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Target-Triggered Three-Way Junction Structure and Polymerase/Nicking Enzyme Synergetic Isothermal Quadratic DNA Machine for Highly Specific, One-Step, and Rapid MicroRNA Detection at Attomolar Level

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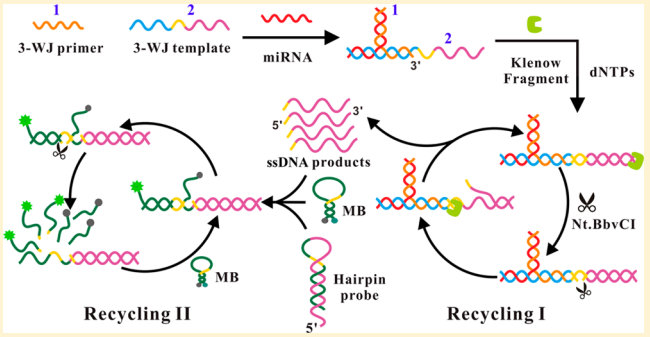
Supporting Information

ABSTRACT: MicroRNAs (miRNAs) play important roles in many biological processes and are regarded as promising cancer biomarkers. Herein, a highly specific, one-step, and rapid miRNAs detection strategy with attomolar sensitivity has been developed on the basis of a target-triggered three-way junction (3-WJ) structure and polymerase/nicking enzyme synergetic isothermal quadratic DNA machine (ESQM). To this end, 3-WJ probes (primer and template) are designed to selectively recognize target miRNA and form the stable 3-WJ structure to trigger ESQM, resulting in a high quadratic amplified signal. A high specificity is demonstrated by the excellent discrimination of even single-base mismatched homologous sequences with mismatched bases in varied locations (close to the 3′-end, the 5′-end, and the middle). In addition, a low detection limit down to 2 amol was achieved within 30 min. This sensitivity is much higher than those of most linear amplification-based approaches and is even comparable to those of some exponential amplification-based methods. Furthermore, the applicability of this method in complex samples was demonstrated by the analysis of cancer cell small RNA extracts, results of which were in good agreement with those obtained by a commercial miRNA kit and previously published data. The miRNA with a 3′ end modification (2′-O-methylation), such as plant miRNA, was also successfully detected, confirming the good universality of the proposed strategy. It is worthwhile to point out that several well-established methods using miRNA as primer for polymerization reaction are of relatively poor performance in the analysis of these modified miRNA. Therefore, these merits endow the developed strategy with powerful implications for biological research and an effective diagnostic assay.



MicroRNAs (miRNAs) are a class of endogenous, nonprotein coding, small, single-stranded RNAs.[1,2] They are found in many eukaryotes including plants and animals. Until now, over 24 500 miRNA sequences and corresponding annotations have been found and published in the miRNA database miRBase.[3] As negative post-transcriptional regulators, miRNAs can form an active RNA-induced silencing complex (RISC) with several relevant proteins to regulate the expression of target genes, which either decrease the stability of mRNAs (mRNAs) or suppress the translation process.[4−6] It is reported that miRNAs are involved in the regulation of over 60% of human protein-coding genes, and they play critical roles in many physiologic processes.[1,5,7] Accumulating evidence has revealed that aberrant miRNA expression level in the tissue and blood samples of patients is associated with various human diseases such as cancer and cardiovascular diseases.[8−10] Due to the above properties, miRNA has been an emerging class of clinically important biomarker for the early diagnosis. Consequently, it is of great significance and urgent need to develop rapid, specific, and sensitive methods for miRNA detection.

Because miRNAs are short (18−25 nt) and of low abundance in total RNA samples with many highly homologous (even single-base mismatch) sequences,[2] the sensitive and selective analysis of miRNA has always been a challenge. Some conventional methods, including Northern blotting,[11] microarray,[12] and quantitative real-time PCR (qRT-PCR),[13,14] have been well developed for miRNA detection. Though Northern blotting is used as the standard method for miRNAs profiling studies, it is labor-intensive and time-consuming with poor

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sensitivity. These intrinsic drawbacks limit its application in clinical diagnosis. Microarray is of high-throughput, but it is not satisfactory, owing to the shortcomings of high operation expense, low sensitivity, poor reproducibility, and specificity. Ascribed to its ultrasensitivity and high specificity, qRT-PCR has been widely employed for the detection of miRNA in cancerous cell lysates and clinical samples. Unfortunately, an essential reverse transcription process of the miRNA is required before the PCR amplification. The complicated and tedious operation undoubtedly increases the experimental cost and RNase contamination, probably causing a false-negative result. Moreover, PCR-based methods must use the costly thermal cycler for the high-precision control of temperature cycling, which makes it difficult to perform in point-of-care testing.

In recent years, several promising isothermal signal amplification strategies have been introduced for the miRNA assay, such as loop-mediated isothermal amplification (LAMP),[15] exponential amplification reaction (EXPAR),[16−19] and rolling circle amplification (RCA).[20−22] Li and co-workers[15] have proposed an ultrasensitive detection method on the basis of LAMP by using target miRNA as primer. Though it can be carried out in a one-step sensing system, this method suffers from a lengthy assay time (90 min) and complicated design with three DNA primers and one long template (198 nt). EXPAR is first devised by Galas and co-workers for the rapid and efficient amplification of short oligonucleotides (8−16 bases).[23] It has been successfully demonstrated by the Li group[18] that EXPAR is suited for miRNAs real-time detection, which could be accomplished in 30 min with ultrahigh sensitivity (about 15 aM). Some improved approaches combining silver nanocluster[16] or quantum dots[17] with EXPAR were successively developed by different research groups. The detection limits can be even down to 2 aM and 0.1 aM, respectively. However, these EXPAR-based methods are mainly limited by the strong nonspecific background amplification, which mainly comes from interactions between the template and DNA polymerase in the absence of target (as primer in these works). Also, they can only efficiently discriminate target from 3′terminus mismatched sequences and presented unsatisfactory specificity to the homologous sequences with mismatched bases in the 5′-terminal and the middle.[18] Among isothermal amplification strategies, RCA-based methods have been widely used to detect miRNA in different samples owing to its simplicity, robustness, and specificity. In these methods, target miRNA was generally regarded as template for the ligation of the padlock probe to be circular DNA and then functions as the primer to initiate the RCA reaction by a highly processive DNA polymerase.[21] Due to the introduction of the ligation reaction, these methods significantly improved the specificity even to discriminate single-base mismatched together with high sensitivity (fM level).[20−22] Nevertheless, RNA ligase, instead of DNA ligase, is used to ensure the high sequence specificity, whereas RNA ligase presents relatively low ligation efficiency compared to DNA ligase.[21] The compromise between specificity and sensitivity may be the cause of the lengthy detection times (over 6 h). Additionally, the DNA polymerase (Phi 29 DNA polymerase) involved in RCA possesses an inherent 3′ → 5′ proofreading exonuclease activity acting on single-stranded (ss) RNA,[24−26] which might lead to a false-negative result. Recently, on the basis of DNA strand polymerization reaction[27] or strand displacement amplification (SDA),[28,29] some novel miRNA detection methods were also reported with high sensitivity. Nevertheless, the selectivity of SDA-based methods is also relatively poor. It is worthwhile to point out that miRNA was employed as a primer to start the amplification reaction in all LAMP-, EXPAR-, RCA-, and SDA-based methods. This may make these methods unapplicable in the analysis of plant miRNAs and other small RNAs of 3′-end modification,[30−32] which results from the inhibition of polymerase-catalyzed DNA synthesis by the modification in 3′ terminal.[24,33,34] Therefore, there is still an ugent need for the development of highly specific, highly sensitive, and one-step miRNA detection method with good universality.

Herein, on the basis of target-triggered three-way junction (3-WJ) structure[35] and polymerase/nicking enzyme synergetic quadratic isothermal DNA machine (ESQM), we reported a highly specific and rapid miRNA detection method with attomolar sensitivity. By the hybridization with both 3-WJ primer and 3-WJ template, target miRNA can specifically form stable 3-WJ structure, which will trigger ESQM to afford a high quadratic amplified signal. The proposed strategy has high sequence specificity to distinguish varied homologous sequences even with single-base mismatch in different positions from near 3′ end to 5′ terminal. It was performed in a one-step reaction of short assay time (30 min) with a low detection limit (2 amol) and a large dynamic range (5 orders of magnitude). The practical application of this strategy in a complex biological sample was demonstrated by the good performance in cell small RNA extracts, results of which are in good agreement with those obtained by qRT-PCR and previously published data.[36,37] Moreover, this method can be used for the efficient detection of miRNAs with 3′ end modification such as plant miRNAs, detection of which are of low efficiency by most reported strategies using miRNA as primer for polymerization reaction.

EXPERIMENTAL SECTION

Materials and Reagents. HPLC-purified miRNAs, the deoxynucleeotide triphosphates (dNTPs) mix, RNase inhibitor, and commercial RNA extraction kits (RNAiso for small RNA) were purchased from Takara Biotechnology Co., Ltd. (Dalian, China). The other oligonucleotides were synthesized by Sangon Biotechnology Co., Ltd. (Shanghai, China). All the sequences are listed in Table S1 (Supporting Information). Klenow Fragment (exo$^-$), Nt.BbvCI, and the corresponding buffers were obtained from New England Biolabs Ltd. (Beijing, China). All other solutions were prepared and diluted by RNase-free water.

Fluorescence Measurement. Fluorescence measurements were carried out by using a FluoroMax-4 fluorescence spectrometer (Horiba Jobin Yvon, Edison, NJ). The emission spectra were obtained from 510 to 600 nm with an excitation wavelength of 495 nm at room temperature in steps of 1 nm. The fluorescence intensity at 518 nm was chosen as the optimal experimental conditions to evaluate the performance of our proposed system. Both the excitation and emission slit widths were set at 5 nm.

MiRNA Detection Based on 3-WJ Structure and ESQM. Various concentrations of miRNA were added in 20 μL of 1× NEBuffer 4 containing 10 nM 3-WJ primer, 10 nM 3-WJ template, 50 nM hairpin probe, 250 nM MB, 2.5 units of Klenow Fragment, 0.8 units of Nt.BbvCI, 16 units of RNase inhibitor, and 0.2 mM dNTPs. Subsequently, the solutions were incubated at 37 °C for 30 min prior to the fluorescence measurement.

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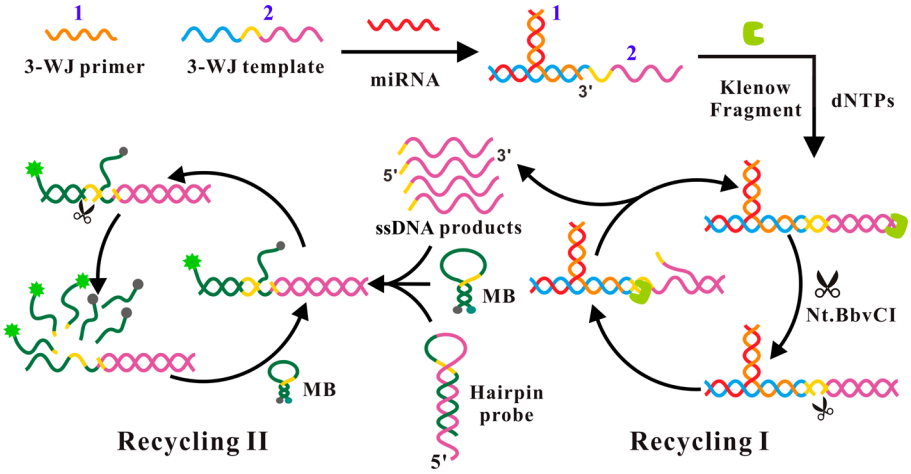
Scheme 1. Schematic Illustration of miRNA Detection Based on Target-Triggered 3-WJ Structure and ESQM[a]



[a]Target miRNA can form stable 3-WJ structure with 3-WJ primer and 3-WJ template, and then it can trigger ESQM including Recycling I and Recycling II.

Gel Electrophoresis. Products of miRNA-triggered ESQM were analyzed by 20% nondenaturating polyacrylamide gel electrophoresis (PAGE) in 1× TBE buffer (90 mM Tris −HCl, 90 mM boric acid, 2 mM EDTA, pH 7.9) at a 45 V constant voltage for 3 h at room temperature. The gel was silver-stained and imaged by a digital camera.

Isolation of MiRNA and Cell Sample Analysis. HeLa cells, A549 cells, and MCF-7 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum and 100 U mL^{-1} penicillin−streptomycin at 37 °C in a humidified 5% CO_2 incubator. The small RNA was extracted from cultured cancer cells by a RNA extraction kit from Takara (RNAiso for small RNA) according to the manufacturer's procedures.

RESULTS

Principle of Target-Triggered 3-WJ Structure and ESQM for MiRNA Assay. As illustrated in Scheme 1, 3-WJ probes (primer and template) are involved in this proposed sensing strategy. They are designed to be 12 and 10 bp complementary with target miRNA, respectively. In the absence of target, no 3-WJ structure is generated. Though having 6 bp complementary sequences, 3-WJ primer and 3-WJ template cannot steadily associate with each other due to the low melting temperature ($T_m \approx 18$ °C) compared to the reaction temperature (37 °C). As a result, the dissociative 3-WJ primer is disabled to initiate ESQM. Oppositely, when target miRNA is present, 3-WJ primer and 3-WJ template can each hybridize with miRNA in close proximity to form the stable 3-WJ structure, resulting in the trigger of ESQM. In detail, the 3-WJ primer in the stable 3-WJ structure can start DNA polymerization reaction along the 3-WJ template in the presence of DNA polymerase Klenow Fragment and dNTPs as "fuel substrate". A recognition sequence (highlighted in yellow) for nicking enzyme Nt.BbvCI is contained in the 3-WJ template. Nt.BbvCI will nick the new synthesized DNA strand on 3-WJ template to produce a 3′-OH, which initiates other DNA polymerization process with the displacement of the ssDNA product. Following these processes (polymerization, nicking, and strand displacement), multiple ssDNA products are generated (Recycling I). These products then can open the hairpin probes via complementary hybridization. By hybridizing with MB, the opened hairpin probe can form DNA duplex structure that contains the full recognition site for Nt.BbvCI. After nicking the MB on the duplex structure, the opened hairpin probe is released as well as the separation of fluorophore from the quencher. The dissociated opened hairpin probe then hybridizes with another uncleaved MB and performs the next cycle of nicking, leading to an amplification signal (Recycling II). Eventually, each target can successfully afford a high quadratic amplified signal via miRNA-triggered 3-WJ structure and ESQM. It is worthwhile to note that the sensing system is carried out in a one-step and isothermal reaction and does not require excess enzymes and complex buffer optimization compared to basic SDA process.

Feasibility and Design of 3-WJ Probes. Due to the significant roles in cellular processes and human diseases,[38−41] miRNA let-7b is selected as a model analyte. Both the 3-WJ primer and 3-WJ template are designed to be 12 and 10 bp complementary to let-7b, respectively. The formation of stable 3-WJ structure by mixing let-7b and 3-WJ probes was demonstrated by the melting curve analysis using SYBR Green I as fluorescence signal (Figure S1). Subsequently, we evaluated whether this stable 3-WJ structure can trigger ESQM. As presented in Figure 1A, the addition of let-7b in the sensing system induced a high fluorescence signal, whereas extremely low fluorescence intensity was observed in the absence of target. To confirm the amplification efficiency of ESQM, a control experiment of basic SDA process was carried out. It can be seen that target-triggered fluorescence signal of ESQM is much higher than that of basic SDA process. A high signal enhancement of 13.1-fold was observed in ESQM, whereas only an 8.4-fold signal increase was achieved by the basic SDA. Furthermore, we theoretically calculate the recycle process of linear (basic SDA) and quadratic amplification system (ESQM) (Table S2 and Figure S2). Their signal equations are described as $Y_1 = N$ for basic SDA and $Y_2 = 0.5N^2 - 0.5N$ for ESQM, where Y_1 and Y_2 represent the signal intensity and N is the cycle number (positive integer). When N is greater than 3 (an enough reaction time), Y_2 is always greater than Y_1. These above results are accordance with the theoretical analysis, and they indicate that ESQM can provide a higher signal by quadratic amplification than basic SDA.

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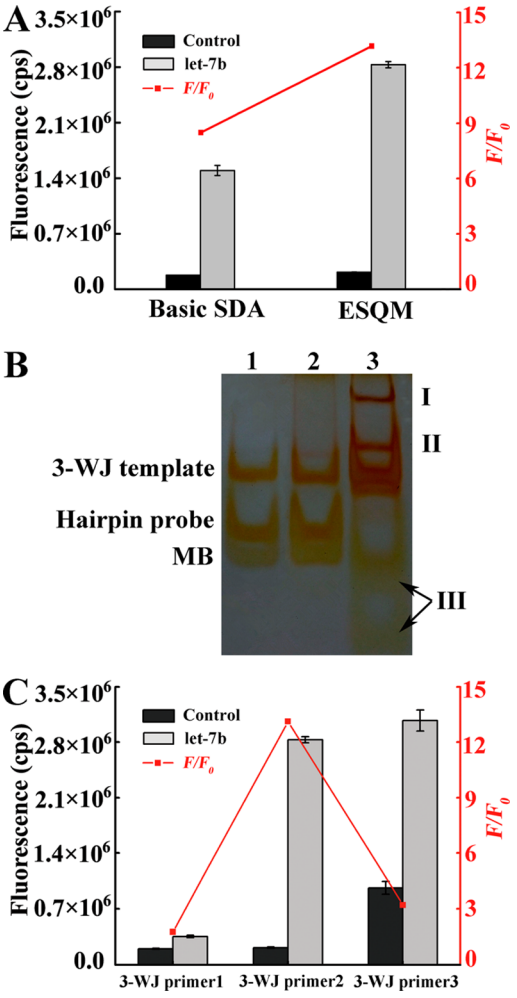

Figure 1. Confirmation of target-triggered signal amplification and investigation of 3-WJ primer structure. (A) The fluorescence responses of ESQM and basic SDA in the presence of 10 nM let-7b, with corresponding backgrounds (control, without let-7b), respectively. The reaction mixture of basic SDA contains 3-WJ basic SDA template, 3-WJ primer, and MB without hairpin probe. The 3-WJ basic SDA template (Table S1) is designed to generate the SDA product which is complementary with MB. (B) Gel electrophoresis analysis: lane 1, marker; lane 2, ESQM without let-7b; lane 3, ESQM with let-7b. I, II, and III represent the 3-WJ structure (complete duplex by polymerization), DNA duplex (opened hairpin probe hybridized with MB) and cleavage products of MB, respectively. (C) Effect of 3-WJ primer length on the fluorescence response of the sensing system. F_0 and F represent the fluorescence intensity in the absence and presence of let-7b, respectively. The error bar indicates the standard deviation of three independent experiments.

Gel electrophoresis analysis was also performed by the sensing system. No new products were observed in the absence of let-7b (lane 2, Figure 1B). However, several product bands including 3-WJ structure (complete duplex by polymerization), DNA duplex (opened hairpin probe hybridized with MB), and cleavage products of MB were obviously presented in the presence of let-7b (lane 3, Figure 1B). All these data suggested that only the addition of target miRNA could form the 3-WJ structure and triggered the quadratic signal amplification catalyzed by DNA polymerase and nicking enzyme.

We then investigated the effect of the 3′ length of 3-WJ primer complementary to 3-WJ template. Three different 3-WJ primers, each having 5, 6, and 7 bp complementary to 3-WJ template, were evaluated. It can be seen in Figure 1C that the 3-WJ primer 3 induced a relatively high fluorescence signal even in the absence of target. It should be pointed out that 3-WJ primer 3 only has 7 bp complementary to 3-WJ template, and the T_m value is estimated to be ~20 °C, which was lower than the reaction temperature of 37 °C. The high background signal is perhaps ascribed to the formation of a transient duplex hybrid between 3-WJ primer 3 and 3-WJ template. In addition, DNA polymerase recognized this transient duplex and then performed the quadratic amplification. On the other hand, when 3-WJ primer 1 was used, there is no significant fluorescence signal observed even in the presence of let-7b. Therefore, 3-WJ primer 2 is chosen for the following experiments.

Optimization of Several Important Conditions. To obtain the highest sensing performance, the molar ratio of 3-WJ primer to 3-WJ template, concentration of nicking enzyme, and reaction time were optimized, respectively. As we expected, a higher ratio of 3-WJ primer to 3-WJ template leads to a higher fluorescence signal (Figure S3A). However, a relatively strong fluorescence intensity of background was also observed as this ratio increased. Due to the highest F/F_0, the molar ratio of 1:1 was employed. Nicking enzyme participated in both amplified processes, and its concentration is significant in the amplification reaction. As presented in Figure S3B, a high concentration of Nt.BbvCI led to a strong fluorescence response. Unfortunately, the fluorescence intensity without target was also enhanced with the increase of Nt.BbvCI concentration. This unwanted background signal mainly resulted from the cleavage of a few MBs which form DNA duplex with hairpin probe by the competing hybridization. According to the highest F/F_0 value, 0.8 unit of Nt.BbvCI was selected as the optimized concentration. The fluorescence responses in different reaction time were also recorded and depicted in Figure S3C. As the time prolonged, the fluorescence intensity of both signal and background increased gradually. Taking into account the balance between sensitivity and velocity, 30 min was chosen for the subsequent experiment.

Detection of Let-7b Based on Target-Triggered 3-WJ Structure and ESQM. With the suitable design of 3-WJ probes and other optimized sensing conditions, different concentrations of let-7b were added to the sensing system to investigate the sensitivity. The relevant fluorescence spectra are shown in Figure 2A. As expected, a gradual increase of fluorescence intensity was observed by increasing the let-7b concentrations in the range of 0−20 nM. Figure 2B presented the relationship between the fluorescence intensities at 518 nm and the concentrations of let-7b. As the inset of Figure 2B depicted, the fluorescence intensity is linearly dependent on the logarithm of let-7b concentration ranging from 1 pM to 50 pM. The correlation equation is $Y = 5.228 + 0.701 \lg X$, where Y is the fluorescence intensity ($\times 10^5$ cps) and X is the let-7b concentration. Based on 3 times the standard deviation over the blank response, the detection limit is 100 fM (2 amol in 20 μL). The sensitivity is superior to those of most fluorescence methods by nonexponential signal amplification[42−44] and even comparable to several exponential amplification-based approaches.[15,19,45] It is worthwhile to point out that such a high sensitivity was achieved within a much shorter assay time of 30 min than those of these exponential amplification-based methods described above.

The discrimination of differences between miRNA family members is of great importance for understanding their

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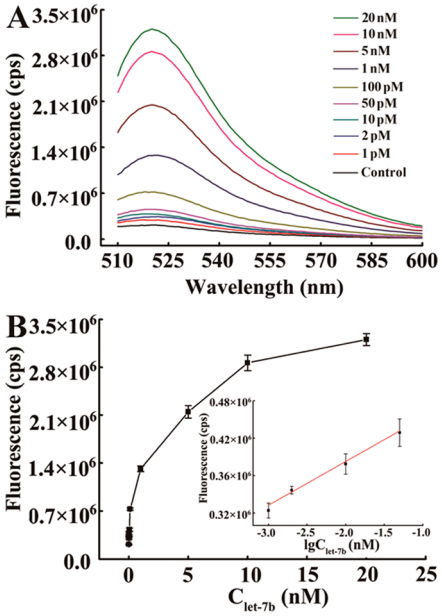

Figure 2. Performance of miRNA detection based on target-triggered 3-WJ structure and ESQM. (A) Fluorescence spectra response to different concentrations of let-7b. (B) Changes of fluorescence intensity at 518 nm upon the addition of different concentrations of let-7b. The inset displays the linear correlation between the fluorescence intensity and the logarithm of let-7b concentration in the range of 1−50 pM.

biological functions and relationship with human diseases. However, it has been always challenging due to their high sequence similarity. To evaluate the specificity of our strategy, several members of the let-7 family (let-7a, let-7c, let-7d, let-7e, and let-7f) were selected. As shown in Figure 3A, all of these homologous sequences led to unremarkable fluorescence responses which were nearly the same as that of background signal and much lower than that of let-7b. Gel electrophoresis analysis also supported the same result in Figure 3B. The new bands representing the generation of 3-WJ structure (complete duplex by polymerization), DNA duplex (opened hairpin probe hybridized with MB), and cleavage products of MB were observed in only lane 1 (with let-7b), indicating the selective formation of 3-WJ structure and the following trigger of ESQM. Also, the selectivity data of conventional duplex template for the same target has been collected and depicted in Figure 3C. It can be seen that the fluorescence responses of all homologous sequences are comparable with that of target miRNA. To make a detailed comparison, the fluorescence enhancements of different sequences by both designs (3-WJ probes and duplex template) are listed in Table S3. The fluorescence enhancement of let-7b is about 13.8−40.9-fold higher than those of other homologous sequences using 3-WJ probes, whereas it is only 1.1−1.6-fold by duplex template. Therefore, all the results demonstrated the highly selective detection of let-7b compared to its family sequences. In detail, these selected homologous sequences have one to four mismatched bases in varied sequence locations (close to 3′-end, 5′-end, and middle). The high specificity to homologous sequences with mismatch located in different sequence positions is owing to 3-WJ structure which is thermosensitive. In this design, only single-base mismatch (regardless of whether located in 3′ and 5′ terminal or middle) can lead to the failure of forming the stable 3-WJ structure.

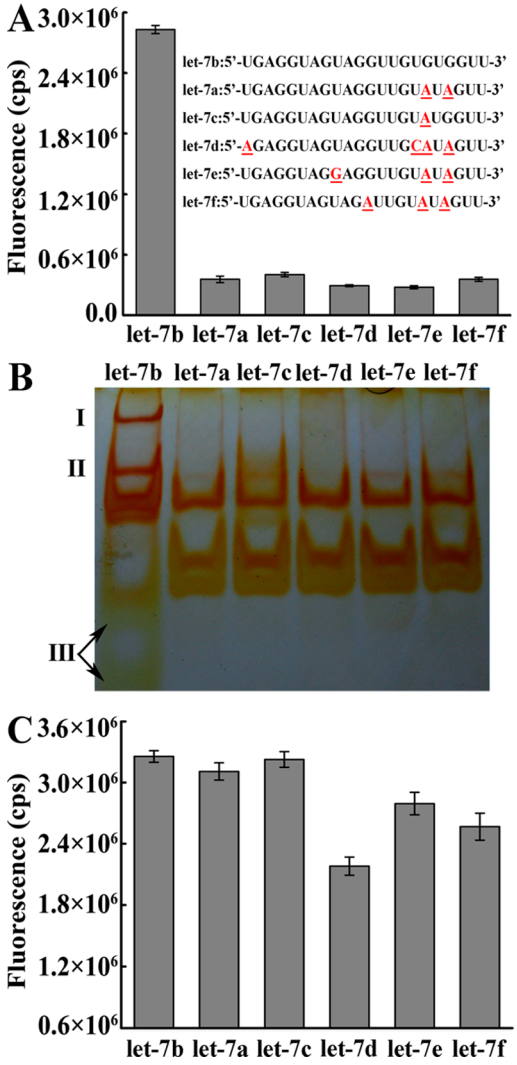

Figure 3. Selectivity of the sensing strategy for let-7b detection over other homologous sequences. (A) Fluorescence intensity to the addition of different inputs. The concentrations of all miRNAs are each 10 nM. (B) Corresponding gel electrophoresis analysis result of (A). I, II, and III represent the 3-WJ structure (complete duplex by polymerization), DNA duplex (opened hairpin probe hybridized with MB), and cleavage products of MB, respectively. (C) Selectivity of a conventional duplex template for let-7b detection over other homologous sequences by changing the 3-WJ probes to a duplex template for the same target. The concentrations of all miRNAs are each 10 nM.

We further investigated the feasibility of this method to real samples by testing the total small RNA extracts from three human cancer cell lines (HeLa, MCF-7, and A549). The same samples were also analyzed by a commercial qRT-PCR kit, respectively. As shown in Figure 4, the results obtained by the proposed strategy were in good agreement with those of qRT-PCR kit and were also consistent with previously published data.[36,37]

Moreover, the excellent universality of 3-WJ probes and ESQM sensing system for miRNAs in different species is confirmed by the detection of plant miRNA, which is 2′-O-methylation at the 3′terminal. The test was also carried out by the detection system of duplex template and ESQM to make a comparison. As presented in Figure 5, the fluorescence response of *Arabidopsis thaliana* miRNA156a (ath-miR156a)

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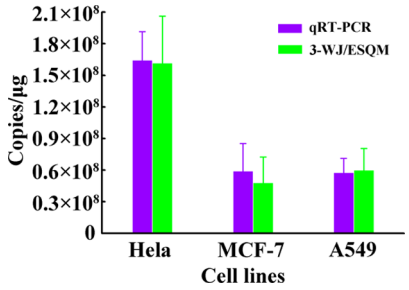

Figure 4. Analysis of miRNA in total small RNA extracted from cultured cells. The error bar indicates the standard deviation of three independent experiments.

is as high as that of corresponding DNA target with the same sequence using 3-WJ probes.

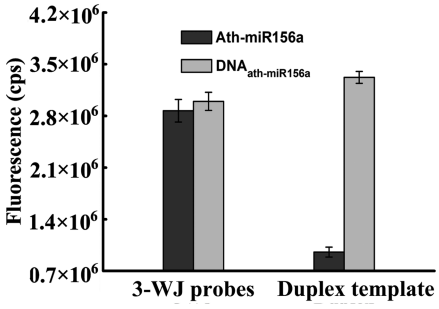

Figure 5. Results of 2′-O-methyl-modified miRNA detection by both 3-WJ probes and conventional duplex template systems. The concentrations of targets are 10 nM. Other conditions are the same as optimized conditions in the text.

However, it is much lower than that of corresponding DNA target by the duplex template. It is noteworthy that let-7b (without 3′terminal modification) induced similar fluorescence response as that of DNA target with the same sequence by the duplex template (Figure S5). The relatively poor fluorescence response of ath-miRNA156a can be ascribed to the inhibitory influence on DNA synthesis by 2′-O-methylation modification at 3′terminal.[33,34] Therefore, all these results indicated the 3-WJ probes of the developed system shows more excellent performance for 3′ modification miRNA rather than conventional duplex template.

DISCUSSION

In this paper, target miRNA can form stable 3-WJ structure with 3-WJ probes and initiate the quadratic signal amplification named ESQM. The detection reaction can be performed in a one-step and isothermal format within a short assay time (30 min). Some merits including high specificity, good universality, and high sensitivity are achieved by the proposed strategy.

MiRNAs are always of high sequence homology among family members, and they play different roles in cell cellular functions and human diseases. The excellent discrimination of the homologous sequences is necessary for the analysis of total RNA samples which is of great importance in biological research and clinical diagnosis. In 2011, Faridani and co-workers have demonstrated that the expression level of let-7b in HeLa cell is at most 5-fold higher than those of let-7c and let-7d, and is slightly lower than that of let-7a which is the same as that of let-7f.[46] According to the result, the discrimination of let-7 family is not an easy work. Though some methods based on EXPAR[16,18] or SDA[28] have been well-established with high sensitivity (fM to aM level), they presented poor sequence specificity. For example, the EXPAR-based method[18] suffers from the strong interference by let-7d, let-7e, and let-7f for the detection of let-7a. Furthermore, the SDA-based method[28] failed to distinguish let-7a from both let-7c and let-7e. These results are mainly due to the use of miRNA as primer to initiate the amplification reaction, and the homologous sequences with mismatched base in the middle and 5′ terminal can also trigger the amplification reaction. Application of these methods in total RNA samples may cause false-positive results. On the other hand, RCA-based methods[20−22] are of high specificity owing to the ligase-catalyzed ligation using miRNA as template, and they have been widely employed for the analysis of cell RNA extract and serum samples. Unfortunately, the introduction of ligase leads to complicated processes (at least two steps) and lengthy reaction time (over 6 h). Moreover, having the 3′ →5′ proofreading exonuclease activity on ssRNA,[25] phi 29 DNA polymerase used in RCA can hydrolyze target miRNA which may cause the false-negative result. In our strategy, 3-WJ probes were used to greatly improve sequence specificity. MiRNA can interact with 3-WJ primer and 3-WJ template relying on hybridization between short complementary sequences (12 and 10 bp), which was thermosensitive. Even a one-base mismatch could severely disrupt this cooperative interaction no matter where the mismatched base was located. By using let-7 family as models, the highly sensitive mismatch discrimination was successfully demonstrated.

A miRNA detection method with excellent universality for miRNAs in all species and even other small RNAs is significant and promising. Recently, an intriguing discovery has been found that the 3′ end nucleotide of miRNAs in plants is methylated at its 2′-OH.[30] Numerous studies have also proven the fact that other small, noncoding RNAs including small interfering RNA (siRNA) in plants, Piwi-interacting RNA (piRNA) in animals, and both siRNA and piRNA in insects present the same terminal modification as plant miRNAs.[30−32] This modification is regarded to protect these small RNAs from 3′ end uridylation and attack by nuclease, which have profound implications in their cellular function. However, this 2′-O-methyl group makes miRNA low efficiency in the tailing reaction catalyzed by poly(A) polymerase.[34] Some DNA polymerases have been demonstrated to be unable to use RNA containing the 2′-O-methyl group as primer to initiate DNA polymerization.[24,33] In this work, we have demonstrated 2′-O-methylation at 3′end inhibited DNA synthesis. Therefore, lots of detection methods that take miRNA as primer for DNA or RNA synthesis are probably of low efficiency in the analysis of theses modified small RNAs. These methods include EXPAR, RCA, SDA, LAMP, and poly(A) miRNA-based RT-PCR. Oppositely, the 3-WJ primer is used to recognize target miRNA and initiate DNA polymerization in this work. As we have confirmed, the modification in miRNA shows no interference on the signal response with 3-WJ probes, whereas it significantly inhibited the DNA synthesis using duplex template. In theory, the proposed strategy could be applicable in all kinds of small RNAs whether they are modified or not. In addition, the sensing system for let-7b could be readily expanded for other miRNA detection by only changing 3-WJ probes, which is of great convenience and low cost.

High sensitivity is another merit of our method. The sensitivity achieved here is much higher than those of most nonexponential signal amplification-based approaches and is

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even comparable with those of several exponential amplification-assisted assays. By testing the total small RNA extracts from different cell lines, we demonstrated the practicability of the developed method in real complex samples with consistent results obtained by qRT-PCR.

In summary, we proposed a rapid, highly specific, and highly sensitive miRNA detection strategy based on target-triggered 3-WJ structure and ESQM. Combined with 3-WJ template and primer, target miRNA will selectively form the stable 3-WJ structure and trigger ESQM. The introduction of 3-WJ probes can readily distinguish homologous sequences with mismatches in varied sequence locations, which is relying on the thermosensitive hybridization between short oligonucleotides. It also results in the feasible analysis of 3′-terminal modified miRNA such as plant miRNA, owing to the using of 3-WJ primer rather than miRNA as primer for polymerization reaction. Without separation and troublesome procedures, the low detection limit of 2 amol is obtained in a one-step format within 30 min. Total small RNA extracted from cancer cell lines were tested by the proposed strategy, and the results were comparable to those obtained from a commercial qRT-PCR kit. Ascribed to these superiorities, the developed methods for miRNA assay makes a significant contribution to future improvements in biological research and clinical diagnostics.

■ ASSOCIATED CONTENT

Supporting Information

Additional information as noted in the text. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

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Notes

The authors declare no competing financial interest.

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