Communications

Highly Sensitive Determination of microRNA Using Target-Primed and Branched Rolling-Circle Amplification**

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MicroRNAs (miRNAs) are short endogenous noncoding RNAs (19–23 nucleotides (nt)) that regulate fundamental cellular processes through the modulation of gene expression.[1] Moreover, specific changes in miRNA expression patterns are associated with various diseases,[2] especially cancers.[3] Since 2001, intense research has been carried out worldwide on the biology of miRNAs.[4] the detection of miRNA plays a critical role for further understanding the biological functions of miRNAs as well as early diagnosis of diseases and discovery of new targets for drugs.[4,5] Several unique characteristics of miRNAs, including their small size, sequence homology among family members, and low abundance in total RNA samples, make them difficult to analyze.[5] The northern blot technique is currently used as the standard method for miRNA analysis.[6] However, owing to its low sensitivity and labor-intensive steps, the method requires large amounts of sample and is very time consuming. Microarray technology offers a way to analyze multiple samples simultaneously.[7] Nevertheless, its sensitivity and specificity for detecting miRNA are not satisfactory.[8] Recently, several amplification strategies for miRNA detection on microarrays using chemical or enzymatical modifications have been proposed to improve the sensitivity, such as a fluorescence labeling assay[9] and nanoparticle-amplified detection.[10] However, these modifications require complex protocols. Several homogeneous methods have been proposed recently for miRNA analysis, such as RT-qPCR,[11] the modified invader assay,[12] and ribozyme-based signal amplification.[13] However, the short length of miRNAs make their experimental design very sophisticated, and the doubly fluorescence-labeled probes or the locked nucleic acid (LNA) used in the method listed would make miRNA detection quite expensive. More recently, our group has also developed a simple and homogeneous miRNA assay based on fluorescence resonance energy transfer with a cationic conjugated polymer. Unfortunately the sensitivity of this assay is not sufficient for the detection of low-abundance miRNAs.[14]

As a result of its simplicity, robustness, specificity, and high sensitivity, rolling-circle amplification (RCA) has become increasingly popular in the detection of DNA, RNA, and proteins.[15] RCA offers an exquisite strategy for detecting miRNAs because the short miRNAs are suitable to be used as templates for ligation of the padlock probes and can subsequently prime the isothermal RCA reaction. Jonstrup et al. have pioneered miRNA detection based on RCA.[16] However, the RCA-based assay still has several unresolved issues, which can limit its widespread application. First, the specificity for discriminating a one-nucleotide difference among related miRNA family members has not yet been demonstrated. Furthermore, the detection of the RCA products requires separation by gel electrophoresis and radioactive-band measurements. Thus, the assay suffers from high labor intensity and inherent safety problems (e.g., health hazard and waste disposal problems).

Herein we report that T4 RNA ligase 2 can greatly improve the specificity for the ligation of padlock probes by using miRNA as the template so that the RCA-based assay can clearly discriminate one-nucleotide differences between miRNAs. In addition, by introduction of a second primer complementary to the RCA products, which results in a branched rolling-circle amplification (BRCA) reaction,[17] the RCA products can be sensitively determined in a homogeneous manner by using SYBR Green 1 (SG) as the fluorescence dye. Therefore, the proposed assay is practical for highly sensitive detection of miRNAs with simple processes.

An outline of the designed procedure is shown in Figure 1. The eleven 5′- and 3′-terminal bases of the padlock probe are designed to be complementary to the half sequence of 22 nt miRNA target (let-7a). Therefore, the padlock probe can be specifically ligated and circularized with the miRNA as the template in the presence of T4 RNA ligase 2. Subsequently, the miRNA can act as the primer to initiate RCA reaction by the high-displacement activity of the phi29 DNA polymerase. Furthermore, a second primer, which is complementary to every tandem sequence on the RCA product, can be extended to displace downstream growing strands and prime the BRCA reaction. The BRCA products, which include large amounts of single-stranded (ss) DNA and double-stranded (ds) DNA of various lengths, can be directly detected by the SG dye. As a result, the robust BRCA reaction combined with the quantification of SG fluorescence enables the detection of miRNA with high sensitivity and high specificity. The BRCA reaction primed by target miRNA can be verified by agarose gel electrophoresis (see the Supporting Information).

Because of the high similarity of miRNA sequences, the specificity of miRNA assay for discriminating one-base differences between miRNAs is in great demand to better understand the biological functions of individual miRNAs. To demonstrate the specificity of the miRNA assay, members of the let-7 miRNA family—let-7a, let-7b, and let-7c—are ideal models because they have the same length and their sequences differ by only one or two bases. In addition, their expression levels are closely associated with cell development and human cancers.[18] Because the specificity of the proposed assay depends on the ligation reaction, where the padlock probe is specifically ligated by using target miRNA as the template, it is crucial in RCA-based miRNA detection to select the ligase for sealing the termini of padlock probe to be perfectly complementary to the miRNA. Although T4 DNA ligase has been used to ligate the padlock probe templated by RNA,[15b,c,16] as shown in Figure 2a it has poor specificity to

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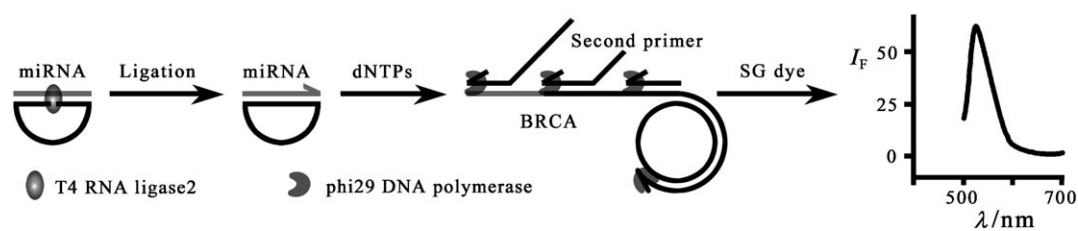Fax: (+86) 312-507-9403
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Figure 1. Illustration of the target-primed BRCA reaction and fluorescence detection of miRNA; see the text for details.
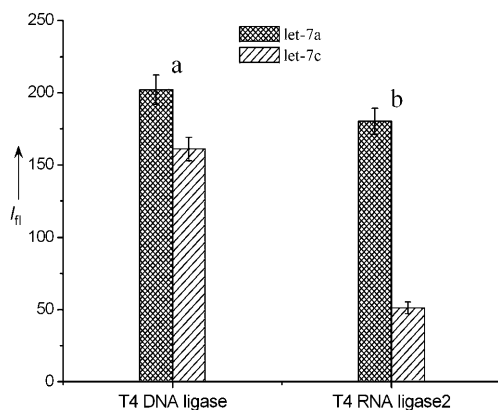

Figure 2. Comparison of the specificity of T4 RNA ligase 2 and T4 DNA ligase. The ligation reactions were conducted with 10 nM padlock probe and 0.1 nM let-7a and let-7c, and were performed at 37°C for 90 min. The fluorescence intensities were measured by addition of SG dye in BRCA products. Error bars were estimated from three replicate measurements.

Figure 3. Effects of the ligation reaction temperature on the specificity of miRNA detection. The ligation reactions were performed with 10 nM padlock probe and 0.1 nM let-7a, let-7b, and let-7c in a reaction volume of 10 μL at different temperatures. Inset shows the fluorescence spectra of BRCA reaction products where the padlock probes were ligated at 39°C. The other experimental conditions were the same as described in Figure 2.

discriminate a single-base difference between let-7a and let-7c; the fluorescence intensity produced by let-7a is only 1.2-fold of that produced by let-7c using the padlock probe perfectly complementary to let-7a. The poor specificity probably results from its template-independent ligation characteristic.[19] Alternatively, we found T4 RNA ligase 2, which is generally used as an efficient catalyst of RNA ligation in double-stranded RNAs or RNA/DNA hybrids,[20] exhibits higher specificity than T4 DNA ligase in ligating the padlock probe. When T4 RNA ligase 2 and the padlock probe perfectly complementary to let-7a are used to perform the miRNA-primed BRCA reaction at 37°C, the fluorescent signal produced by let-7a is approximately 3.5-fold higher than that of let-7c (Figure 2b).

In addition to the ligase used, another critical factor to affect the specificity of miRNA assay is the temperature of the ligation reaction. We performed the ligation reaction of the padlock probe perfectly complementary to let-7a at 37–40°C using let-7a, let-7b, and let-7c as the template. After the BRCA reactions, one can see from Figure 3 that the resulting fluorescence intensities gradually decrease, but the specificity to detect let-7a can greatly be improved by increasing the temperature. At a ligation temperature of 39°C, the fluorescent intensity of let-7a is approximately 50-fold and 7-fold higher than that of let-7b and let-7c, respectively. It is noticeable that let-7a and let-7c differ by only one base near the 3′ end of the miRNAs (base A in let-7a, base G in let-7c),
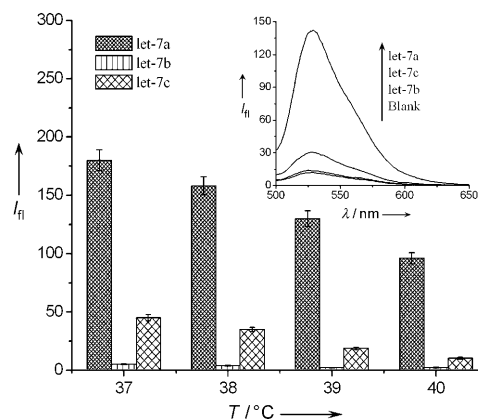
and the interaction energy between T-A and T-G is similar. Therefore, it is difficult to discriminate let-7a and let-7c by any hybridization-based method,[8] only the method based on fluorescence correlation spectroscopy yields a 3-fold difference in signal intensity between let-7a and let-7c.[21] Thus, our proposed method demonstrates high specificity.

The relationship between the fluorescent response and the concentration of target miRNA (let-7a) was investigated. The fluorescence intensities increased remarkably with the increase of let-7a concentration over the range of 0.025 to 2.5 pM. There was a good linear correlation between the relative fluorescence intensity and the amount of let-7a in the concentration range of 0.025–1 pM. The correlation equation was $I = 5.7 + 251.7\,C$ (pM) and the correlation coefficient was $r = 0.9994$. The detection limit (3σ, $n = 11$) was estimated to be 10 fM (i.e. 6 amol in 600 μL sample). The linear dependence of fluorescence intensities on miRNA concentration indicates that the proposed method is suitable for accurate quantitation of miRNA. A series of seven repetitive measurements of 0.25 pM let-7a was used for estimating the precision; the relative standard deviation was 4.3%, suggesting good reproducibility of this miRNA assay. The assay was applied to the quantitation of let-7a content in total RNA samples extracted from healthy human lung tissue (see the Supporting Information).

In summary, we have developed a simple and sensitive method for the quantitative detection of miRNA based on a target-primed BRCA reaction coupled with fluorescence quantification. In contrast to previous reports, the proposed method does not require any isolation steps, which should significantly reduce the cost and simplify the manipulation for miRNA determination. Moreover, when T4 RNA ligase 2 is used, high specificity can be achieved to clearly discriminate one-base differences between miRNAs. In addition, in situ detection is desirable for better insight into regulatory roles of miRNAs. Microarray-based methods are not suitable for in situ detection since washing steps are required.[5] With the target-primed RCA reaction, the RCA products can anchor to the target and the detection signals remain colocalized with the target. Therefore, this method holds great potential for in situ detection of miRNA in specific tissues.[15d, 22] Furthermore, RCA methods have been applied to nucleic acid detection on microarrays,[23] in individual cells,[15d] and in single DNA molecules.[22] The RCA products can also be detected by sensitive chemiluminescent and colorimetric methods.[24] Therefore, the proposed RCA-based miRNA assay should open new avenues for miRNA detection on high-throughput platforms or in single cells by various detection techniques.

Experimental Section

The fluorescence measurements were performed with a Hitachi F-4500 spectrofluorimeter (Tokyo, Japan) equipped with a xenon lamp. A Biometra T1 thermocycler (Germany) was used to control the temperature of the ligation and RCA reactions. Gel electrophoresis images were acquired with a VersaDoc 4000 imaging system (Bio-Rad, USA).

T4 DNA ligase and T4 RNA ligase 2 were purchased from New England Biolabs. Phi29 DNA polymerase was purchased from Epicentre Technologies (Madison, WI). SG dye (20×) was purchased from Xiamen Bio-vision Biotechnology Co. Ltd. (Xiamen, China). Human lung total RNA (100 µg/100 µL) was purchased from Ambion. All of the synthetic DNA, RNA (see the sequences listed in the Supporting Information), diethyprocarbonated(DEPC)-treated water, deoxynucleotides (dNTPs), and ribonuclease inhibitor used in this study were obtained from TaKaRa Biotechnology Co. Ltd. (Dalian, China). All of the oligonucleotides were purified by HPLC, except the second primer which was purified by PAGE. All solutions were prepared in DEPC-treated deionized water.

For the ligation reactions with T4 RNA ligase 2, the reaction mixture consisted of ligation buffer (50 mM Tris-HCl, pH 7.5, 2 mM MgCl$_2$, 10 mM dithiothreitol (DTT), 400 µM ATP), 2 U of T4 RNA ligase 2, 40 U of ribonuclease inhibitor, 10 nM padlock probe, and an appropriate amount of the target miRNA in a reaction volume of 10 µL. For the ligation reactions with T4 DNA ligase, the reaction mixture consisted of ligation buffer (10 mM Tris-HCl, pH 7.5, 10 mM MgCl$_2$, 10 µM ATP), 400 U of T4 DNA ligase, 40 U of ribonuclease inhibitor, 10 nM padlock probe, and an appropriate amount of target miRNA in a reaction volume of 10 µL. Before the ligase and ligation buffer were added, the reaction mixture was heated at 55 °C for 5 min. After the reaction mixture had been annealed at 39 °C for 30 min and cooled to room temperature, the ligase and the buffer were added and the reaction mixture was incubated at 39 °C for 90 min.

The product of the ligation reaction was added to the 10 µL BRCA reaction mixture containing 80 mM Tris-HCl (pH 7.5), 100 mM KCl, 20 mM MgCl$_2$, 10 mM (NH$_4$)$_2$SO$_4$, 8 mM DTT, 500 µM of each dNTP, 0.2 µM second primer, and 20 U of phi29 DNA polymerase.

The BRCA reactions were performed at 30 °C for 6 h and terminated by incubation at 65 °C for 10 min.

Aliquots of 6 µL BRCA product and 4 µL SG (20× concentrate) were combined in a 1.5 mL centrifuge tube and diluted to 600 µL with 10 mM phosphate buffer (pH 7.5). After incubation for 10 min at room temperature, the fluorescence spectra were measured in a 1 × 1 cm quartz cuvette. The excitation wavelength was 480 nm, and the spectra are recorded between 500 and 700 nm. The fluorescence emission intensity was measured at 530 nm. The relative fluorescent intensity was the net intensities produced by the miRNAs, where the background signal recorded in the absence of miRNA had been subtracted for each value.

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