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Original Article

Quantitative analysis of miRNAs using SplintR ligase-mediated ligation of complementary-pairing probes enhanced by RNase H (SPLICER)-qPCR

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Here, a method using SplintR ligase-mediated ligation of complementary-pairing probes enhanced by RNase H (SPLICER) for miRNAs quantification was established. The strategy has two steps: (1) ligation of two DNA probes specifically hybridize to target miRNA and (2) qPCR amplifying the ligated probe. The miRNA-binding regions of the probes are stem-looped, a motif significantly reduces nonspecific ligation at high ligation temperature (65°C). The ends of the probes are designed complementary to form a paired probe, facilitating the recognition of target miRNAs with low concentrations. RNase H proved to be able to stabilize the heteroduplex formed by the probe and target miRNA, contributing to enhanced sensitivity (limit of detection = 60 copies). High specificity (discriminating homology miRNAs differing only one nucleotide), wide dynamic range (seven orders of magnitude) and ability to accurately detect plant miRNAs (immune to hindrance of 2′-O-methyl moiety) enable SPLICER comparable with the commercially available TaqMan and miRCURY assays. SYBR green I, rather than expensive hydrolysis or locked nucleic acid probes indispensable to TaqMan and miRCURY assays, is adequate for SPLICER. The method was efficient (<1 h), economical ($7 per sample), and robust (able to detect xeno-miRNAs in mammalian bodies), making it a powerful tool for molecular diagnosis and corresponding therapy.

INTRODUCTION

MicroRNAs (miRNAs) are a class of short non-coding RNAs (approximately 19–24 nt), serving as important gene regulators both in animals and plants by pairing to mRNAs to direct their post-transcriptional repression.[1,2] By targeting mRNAs, miRNAs can impact translation, in turn modulate diverse biological processes, including cell differentiation, proliferation, apoptosis, and immune response.[3–6] In addition to their intracellular functions, miRNAs are also discovered with abilities to carry on intercellular communications.[7] Mounting evidence has revealed that the abnormal expression of miRNAs is closely related to many diseases, especially in carcinogenesis and the progression of cancers. Based on this, changes in miRNA expression patterns have been used as biomarkers for diagnosis and prognosis of cancers and other major diseases.[8,9] Recently, cross-kingdom regulatory effects of plant miRNAs have been reported by several independent groups, suggesting that exogenous miRNAs could exert their functions on humans and animals by regulating gene expressions and metabolic processes.[10–13] Therefore, comprehensive profiling and accurate quantification of miRNAs from biomedical samples are essential for understanding their biological roles, as well as for developing rigorous diagnostic methods against various types of diseases.

Northern blotting was first developed to probe miRNAs by hybridizing them with cDNA oligomers labeled by ^{32}P-nucleotides or fluorophores.[14] The method requires large amount of total RNA and has limited sensitivities for miRNAs of low copy numbers.[15] Later, microarrays are fabricated for simultaneously profiling dozens of miRNAs on one chip.[16] However, the desired accuracy is still hard to achieve due to non-specific binding and inadequate S/N ratios when dealing with less-abundant miRNAs.[17] Nanostring nCounter uses enzymatic labeling of the miRNA before detection.[18] The method requires ligation of miRTag oligonucleotide to 3' end of a miRNA to form an RNA/DNA duplex, a process allows multiplex analyzing specific miRNAs by decoding the linear arrangement of different colored fluorescent dyes. However, variation existed in the efficiency of labeling the 3' end of RNA, and quantitative analysis cannot be realized for its poor dynamic range. Overall, these methods are suitable for mining new miRNAs, as to the job of accurate quantification, they are considered incompetent for the biased labeling processes and the limited signal output.[19]

The preferred method for quantitative analysis of nucleic acids is qPCR. Because miRNAs cannot be directly amplified *in vitro* like DNAs using PCR, reverse transcription (RT)-qPCR is the common

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method to quantify miRNAs, which requires the conversion from miRNA to cDNA catalyzed by RT before qPCR reactions. Currently, two strategies are considered valid and generally used in laboratories worldwide. One of them is adding a poly(A) tail to a miRNA to serve as the initial site for RT.[20] Although this strategy is widely applied for commercially available kits (miRCURY platform) provided by Qiagen, it is also unable to distinguish pre-miRNAs from mature miRNAs.[21] Besides, the secondary structures of miRNAs dramatically affect the efficiency of poly(A) polymerase, resulting in biased RTs.[22] In addition, plant miRNAs are subjected to 2′-O-methylation at 3′-terminal during their biogenesis. The moiety imposes steric hindrances on polyadenylation process, decreasing the sensitivity of miRNA quantification.[23] To solve these problems, another strategy using a hairpin RT primer was presented.[24] It features a short 3′ extension complementary to the target miRNA as a primer for cDNA synthesis by RT. The synthesized cDNA, serving as the template for subsequent qPCR, is merely composed of the RT primer and the sequence complementary to target miRNA. Therefore, the length and locations for qPCR primers are limited. Since reverse transcriptases (RTs) cannot withstand elevated temperatures, RT reactions for the two strategies have to be performed at lower temperature (37°C or 42°C), imposing a challenge to distinguish highly homologous miRNAs (differ in one or two nucleotides).[25,26] More important, the miRNA sequence could be degraded by RNase H domain on most RTs, resulting in false-negative results for miRNAs with low abundance.[27]

To overcome this, a series of isothermal amplification techniques free from RTs have been established.[28] These methods seem to be simple, fast, and able to detect miRNAs in a wide dynamic range, but kits based on the isothermal methods are not commercially available so far. In addition, a lack of correlation between reaction speed and analytical sensitivity for isothermal reactions, as pointed out by Prof Ismagilov from the California Institute of Technology, that samples of less miRNA copies could reach signal threshold before can samples of high copy numbers, overshadows the practical applications of the isothermal techniques, especially for real-time monitoring.[29] Recently, methods using droplet digital PCR, phage-mediated counting, and DNA-modified gold coated magnetic nanoparticles were reported to quantify miRNAs.[30–32] Although these methods were accurate and efficient, high costs, complicated operations, and dependence on sophisticated apparatus stand in the way for wide acceptance. While flawed, qPCR-based strategies that balance accuracy and cost remain the first choice of research for miRNA analysis. Kits and solutions for qPCR are now well-developed and commercially available. Thus, efforts to establish ideal strategies for miRNA quantification mainly lie in how to precisely and efficiently convert miRNA to DNA. Compared with RT, which needs multistep enzymatic reactions to catalyze the transition from miRNA to cDNA, ligation can fulfill that in just one reaction by sealing two DNA probes in the presence of target miRNA. As a proof of concept work, Li et al.[33] successfully quantified the miRNAs taking advantage of the ligation by T4 DNA ligase. However, the affinity of the ligase to the heteroduplex formed by target miRNA and DNA probes is low (K_m = approximately 300 nM), which seriously impairs the sensitivity (limit of detection [LOD] = 10^5 copies). The problem remained unsolved until the advent of SplintR ligase, an enzyme cloned from *Chlorella* virus by New England Biolabs, which was ideally suitable for RNA-splinted DNA ligation for its faster maximum turnover rate (>20 folds) and lower K_m (1 nM).[34] Powered by that, Jin et al.[35] developed a two-step strategy by combining miRNA-splinted DNA ligation and qPCR reaction for miRNA detection. The strategy has proved to be robust, only with two flaws: low sensitivity (LOD = 10^3 copies), dependence on expensive double-quenched DNA probes to enhance specificity.

In this work, we presented SplintR ligase-mediated ligation of complementary-pairing probes enhanced by RNase H (SPLICER) for quantitative analysis of miRNAs. The strategy is composed of two steps: (1) ligation of two DNA probes specifically hybrid with target miRNA and (2) qPCR amplifying the ligated DNA probe. The ligation by SplintR ligase is highly specific, as miRNAs with one nucleotide difference can be distinguished. The probes are elaborately designed with the stem-loop motif at the miRNA binding parts to rule out the hybridization with non-target miRNAs. In addition, the ends of the probes are designed complementary. By intermolecular paring, the two probes can form a regional duplex structure, which is conducive to the rapid ligation. Interestingly, RNase H, a ribonuclease that degrades the RNA strand in a DNA/RNA heteroduplex, was found with the ability to stabilize the heteroduplex for ligation, contributing to the enhanced sensitivity at aM level (60 copies). One of the probes is modified with 5′-biotin; therefore, the ligated probe can be isolated using streptavidin magnetic beads (SMBs). This process enriches the ligation products, and eliminates the interference of background molecules that might cause false-positive or -negative results. Quantitative analysis of miRNAs is realized by the established strategy, which also proved robust for detecting xeno-miRNAs in mammalian bodies. Since the ligation step has provided enough specificity, an inexpensive SYBR Green-based qPCR system could be applicable for subsequent qPCR reaction. Compared with the two widely used RT-qPCR systems from Qiagen and Thermo Fisher Scientific, the current method is economical, accurate, and ultrasensitive, making it a promising molecular diagnosis method for miRNA-related researches.

RESULTS

Principle of SPLICER-qPCR

The schema of SPLICER-qPCR can be seen in Figure 1. The probes consist of three parts. One is the stem-looped miRNA binding region for target miRNA recognition. Flanked by that, are sequences for qPCR primers. The ends of the two probes (probe 1 and probe 2) are complementary, which allows them to form a paired probe before ligation. The nick in the heteroduplex consisting of the target miRNA and the paired probe is sealed by SplintR ligase with the help of RNase H. In some application scenarios, the ligated probe can be enriched using the SMBs that bind the biotin moiety tethered on probe 1. Finally, the ligated probes are quantified using SYBR Green-based qPCR

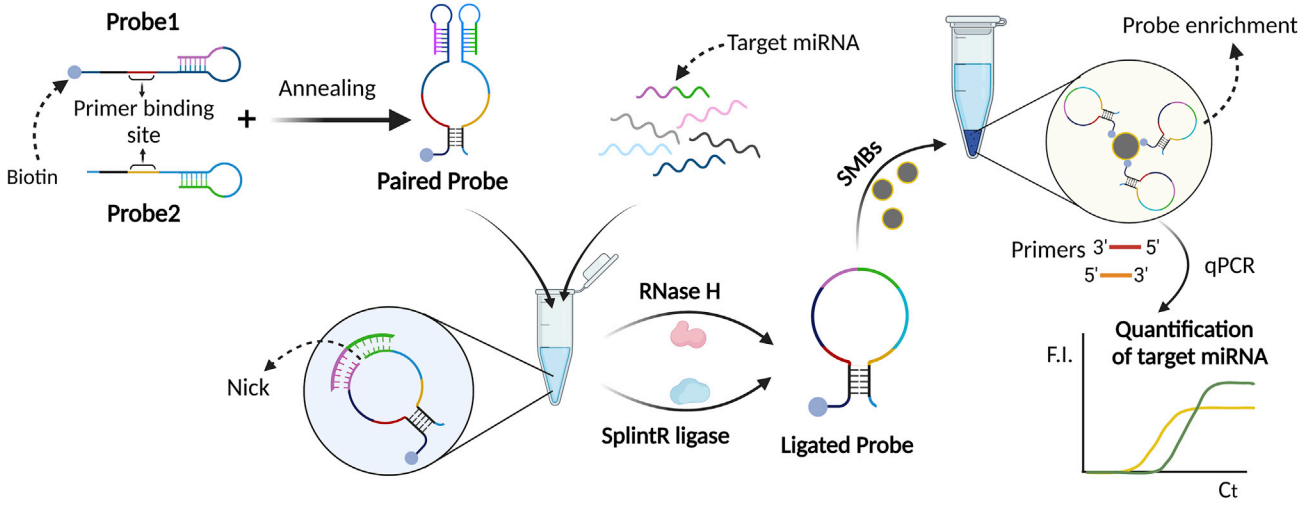

Figure 1. Scheme of SPLICER-qPCR. Probe1 and probe2 are designed regional complementary, so a paired probe could be prepared by annealing the two probes

The target miRNA hybridizes with the paired probe to form a heteroduplex with a nick, which is then ligated by SplintR ligase with the aid of RNase H. SMBs are applied in certain application scenarios to remove the background molecules including the non-target miRNAs. The enriched ligated probes proceed to qPCR reactions to realize the quantification of the target miRNA.

system on a typical qPCR thermocycler. The sequence information of the probes is listed in Table 1.

Probe design for the specificity and sensitivity of ligation

Here, miR159, a plant miRNA discovered with cross-kingdom chemoprevention effects against breast cancer, was selected to validate the method.[11] The process of miRNA recognition was sketched in Figure 2A. The two probes are intramolecularly stem-looped at the ends, which is able to eliminate the bindings from non-target miRNAs, as well as the primaries and the precursors of the target miRNAs. The terminal nucleotide of the stem region was non-complementary, a design augments the effect of DNA breathing to facilitate the fast pairing with target sequences.[36] Only in the presence of miR159, whose sequence is perfectly complementary to the stem parts, can the structure unfold and then form a heteroduplex serving as a substrate for ligation. To investigate the ligation specificity by SplintR ligase, seven variants of miR159 were synthesized (Figure 2B). Previous studies reported that SplintR ligase worked at 37°C.[34,35,37] To improve ligation specificity, we tried to apply it at higher temperature. The ligation was performed at 45°C, 65°C, and 85°C for 5 min, and 1 μg yeast RNA was included to stimulate the complex background of biological samples. Afterward, the products were resolved on denaturing-PAGE gels with 7 M urea. As shown in Figure 2C, substantial ligation products for miR159 were observed at all the tested temperatures, indicating the feasibility of high-temperature ligation by SplintR ligase. Considering the limited sensitivity of electrophoretic analysis, the ligation products were quantified by qPCR. The results showed that more than 5 nM ligation products of miR159 were detected at 45°C and 65°C. In contrast, the ligation products of seven variant miRNAs were less than 100 pM, which is 100-fold lower than that of miR159. With the increase in the ligation temperature (from 45°C to 85°C), products from the variant miRNAs significantly decreased, confirming the fact that elevated temperature is conducive to ligation specificity.[35] When the temperature reached 85°C, products of miR159 was observed with a slight decrease. This could be ascribed to denature of the ligase and dissociation of the heteroduplex at such high temperature. To balance efficiency and specificity, ligation at 65°C was selected for the subsequent assays. Granted, linear probes (without stem-loop structures) can also mediate the miRNA-splinted ligation.[33] Nonetheless, non-specific products were observed when linear probes were applied (Figure S1 and Table S1). One explanation is that a non-target miRNA and the linear probes could form a transient complex during ligation. Although the heteroduplex with mismatches is unstable, the temporary structure could be recognized and connected by ligase, resulting in the non-specific product. In contrast, the miRNA-binding regions here are stem-looped (Figure 2A). The intermolecular pairing precludes the transient binding of non-target miRNAs, while only the target sequence can form a heteroduplex with the probes, a feature that guarantees the high specificity of the ligation reaction.

Next, we evaluated the sensitivity of the strategy using miR159 subjected to gradient dilution. Fixed concentration of the paired probe was used for all the ligation reactions. The added yeast RNA (1 μg), simulating the complexity of biological samples, was more than 20,000-fold to that of the target miRNA (w/w). As shown in Figure 2D (left), as low as 100 aM of miR159 (600 copies in 10 μL ligation system) was detected with fixed 5 μM of the paired probe. The paired probe has significantly enhanced the detection sensitivity, as evidenced by the assay using two separated probes (5 μM each) shown in Figure 2D (right), where the LOD only achieved at 1 fM (6,000 copies in a 10-μL ligation system). The predicted secondary

Table 1. Sequence information of the miRNA and DNA oligomers used for quantification[a]

Name	Sequence (5' → 3')	Length (nt)
bol-miR159	p-UUUGGAUUGAAGGGAGCUCUAm	21
Probe 1	b-AAAAAAAAA*gacgcgtcgctc*GCGGCCG CTTACA***TTCCACGCTGTATTAAAGTC***; GTCGGGAGCCAGGCTATTAC**TAGAG CTCCCT**	86
Probe 2	p-**TCAATCCAAA**GGTATCCAGGGGG ATTGC***AAGTGCGATACGAATAGGC;*** ATTCGAAGCAT*gagcgacgcgtc*ACAT	74
Forward primer	TTCCACGCTGTATTAAAGTC	20
Reverse primer	GCCTATTCGTATCGCACTT	19

p-, phosphorylation modification; m, 2' O-methyl modification at the 3' terminal; b-, biotin moiety tethered on probe 1.

[a]"bol" is the abbreviation of the plant, broccoli (*Brassica oleracea*). The bold nucleotides on denote the regions complementary to the target miR159. Intramolecular hairpin in each probe can be formed by the underlined sequences. The italic lowercase nucleotides on the two probes are complementary, by which the two probes can associate. The two primers are designed to target the bold italic sequences.

structure of the paired probe was shown in Figure S2, whose existence was also validated by PAGE analysis (Figure S3). Placing the two probes in adjacency can guarantee rapid formation of the DNA/miRNA duplex once the target miRNA is captured by one of the probes, contributing to the ultra-sensitive detection of miRNAs with low concentrations. In hope of further improving the sensitivity, ligation reactions with elongated time (10 min and 15 min) were performed. However, both of them failed to detect miR159 lower than 100 aM, and even ligation of 15-min backfired with a LOD of higher than 1 fM (Figure S4). This suggested that short time ligation (5 min) was sufficient for the ligation-mediated strategy; to improve the sensitivity, alternative methodologies should be considered.

RNase H enhances the performance of SPLICER-qPCR

The previous studies suggested that RNase H, a ribonuclease that digests the RNA sequence in a DNA/RNA hybrid, was able to accelerate the formation the heteroduplex under thermodynamic-favored conditions.[38,39] In our previous work, we found that RNase H stabilized RNA/DNA hybridizations even at temperatures above their T_m.[40,41] Inspired by this, we risked an idea of introducing RNase H to the ligation system to see if it could help the paired probe capture target miRNAs. First, ligation containing RNase H and 5 μM of miR159 that can be analyzed by electrophoresis was carried out to provide visualized results. To our relief, RNase H did not diminish the ligation products, as the ligated probe was observed for all the reactions (Figure 3A). By measuring the intensities of the bands, relative amounts of the ligation products were obtained. When RNase H of 0.02 U was introduced, more products were generated than that without the enzyme (0 U). This indicated that a suitable amount of RNase H could facilitate the interaction between the probes and the miRNA, resulting in the increased ligation products. Next, sensitivity of the ligation-mediated strategy with the addition of 0.02 U RNase H (the highest product in Figure 3A) was reevaluated. As shown in Figure 3B, LOD of miR159 was achieved at 10 aM (60 copies in a 10-μL ligation system), with a dynamic range covering seven orders of magnitude (10 pM–10 aM), suggesting that the conducive effect of RNase H was also applicable for miRNAs with low concentrations. The capability of the RNase H-enhanced strategy to discriminate highly homologous miRNA was also tested. These sequences were of high similarity, as some of them (miR159e) only differ by one nucleotide from that of miR159, posing a big challenge for specific ligation and quantification. Concentrations of the miRNAs were 50 pM and the reactions without RNase H were performed in parallel for comparison. As shown in Figure 3C, the strategy demonstrated a good specificity in the presence of RNase H, as the ligation product of miR159 was four orders of magnitudes higher than that of the non-target sequences. miR159-e, the sequence with one mutated nucleotide (Figure 2B), merely delivered product of 6.7 fM, less than 1/1,000 of that of miR159. Interestingly, RNase H significantly increased the ligation product of miR159 ($p < 0.01$), leaving the products of non-target sequences (miR159-a-miR159-g) unaffected. This indicates that RNase H selectively recognizes the perfectly matched DNA/miRNA duplex, a process that can promote the specific ligation by SplintR ligase, and, therefore, eliminates false-positive results from miRNAs of high similarity.

Quantification of plant miRNAs by SPLICER-qPCR

The levels of plant-derived miRNAs (miR156, miR159, miR168a) in fresh broccoli were analyzed by the SPLICER-qPCR (referred to as SPLICER in the following) to evaluate its performance in practical applications. The sequence information can be found in Table 1 and Table S3, and the concentrations of the extracted RNAs are shown in Table S4. For comparison, Qiagen's miRCURY qPCR system and Thermo Fisher's TaqMan Assays (referred to as miRCURY and TaqMan in the following) were applied. Aliquots from the same total RNA were used as samples for the three methods. Each sample was tested in triplicate at the cDNA synthesis step to assess the repeatability. The results were expressed as quantification cycle (C_q) values and standard deviations shown in Figure 4A. The three miRNAs were determined as high (miR156), moderate (miR168a), and low (miR159) levels by SPLICER, in accordance with the results from miRCURY and TaqMan. Low standard deviations were observed for all the three methods, suggesting that the repeatability of SPLICER was comparable with that of miRCURY and TaqMan. Furthermore, relative quantifications of the three miRNAs were performed by SPLICER, using miR2911 as a spike-in (Figure S5). Relative levels of the miRNAs were obtained by normalizing their C_q values to that of miR2911. The expression profile agreed with that in Figure 4A, indicative of the practicability of SPLICER for relative quantification.

To compare the sensitivities of the three methods, the total RNA was subjected to gradient dilution, and miR159 levels in the diluted solutions were analyzed. As shown in Figure 4B, SPLICER was more sensitive than miRCURY and TaqMan at all the tested concentrations. SPLICER was able to detect the presence of miR159 in the samples with 1,000-fold dilution, while both miRCURY and TaqMan only achieved the detection in the 10-fold diluted samples. The superior sensitivity of SPLICER can be ascribed to the fact that ligation is

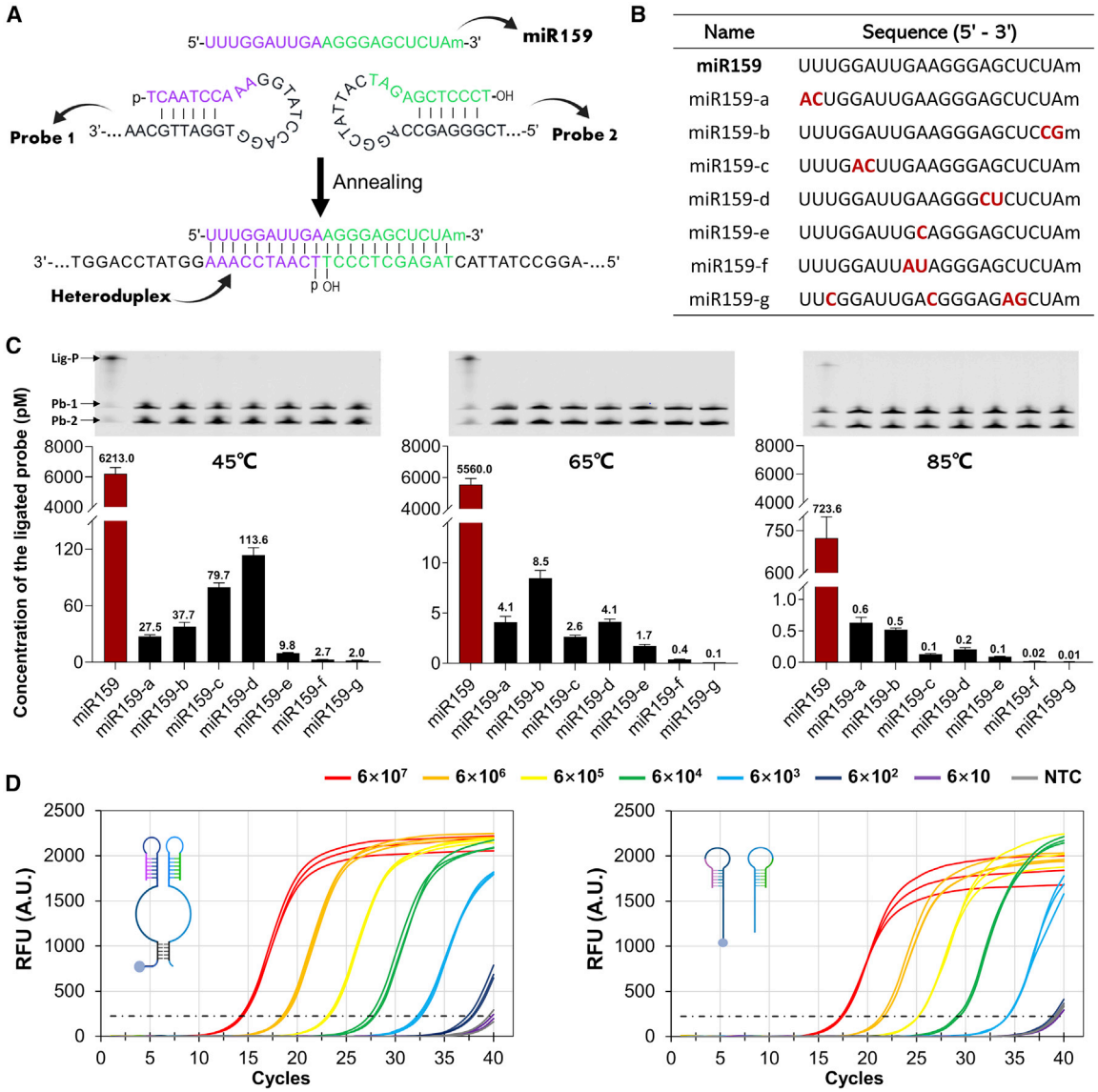

Figure 2. Quantitative detection of miRNA159 by the ligation-based method

(A) Schematic binding process of miR159 and two specific probes. (B) Sequence of miR159 and its variants. The mutated nucleotides from miR159 were marked in red. "m" indicates 2′-O-methyl modification. (C) Specificity of the miR159-splinted ligation at different temperatures. Ligation reactions were performed using probe 1 and probe 2 that exclusively target miR159 sequence. Urea denaturing PAGE (top) and qPCR (bottom) were employed to analyze the ligation products. "Lig-P," "Pb-1," and "Pb-2" denote ligated probe, probe 1, and probe 2. The ligation system contains 2 μL miRNA (5 μM), 3 μL the probes (5 μM each), 1 μL yeast RNA (1 μg/μL), 1 μL 10× SplintR buffer, and 2.5 μL RNase-free water. The solution was incubated at 95°C for 5 min and slowly cooled down to the ligation temperature, followed by the addition of SplintR ligase (0.5 μL) and incubated for 5 min at the designated temperatures. Results of three independent reactions were summarized and shown in the bar charts. (D) Sensitivities of the ligation-based method using the paired (left) and the separated probes (right). Probe 1 and probe 2 were annealed beforehand to form the paired probe by complementary sequences. Gradient diluted miR159 solutions (6×10^7–6×10 copies) added with yeast RNA served as the templated for quantification, and each sample was tested in triplicate. The ligation system was as described in (C), and the reactions were performed at 65°C for 5 min, followed by ligase inactivation and qPCR as described in the Materials and methods section.

more efficient than RT for cDNA generation, since only one chemical reaction (linking the two probes) is needed to finish the conversion from miRNA to cDNA.[35,42] Next, the specificity of the three methods was evaluated by a spike-in assay using miR159-e, a similar miRNA differing one nucleotide from miR159. Certain amount of miR159-e was added to the 1,000-fold diluted total RNA to guarantee its concentration was 1 pM. Aliquots of the prepared solution were analyzed by the methods. As shown in Figure 4C, the solutions spiked with miR159-e failed to generate detectable signals for SPLICER, indicative of its high specificity to discriminate highly homologous sequences in

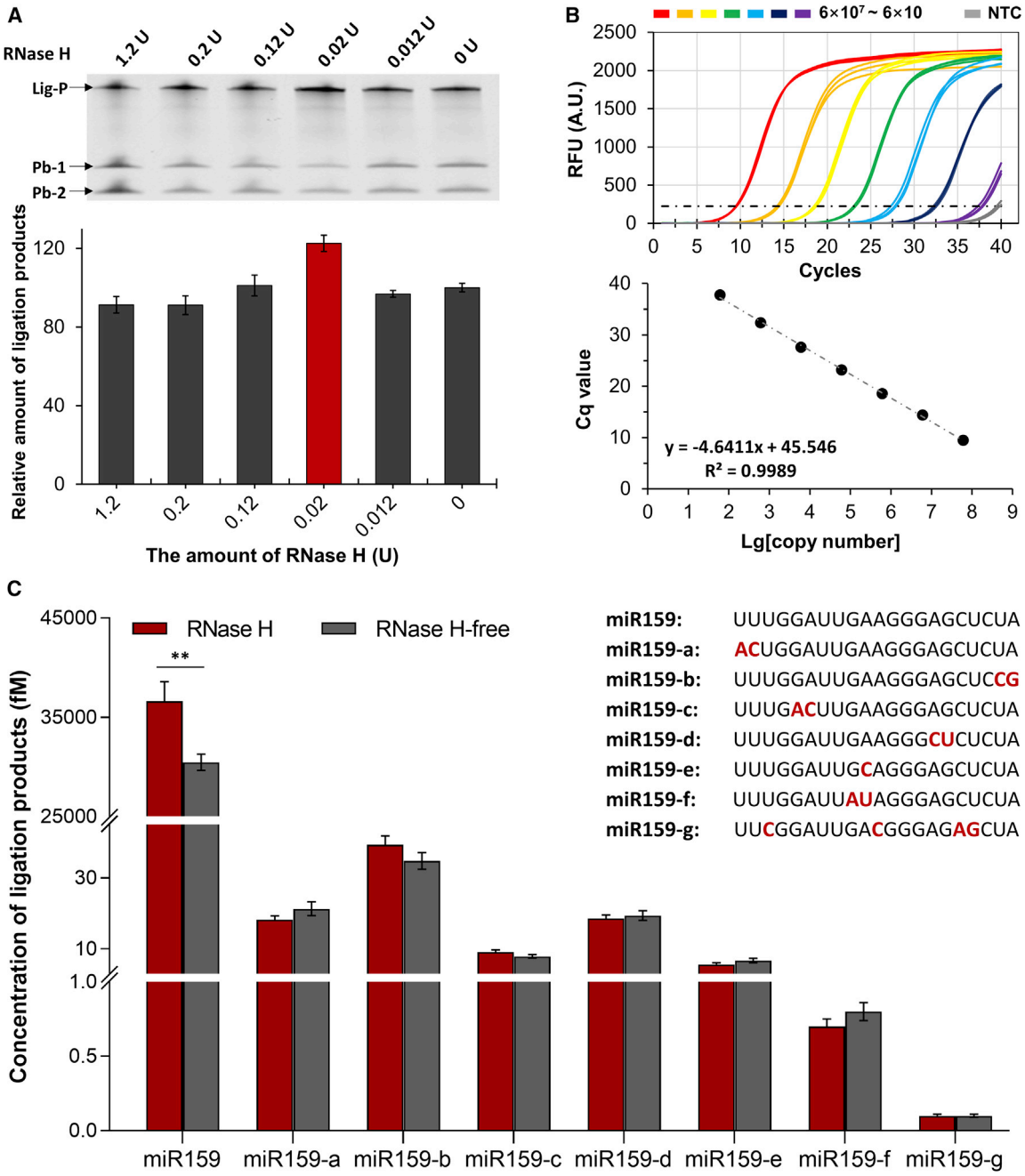

Figure 3. Addition of RNase H improves the sensitivity and the specificity of SPLICER-qPCR

(A) Dosage effect of Rnase H on the ligation products. Rnase H varying in amount (1.2–0.012 U) was introduced to the ligation system. The products were analyzed by urea denaturing PAGE (top), and their relative amounts (bottom) were determined by comparing with that of ligation without Rnase H (0 U). "Lig-P," "Pb-1," and "Pb-2" denote ligated probe, probe 1 and probe 2. The ligation system was firstly set up including: 2 μL miRNA (5 μM), 3 μL paired probes (5 μM each), 1 μL yeast RNA (1 μg/μL), and 1 μL 10× SplintR buffer. The mixture was heated at 95°C for 5 min and cooled down to 65°C, before the addition of 2.5 μL RNase H (varying in amount) and 0.5 μL SplintR ligase. The ligation was for 5 min, and terminated by incubation at 95°C for 5 min. (B) Sensitivity of SPLICER-qPCR for quantitatively detecting miR159 in the presence of RNase H. RNase H of 0.02 U was included in ligation of miR159 varying in concentration. Each sample was tested in triplicate, and the mean values were used to plot the standard curve. (C) Effects of RNase H on the specificity of SPLICER-qPCR. The paired probe targeting miR159 was used in ligation reactions for miR159 and its variants (mutated nucleotides marked in red). For ligations with RNase H, 0.02 U was applied. The amount ligation products were determined by qPCR. Results of three independent reactions were summarized and shown in the bar charts. "**" represents an extremely significant difference with $p < 0.01$.

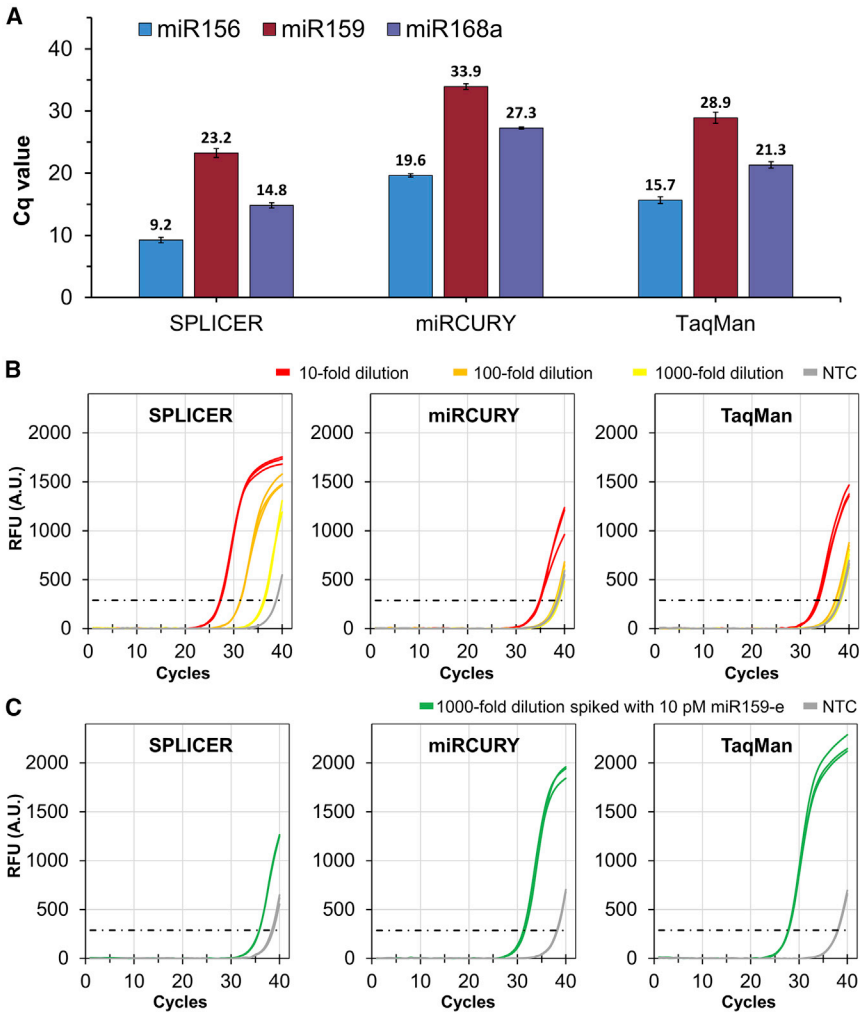

Figure 4. Detection of the plant-derived miRNAs using SPLICER, miRCURY and TaqMan

(A) Quantitative detection of miR156, miR159, and miR168a from broccoli. The total RNA was extracted from fresh broccoli, and aliquots from the same solution were analyzed by the three methods. Results of three independent reactions were summarized and shown in the bar charts. (B) Sensitivities of the three methods to detect the plant-derived miR159 from the diluted RNA extracts. The total RNA extracts subjected to 10 times gradient dilution were used as templates to quantify miR159. Each sample was tested in triplicate. (C) Specific detection of the plant miR159 accompanied with high concentration of miR159-e. Probes and primers of three methods were specific to miR159. miR159-e, which has one mutated nucleotide from miR159, was used as a highly similar sequence to evaluate detection specificity. Each sample was tested in triplicate. Sequence information: miR159 (5′-UUUGGAUUGAAGGGAGCUCUA-3′), miR159-e (5′-UUUGGAUUGCAGGGAGCUCUA-3′), the underlined letter indicates the mutated nucleotide.

biological samples. In contrast, false-positive results were observed for miRCURY and TaqMan, as C_q values of miR159-e were determined as 31.4 and 28.6, respectively, distinctive from that of non-template controls (NTCs). The discrimination ability of SPLICER lies in three aspects: (1) ligation at elevated temperature (65°C) where hybrid formation between probes and mismatched-miRNAs is not allowed, (2) stem-loop structures on the probes eliminate binding of non-target miRNA, and (3) RNase H selectively stabilizes the perfectly matched DNA/miRNA duplexes.

Detection of miRNAs from mouse tissues

The performance of SPLICER to analyze animal miRNAs was also assessed by detecting miR-122, miR-24, and let-7a from different mouse tissues. Probes and primers used for the assays were listed in Table S3. Again, miRCURY and TaqMan were used for comparison. The total RNAs were extracted from brain, heart, liver, lung, kidney, and muscle by the TRIzol-based assay, and their concentrations were shown in Table S4. Aliquots of the total RNAs were analyzed by the three methods respectively. Relative expression levels of the three miRNAs across the six tissues were expressed as the fold-changes calculated from the C_q values. The lowest expression of a miRNA in a specific tissue was set as base level. As shown in Figure 5A, the presence of the miRNAs was detected by the all three methods. The fold-changes of miR-122 and miR-24 determined by SPLICER were consistent with that by miRCURY and TaqMan, demonstrating that SPLICER is reliable to detect miRNA levels in experimental animals. As revealed by SPLICER, Let-7a was highly expressed in heart, miR-122 was found with good accumulation in liver, and miR-24 was observed with moderate expressions across the six tissues, except for liver. This agreed with previous reports demonstrating that expression profiles of miRNAs varied in different tissues and organs.[43–45] In addition, SPLICER proved to be able to differentiate between precursor miRNA and mature miRNA. When the same amount of the two oligomers were quantified, the C_q value of let-7a was 13.31 cycles lower than that of pre-let-7a (Figure S6). This indicated that pre-let-7a contributed less than 0.01% background signal when coexisted with equal mole of let-7a, the mature miRNA sequence.

Correlations between the results obtained from the three methods were plotted based on ΔC_q of the miRNAs. The ΔC_q of a given miRNA was calculated by subtracting the C_q value of a tissue from that of the tissue where the lowest expression was observed. SPLICER was observed with good correlations with both miRCURY and TaqMan (Figure 5B), as evidenced by the high R values ($R_{SPLICER\text{-}miRCURY}$ = 0.9648; $R_{SPLICER\text{-}TaqMan}$ = 0.9585). This suggested that profiling of the miRNAs was not method specific; thus, the relative expression of miRNAs can be accurately determined by any of them. Given that the quantification

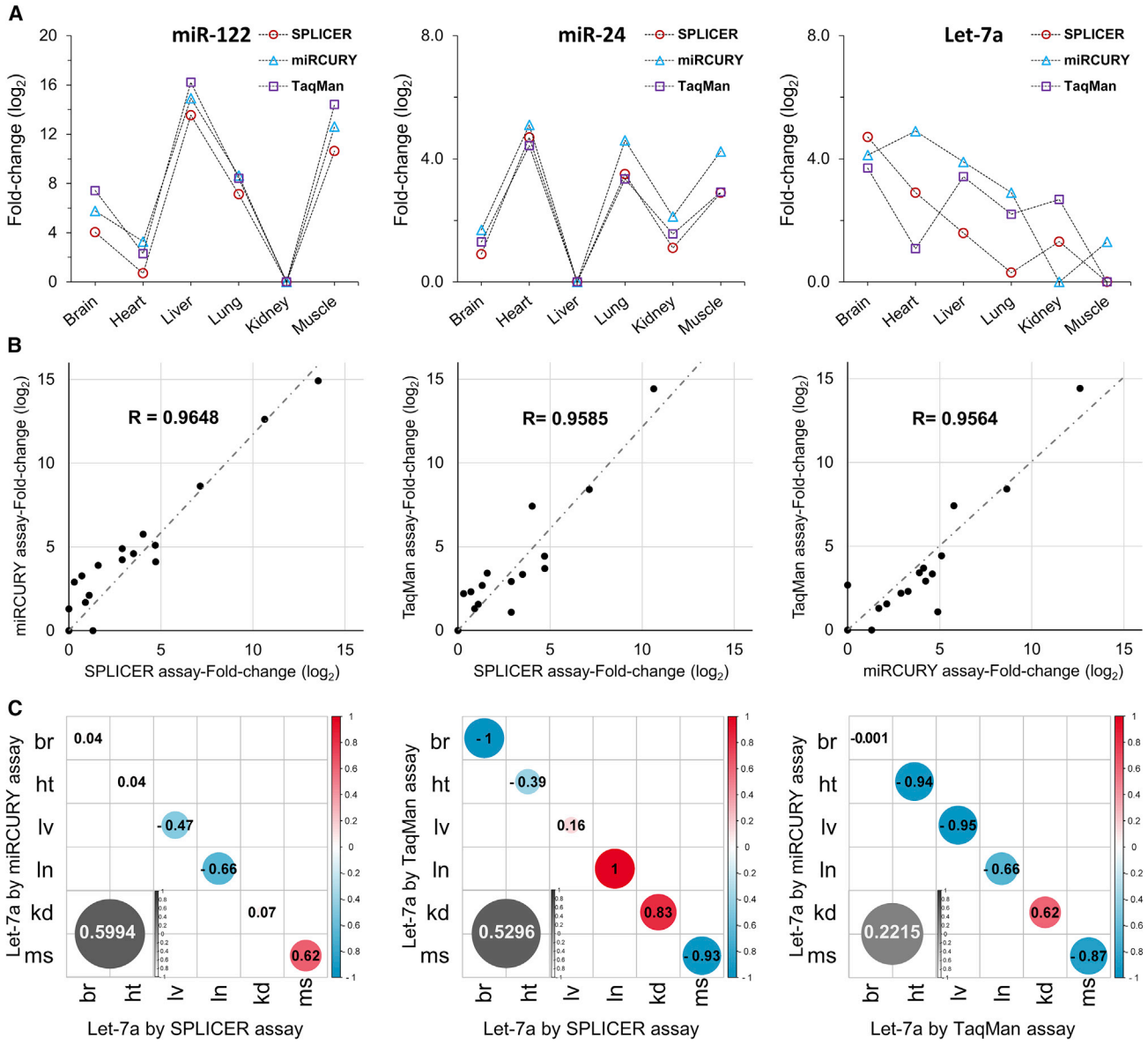

Figure 5. Analysis of miRNAs from different mouse tissues using SPLICER, miRCURY, and TaqMan

(A) Relative expression of miR-122, miR-24 and Let-7a in mouse tissues as quantified by the three methods. The fold-change of a miRNA determined by one method was calculated based on the C_q values, using the tissue that has the lowest level of the miRNA as base level. Each sample was tested in triplicate, and the mean values were used for plotting. (B) Correlations between the quantitative results from the three methods. Pearson correlation coefficient (R) was used to evaluate the relevance between any two methods. The linear regression curves based on the bivariate distribution were expressed as the gray dotted lines. (C) Correlations between quantification results of Let-7a from different mouse tissues. The total correlation coefficients of Let-7a were expressed by gray circles varying in hue. Red, white, and blue circles indicate positive, weak, and negative correlations, respectively.

results of Let-7a varied appreciably among different strategies (Figure 5A), to further interpret this, the results of Let-7a were dissected. As can be seen in Figure 5C, low correlations were observed for let-7a ($R_{SPLICER\text{-}miRCURY}$ = 0.5994; $R_{PLICER\text{-}TaqMan}$ = 0.5296; $R_{TaqMan\text{-}miRCURY}$ = 0.2215), indicating that let-7a was primarily responsible for the inconsistence between the strategies. Unlike miR-122-5p and miR-24-5p (quantification results were consistent among the three methods), let-7a-5p is a member of the let-7 family that includes 21 homologous sequences.[46] Some of them, for example, let-7c-5p, let-7e-5p, and let-7f-5p, only differ one nucleotide from that of let-7a-5p (Table S5). Measuring a miRNA that has close siblings is more complicated in terms of specificity. The three methods use different means to discriminate non-specific sequences. This could account for significant variations in the results of measurements and, as

a consequence, low correlations for the miRNA of many homologous sequences. The relative quantification of miR-24 was performed by using miR-192 as the endogenous control, based on which the qPCR results of miR-24 were normalized (Figure S7). The expression profile of miR-24 across six tissues was consistent with that shown in Figure 5A, demonstrating the applicability of SPLICER for relative quantification.

Quantitative detection of exogenous plant miRNA in animal circulation

In the recent decade, plant miRNAs were discovered with regulatory effects on mammalian bodies. Although it was controversial, more and more studies supporting the cross-kingdom functions of exogenous miRNAs were reported. To further elucidate this, the ability to precisely reflect the level of exogenous miRNA in animal or human circulation is necessary. Hence, analysis of miR159 (a plant-specific miRNA) in rat peripheral circulation by SPLICER was conducted, while using miRCURY and TaqMan for comparison. The miR159 oligomer was introduced to rat circulation by intravenous injection on tail. The peripheral blood was then collected at different time points, followed by the miRNA quantification. Since one of the probes for SPLICER was modified by a biotin moiety, SMBs could be applied here to eliminate massive background biomolecules from rat blood. As shown in Figure 6A, SPLICER powered by SMBs was able to detect the presence of plant miR159 in rat at the four time points. According to the standard curve (Figure 6B), absolute amounts of miR159 were obtained. The level of the plant miRNA dramatically decreased with the time course (Figure 6C). However, a certain amount of miR159 (1.5 fM) survived after being in rat blood for 4 h. This agreed with the previous reports that miR159 was able to withstand RNase digestion under unfavorable oral and gastrointestinal conditions.[47,48] The quantification of miR159 by miRCURY was undesirable in terms of sensitivity and dynamic range. The method failed to detect the presence of miR159 after the injection for 2 h (Figure 6A), and the valid dynamic range (R^2 = 0.9871) only covered five orders of magnitude. The poor performance could be ascribed to 2′-O-methyl group on 3′ terminal of plant miRNA catalyzed by (HEN1), a small RNA methyltransferase.[49] The modification inhibits the addition of nucleotides to the 3′ end of miRNA by polyadenylate polymerase, resulting in the limited sensitivity. This is consistent with the previous study suggesting that the polyadenylation-based qPCR methods are not ideal solutions for plant miRNA quantification.[50] TaqMan assayed here was able to detect the presence of miR159 for 4 h, achieving the same LOD with that of SPLICER. Yet its dynamic range was limited, suggesting the inadequacy of the method for plant miRNAs with low abundance. In addition, the C_q value of miR159 at 4 h was close to that of NTC (ΔC_q = 1.24), and all the C_q values were higher than that by SPLICER. The delayed signals may be caused by the inhibitory factors from biological samples and the chemical reagents used for RNA extraction, which would increase the tendency of false-negative results for plant miRNA characterization using TaqMan assays.

DISCUSSION

The precise quantification of miRNAs has long been a challenging work, given their short length, high sequence similarity, and variable modifications.[25] Apart from that, the complexity of biological samples where miRNAs are surrounded by tremendous macromolecules imposes substantial inhibitions and interferences on the quantitative analysis.[51] In this study, a ligation-based quantification method named SPLICER-qPCR (SPLICER) was established, which has been demonstrated with great specificity and sensitivity for quantitively analyzing miRNAs from both animal and plant specimens. Like most analytical methods for miRNAs, SPLICER consists of two steps: first, converting the presence of target miRNAs into cDNAs, and second, amplifying the cDNAs in a quantitative manner. One of the advantages of SPLICER is using ligation rather than RT to synthesize the cDNA. The RT step, although required as the essential information by MIQE guidelines, is generally considered as the most problematic step for miRNA quantification, since it could be easily affected by RNA input, priming strategy, and background nucleic acids in samples.[52–54] In addition, the choice of RT can dramatically affect quantification results, making it hard to compare results obtained from different studies.[55,56] For ligation, the transformation from miRNA to cDNA can be realized only by one reaction, that is linking the phosphodiester bond between probe 1 and probe 2 (Figure 1). Probes with fixed concentrations proved to be capable of detecting miRNAs that drastically vary in abundance (Figures 2D and 3B). When dealing with miRNAs with extremely high abundance (which is quite rare), diluting the sample could be applied. For target miRNAs with a low abundance, the high concentrations of the probes are conducive to recognising and capturing the miRNA sequences, thus guaranteeing the sensitivity of SPLICER. Another strength of the method is the use of SMBs, which can eliminate the background interference and facilitate cDNA probes enrichment, thus contributing to the enhanced sensitivity for the miRNA quantification (Figure 6).

qPCR is the most reliable and precise technique to determine the absolute copy numbers of DNAs and RNAs so far. It requires the amplification of the target sequence to record the fluorescence signal directly proportional to the amount of target sequences. However, a miRNA cannot be directly amplified because of the RNA nature, as well as its short length that renders primer design targeting its cDNA without overlap impossible.[57] In SPLICER, the problem was solved by transforming the presence of a miRNA into the ligated paired probe in a one-to-one fashion (Figure 2A). The design of primers is made easier since the length and the sequence of the probes can be freely adjusted. The probes used in SPLICER are stem-looped. Granted, linear probes (without stem-loop structures) can also mediate the miRNA-splinted ligation.[33] Nonetheless, non-specific products were observed when linear probes were applied (Figure S1, Table S1). One explanation is that a non-target miRNA and the linear probes could form a transient complex during ligation. Although the heteroduplex with mismatches is unstable, the temporary structure could still be recognized and connected by ligase, resulting in the non-specific product.[58] In contrast, the miRNA-binding regions on the two probes used in SPLICER are stem-looped (Figure 2A). The intermolecular pairing precludes the transient binding of non-target miRNAs, only target sequence can form a heteroduplex with the probes, a feature that guarantees the high specificity of the ligation reaction. In addition, the paired probe containing two

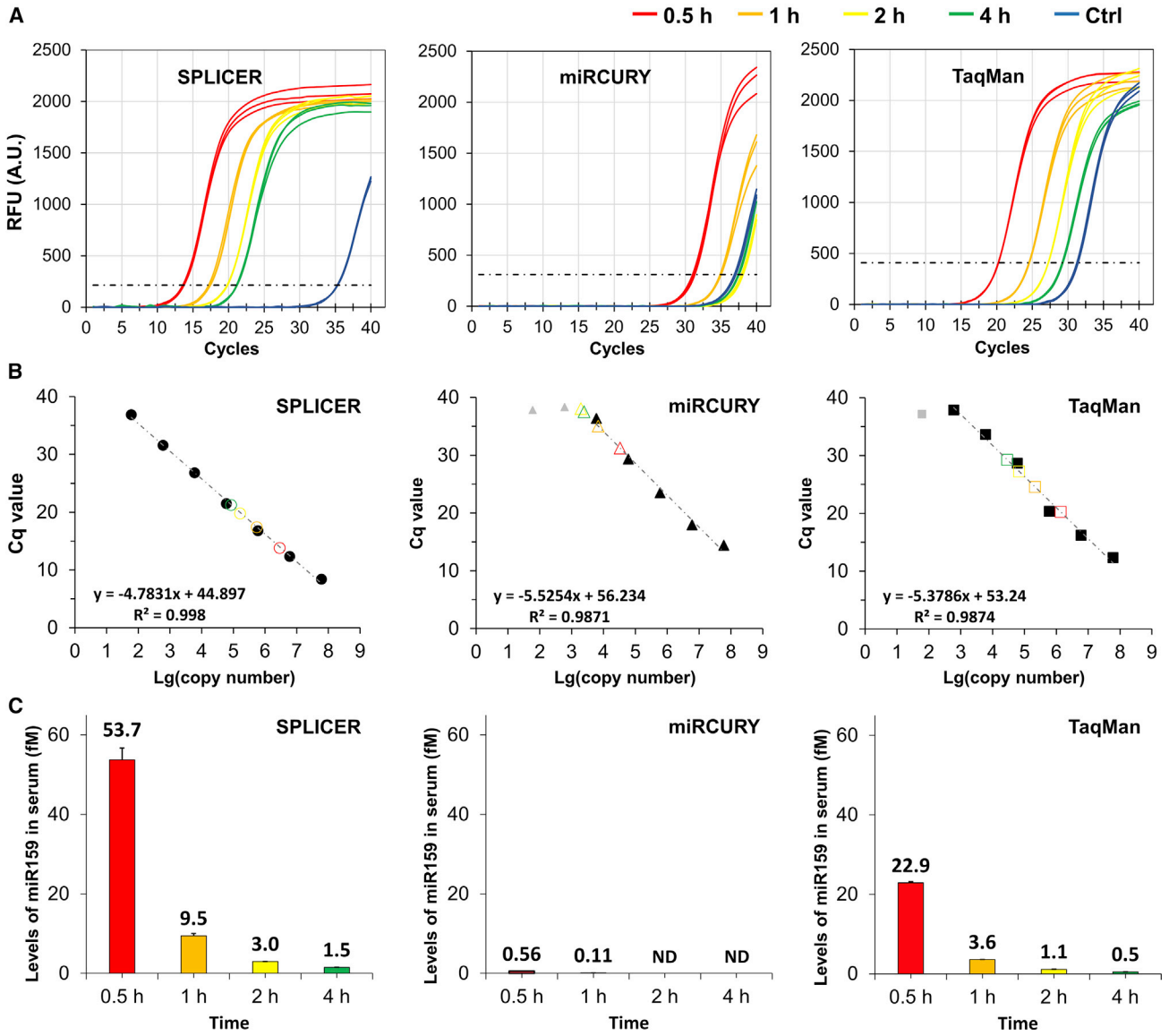

Figure 6. Quantitative detection of exogenous miR159 in rat circulation by SPLICER, miRCURY, and TaqMan

(A) Time course amplification curves of miR159 in rat peripheral blood introduced by tail vein injection. (B) Dynamic ranges and standard curves of miR159 from the three methods. Data points considered as outliers from dynamic range were marked in gray. (C) Levels of exogenous miR159 in rat serum at 0.5, 1, 2, and 4 h after the tail vein injection. Data were obtained based on the C_q values and standard curves presented in (B). ND, not detected. Each sample was tested in triplicate, and the mean values and the standard deviations were used for plotting the bar charts. Operation of SPLICER was as follows. The ligation system: 2 μL template miRNA, 3 μL mixed probe 1, probe 2 (5 μM each), 1 μL yeast RNA (1 μg/μL), and 1 μL 10× SplintR buffer. The mixture was heated at 95°C for 5 min and slowly cooled down to 65°C, followed by adding RNase H (2.5 μL) and SplintR ligase (0.5 μL), and incubation at 65°C for 5 min. The ligation reaction was terminated at 95°C for 5 min before the qPCR reaction as detailed in the Materials and methods section.

probes with complementary ends is applied. The high sensitivity was achieved by using the special design, as evidenced by the low LOD for miRNA quantification (Figures 2D and 3B). In some cases, the term limit of quantification (LOQ) is used instead of LOD to describe the lowest amount of measurand in a sample that can be quantitatively determined.[59] Here, the two terms denote the same substance, that is, the lowest copy number of a miRNA that can be detected by given strategy for the purpose of quantification. In theory, miRNAs with copy numbers lower than the LOD cannot be detected. In practice, detections of miRNAs below the LOD are considered with low credibility and not recommended as valid data for quantification. The statistics definition of the LOD is a parameter with probability, which means sometimes no exact value of the LOD is obtained. In experimental and clinical scenarios, the LOD and LOQ are interchangeable. For

Table 2. Comparison of the three methods for miRNA quantification[a]

Name	cDNA synthesis strategy	Sensitivity	Dynamic range	Melting curve analysis	Exogenous plant miRNA quantification	Analysis duration	Cost/sample
TaqMan	Hairpin RT primer	10^2–10^3	5–6 logs	no	Acceptable	1.5 h	$16
miRCURY	Poly(A) tailing	10^2–10^3	5–6 logs	no	Not recommended	>2 h	$32
SPLICER	probe ligation	10–10^2	6–7 logs	yes	Suitable	<1 h	$7

[a]All the information was gathered from previous reports and the results from the current study. Analysis duration was the period from extracted RNAs to qPCR results. Cost per sample was calculated by all the reagents or kits used in the RT steps and the qPCR reactions.

mechanism studies using mathematical approaches, the LOQ is recommended.

Plant miRNAs are subjected to 2′-O-methylation at 3′ terminals during their biogenesis.[60] The presence of the modification could inhibit the progress of polyadenylation that is the prerequisite for all the poly(A)-tailed RT-qPCR protocols (e.g., miRCURY, Qiagen), resulting in the impaired sensitivity as reported previously.[50] In SPLICER, the ligation takes place in the middle of miRNA sequences, immune to the negative effects caused by the 2′-O-methyl group. The ligation system of SPLICER is simple, and its volume is small. All of the 10-μL ligation products can be used as the template for qPCR reaction, contributing to high sensitivity when dealing with miRNA of low copies. In contrast, when using TaqMan and miRCURY, only portions of cDNA products are allowed to proceed to qPCR, which could cause false-negative results. This stands a good chance of false negative results for less-abundant miRNAs. Given the fact that miRNAs are short in length (average 22 nt), the only way to achieve high selectivity is to use identity information of a miRNA sequence as much as possible.[61] In TaqMan and other protocols using a hairpin RT primer, only a part of a miRNA sequence (7–9 nt) was involved in target recognition, which could result in tremendous non-target cDNA products. As to miRCURY and the related methods that polyadenylate miRNAs for quantification, the RT step provides no selectivity because all the RNAs in one sample are transcribed into cDNAs.[62] Therefore, the SYBR Green-based miRCURY kits, which are less expensive, are not recommended by the vendor on account of the limited specificity. To guarantee the specificity of quantification, both TaqMan and miRCURY apply the hydrolytic probe (a short cDNA contains fluorophore and quencher) in their qPCR reactions. The probes are not only expensive, but also make monitoring the specificity of the qPCR reaction by melting curve analysis impossible.[57] In SPLICER presented in this study, all the nucleotides perfectly complementary to the probes are participating in the specific ligation (Figure 2A), which was able to discriminate the non-target sequence with one mutated nucleotide (miR159-e), as evidenced by the results in Figure 2C. Since the ligation has provided enough specificity, a simple SYBR Green-based qPCR system is competent here for accurate miRNA quantification. The costs of the three methods were compared in Table 2. SPLICER is able to deliver results within 1 h using less than $7. In contrast, cost per sample is doubled ($15.90, TaqMan) and even quadrupled ($32.30, miRCURY), because of the expensive probes they applied (miRNA-specific TaqMan MGB probe, locked nucleic acid [LNA] hydrolysis probe).

The SplintR ligase used in SPLICER is a newly developed DNA ligase that catalyzes the ligation of two DNA strands splinted by an RNA oligomer.[34] According to the user manual, the optimal temperature for SplintR ligase was 25°C. Here, we applied it at 65°C for ligation. One may doubt that the ligase was deactivated at such high temperature. In fact, the perfectly matched RNA/DNA duplex (consisting of the target miRNA and the probes) could be rapidly recognized and ligated by the ligase in a very short time, long before the ligase reaches its half-life and loses all of its activity.[63] Meanwhile, duplexes formed by non-target miRNAs and the probes become extremely unstable at such a high temperature, decreasing their chances of successful ligation, resulting in the enhanced specificity as demonstrated in Figure 2C. The SPLICER established in this study has also proved with the ability to detect isomiRs (Figure S8). Six miR-21 isoforms with length 1–2 nt longer or shorter at the 3' or 5' ends than the canonical miR-21 sequence were detected by SPLICER. Except for the two short isoform sequences, the C_q values of the isomiRs were statistically equal to that of miR-21, suggesting that SPLICER was able to profile full isomiR family that execute similar biological functions with appreciable significance.[64]

One of the novelties of SPLICER is using RNase H to facilitate the ligation of the probes mediated by target miRNA. The nuclease was introduced to the ligation system and incubated at 65°C, much higher than 37°C, its optimal temperature. And the results showed that it has conducive effects on the quantification sensitivity (Figure 2D), without lowering the specificity (Figure 3C). The crystal structure of RNase H revealed that the nuclease has both binding domain and catalytic domain.[65] The former is responsible for recognizing RNA/DNA duplex, while the latter degrades the RNA strand in a non-specific manner.[66] The catalytic domain is susceptible to the change of environment as reported previously.[63,67] Therefore, the high temperature (65°C) used in this study could attenuate its hydrolytic activity, while the binding domain is still functional, which stabilizes the heteroduplex consisting of miRNA and two probes, contributing to the enhanced detection sensitivity (Figure 4B). RNase H activity is an integral part of RTs. During cDNA synthesis, RNase H domain removes RNA template, followed by the polymerization of plus DNA strand to form duplex DNA.[68] Hence, the accuracy of RNA quantification could be impaired as a result of the RNase H activity. AMV, a commercially available RT product with relatively high RNase H activity, was reported with low cDNA yield and limited sensitivity.[55] In contrast, RNase H is a favorable component for the performance of SPLICER-qPCR, mainly because the ligation reaction

is simple and can be finished in one step. In contrast, the process of RT is complicated, involving multiple steps that take a long time. This gives RNase H domain the opportunity to degrade RNA templates, leading to the variation of results obtained from the RT-based methodologies.

Target miRNA in the RNA extraction coexists with massive background molecules. These molecules are reagents used for the RNA extraction and co-purified components from the biological samples. Their presence frequently inhibits the subsequent qPCR reaction, resulting in an unacceptable LOD.[69] SMBs were used here to help detect the plant miRNA in rat circulation. One of the SPLICER probes is tethered with biotin, by which the ligated probe can bind to the SMBs and being isolated using magnetic separation, a process that removes the background molecules from the qPCR system, leading to the wide dynamic range of SPLICER as shown in Figure 6B. As to TaqMan, biotin moiety cannot be tethered on the RT primer for its stem-looped structure, rendering the use of SMBs impossible. In the miRCURY system, although the poly(T) RT primer can be biotinylated, magnetic separation using SMBs cannot eliminate the non-target cDNA sequences, because all the RNA molecules are non-selectively reverse transcribed into cDNAs. In other words, the RT step of the miRCURY system does not provide any specificity, even when a biotinylated RT primer is used. The unique probe structure of SPLICER enables it to introduce SMBs when target miRNAs are accompanied with tremendous biomolecules. If the background interference from samples is not serious, SMBs are not used to decrease costs.

Conclusion

SPLICER-qPCR was established in this study to quantitatively analyze miRNAs in biological samples. The method was demonstrated with high specificity (discriminating homology miRNAs differing only one nucleotide), good sensitivity (LOD of 60 copies), and wide dynamic range (covering seven orders of magnitude). Ingenious probe design, high ligation temperature, and introduction of RNase H account for the robustness of SPLICER-qPCR, which is especially suitable for detecting exogenous plant miRNAs in animal tissues as powered by SMBs. Owing to complete ligation of two DNA probes within 10 min, the whole process can be finished less than 1 h. Besides, SYBR Green I, rather than expensive TaqMan or LNA probes that are integral parts of TaqMan or miRCURY assay, was sufficient for miRNA quantification by SPLICER-qPCR. These two merits significantly increase the efficiency and decrease the cost of the method, paving the way for its widespread application. By using a better RNA extraction method and more powerful qPCR system, greater performance and high-throughput analysis can be expected from SPLICER-qPCR. The related works are now in progress.

MATERIALS AND METHODS

Oligomers and reagents

The miRNA oligomers were ordered from Genscript Biological Technology Co. Ltd., and the DNA oligomers used as probes or primers were synthesized by Sangon Biotech Co. Ltd. under RNase-free conditions. SplintR ligase and SMBs were provided by New England Biolabs. RNase H, SYBR Green I Nucleic Acid Gel Stain and Yeast RNA (10 μg/μL) were purchased from Life Technologies. TB Green Fast qPCR Mix was obtained from Takara Bio.

TaqMan MicroRNA RT Kit (Catalog no. 4366596), TaqMan miRNA Assays (Catalog no. 4440886), and TaqMan Universal Master Mix II (Catalog no. 4440040) were provided by Thermo Fisher Scientific. The assay ID of TaqMan miRNA Assays were bgy-miR156 (471769_mat), bol-miR159 (000338), osa-miR168a (007594_mat), mmu-miR-122 (002245), mmu-miR-24 (000488), and mmu-let-7a (000377).

The miRCURY LNA RT Kit (Catalog no. 339340) for RT on miRNAs, miRCURY LNA probe PCR Kit (Catalog no. 339371) for qPCR reaction, and miRCURY LNA miRNA PCR Assay (Catalog no. 339306) for specific primer, were purchased from Qiagen. All the three kits were used in one experiment to realize the quantification of miRNAs. The universal poly(T) primer was included in the miRCURY LNA Probe PCR Kit, while the forward specific primers were provided by the miRCURY LNA miRNA PCR Assay. The identifiers of the miRCURY LNA miRNA PCR Assay were bgy-miR156 (YP02112257), bol-miR159 (YP02100546), osa-miR168a (YP02100652), mmu-miR-122 (YP00205664), mmu-miR-24 (YP02103215), and mmu-miR-let-7a (YP00205727).

Ligation assay

The ligation system contained 2 μL miRNA (5 μM), 3 μL mixed probe 1 and probe 2 (5 μM each), 1 μL yeast RNA (1 μg/μL), 1 μL 10× SplintR buffer, and 2.5 μL RNase-free water. The solution (final volume, 9.5 μL) was incubated at 95°C for 5 min and slowly cooled down to the ligation temperature on a thermal cycler. Then SplintR ligase of 0.5 μL was introduced, and the ligation was carried out at designated temperatures for 5 min, terminated by heating the mixture at 95°C for 5 min. The ligation products were analyzed by PAGE gel, or used as templates for qPCR.

For ligation including RNase H, the enzyme was diluted to certain concentrations using 1× RNase H buffer. The ligation reaction was set up, including 2 μL miRNA (5 μM), 3 μL mixed probe 1 and probe 2 (5 μM each), 1 μL yeast RNA (1 μg/μL), and 1 μL 10× SplintR buffer. The resulting mixture was heated at 95°C for 5 min and slowly cooled down to 65°C, followed by adding 2.5 μL RNase H and 0.5 μL SplintR ligase. The subsequent operation was performed as same as described above.

qPCR

qPCR was performed in a total volume of 20 μL containing 1× TB Green Fast qPCR Mix, 0.4 μM forward and reverse primer, 1 μL TB Green dye, and 7 μL ligation products. For ligation products of high concentrations (>1 pM), the products were diluted by 10 or 100, and then 7 μL was used. Each reaction was performed in triplicates on a CFX96 Real-Time PCR Detection System with the procedure: pre-denature at 95°C for 30 s (activating Hot Start Taq

DNA Polymerase), followed by 40 cycles of 95°C for 10 s and 60°C for 15 s. Reaction specificity was assessed by melting curve analysis immediately after the qPCR. All results were interpreted by using Bio-Rad CFX Manager 3.0 software.

Quantitative analysis of plant- and animal-derived miRNAs

Three plant-derived miRNAs (miR156, miR159, and miR168a) were quantified by the established method. Total RNAs were extracted from fresh broccoli using frozen-grinding in liquid nitrogen, before the extraction using TRIzol reagent (Invitrogen) according to the user guide. The amount and the purity of the extracted RNA were measured using the NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific). Then aliquots of the RNA solutions were subjected to miRNA quantification using probes and primers targeting to the three miRNAs. For comparison, the same RNA extracts were also analyzed by miRCURY assay and TaqMan assay, respectively. The two assays were performed stringently according to the manufacturer's instructions. The results from qPCR reactions were analyzed by Bio-Rad CFX Manager 3.0 software.

For animal-derived miRNAs, miR-122, miR-24, and let-7a from different mouse tissues were quantitatively analyzed. First, samples from brain, heart, liver, lung, kidney, and muscle on skeletal were dissected, followed by total RNA extraction using the protocol described for plant miRNA extraction. The extracted RNAs were analyzed on the NanoDrop 2000, and aliquots of the RNA solutions were subjected to miRNA quantification using probes and primers targeting miR-122, miR-24 and let-7a. For comparison, miRCURY assay and TaqMan assays were used, and performed stringently according to the manufacturer's instructions. All the results were interpreted by using Bio-Rad CFX Manager 3.0 software. Pearson correlation coefficients were calculated based on the variations of C_q values from the three methods.

Quantification of exogenous plant miRNA in animal circulation

All animal experimental operations were permitted by The Committee on Care and Use of Laboratory Animals of Shaanxi Normal University (Shaanxi, China, C31871752). To evaluate the ability of detecting exogenous miRNAs in animal circulation, rat tail-vein injection using miR159 was designed. The SD rats (10-week age) were randomly divided into five groups (n = 3). Four were intravenously injected with 0.4 mL miR159 oligomer (400 pmol), and the control group was injected with same volume of saline solution. Peripheral blood was collected at designated time points, and total RNA in serum was extracted using TRIzol LS reagent (Invitrogen) according to the user guide. The RNAs from the same group were blended to minimize individual variation, and their contents were determined as described above. To perform the absolute quantification, miR159 oligomers were subjected to gradient dilution to plot standard curves. The samples were subjected to ligation reaction as described above, followed by addition of 5 μL SMBs (4 mg/mL). The solution was thoroughly mixed and kept at room temperature for 5 min, before magnetic separation to remove the supernatant. The SMBs loading the ligated probe were resuspended by 10 L 1× SplintR buffer, then qPCR reactions were performed as described above. For comparison, miRCURY assay and TaqMan assay were applied, using the same RNA extracts. The experiments were carried out according to the user manuals. The Bio-Rad CFX Manager 3.0 software was used to analyze all the results delivered by the three methods.

Statistical analyses

All experiments were independently repeated at least three times, and the results were expressed as means ± standard deviations. A significant difference was assessed by one-way ANOVA (GraphPad Prism 9.4.0) and Tukey's HSD post hoc tests. The a p value of less than 0.05 was considered as statistically significant, and a p value of less than 0.01 was considered extremely significant.

DATA AVAILABILITY STATEMENT

Data that support the findings of this study are available from the corresponding author (X.W. and X.Y.), upon reasonable request.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.omtn.2022.12.015.

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AUTHOR CONTRIBUTIONS

X.W. and X.Y. designed the study. X.Q., K.X., and X.W. performed the experiments. H.T., Y.L., and B.Q. validated the results. Y.Z. and X.W. drew the figures. X.Q. and X.W. drafted the manuscript. Y.Z. and X.Y. provided the suggestion for the manuscript and proofed the final draft. All authors approved the submitted manuscript.

DECLARATION OF INTERESTS

The authors declared no conflict of interest.

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