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A dumbbell probe-mediated rolling circle amplification strategy for highly sensitive microRNA detection

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ABSTRACT

We herein report the design of a dumbbell-shaped DNA probe that integrates target-binding, amplification and signaling within one multifunctional design. The dumbbell probe can initiate rolling circle amplification (D-RCA) in the presence of specific microRNA (miRNA) targets. This D-RCA-based miRNA strategy allows quantification of miRNA with very low quantity of RNA samples. The femtomolar sensitivity of D-RCA compares favorably with other existing technologies. More significantly, the dynamic range of D-RCA is extremely large, covering eight orders of magnitude. We also demonstrate miRNA quantification with this highly sensitive and inexpensive D-RCA strategy in clinical samples.

INTRODUCTION

MicroRNAs (miRNAs) are a class of short (19–24 bases), endogenous non-coding RNAs that mediate post-transcriptional gene regulation by base pairing to the 3′ untranslated region of messenger RNAs. These miRNAs are regulated temporally and spatially, and involved in cell proliferation, differentiation and apoptosis (1–3). Over the past decade, in particular, studies on miRNA expression profiles have shown that aberrant expression exits in human cancer cell lines (4,5) and primary cancers (6–9). The close correlation between dysregulation of miRNA expression and human cancers has intrigued intense interest to explore the possibility of using miRNAs to classify human cancers, and for utilization as cancer-specific biomarkers for clinical diagnostics (10).

There is increasing interest in developing new methods for nucleic acid detection that has high significance in genetic analysis, molecular diagnostics and rapid screening of infectious diseases (11–16). Particularly, motivated by the above-mentioned miRNA studies, various strategies for the detection of miRNAs have been proposed, such as real-time quantitative PCR (17,18), locked nucleic acid (LNA)-based northern blot (19), microarray or bead-based flow-cytometry (20–24), array-based Klenow enzyme assay (25) and miRNAs serial analysis of gene expression (26). These novel strategies involve methodological improvement on design and labeling of primers or probes, chemical modification of nucleotides and incorporation of signal amplification. More recently, an isothermal amplification method designated as rolling circle amplification (RCA), was employed for sensitive detection of miRNAs. Due to the simplicity, robustness and high signal amplification of RCA, it has been popularly employed in DNA (15,27) and protein detection (28).

Several novel methods for miRNA detection by using padlock probe-based RCA have been reported (29,30). Jonstrup *et al.* (29) first reported an RCA-based protocol for miRNA detection, which nevertheless relied on time-consuming northern blotting. Cheng *et al.* (30) improved this method by introducing a second primer, and then RCA products were analyzed via fluorescence from an intercalative dye, SYBR Green I (SG). In addition to the sensitivity, selectivity is the other important factor in miRNA detection. Several previous reports have employed a stem–loop primer (31), LNA modification (24) and high-stringency enzymes (30) to improve the specificity of miRNA detection. In this work, we propose a dumbbell probe (32)-mediated RCA (D-RCA) strategy for high sensitivity and selective miRNA detection

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The authors wish it to be known that, in their opinion, the first two authors should be regarded as joint First Authors.

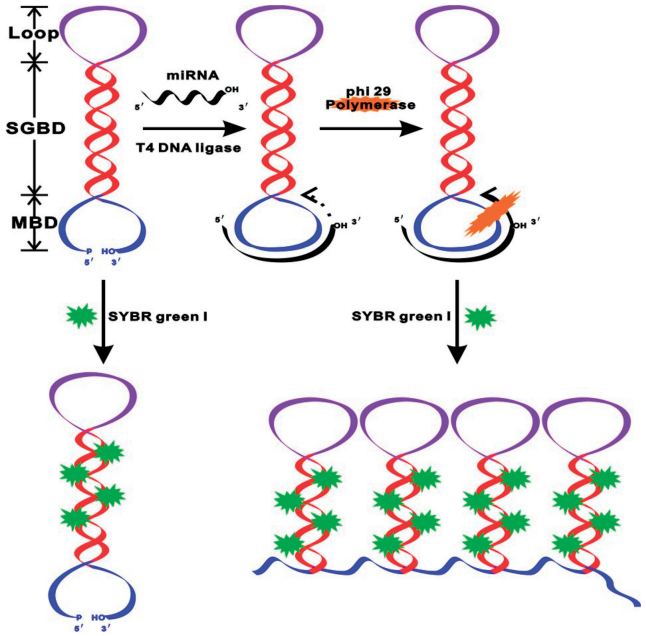

Figure 1. The D-RCA strategy for miRNA detection. A dumbbell probe contains three domains, an MBD, an SGBD and a loop domain. The binding of miRNA to MBD initiates RCA in the presence of T4 DNA ligase and phi29 polymerase, which generates a long DNA sequence that contains many SGBD for an amplified fluorescent readout.

(Figure 1). We aim to improve the sensitivity of miRNA detection via RCA, and the selectivity by using the dumbbell probe to reduce non-specific amplification.

MATERIALS AND METHODS

Oligonucleotides and total RNA

Oligonucleotides were purchased from Invitrogen. The sequences for the interrogated miRNA are shown in Supplementary Table S1, and the sequences of all hairpin probes are listed in Supplementary Table S2. These probes were chemically synthesized with a phosphate at the 5′ end and purified by polyacrylamide gel electrophoresis (PAGE) (Invitrogen). Total RNA from colon tumor tissue (CTT) and normal adjacent tissue (NAT) were obtained from Ambion.

Ligation reactions

The ligation reaction was carried out with 20 µl of a reaction mixture containing 1× ligation buffer [40 mM Tris–HCl, 10 mM $MgCl_2$, 10 mM Dithiothreitol (DTT), 500 µM ATP (pH 7.8), 10 units of T4 DNA ligase (Fermentas), the dumbbell probe and 2 µl of synthetic miRNA]. Before adding T4 DNA ligase and ligation buffer, the oligonucleotide mixture was denatured at 65°C for 3 min, and cooled slowly to room temperature over a 10-min period. After annealing, T4 DNA ligase and ligation buffer were added to the mixture and incubated at 37°C for 2 h.

RCA reactions

For RCA, the 20-µl ligation product was mixed with 4 µl 10× reaction buffer [330 mM Tris–acetate, 100 mM $Mg(Ac)_2$, 660 mM Potassium Acetate (KAc), 1% Tween 20 and 10 mM DTT (pH 7.9), 2 µl (10 u/µL) phi29 DNA polymerase (Fermentas), 8 µl 10 mM dNTPs mixture (Takara) and RNase-free water]. The reaction mixture was incubated at 30°C for 6 h.

Measurement of fluorescent spectra

The RCA amplification product was mixed with 10 µl 10× SG dye (Invitrogen) and diluted to final volume of 200 µl with 10 mM PBS (pH 7.4). The fluorescent spectra were measured using a spectrofluorophotometer. The excitation wavelength was 497 nm, and the spectra are recorded between 507 and 650 nm. The fluorescence emission intensity was measured at 530 nm.

RESULTS AND DISCUSSION

We designed a dumbbell probe with two stem–loop structures as a recognition element for the specific detection of miRNA with high sensitivity (Figure 1). The dumbbell probe contains three domains, a miRNA-binding domain (MBD), a SG-binding domain (SGBD), and a loop domain. The specific probe-target binding occurs at the MBD, which contains two segments that are designed to be complementary to the target miRNA. MBD-miRNA binding leads to a nick-containing duplex, which is closed to form a loop in the presence of T4 DNA ligase. The SGBD is the signaling domain that is a segment of duplex. SG can preferably bind to the SGBD duplex via intercalation (33), leading to strong fluorescent signals. The loop domain is a spacer region, which provides a covalent linker between the duplex. Importantly, when miRNA binds to the MBD that closes the dumbbell with the action of ligase, phi29 DNA polymerase can initiate the RCA reaction that replicates numerous dumbbell probes.

The RCA products were quantified via the fluorescent intensity of SG. We reason that the dumbbell probe could improve both the sensitivity and the specificity of miRNA detection. The fact that one SGBD domain can bind to many molecules of SG offers additional signal amplification for miRNA detection. On the other hand, the presence of the stem–loop structures in the dumbbell probe brings about high conformational constraint, and the competition between the stem region and the loop/target binding could significantly improve the specificity (17,34).

The inherent binding ability of the dumbbell probe with SG was first evaluated. The emission peak of SG appeared at 530 nm when the concentration of dumbbell probes reached 100 nM (Supplementary Figure S1). This fluorescent emission is due to the preferable intercalative binding of SG to dsDNA (33). Background suppression is important for ultrasensitive nucleic acid detection (35). Given that signal output in this strategy is based on the fluorescent readout of SG intercalated into SGBD, it is important to optimize the dumbbell probe concentration to

improve the signal/background ratio of RCA. We found that RCA signals increased along with the concentration increment of *D-12s-miR21* probe. For example, when the concentration of the *D-12s-miR21* probe is ~1–10 nM, fluorescent signals for low-concentration targets (1 fM to 100 pM) were almost indistinguishable from the background, leading to relatively poor sensitivity as compared to the use of a 50-nM probe (Supplementary Figure S2). In addition, excessive dumbbell probe (250 nM) significantly increased the background as well, which decreased the signal: background ratio, and more importantly, made it difficult to quantify miRNA concentrations. Moreover, the stem length of the hairpin probe is also a key to the improvement of signal/background ratio of RCA since the fluorescent intensity of SG becomes higher and higher, proportional with the length increase of SGBD (33). Since the *D-24s-miR21* probe with 24-bp stem structure has higher binding capacity for SG than that observed for the 12-bp stem structure (Supplementary Figure S1), we found that the 24-bp stem of the dumbbell probe caused a large background signal (Supplementary Figure S2).

Subsequently, we compared dumbbell probe-mediated RCA with linear-probe-mediated RCA to reveal the advantages of the D-RCA strategy (Figure 2 and Supplementary Figure S3). As observed for linear probe-mediated RCA, the signal was very low until the target concentration reached 1 pM. In contrast, the signal of dumbbell probe-mediated RCA was significantly higher than negative control when the target concentration was only 1 fM, exhibiting a sensitivity improvement by three orders of magnitude.

Since miRNA families (e.g. *let-7* family) often possess closely related sequences with high homology (1- or 2-nt difference) (36), it is thus important to evaluate the sequence specificity of our D-RCA strategy for miRNA analysis. We designed the sequence-specific probes for *hsa-let-7d*, *hsa-let-7e* and *hsa-let-7f* in the *let-7* family, which differed by only 1 or 2 bases from each other (Supplementary Table S2). Importantly, we found that the specific dumbbell probes led to significantly higher fluorescence intensity than non-specific probes ($P < 0.05$), with an improvement by at least 3-fold for all interrogated *let-7* miRNAs (Figure 3a and Supplementary Figure S4). Of note, the 1-base sequence specificity was poor when T4 DNA ligase was employed in the previous report (30), whereas use of T4 RNA ligase 2 realized high-specificity in the assay. The marked improvement of sequence specificity even with T4 DNA ligase in our work clearly shows the high stringency of the stem–loop structure in the probe, which brings about high sequence specificity due to the conformational constraint of the stem–loop (17,34).

Dynamic range is also important for accurate quantification of miRNA since the miRNA expression level varies

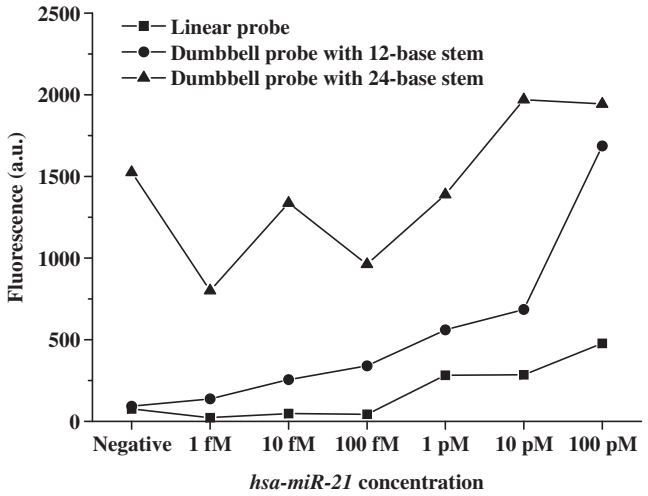

Figure 2. Comparison of the detection performance by using either linear or dumbbell probes (12- and 24-base stems).

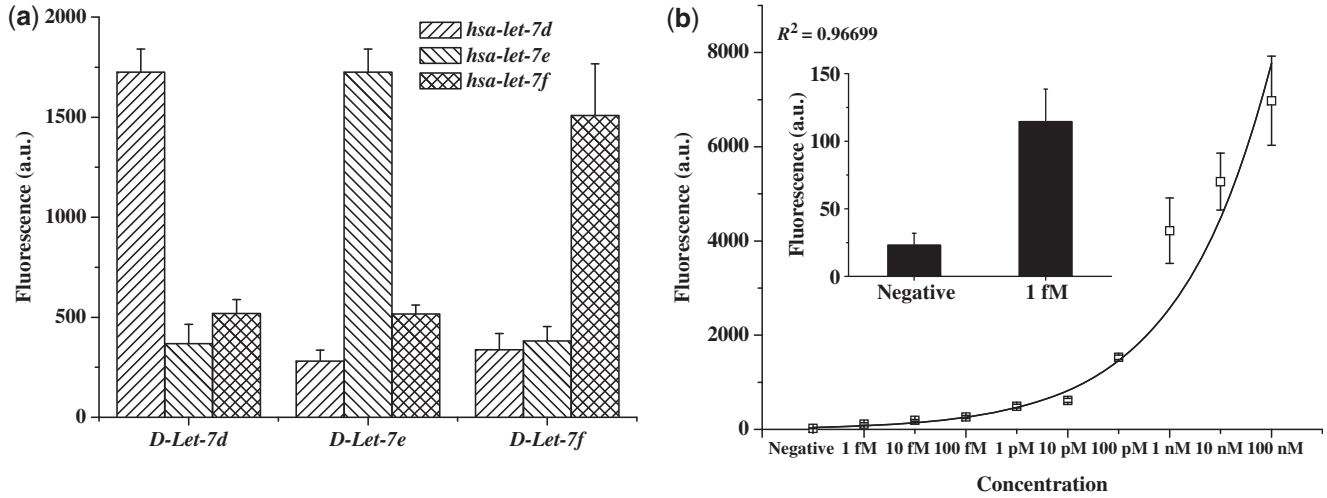

Figure 3. The performance of the D-RCA-based miRNA detection. (**a**) Three miRNAs, *hsa-let-7d*, *7e* and *7f* of 100 pM were employed to reveal the selectivity for a 1- or 2-base mismatch. (**b**) The concentration profile for the detection of miRNA *hsa-miR-21* was in the range 1 fM to 100 nM. Data were collected from at least three independent sets of experiments. Error bars (SD) were estimated from at least three independent measurements. The squared correlation coefficient (R^2) was analyzed by sigmoidal fit.

greatly (10–50 000 copies/cell) in different cell lines and tissues (17,36,37). To evaluate the dynamic range of D-RCA, synthetic *hsa-miR-21* was serially diluted to concentrations ranging from 1 fM to 100 nM and targeted with our D-RCA method (Figure 3b). Significantly, the fluorescence intensity gradually increased along with *hsa-miR-21* concentration, leading to an impressively large dynamic range that spans eight orders of magnitude (1 fM to 100 nM). We also note that the fluorescence signal was significantly larger than the background ($P < 0.05$), even when the concentration of *hsa-miR-21* was as low as 1 fM (>3 SD; Figure 3b, inset), suggesting that the detection limit of D-RCA method was at least 1 fM. In this regard, the D-RCA method has remarkable advantage over previously reported methods (Table 1).

Given the high sensitivity, sequence specificity and large dynamic range of our D-RCA method, we attempted to employ this novel strategy to perform miRNA analysis in clinical samples. Recent studies have provided strong evidence that *hsa-miR-21* is a biomarker candidate in colon cancer, which has significantly higher expression in CTT than in NAT (9). We then employed D-RCA to quantify the expression level of *hsa-miR-21* in colon tumor and NAT. The samples (+L) and negative controls (−L) were performed either in the presence or in the absence of T4 DNA ligase. We found that the expression levels of *hsa-miR-21* in CTTs were significantly higher than that detected in NAT ($P < 0.05$; Figure 4). The differentiation ratios of expression levels between CTT and NAT were 1.5, 2.0 and 2.3 in total RNA sample of 100 pg, 1 ng and 50 ng, respectively, with the dumbbell probe at a concentration of 50 nM. When the concentration of the dumbbell probe was decreased to 25 nM, we could still effectively differentiate CTT from NAT, which led to differentiation ratios of 2.7, 2.0 and 1.5 in a total RNA sample of 100 pg, 1 ng and 50 ng, respectively. Also of note, the background signals for negative controls were significantly lower than those observed in samples. It is thus important to note that our D-RCA method can reliably quantify miRNA directly in total RNA samples as few as 100 pg, which provide a highly sensitive approach for clinical diagnostics.

Table 1. The comparison for existing miRNA analysis methods

Methods	Detection limit	Total RNA	Dynamic range	Cost	References
D-RCA	20 zmol	100 pg	8	low	This work
Branched RCA	8.5 amol	3 ng	3	low	(30)
Padlock RCA-blotting	0.5 amol	3 ng	4	high	(29)
Stem–loop RT-PCR	0.2 zmol	25 pg	7	high	(17)
Microarray	0.2 amol	120 ng	4	high	(23)
Bead-based assay	1 amol	100 ng	4	high	(20)
LNA-based blotting	1 fmol	2.5 μg	4	high	(19)

CONCLUSION

The D-RCA strategy described in this work shows promising capabilities of specific and sensitive analysis for miRNA expression. The advantages of D-RCA include: (i) the dumbbell probe integrates target-binding, amplification and signaling within one multifunctional design; (ii) the sensitivity of D-RCA compares favorably with other existing technologies that allows quantification of miRNA with a very low amount of RNA sample. More significantly, the dynamic range of D-RCA is extremely large, covering eight orders of magnitude; (iii) RCA is an isothermal amplification protocol that does not rely on expensive devices (e.g. PCR machine), which significantly reduces the cost of D-RCA assays. We expect that this highly sensitive and inexpensive D-RCA strategy will become a promising miRNA quantification method in clinical diagnostics.

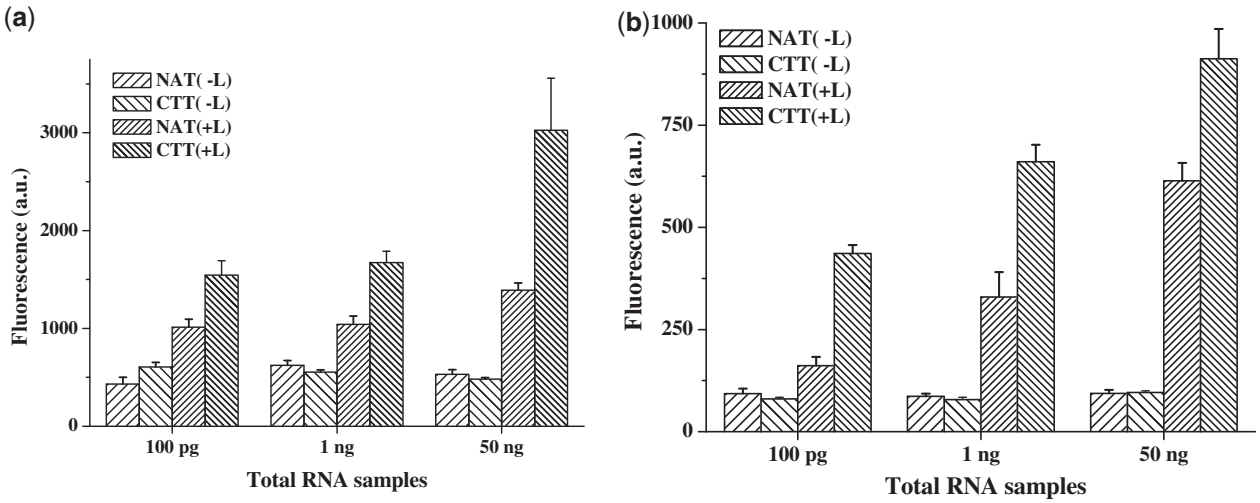

Figure 4. The expression levels of *hsa-miR-21* in total RNA samples with the dumbbell probe of (**a**) 50 nM and (**b**) 25 nM. Negative controls (−L) were performed with identical conditions in total RNA samples (+) without adding T4 DNA ligase. Statistical analysis was performed by paired *t*-test. Error bars (SD) were estimated from three independent measurements.

SUPPLEMENTARY DATA

Supplementary data are available at NAR Online.

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Conflict of interest statement. None declared.

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