analytical chemistry

Technical Note

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Label-Free MicroRNA Profiling Not Biased by 3′ End 2′-O-Methylation

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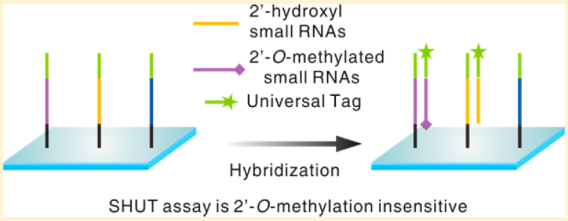
Supporting Information

ABSTRACT: Accurate quantification of miRNA expression level is essential to the study of its biology, and many cutting-edge technologies have been developed to accommodate this need. Yet most of them were designed primarily for the "regular" RNAs such as animal miRNAs and may overlook the fact that plant miRNAs and many other small noncoding RNAs are 2′-O-methylated at the 3′ end nucleotide. According to our experimental data and previous reports, this structural variation is detrimental to the effectiveness of the commonly used enzymatic labeling methods, leading to strongly biased results (~24-fold difference). Herein, we demonstrate that our Stacking-Hybridized Universal Tag (SHUT) microarray assay is well suited for unbiased profiling of both normal and methylated small RNA species. The detected signals of small RNAs with 2′-hydroxyl and 2′-*O*-methyl 3′ ends are highly consistent (no significant difference at $\alpha = 0.01$ level). For specificity, the presented method edges over others by its unique ability to discriminate single-base difference at or near the 5′ end. Notably, as compared to many delicate techniques, this enzyme-free and label-free approach requires much less reagent and manipulation, benefiting the SHUT-based applications with more efficient workflow and highly reproducible results.



MicroRNAs (miRNAs) are a class of small noncoding RNAs (typical length 22 ± 4 nucleotides) that play fundamental roles in multiple biological processes in animals, plants, and viruses.[1] To date, over 18 000 miRNAs in 168 species have been reported,[2] and the number keeps growing. High-throughput methods are essential tools for biologists to analyze large numbers of these small RNA molecules. At present, oligonucleotide microarrays and deep sequencing are widely employed for multiplexed or genome-wide analysis of miRNAs.[3] Successful applications of these high-throughput technologies have fueled research on miRNAs of many animal species in the past decade.

However, this general picture is slightly different for plant miRNA profiling. An intriguing biochemical fact has been uncovered in 2005 that the biogenesis of plant miRNAs consists of an additional step: the ribose of the 3′ end nucleotide is methylated at its 2′ hydroxyl group.[4] Later studies have revealed the essentiality of the methyl group in protecting plant miRNAs from addition of uridine-rich tails to the 3′ terminus and subsequent degradation.[5] Interestingly, many other small noncoding RNAs, such as small interfering RNA (siRNA), heterochromatic small interfering RNA (hc-siRNA), *trans*-acting siRNA (ta-siRNA), and natural antisense short interfering RNA (nat-siRNA) in plants, siRNA and Piwi-interacting RNA (piRNA) in insects, and piRNA in animals are also known to be 2′-O-methylated at the 3′ end.[6] The 2′-O-methylation of small RNAs may have a similar biological function as to protect the terminal nucleotide from exoribonuclease attack.[7]

Although biologically crucial, 2′-O-methylation of small RNAs seems to be chemically trivial, especially for the purpose of profiling. Since the RNA sequence is by no means altered by the methylation, analytical biochemists could just assay them in the same way as they profile the nonmethylated species. Yet quite unexpectedly, in practice these tiny methyl groups have raised serious challenges for available high-throughput assay platforms (microarray and sequencing), and in some cases they may produce highly biased data.[8] The cause of failure could be revealed by an examination of their working mechanisms. Currently, enzymatic labeling or ligation is an indispensable step for most commercial miRNA profiling assays.[9] There are two major kinds of enzymes involved: polyadenylate polymerase (PAP) and T4 RNA ligase. PAP is generally used to append a poly(A)-tail to the 3′ end of the miRNA for downstream labeling (Affymetrix, Invitrogen, Ambion)[3,10] (Figure 1a) or amplification (Illumina),[11] while T4 RNA ligase is used where oligonucleotides need to be added to the 3′ end of the miRNA to serve as a fluorescently labeled substrate (Agilent)[12] or an adaptor for small RNA library creation and sequencing.[13] Unfortunately, several studies have shown that the 3′ end 2′-O-methyl group prevents the miRNA from being an effective

Received: May 30, 2012
Accepted: July 6, 2012
Published: July 6, 2012

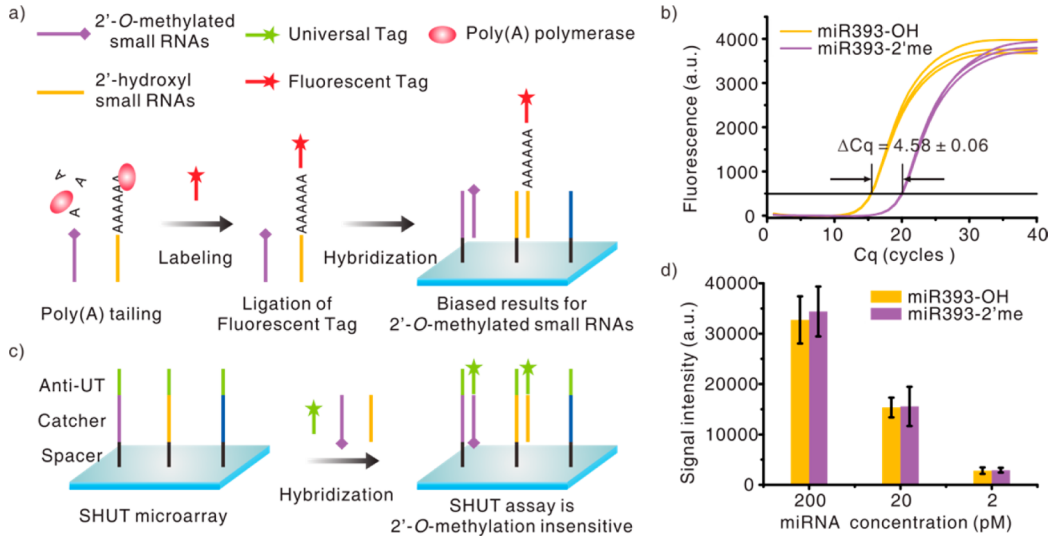


Figure 1. (a) Poly(A) polymerase-based enzymatic labeling methods for miRNA microarray (Affymetrix, Invitrogen, etc.). After poly(A) tail addition at the 3′ end of miRNA, a fluorescent tag sequence is ligated to the 3′ end of the poly(A) tail. The tagged miRNAs are then hybridized to the microarray. However, poly(A) tailing reaction is hindered by the 2′-O-methyl group on the terminal nucleotide. (b) Quantification of 2′-O-methyl 3′ end nucleotide bias using qPCR. The quantification cycle (C_q) for amplification between adenylated tailing of unmodified (miR393-OH) and 2′-O-methylated miRNAs (miR393-2′me) is 15.56 ± 0.04 and 20.14 ± 0.05, respectively. (c) Schematic representation of the SHUT assay. When the target small RNAs binds to the catcher sequence, the hybridization of the universal tag (UT) gains extra stability from the base stacking effect. It is insensitive to 2′-O-methylation at the 3′ end of small RNAs since we can use the nonmethylated end as the stacking site. (d) SHUT assay results of unmodified (miR393-OH) and 2′-O-methyl-modified miRNAs (miR393-2′me) with different concentrations (200 pM, 20 pM, 2 pM). The background signals have been subtracted. Error bars represent the standard deviation in triplicate.

initiator for the poly(A) polymerase-guided tailing reaction and inhibits the ligation activity of the T4 RNA ligase.[5b,8] Although Exiqon and Febit assays do not use these two enzymes, other enzymes they used also have to directly interact with the 3′ end nucleotide of miRNAs where the methylation might hinder the catalytic reactions.[14] Therefore, for plant miRNA samples, the data produced by many enzyme-based assays are methylation-biased.

On the basis of the above observation, we argue that at least three issues must be taken into serious consideration when using enzymatic commercial technologies to quantitate end-methylated small RNAs: (1) compromised detection sensitivity due to impeded enzymatic reactions; (2) it would be risky to draw any conclusion by comparison of the expression data of regular and end-methylated species; (3) most importantly, even for the same methylated small RNA, there is no solid ground for direct comparison of results from different assays, since it is very unlikely in any realistic scenario that the performances of the enzymatic reactions used were affected to an identical extent.

On the other hand, several emerging label-free methods are potentially insensitive to the 3′ end modification, such as electrochemical device,[15] enzyme-based colorimetric detection,[16] surface plasmon resonance (SPR),[17] surface-enhanced Raman spectroscopy (SERS),[18] and silicon nanowire,[19] etc. Unfortunately, so far there is no published data to support this assumption. Moreover, the applicability of these methods is far from practical usage as they have yet to overcome severe technical limitations such as poor multiplicity, low sensitivity, small dynamic range, or dependence on special instruments. As to chemical labeling methods which may also be unaffected by the 3′ end modifications, the heterogeneous labeling efficiency toward different nucleotides results in another sort of bias.[17] Thus, novel solutions are required to better deal with the 2′-O-methylation interference.

Here, we demonstrated that a fluorescent microarray-based, label-free profiling method developed by our group termed Stacking-Hybridized Universal Tag (SHUT) assay[20] is bias-proof to the end-nucleotide 2′-O-methylation of small RNAs. Previously, we have applied it to unmethylated miRNA detection and proved that it allows direct use of total RNA with high sensitivity (20 fM) and specificity (sharp discrimination of single-base mismatch at 5′ and 3′ ends).[20] Now, with all these key analytical features remaining the same when applied to the 3′ end methylated small RNAs, this method is found to be configurable to work in a methylation-insensitive manner: the detection signal is correlated only to the target concentration, no matter if the end nucleotide is modified or not.

EXPERIMENTAL SECTION

Polyadenylation, Reverse Transcription, and qPCR. In order to compare the efficiency that PAP adds poly(A)-tails to 2′-hydroxyl (miR393-OH) and 2′-O-methyl 3′ ends (miR393-2′me), both of them with same initial RNA input (10 μg) were polyadenylated by poly(A) polymerase (Ambion, Austin, TX) at 37 °C for 1 h in a 25 μL reaction mixture following the manufacturer's directions. Then, 0.5 μg of polyadenylated products were reverse transcribed by 1 U of M-MuLV reverse transcriptase (NEB, Hitchin, U.K.) with 1 μM Poly(T) adapter in a final volume of 10 μL according to the manufacture's protocols. qPCR of miRNAs was carried out using corresponding miRNA sequence as a sense primer and an antisense adaptor primer (details in the Supporting Information).

For qPCR verification of the tissue profiling experiments, the process was similar to the above experiment except that 0.5 μg of total RNA from each plant tissue was used as input material, and stem-loop RT primers were used in reverse transcription. The 5.8S rRNA was used as the endogenous control for

dx.doi.org/10.1021/ac301360z | Anal. Chem. 2012, 84, 6361−6365

normalization, owing to its stable expression across all these tissues. The sequences of the primers used in this work were listed in Table S1 in the Supporting Information.

Microarray Detection of miRNAs. Hybridization of miRNAs (TAKARA, Dalian, China) (Table S1 in the Supporting Information) with surface-bound probes was performed in a hybridization chamber (Agilent G2534A, Santa Clara, CA). Synthetic ssRNA oligonucleotides with either 2′-hydroxyl (miR393-OH) or 2′-*O*-methyl 3′ ends (miR393-2′me) at various assigned concentrations and 200 nM Cy3-labeled reporter molecule (UT) in 45 μL of hybridization solution (5× SSC and 0.2% SDS) were incubated with the arrayed glass slides in a hybridization oven (Agilent 2545A, Santa Clara, CA). After overnight (16 h) hybridization at 42 °C, the arrays were washed in 5× SSC and 0.1% SDS at 30 °C for 6 min and then washed for 3 min twice at room temperature in 0.2× SSC. The slides were immediately dried on a slide centrifuge and scanned using a LuxScan 10 K Microarray Scanner (CapitalBio, Beijing, China) at a fixed power and PMT gain settings through a single-color channel (532 nm wavelength).

For sensitivity and specificity experiments, synthetic 2′-O-methyl-modified miRNAs (miR169a and miR157a) at various assigned concentrations were tested. Single mismatch near the 5′ end of miRNAs (200 pM miR157a, miR157d, and miR169i) were hybridized with capture probes. The Zip-5 probe was included in detection probes to estimate fluorescence background. For miRNA profiling, 2.0 μg of total RNA isolated from leaf, stem, and 2-week-old seedling tissues of *Arabidopsis thaliana* (Columbia accession) was directly used. Zip-5 RNA was used as an external control. The process of these assays was similar to that of the 2′-*O*-methylated miRNA detection (see the Supporting Information for microarray fabrication and data processing).

RESULTS AND DISCUSSION

For the purpose of experimentally determining the impact of 3′ end 2′-O-methylation on the most commonly used PAP enzyme, we compared the efficiencies of poly(A)-tail addition by PAP to miR393-2′me (an *Arabidopsis thaliana* miRNA, 2′-O-methyl-modified) or to miR393-OH (the unmethylated counterpart) using qPCR. The results showed that the amplification efficiency of miR393-OH and miR393-2′me was 99.8% and 99.9%, respectively (Figure S1 in the Supporting Information), and the C_q value of miR393-2′me was 4.58 ± 0.06 smaller than that of miR393-OH's (Figure 1b), indicating the methylated 3′ end nucleotide was adenylated 23.9-fold less efficiently than the unmodified 3′ end. Consequently, only about 4.2% of the methylated miRNA may get labeled as compared to its nonmethylated counterpart. Such a low labeling rate is detrimental for accurate profiling of the methylated small RNAs. On the other hand, the T4 RNA ligase widely used for small RNA sequencing has recently been reported yielding an inconsistent ratio of ligation for nonmethylated and methylated small RNAs, despite optimization of reaction parameters.[8] Both this report and our data above suggest the liability of conventional enzyme-based methods to bias when applied to methylated small RNAs; eventually this may lead to flawed results (most likely to contain a false-negative error).

Our detection scheme based on the "base-stacking hybridization" mechanism is illustrated in Figure 1c (details in the Supporting Information). Since this strategy requires neither labeling of miRNAs nor participation of any enzyme and uses the normal 5′ end nucleotide instead of the methylated 3′ end one for base-stacking interaction, it is completely immune to the methylation-caused biases. This was experimentally confirmed by assaying a nonmethylated small RNA (miR393-OH) and its 3′ end 2′-O-methylated counterpart (miR393-2′me) at concentrations of 200 pM, 20 pM, and 2 pM. As we expected, the detected signal of both small RNAs at the same concentration showed no significant difference at $\alpha = 0.01$ level (Figure 1d), indicating the SHUT assay is insensitive to the 3′ end methylation.

It is reasonable to check if the hybridization efficiency was indeed affected when the extra 3′ end methyl group was placed at the base-stacking site. We did this by using the same pair of SHUT probes from Figure 1c with the catcher sequence reversed and 3′ end Cy3-labeled UT (designated UT′). This leads to the presence of the methylated end of miR393-2′me at the stacking site (Figure S2 in the Supporting Information). The result showed no significant difference of detection signals within the uncertainty of measurement ($\alpha = 0.05$). It should also be pointed out that the intensity values are close to the corresponding ones in Figure 1c. As a preliminary evaluation, it indicates that the influence of the methyl group at the stacking site to the stability of base-stacking effect might be negligible. However, more tests are needed before applying this "reversed" SHUT design to 3′ end methylated RNA species. Consequently, the "methyl-free" base-stacking scheme (Figure 1c) was used throughout the remaining work. It is noteworthy that other 5′ end modifications, such as 5′-triphosphates and 5′-7mGppp-cap, are also known to be refractory to standard enzymatic treatments.[8] Although a similar test of their influence on the stacking interaction is not applicable due to the lack of commercial providers of synthetic RNAs with such modifications, we speculate that the potential interference could be circumvented in the same way by employing the modification-free 3′ end as the stacking site.

Another challenge in measuring miRNA levels arises from the existence of homologous miRNAs, which typically varies in 1−3 nucleotides but nevertheless exhibit differential expression patterns.[21] A survey of the specificity of SHUT assay has been conducted in our previous work and it demonstrated an excellent discerning power for a single-base difference at both ends of small RNAs, which many current assays are not capable of. In this study, we further tested the selectivity of the SHUT assay toward end base insertion/deletion and a single base difference at the second and third position from the 5′ end. We first tested the single-base variations near the 5′ end by detecting two plant miRNAs (miR157a, miR157d) whose only difference is one base longer/shorter than each other at the 5′ end (Figure 2a). The signals decreased sharply (higher than 82%) for the mismatch miRNAs. Next, we synthesized three probes (miR169i-1, miR169i-2, miR169i-3) which artificially introduced a mismatch (on the first, second, and third base from the 5′ end, respectively) and one probe (miR169i) complementary to the target miRNA to detect miR169i (Figure 2b). The cross-hybridization rate was 7%, 2%, and 16%, respectively. These results indicate that the mismatch base near the stacking site effectively disturbs the base-stacking interaction, making the assay very sensitive to these mismatches.

As the sensitivity and dynamic range data of the SHUT were obtained using nonmethylated human miRNAs in our previous study, we assayed two synthetic methylated miRNAs (miR169a

dx.doi.org/10.1021/ac301360z | *Anal. Chem.* 2012, 84, 6361−6365

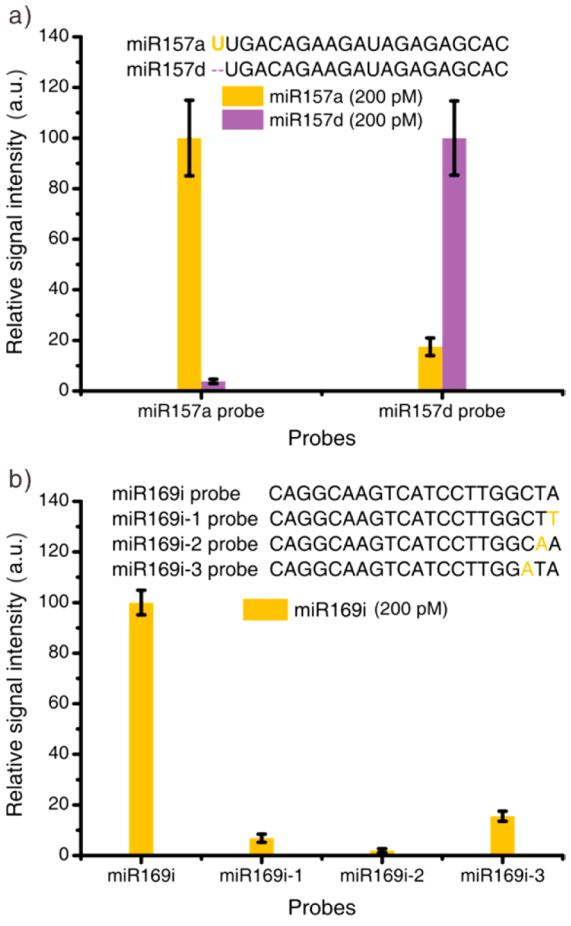


Figure 2. Specificity of the SHUT assay. (a) Discrimination of 2′-O-methyl-modified miRNAs with one base longer/shorter at the 5′ end. (b) Discrimination of 2′-O-methyl-modified miRNAs with a single-base difference from the 5′ end. The 5′ spacer and 3′ anti-UT of probe sequences are omitted in this drawing. Positions of miRNAs or probes having different bases are highlighted. Error bars represent the standard deviation in triplicate.

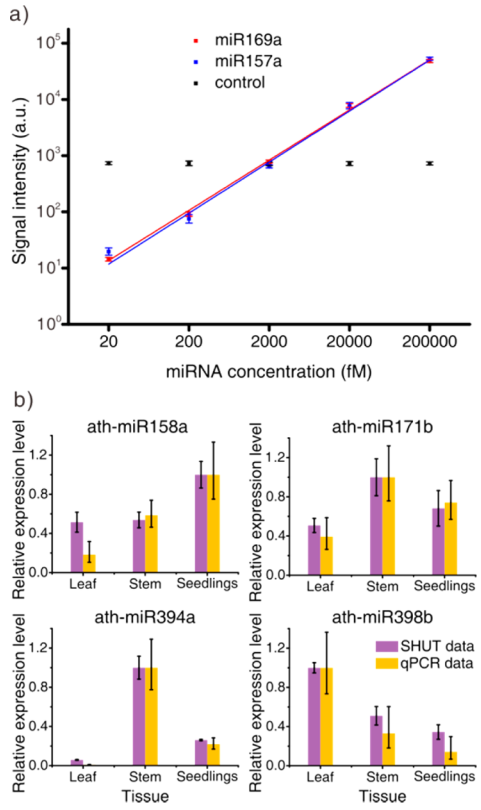


Figure 3. (a) Sensitivity and dynamic range of the SHUT assay. Two 2′-O-methyl-modified miRNAs (miR169a and miR157a) are assayed at concentrations of 20 fM−200 pM. Methylated miR393a (2 pM) is added as a control. (b) Expression level of four miRNAs (miR158a, miR171b, miR394a, and miR398b) in leaf, stem, and seedling tissues of *Arabidopsis thaliana*. Error bars represent the standard deviation in triplicate.

and miR157a) at concentrations ranging from 20 fM to 200 pM, with one methylated miRNA (miR393a) at a fixed concentration of 2 pM spiked-in as an external control (Figure 3a). A good signal-concentration linearity was obtained for these two miRNAs: the correlation coefficients is 0.9984 for miR169a and 0.9921 for miR157a. The linear dynamic range exceeds 4 orders of magnitude. Thus, the proposed method is suitable for accurate quantitation of plant miRNAs in a wide range of concentrations. The estimated detection limit of 20 fM is comparable to some of the most sensitive methods.[22]

As a further validation of the method, we then examined the expression profiles of four miRNAs (miR158a, miR171b, miR394a, and miR398b) in three tissues (leaf, stem, and seedling) of *Arabidopsis thaliana*. Total RNA (2 μg) extracted from each tissue was applied directly to the microarray hybridization solution. The obtained expression profiles were compared with the results from qPCR with stem-loop reverse transcription (which is not affected by 2′-O-methylation as it requires no poly(A) tailing or other enzymatic ligation procedure),[23] and the two were found to agree with each other satisfactorily (Figure 3b).

CONCLUSIONS

In summary, the SHUT method first and successfully addressed the 2′-O-methylation-related detection biases for small RNAs, one of the most challenging and unsolved issues among current high-throughput miRNA detection technologies. Moreover, this method surpasses many current technologies by efficiently discriminating single-base differences at the 5′ end of small RNAs. Finally, the SHUT assay requires only one hybridization step, allows direct use of total RNA without small RNA enrichment or labeling, and does not require the tailor-made locked-nucleic acids (LNA) probes. All of these reduce the expense on reagents, labor, and time. The SHUT assay is fully capable of paving a new avenue toward reliable and efficient profiling of methylated small RNAs.

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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Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We are grateful to Prof. Hongwei Ma for his assistance in microarray printing and scanning. This work is supported by the National Natural Science Foundation of China (Grant No. 60701018) and the Natural Science Foundation of Jiangsu Province (Grant No. BK2011361, SBK201240786).

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