Accurate Detection of Target MicroRNA in Mixed Species of High Sequence Homology Using Target-Protection Rolling Circle Amplification

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ABSTRACT: The close relationships of miRNAs with human diseases highlight the urgent needs for miRNA detection. However, the accurate detection of a target miRNA in mixed miRNAs of high sequence homology presents a great challenge. Herein, a novel method called target-protection rolling circle amplification (TP-RCA) is proposed for this purpose. The protective probe is designed so that it can form a fully complementary duplex with the target miRNA and can also mismatch duplexes with other nontarget miRNAs. These duplexes are treated with a single-strand-specific nuclease. Consequently, only the target miRNA in a perfect-match duplex can resist the cleavage of nuclease, whereas the nontarget miRNAs in mismatched duplexes will be digested completely. The protected target miRNA can be detected using RCA reactions. MicroRNA let-7 family members (let-7a–let-7f) and nuclease CEL I were used as proof-of-concept models to evaluate the feasibility of the TP-RCA method under different experimental conditions. The experimental results show that the TP-RCA method can unambiguously detect the target let-7 species in mixtures of let-7 family members even though they may differ by only a single nucleotide. This TP-RCA method significantly improves the detection specificity of miRNAs.

INTRODUCTION

MicroRNAs (miRNAs) are a class of endogenous, noncoding RNAs with a length of 18–25 nucleotides. These small RNAs can regulate post-transcriptional expression by binding the open reading frame or 3′-UTR regions of mRNAs. Thus, as key epigenetic regulators, miRNAs play important roles of cell proliferation, differentiation and apoptosis. Sensitive, accurate, and convenient miRNA detection presents a great technical challenge because miRNAs possess unique features including a small size, low abundance, and high-sequence homology. For example, let-7 family members have only one or two nucleotide differences from each other. A similar phenomenon has also been observed for the miR-200 and miR-141 subfamilies. The most commonly used method for miRNA detection is real-time polymerase chain reaction (RT-qPCR). In the strategy, a tail of a hairpin-shaped primer first hybridizes with the 3′-region of the target miRNA for reverse transcription, and a pair of primers is then used to amplify the reverse transcripts. Subsequently, these amplicons undergo a thermal-cycle amplification process. Although RT-qPCR has been used for years and often considered the gold standard for miRNA detection, it has an intrinsic drawback with regards to detection specificity. The tails of the hairpin primers are only a few nucleotides long and cannot efficiently discriminate different miRNAs, in particular, those miRNAs with one or two nucleotide variations near the 3′-end.

A decade ago, rolling circle amplification (RCA), as an isothermal nucleic acid amplification method, was developed, and its working mechanism suggests that it appears to be more suitable for miRNA detection. Without reverse transcription, the target miRNA can be used as the primer and prolonged by phi29 DNA polymerase to produce a single-strand DNA chain consisting of millions of tandem repeats complementary to the circular template (CT). The RCA methods can provide great operational simplicity and very high detection sensitivity under a constant temperature. Various RCA-based protocols have been extensively applied, in which padlock RCA is the most widely used in high-sensitivity miRNA detection. However, data suggest that the padlock probe format could not resolve the problem of the accurate identification of each let-7 member using conventional RCA-based methods because of their high sequence homology at the 3′-regions.

To achieve the highly accurate detection of miRNAs, we propose a novel strategy named target-protected RCA (TP-RCA) (Figure 1). The key step of this proposed TP-RCA is the full hybridization of the protective probe with the target miRNA and the elimination of all of the nontarget miRNAs by treatment with a single-strand specific nuclease (SSS-nuclease). In this study, we investigated the cleavage efficiencies of a SSS-nuclease.
nuclease on one nucleotide mismatch site in RNA/DNA duplexes, and evaluated the ability of TP-RCA to distinguish the target miRNA from other family members with high sequence homology. Experimental results provide convincing evidence that this TP-RCA strategy can identify the target miRNAs unambiguously at one-nucleotide resolution, a much better outcome than previously reported data.

RESULTS AND DISCUSSION

Principle of the TP-RCA Strategy for miRNA Detection. The proposed TP-RCA for miRNA detection is schematically illustrated in Figure 1. A protective DNA probe is designed to be fully complementary to the target miRNA. First, the protective probe is mixed with a sample containing target miRNA and other nontarget miRNAs (known as interferences), and the protective probe can form a perfect-matched DNA/RNA duplex with the target miRNA and many mismatched DNA/RNA duplexes with nontarget miRNAs. Next, the single-strand specific nuclease CEL I is applied to the sample solution. All of the mismatched DNA/RNA duplexes will be cleaved into small fragments, which eventually will be further hydrolyzed to dNTPs and NTPs. In contrast, the perfectly matched DNA/RNA duplex is able to resist the cleavage of CEL I and survive in the sample solution. The sample is then heated to inactivate the nuclease. Finally, the nuclease-treated sample was analyzed using the RCA reaction. As expected, only the protected target miRNA can serve as the primer to initiate the RCA reaction.

Nuclease Cleavage of RNA/DNA Heteroduplexes. To validate the proposed TP-RCA method, we first examined the cleavage capability of sss-nuclease CEL I. CEL I has not been commonly used as a single-strand specific nuclease to eliminate single-strand nucleotides in comparison with other single-strand specific nucleases (such as mung bean nuclease and RNase One ribonuclease). CEL I can recognize the single-stranded features (mismatches, insertions, deletions, etc.) of duplexes. Practically, CEL I was used to accurately identify a variety of mutations and polymorphisms in the human BRCA1 gene. Later, CEL I was developed for use in a commercial assay Surveyor for simple and flexible mapping of both known and unknown mutations and has been used to perform high-throughput screening of induced point mutations in Arabidopsis and Lotus. Fortunately, its unique feature made it more suitable for mismatch detection in double-strand nucleotides, which is one of the key features in the proposed TP-RCA method. Furthermore, the intrinsic components of the CEL I reaction buffer are similar to those of the phi29 reaction buffer. It requires divalent metal ions (Mg2+) for its enzymatic activity. On the other hand, the pH values of the reaction buffer of S1 nuclease and mung bean nuclease are 4.5 and 5.0, respectively, which could influence the following RCA reactions significantly, but CEL I demonstrates the best performance under optimal conditions (pH range of 6 to 9 and temperature of 40 to 65 °C). Considering all of these factors, CEL I was considered the more conducive nuclease for the TP-RCA methods. However, CEL I cleavage on RNA/DNA heteroduplexes has never been characterized. Accordingly, we tested CEL I cleavage on a series of mismatched RNA/DNA duplexes.

Figure S1 shows the electrophoresis image of the RNA/DNA duplexes of mismatches cleaved by CEL I. As shown, in the optimal temperature range of CEL I, the efficiency of enzyme digestion is directly proportional to the temperature. However, high temperature (65 °C) will gradually hydrolyze the complete complementary double chains. In addition, at the same temperature, the efficiency of enzyme digestion is directly proportional to the reaction time, but a longer reaction time (>60 min) will also cleave the complete complementary double chains. Some differences in CEL I cleavage on the DNA/DNA and RNA/DNA of mismatches have been observed, and the cleavages have better outcomes when the reaction is performed at a temperature close to the $T_m$ of the DNA/RNA duplex. Previous studies showed that CEL I exhibited a mismatch cleavage preference in the order of C/C > C/A ~ C/T > G/G > A/A > T/G ~ G/A > T/T for unknown reasons. In this study, CEL I showed the following cleavage trend: $dT/rG$ ~ $dA/rG$ > others > $dT/rU$ ~ $dT/rG$ ~ $dG/rU$. These cleavage differences must be attributed to the intrinsic enzyme–substrate recognition and relevant cleavage mechanism of CEL I nuclease.

When optimizing the experimental conditions for CEL I practical application in TP-RCA, two factors were considered. First, in the current CEL I digestion experiments, RNA/DNA duplexes are 30 oligonucleotides long. In the proposed miRNA detection experiments with TP-RCA, the RNA/DNA duplexes are 22 nucleotides long in general; thus, the cleavage temperature should be adjusted accordingly. Second, many studies have reported that RNA/DNA heteroduplexes are usually more stable than DNA/DNA duplexes. To achieve high-quality cleavage efficiency of CEL I, we tested different temperatures around the melting temperature ($T_m$) of the 22-mer RNA/DNA heteroduplexes and decided to perform CEL I hydrolysis at 55 °C for 60 min in the following experiments (Figure 2).

Feasibility Evaluation of the Double-Strand Primers for the RCA Reaction. In RCA-based miRNA detection, miRNAs usually hybridize directly with the CT and serve as
primers for RCA reactions. In our proposed TP-RCA strategy, the target miRNA is protected by corresponding protective probes. Therefore, it is necessary to prove that the protected target miRNA in a duplex form is still able to initiate the RCA reactions. Single-strand let-7a and double-strand let-7a/pro-7a at different concentrations were employed for this purpose. In the experiment, let-7a at 100 nM and pro-7a at 100 nM were mixed at 20 °C, heated at 95 °C for 20 min, and cooled to room temperature slowly. The duplex of let-7a/pro-7a was then diluted to different concentrations and mixed with CTs for RCA reactions. As shown in Figure 3a, both single-strand let-7a and double-strand let-7a/pro-7a are capable of initiating RCA reactions and producing fluorescence signals. Generally, RCA fluorescence signals produced by single-strand let-7a increase almost linearly as the RCA reaction progresses, and the signal intensity is positively proportional to the primer concentration in the range of 5−500 pM ($R^2 = 0.969$) (Figure 3b). The RCA reactions initiated by the let-7a/pro-7a duplex demonstrated fluorescence curves, and their fluorescence signals were also positively correlated with the concentrations of the let-7a/pro-7a duplex ($R^2 = 0.981$) (Figure 3c).

When determining the reaction temperature of phi29, we referenced some publications; some studies used 30 °C,$^{14−16}$ and others used 37 °C.$^{17−22}$ After experimental parameter optimization, we decided to use 37 °C as the reaction temperature of phi29 because it provides better results.

The results that the let-7a/pro-7a duplex could initiate RCA reactions at 37 °C are slightly surprising, but they are interpretable. It should be kept in mind that the duplex formation of two complementary oligonucleotides is a dynamic process; that is, base pairs at either end of short duplexes are always in the equilibrium formation−dissociation process. Once the 3′-end of the protected target (let-7a) becomes single-stranded and transiently hybridizes with the CT, it could start the RCA reactions because phi29 DNA polymerase possesses a high binding affinity to the 3′-end of the protected target on the CT and a high polymerization capability at 37 °C. Careful examination of the RCA fluorescence curves provides further evidence. The RCA fluorescence curves initiated by a single-stranded primer (let-7a) exhibit normal RCA fluorescence curves, whereas those initiated by double-stranded primers (let-7a/pro-7a) show S-shaped curves, which indicates that these RCA reactions are in a branched-RCA amplification mode. After the protected miRNAs hybridize with the CT, they will start the RCA reaction, and the released protective probe will act as the second primer to hybridize with the repeated sequences of the RCA products and turn into branched RCA reactions. There is no question that the RCA reaction initiated by let-7a/pro-7a duplexes is a more complex nucleic acid amplification process than we thought at this moment, and more comprehensive investigations are needed. Nevertheless, these results confirmed that the protected miRNAs in the duplex forms can be used as primers to initiate RCA reactions for miRNA detection and laid a foundation for the following experiments.

**Assessment of the TP-RCA Detection Specificity.** In conventional RCA detection, all of the target and nontarget miRNAs can directly hybridize with the CT and serve as primers for RCA reactions. Thus, the detection specificity solely relies upon the hybridization complementarity. In the TP-RCA method, all of the nontarget miRNAs are eliminated, and only the surviving target miRNA can initiate the RCA reactions, providing enhanced detection specificity. To assess the TP-RCA detection specificity, six members of the microRNA let-7 family (let-7a−let-7f) were used as the intended targets. Six DNA-protective probes (pro-7a−pro-7f) fully complementary to the targets let-7a−let-7f and six CTs (cir-7a−cir-7f) (see Table S1 in Supporting Information) for RCA reactions were synthesized accordingly.

Take detection of let-7a as an example. The protective probe pro-7a was incubated with each of the let-7 species, thus forming six duplexes: one perfect-match duplex (let-7a/pro-7a)
and five mismatched duplexes (let-7x/pro-7a, x ≠ a). After CEL I treatment, each duplex was analyzed using cir-7a as the RCA template. The fluorescence curves of the TP-RCA reactions are shown in Figure 4a: only the let-7a species illustrates an observable fluorescence signal, and other let-7 species exhibit signals similar to the negative control. For comparison, the outcomes of the conventional RCA reactions are given in Figure S3. The detection specificity of pro-7a for let-7a was set as 100%, and those for other let-7 members were measured accordingly. The histograms of pro-7a specificity are presented in Figure 4b. The detection specificities of other protective probes (pro-7b−pro-7f) are given in Figures S4 and S5. The detection specificities of these protective probes summarized in Table 1 show that the proposed TP-RCA method can well discriminate nontarget let-7 members (<10%). These results demonstrate much better specificities than the conventional RCA detection published so far. Xu et al. utilized palindromic padlock probe-based RCA to evaluate the detection specificity of some cancer-related miRNAs. Using let-7a miRNA as a circularization splint and a polymerization primer, their RCA results showed that, in comparison with the target miRNA let-7a, the detection specificity of let-7d (two mismatches), let-7e (one mismatch), let-7f (one mismatch), and miR-21 were 26.7, 36.9, 38.9, and 7.8%, respectively.23 Ye et al. reported the detection of let-7 family members using the RCA-loop-mediated isothermal amplification (RCA-LAMP) method. This method combined padlock-RCA with LAMP to achieve high sensitivity for miRNA detection. When let-7a was considered the target, the interfering signals produced by let-7b, let-7c, let-7d, let-7e, and let-7f were 13.52, 18.8, 11.43, 18.84, and 2.14%, respectively. These interferences were rationalized in terms of the fact that detection specificity is highly dependent on the ligation reaction of the RCA CT.24 Furthermore, a RCA study for detection of differentiating each let-7 species from other family members also showed similar detection specificity.25

In conventional nucleic acid detections (PCR, nucleotide sequencing, microarray, etc.), the detection specificity depends strongly upon the complementarity of the probe and the

| let-7 family members | pro-7a | pro-7b | pro-7c | pro-7d | pro-7e | pro-7f |
|----------------------|--------|--------|--------|--------|--------|--------|
| let-7a               | 100    | 12.84  | 0.00   | 9.12   | 12.84  | 8.56   |
| let-7b               | 1.35   | 100    | 2.60   | 3.56   | 6.60   | 7.21   |
| let-7c               | −3.00  | −0.19  | 100    | 11.33  | 7.46   | 4.73   |
| let-7d               | 8.36   | 10.23  | 5.34   | 100    | 12.68  | 7.22   |
| let-7e               | 6.44   | 4.84   | 3.57   | 7.53   | 100    | 7.53   |
| let-7f               | 3.56   | 4.06   | 2.56   | 5.23   | 8.92   | 100    |
intended target. Some previous studies have shown noticeable detection specificity; however, either only let-7a or some different microRNAs having great nucleotide sequence variations were used as the targets. When the duplex contains only one mismatch (such as miRNA family members), its hybridization behavior could be very close to that of the perfect-match duplex depending upon the chemical nature and position of the mismatch. Thus, it is relatively difficult to discriminate the target precisely from mixtures of high sequence homology. In the proposed TP-RCA strategy, the protective probe and CEL I digestion can amplify the difference from a single-nucleotide mismatch into the survival of the target miRNA and the elimination of interferences. Thus, only the protected target miRNA can be amplified in subsequent RCA reactions using the corresponding CT, providing excellent detection specificity at single-nucleotide resolution.

**Specific Detection of Targeted let-7 Species in Mixtures Using TP-RCA.** In reality, the samples to be analyzed could be cell extracts, body fluids (saliva, urine, cerebrospinal fluid, etc.), circulating blood, or homogenized tissues. These samples contain a small quantity of target and abundant interferences. Some of these interferences might be of high sequence homology with targets and could create unwanted false signals. To satisfy the high detection specificity requirements for practical applications, we evaluated the ability of the proposed TP-RCA method to identify the target miRNAs in mixtures of homologous species. We employed the let-7 family members for this test. Six series of mixtures consisting of different let-7 species in different ratios were prepared. For example, the ratios of let-7a in mix-a1 to mix-a6 decreased stepwise from 100 to 0% when mixing an equal quantity of other let-7 species (let-7b–let-7f). (See “Preparation of Mixture Samples for High-Specificity Detection” for details). For comparison, we used the RT-qPCR method to analyze the same samples mix-a1 to mix-a6. Unfortunately, RT-qPCR results were not satisfactory because the nonspecific amplifications of the intrafamily interferences cannot be eliminated even though mir-93-5p was clearly distinguished as the intended interference (Figure S6). Interestingly, the resolution of RT-qPCR could be much improved when the target-protection process was performed prior to RT-qPCR (Figure S7).

As shown in Figure 4c, after treatment with the protective probe pro-7a and CEL I digestion, fluorescence curves of RCA reactions show that the fluorescence intensities at 90 min decrease, which are proportional to the quantities of let-7a in these mixtures, and its histogram of the fluorescence intensities illustrates a good linearity ($R^2 = 0.95$) (Figure 4d). In Figure 4c, both samples of max-a6 and the negative control contain no let-7a species, while their relative fluorescence intensities are 2.53 and 0.0%, respectively, indicating that other let-7 species indeed create signal interference. Thus, we define that the detection specificity for let-7a is 97.47% (Table 2). In contrast, the fluorescence curves of these same six mixture samples without pro-7a protection were undistinguishable (see Figure S7a). The detection specificities of other protective probes in relevant mixtures are given in Figures S8–S10. The analysis results of other family members were similar and are listed in Table 2. These results provide convincing evidence that the target protection procedure can significantly improve the detection specificity of miRNA detection.

### CONCLUSIONS

In this study, we proposed a TP-RCA method for highly specific miRNA detection. The key step of this method is to use a protective-probe to protect the target miRNA by forming a perfect-match duplex. In contrast, all of the nontarget interferences will be eliminated by a single-strand specific nuclease because their duplexes with the protective probe contain mismatches. As a result, only the protected miRNAs can survive and produce the expected fluorescence signal when using RCA reactions for detection. We used let-7 family members as the model to show significantly improved detection specificity, which was much better than the results reported previously. The proposed TP-RCA method has great potential for applications in fundamental research and clinical diagnosis.

### EXPERIMENTAL SECTION

**Materials.** All oligonucleotides of the PAGE grade in this study were synthesized by Sangon Biotech (Shanghai, China). Phi 29 DNA polymerase and its reaction buffer were purchased from Thermo-Fisher Scientific (Waltham, USA). T4 DNA ligase, exonuclease III, dNTPs and their reaction buffers were purchased from Takara Biotech (Beijing, China). The fluorescence dye SYBR Green II was purchased from Thermo-Fisher Scientific (Waltham, USA). The single-strand nuclease CEL I was purchased from Transgenomic (California, USA).

**Nuclease CEL I Activity Evaluation.** A series of RNA oligonucleotides (30 nucleotides long) and a series of DNA oligonucleotides (30 nucleotides long) were designed to construct RNA/DNA heteroduplexes containing twelve types of RNA/DNA mismatches (dT/rU, dG/rG, dC/rG, dA/rG, dT/rG, dG/rU, dA/rU, dG/rA, dT/rC, dC/rU, dA/rC, and dC/rA). The mismatched position of RNA/DNA duplexes was intentionally designed at the center for the consistency of enzymatic kinetics (Table S1).

Mismatched RNA/DNA heteroduplexes were dissolved using phi29 DNA polymerase reaction buffer (33 mM Tris-acetate with a pH 7.9 at 37°C, 10 mM magnesium acetate, 66 mM potassium acetate, 0.1% (v/v) Tween 20, and 1 mM DTT). The total volume of the reaction system was 20 μL, in which 50 nM RNA/DNA heteroduplexes and 2 μL of CEL I (2 units) were mixed. Enzymatic digestion was performed at 45, 55, and 65°C for 30, 60, 90, and 120 min, respectively. The digestion products were analyzed using 20% polyacrylamide gel electrophoresis.

**Evaluation of the CEL I Cleavage Specificity.** The DNA protective probe pro-7x was synthesized, which is fully complementary to the proposed target let-7x. The target let-7x of interest was diluted to 500 nM. First, 2.0 μL of target, 2.0 μL of probes, 1.0 μL of 10× phi29 DNA polymerase reaction buffer, and 5.0 μL of double distilled water (ddH2O) were

| Target in Mixtures | Linearity ($R^2$) | Detection Specificity (%) |
|--------------------|------------------|---------------------------|
| let-7a             | 0.95             | 97.47                      |
| let-7b             | 0.95             | 94.24                      |
| let-7c             | 0.97             | 87.69                      |
| let-7d             | 0.98             | 93.89                      |
| let-7e             | 0.94             | 97.83                      |
| let-7f             | 0.93             | 100.00                     |
mixed together to ensure the fully complementary hybridization of the target and probe. Then, the digestion reaction was initiated by adding 10 μL of working solution (1.0 μL of 10× phi29 DNA polymerase reaction buffer, 2.0 μL of CEL I and 7.0 μL of ddH2O). This mixture was incubated at 55 °C for 60 min. The digestion product was then heated at 95 °C for 20 min to deactivate CEL I. Finally, the digested product was also prepared.

The curves of RCA reactions were recorded on a Multiplate Reader (Tecan, Switzerland) with excitation at 480 nm and fluorescence emission at 530 nm. The fluorescence signals were collected at intervals of 1 or 2 min, and the gain value was set at 70% manual.

Preparation of Sample Mixtures for High-Specificity miRNA Detection. Six series of samples consisting of different let-7 members were prepared to assess the detection specificity of the proposed TP-RCA strategy. When one of the let-7 family members was specified as the target, the other family members were referred to as interferences. For example, when let-7a was considered as the target, other let-7 members (let-7b, let-7c, ..., let-7f) in equal quantities were mixed as the interference. Mixtures of the target and interference at different ratios were further prepared. Mix-a1 contains 100% of target let-7a, and the ratio of let-7a in mix-a2, mix-a3, mix-a4 decreases stepwise until mix-a6 has 100% of interference. By following the same procedure, a series of mix-b, ..., mix-f were also prepared.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.0c05279.

Details regarding the oligonucleotide sequences, optimization of the CEL I digestion time and temperature, optimization of RCA template cyclization, comparison of the detection specificity of the targeted let-7 species by the normal RCA method and TP-RCA, specificity for the detection of targeted let-7 species by RT-qPCR, comparison of the detection specificity of targeted let-7e in mixtures using RT-qPCR with and without the target protection procedure, and comparison of the detection specificity of mixed let-7 species by the normal RCA method and TP-RCA (PDF)

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ABBREVIATIONS

TP-RCA, target-protection rolling circle amplification; sss-nuclease, single strand-specific nuclease; CT, circular template; Tm, melting temperature

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