration of let-7a accordingly. Meanwhile, a good correlation between the calculated let-7a amounts by the proposed approach and by qRT-PCR was obtained with a correlation coefficient of 0.9967, suggesting that the proposed method could be potentially utilized in clinical practices.

To test the repeatability, the proposed method was applied to detect known amounts (1 and 10 pM) of let-7a from 10 commercial human serum samples. The variable coefficient of calculated let-7a concentration of the 10 samples duplicates were 3.12 and 4.07% (Figure. S3), demonstrating that this

approach has adequate stability for detecting target miRNA in the complex sample.

4. CONCLUSIONS

In summary, we depict here a novel hairpin/DNA ring ternary complex initiated RCA-based approach and successfully applied it for let-7a detection. The method possesses several advantages: (i) the design of hairpin/DNA ring ternary endows the method a high specificity in identifying let-7a from mismatched sequences; (ii) a low limit of detection of 324 aM, which is derived from the target recognition-based signal cycle, RCA, and DNAzyme cleavage-based signal amplification. Compared with former research that focused on improving the accuracy of RCA-based approaches, the proposed approach exhibited a low response to the one-mismatched sequences (24.9%). Despite that the method requires a relatively long reaction time, the design of the hairpin/DNA ring ternary complex-initiated RCA can be expanded for the detection of a diverse of biomarkers, such as DNA gene sequence, proteins, and other biomolecules, providing a new avenue for accurate and sensitive determination of let-7a and showing a promising prospect for the clinical diagnostics of lung cancer.

■ ASSOCIATED CONTENT

ⓢ Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c06227.

> Sequences of the oligonucleotides used in the present study; experimental section; electrophoresis results of the RCA process; fluorescence intensities of the approach when detecting homologous miRNAs of let-7a; and calculated let-7a concentration from sample duplicates (PDF)

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Author Contributions

H.H. and X.Z. contributed equally. Q.D. is the lead corresponding author. H.H. and X.Z. contributed equally to the research, methodology, formal analysis, investigation, data curation, and writing-original draft; M.W. and Q.D.: visualization, writing-review, editing, supervision, and funding acquisition; H.Z., X.L., and P.F.: formal analysis and investigation.

Notes

The authors declare no competing financial interest.

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