Chapter 6

Genome-Wide Identification of MicroRNAs in *Medicago truncatula* by High-Throughput Sequencing

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Abstract

MicroRNAs (miRNAs) are small, endogenous RNAs that play important regulatory roles in development and stress response in plants by negatively regulating gene expression post-transcriptionally. *Medicago truncatula* has been used as a model plant to study functional genomics of legume plants. It has also been widely used to functionally study miRNAs. Identification of miRNAs at the whole-genome level is essential for functional characterization of miRNAs in plants. High-throughput sequencing is a powerful technology to identify miRNAs. In this chapter, the methods used for construction of a small RNA library and high-throughput sequencing involving total RNA isolation, small RNA purification, adapter ligation, reverse transcription, PCR amplification, and Solexa sequencing are described. Bioinformatics and analysis of differential expression of miRNAs including primary disposal, miRNA identification, target prediction, and expression analysis are also discussed. These methodologies associated with identification and functional characterization of miRNAs may provide useful tools for readers to study miRNAs in plants in general and *Medicago truncatula* in particular.

Key words microRNA, *Medicago truncatula*, High-throughput sequencing, Bioinformatics analysis

1 Introduction

There are two most abundant classes of plant small RNAs (sRNAs), endogenous small interfering RNAs (siRNAs), and microRNAs (miRNAs). Of them, miRNAs are one type of endogenous noncoding sRNAs with the approximate length of 21 nt, initially discovered in *Caenorhabditis elegans* as developmental timing regulators [1]. The existence of miRNAs has been widely recognized in organisms including plants, mammals, and viruses. miRNAs have the ability to down-regulate gene expression by targeting corresponding messenger RNAs (mRNAs) in both plants and animals [2, 3].

In plants, the generation of miRNAs is a multistep enzymatic process. The primary transcripts of miRNAs translated by RNA polymerase II are digested to generate pre-miRNAs by DICER-LIKE1 (DCL1). The pre-miRNAs are further digested by DCL1,

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leading to the generation of miRNA/miRNA* duplexes. Mature miRNAs which depart from miRNA/miRNA* are incorporated into the RNA-induced silencing complex (RISC) with argonaute protein. miRNAs direct RISC to bind their target mRNAs for cleaving mRNAs with near-perfect complementarity and/or inhibiting translation of those with lower complementarity [4–8].

It has been widely reported that miRNAs play a regulatory role in diverse biochemical and physiological processes in plants. For instance, miRNAs have been shown to play a role in modulation of those processes associated with growth and development in plants, including seed germination [9], leaf morphogenesis [10, 11], and floral organ and root development [6, 12–15]. In addition, there is increasing evidence demonstrating that miRNAs are closely involved in responses of plants to various abiotic and biotic stresses. These include drought [16–20], cold [21–23], salinity [24], nutrition starvation [25–28], metal ion toxicity [29, 30], oxidative stress [31], submergence [32], UV-B radiation [33, 34], and virus [35, 36].

Identification of miRNAs is an essential step to functionally characterize miRNAs in plants. Identification of miRNAs from plant species was firstly done by the traditional Sanger sequencing of relatively small-size cDNA libraries. Using this method, many conserved miRNAs from different species, including Arabidopsis, rice, and poplar, were identified [37]. However, this method is time-consuming and expensive. Moreover, the expression of the non-conserved miRNAs is often low compared to that of the conserved miRNAs [38]. Therefore, many non-conserved miRNAs cannot be detected using the small-scale sequencing method. The development of high-throughput sequencing technology has overcome these problems, and this technology has successfully been used to identify miRNAs in many plant species such as Arabidopsis [39, 40], rice [41], wheat [42], soybean [43], cotton [44], grapevine [45], and poplar [46].

The application of high-throughput sequencing technology has led to systematical identification of numerous miRNAs involved in regulation of many biological and physiological processes in the legume model plant *Medicago truncatula*. According to the newest miRNA database (miRBase 19, released in Aug 2012), *M. truncatula* has the largest number of precursors and mature miRNAs among the reported plant species [47]. This approach has successfully been used to identify novel miRNAs in *M. truncatula*. For instance, Szittya et al. [48] identified 25 conserved miRNA families and 26 new miRNA candidates from *M. truncatula*. Jagadeeswaran et al. [49] identified eight novel sRNAs, of which four are annotated as legume-specific miRNAs. Moreover, the application of high-throughput sequencing has been further extended to functional characterization of miRNAs in *M. truncatula*. For example, Lelandais-Briere et al. [50] identified many miRNAs

in root nodules of *M. truncatula*. Wang et al. [51] found that ten known miRNA families and eight new miRNAs were responsive to drought stress. Chen et al. [51] identified eight ethylene-responsive miRNAs [52]. In addition, the involvement of miRNAs in aluminum and mercury toxicity has also been reported in *M. truncatula* [29, 30].

Two types of sequencers have been used in high-throughput sequencing, Roche 454 sequencer and Illumina Solexa sequencer. The two sequencers differ in terms of read length and quantities of reads measured. For instance, longer reads (approx. 400 nt) can be generated by Roche 454 sequencer, while the read length produced by Illumina Solexa sequencer is usually 35 nt. In addition, Illumina Solexa sequencer can generate more reads than Roche 454 sequencer. For example, 844,110 reads and 97,028 unique sequences were obtained from *M. truncatula* by Lelandais-Briere et al. [50] using the Roche 454 sequencer. In contrast, a total of 13,683,619 reads and 4,878,445 unique sequences from two libraries of *M. truncatula* were achieved by the Illumina Solexa sequencer [51]. Given that the length of mature miRNA sequences is about 21 nt, high-throughput sequencing by Illumina Solexa sequencer is more appropriate. Therefore, in this chapter, we shall mainly discuss the methods employed in the study of plant miRNA by high-throughput sequencing using the Illumina Solexa sequencer. The method used for sequencing miRNA has been improved from the method of Hafner et al. [53] and Allen et al. [54].

2 Materials

2.1 Plant Materials

To search for novel miRNAs, one type of material from *M. truncatula* is sufficient. However, if the study aims to compare miRNA expression patterns among different organs and/or tissues, or under varying treatments, several plant materials have to be used. In this chapter, we discuss the two types of sample preparation, and refer to as Sample 1 and Sample 2.

2.2 Reagents

1. Trizol.
2. Chloroform.
3. Isopropanol.
4. 75 and 100 % Ethanol.
5. RNase-free ddH_2O.
6. RNase-free 0.4 M NaCl.
7. ssRNA ladder marker.
8. 3′ RNA adaptors: AppTC GTA TGC CGT CTT CTG CTT G-L (p: phosphate; L: 3′ OH blocking group).

9. 5′ RNA adaptors: rGrUrU rCrArGr ArGrUr UrCrUr ArCrAr GrUrCr CrGrAr CrGrAr UrC.
10. 0.1 % BSA.
11. T4 RNA ligase.
12. PEG6000.
13. 0.1 M DTT.
14. dNTPs (2 mM each).
15. SuperScript II Reverse Transcriptase (Invitrogen).
16. 150 mM KOH/20 mM Tris base.
17. 150 mM HCl.
18. 2× Phusion HF master mix (Finnzymes).
19. 5′ Primer: AAT GAT ACG GCG ACC ACC GAC AGG TTC AGA GTT CTA CAG TCC GA.
20. 3′ Primer: CAA GCA GAA GAC GGC ATA CGA.
21. Agarose gel and low-melting agarose gel.
22. 25 bp DNA ladder.
23. Water-saturated phenol (pH 7.8).
24. Phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0).
25. 0.5 M NaOH.

2.3 15 % TBE–Urea Denaturing Polyacrylamide Gel

1. 30 % stock acrylamide solution: 29 g acrylamide, 1 g bis-acrylamide. Make up to 100 mL in distilled water and filter. Stable at 4 °C for months.
2. 10× TBE buffer: Tris base 10.8 g, boric acid 5.5 g, $Na_2EDTA{\cdot}2H_2O$ 0.93 g. Adjust pH to 8.3 and make up to 100 mL.
3. To make 50 mL of 15 % TBE–urea denaturing polyacrylamide gel, add 25 mL of 30 % stock acrylamide solution, 5 mL of 10× TBE buffer, and 21 g urea. Make up to 50 mL, and add 400 μL of 10 % ammonium persulfate, 40 μL *N*,*N*,*N*′,*N*′-tetramethylethylenediamine. Mix gently.

2.4 Buffers

1. 2× Loading buffer: 18 mM EDTA, 0.025 % bromophenol blue, 0.025 % xylene cyanol in formamide.
2. 10× T4 RNA ligase buffer: 500 mM Tris–HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP.
3. 5× First-strand buffer: 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.

2.5 Instruments

1. Tabletop centrifuge.
2. Thermomixer.
3. Electrophoresis system.

4. Agilent 2100 (Agilent).
5. PCR instrument (Eppendorf).
6. UV transilluminator.
7. Solexa sequencer (Illumina).

3 Methods

3.1 Construction of Small RNA Library and High-Throughput Sequencing

The outline of sRNA library construction and high-throughput sequencing are shown in Fig. 1. The description of methods is given in the following section.

3.1.1 Isolation of Total RNAs

Total RNAs were extracted from Sample 1 and Sample 2 using the Trizol reagent (*see* **Notes 1–6**). Before gel purification of sRNAs, we recommend using 50 μg of total RNA per sample. As a rule of thumb, 0.1 g of leaf tissue of *M. truncatula* can yield about 20 μg total RNA.

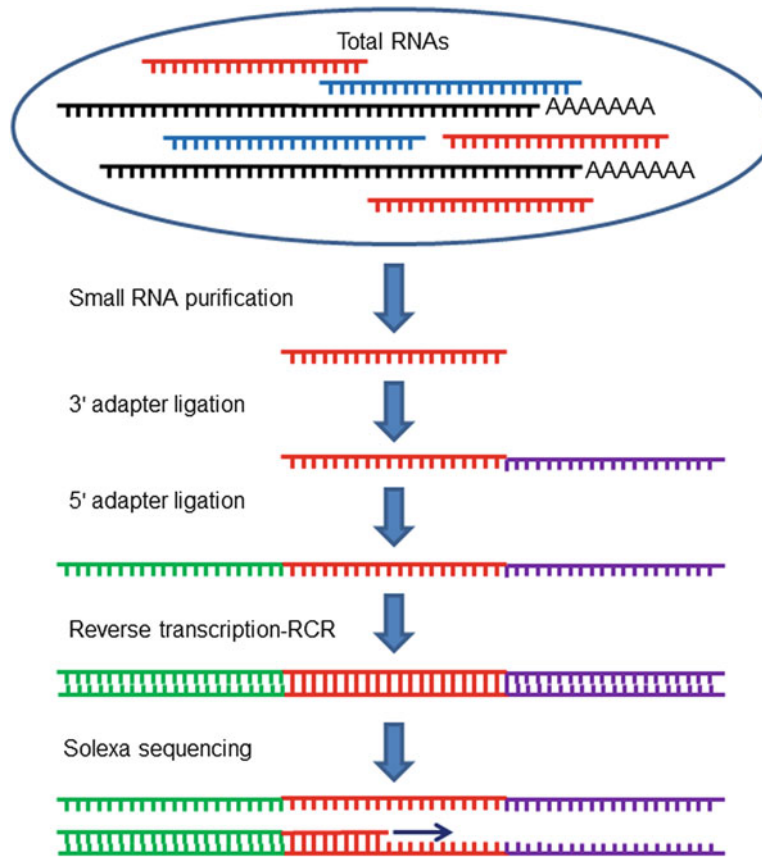

Fig. 1 Outline of small RNA library construction and high-throughput sequencing

1. Add 1 mL Trizol reagent per 0.1 g of sample, which is triturated in a mortar with liquid nitrogen.
2. Incubate the homogenized sample for 5 min at room temperature to permit complete dissociation of the nucleoprotein complex.
3. Add 0.2 mL of chloroform per 1 mL of Trizol reagent used for homogenization. The tube should be capped securely.
4. Shake tube vigorously for 15 s.
5. Incubate for 2–3 min at room temperature.
6. Centrifuge the sample at 12,000 × *g* for 10 min at 4 °C.
7. Place the aqueous phase into a new tube, add 0.5 mL of 100 % isopropanol to the aqueous phase, and mix them gently (*see* **Note 7**).
8. Incubate at room temperature for 10 min.
9. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
10. Remove the supernatant from the tube, and leave the RNA pellet in the tube exclusively.
11. Wash the pellet, with 0.5 mL of 75 % ethanol.
12. Vortex the sample briefly, and then centrifuge the tube at 7,500 × *g* for 5 min at 4 °C. Discard the wash solution.
13. Air-dry the RNA pellet for 5–10 min (*see* **Note 8**).
14. Resuspend the RNA pellet in RNase-free ddH_2O.
15. Determine the concentration and quality of the RNA by Agilent 2100 (*see* **Note 9**).
16. Proceed to downstream application, or store at –70 °C.

3.1.2 Gel Purification of Small RNAs

The total RNA is size-fractionated on a 15 % TBE–urea denaturing polyacrylamide gel and the 18–30 nt sRNAs are then eluted from the excised gel slice.

1. Mix the solution of total RNA with one volume of 2× loading buffer. Denature the RNA by incubation for 30 s at 90 °C, load the RNA and ssRNA ladder marker on a 15 % denaturing polyacrylamide gel, and run the gel at 30 W until the bromophenol blue dye of the gel-loading solution appears within the lower third of the gel (*see* **Notes 10–12**).
2. Dismantle the gel, leaving it mounted on one glass plate. Cut the gel slice containing 18–30 nt sRNAs in smaller pieces according to RNA markers so that they can fit into a preweighed 1.5 mL tube. According to the weight of the gel slices, add 2–3 volumes (v/w) of RNase-free 0.4 M NaCl and elute the sRNAs from the gel by incubating the tube overnight at 4 °C under constant agitation. Collect the supernatant and add 3 volumes of absolute ethanol to precipitate the sRNAs for 2–8 h at 20 °C.

3. Collect the sRNA pellet in a tabletop centrifuge for 15 min at 14,000 × *g* at 4 °C. Remove the supernatant without perturbing the pellet. Air-dry the RNA pellet to evaporate residual ethanol, as it may inhibit the subsequent enzymatic steps.

3.1.3 Adapter Ligation

Adapters joined to the 3′ end and 5′ end of sRNAs allow for RT-PCR amplification.

1. Ligate the 18–30 nt sRNAs with 3′ RNA adapters by T4 RNA ligase. Prepare a reaction mixture for ligation of the 3′ RNA adapters per 1 μg sRNAs by combining the following components:

sRNAs	1 μg
3′ RNA adapters (100 μM)	1 μL
10× T4 RNA ligase buffer	5 μL
0.1 % BSA	3 μL
T4 RNA ligase	40–50 U
PEG6000	Final 25 %
RNase-free ddH_2O	Up to 50 μL

   Mix gently and react at 20 °C for 18 h.
2. Gel purification as described in Subheading 3.1.2.
3. Ligate sRNAs with 5′ RNA adapters: follow the way of ligation of the 3′ RNA adapters.
4. Gel purification following the protocols in Subheading 3.1.2.
5. Dissolve the pellet in 5.6 μL RNase-free ddH_2O for each sample.

3.1.4 Reverse Transcription

1. Denature the RNA and 3′ primer by incubating the tube for 30 s at 90 °C and transfer the tube to a 50 °C incubator.
2. Add the following reaction mix to each sample:

5× First-strand buffer	3 μL
0.1 M DTT	1.5 μL
10× dNTPs (2 mM each)	4.2 μL

3. Add 0.75 μL of Superscript II reverse transcriptase and mix gently. Incubate for 40 min at 42 °C.
4. Add 40 μL of 150 mM KOH/20 mM Tris base and incubate for 10 min at 90 °C to hydrolyze the RNA.
5. Neutralize the solution by addition of 40 μL of 150 mM HCl to pH 7.0–9.5; thus the subsequent PCR is not inhibited (*see* **Note 13**).

3.1.5 PCR Amplification

1. Perform standard 100 μL PCR reactions for each sample by combining the following components (*see* **Note 14**):

2× Phusion HF master mix	50 μL
cDNA	10 μL
5′ Primer (10 μM)	5 μL
3′ Primer (10 μM)	5 μL
RNase-free ddH_2O	Up to 100 μL

   Mix them gently.

2. Use the following cycle conditions for PCR amplification:

Initial denaturation	98 °C	30 s
(15 + *n*) cycles	98 °C 50 °C 72 °C	15 s 85 s 60 s
Final extension	72 °C	10 min
Hold	4 °C	∞

3. Analyze the samples on a 2.5 % agarose gel from the 15th cycle to define the optimal cycle number. The PCR product may appear as a double band with the higher band occurring at the expected length of about 70–80 bp and a lower band corresponding to the 3′ adapter to 5′ adapter ligation products occurring at about 50 bp (*see* **Note 15**).
4. Perform three standard 100 μL PCR reactions, to obtain enough product, with the optimal cycle number (*see* **Note 16**).
5. Purify the PCR product of about 70–80 bp. Run the production of PCR in a 3 % low-melting agarose gel containing a 25 bp DNA ladder for approx. 1 h at 100 V until the marker bands are sufficiently resolved. Excise the band of approximately 70–80 bp on a UV transilluminator. Transfer the gel slice to a 1.5 mL reaction tube and add RNase-free 0.4 M NaCl to a total volume of 500 μL. Melt the agarose at 70 °C for 10 min. Add 1 volume of preheated water-saturated phenol (pH 7.8). Vortex the solutions vigorously and immediately separate the phases by centrifuging at 14,000 × *g* for 5 min in a tabletop centrifuge. Collect the solution at the upper phase and extract it in a new tube with 1 volume phenol/chloroform/isoamyl alcohol (25:24:1, pH 8.0), and 1 volume chloroform. Precipitate the DNA by addition of 2 volumes of absolute ethanol and incubation for at least 1 h at 20 °C. Collect the DNA pellet in a tabletop centrifuge at 14,000 × *g* at 4 °C for 15 min. Remove the supernatant without disturbing the pellet. Air-dry the DNA pellet to evaporate residual ethanol.

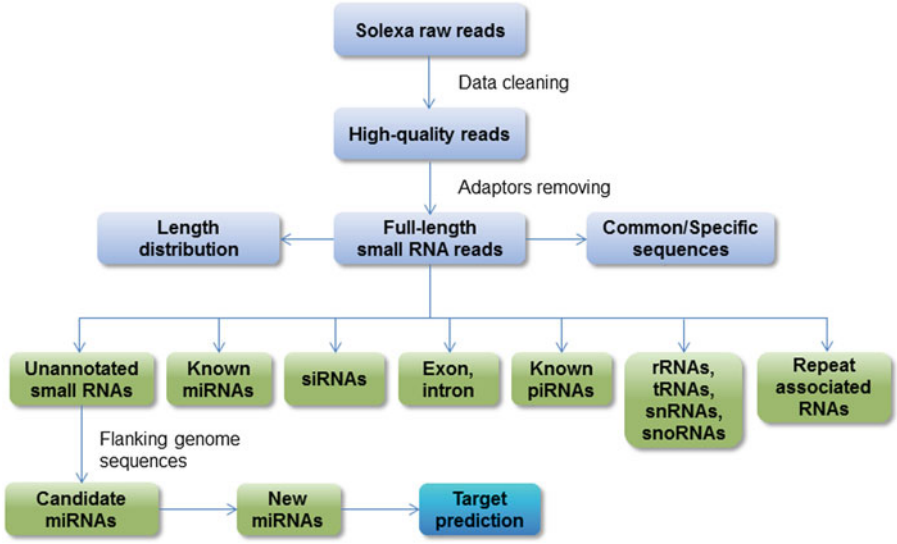

Fig. 2 Flowchart of bioinformatics analysis

3.1.6 Solexa Sequencing

The purified DNA samples are now ready for sequencing by the Illumina Solexa sequencer. The high-throughput sequencing is performed following the manufacturer's instruction.

3.2 Bioinformatics Analysis

Figure 2 shows the procedures sued for bioinformatics analysis. The detailed description of the protocols is given in the following section.

3.2.1 Primary Disposal

1. The raw reads from Solexa sequencing should firstly go through the data cleaning. This includes removal of the low-quality tags and several kinds of contaminants such as tags less than 18 nt, polyA and those tags without adaptors.
2. Remove adaptor sequences of the sRNA reads with exact matches to the adaptor sequences.
3. Analyze the length distribution and common/specific sequences between Sample 1 and Sample 2, including summary of unique tags and total tags.
4. Map high-quality reads to the *M. truncatula* genome sequence (Mt3.5) downloaded from http://medicago.jcvi.org/cgi-bin/medicago/download.cgi/ using SOAP [55] to analyze their expression and distribution on the genome.

3.2.2 Identification of miRNA

1. Remove rRNAs, tRNAs, siRNAs, snRNAs, and snoRNAs from the matched sequences through BLASTn search [56] using NCBI Genbank database http://www.ncbi.nlm.nih.gov/blast/Blast.cgi/ and Rfam database http://www.sanger.ac.uk/Software/Rfam/. Mismatches are not allowed in the above two approaches (*see* **Note 17**).

2. Align the unique sequences with known miRNAs from miRBase 19 (http://www.mirbase.org/) [57].
3. Identify potential candidate miRNAs by folding the flanking genome sequences of unique sRNAs using MIREAP (https://sourceforge.net/projects/mireap/) (*see* **Note 18**). Parameters are set based on the criteria for annotation [58].

3.2.3 Target Prediction

Targets of miRNA are predicted using tools of the psRNATarget (http://plantgrn.noble.org/psRNATarget/) and the srna-tools (http://srna-tools.cmp.uea.ac.uk/plant/) [59], by aligning mature miRNA sequences with genome of *M. truncatula* and *A. thaliana*. Target can be validated by 5′ RACE or degradome analysis that also can be processed by high-throughput sequencing [60].

3.3 Analysis of Differential Expression

1. Normalize the frequency of miRNAs of the two libraries to 1 million by total number of miRNAs in each sample (*see* **Note 19**). Normalized expression = actual miRNA count/total count of clean reads × 1,000,000.
2. Calculate the fold change for each miRNA between Sample 1 and Sample 2. If Sample 1 sets as a control, fold-change = $\log_2$ (Sample 2/Sample 1). A fold-change >0 means that the miRNA expression of Sample 2 is higher than that of Sample 1, while a fold-change <0 indicates that the miRNA expression of Sample 2 is lower than that of Sample 1. Perform statistics analysis according to Poisson distribution. If $P \leq 0.05$, the differences are referred to as significant.
3. Validate the differential expression by real-time quantitative PCR or sRNA northern blot.

4 Notes

1. Soak mortars in 0.5 M NaOH for 30 min, and rinse them thoroughly with water. Then bake them at 180 °C for 8 h to inactivate RNases.
2. Wear disposable gloves. Hands often contain bacteria and molds that can contaminate RNA, and introduce RNases.
3. Use disposable RNase-free plasticware and pipettes for RNA work to prevent cross-contamination with RNases.
4. All of the reagents used in isolation and transcriptional of RNA must be RNase-free.
5. Freeze tissue samples immediately with liquid nitrogen upon collection. The samples must be fresh or stored at −70 °C prior to trituration.

6. Trizol reagent is harmful if handled improperly. Work related to Trizol reagent must be done in a fume hood. If Trizol reagent contacts with skin or eyes, wash the exposed area immediately with copious amounts of water for 15 min and seek medical attention if necessary.
7. Avoid drawing the organic layer into the pipette during removing the aqueous phase.
8. Do not allow the RNA to dry completely, because the solubility of the pellet may be lost.
9. Total RNAs should be isolated again if 28S and 18S bands tested by Agilent 2100 are not distinct and sharp.
10. Avoid overloading the pocket of the gel to ensure good fractionation of the RNAs.
11. Leave at least one well empty between the different samples to avoid cross-contamination.
12. Do not run the gel excessively to ensure that the sample contains sRNAs as small as possible.
13. The pH value of cDNA templates should be between 7.0 and 9.5 so that the PCR is not inhibited. If necessary, readjust the pH by adding base or acid.
14. Perform a negative control to check DNA contamination in the reaction mixture.
15. The number of PCR cycles can be adjusted according to gel analysis of different cycles. The gel might appear as a double band with the higher band at 70–80 bp and a lower band at about 50 bp. Never amplify for more than 30 cycles because it will distort the sRNA ratios.
16. Define the optimal cycle number within the exponential amplification phase of the PCR.
17. In the process of miRNA identification, siRNAs, snRNAs, and snoRNAs have to be removed. However, if these sRNAs are studied, they can be used for further analyses following the protocols similar to analysis of miRNAs described in the above section.
18. The new miRNAs can be named after submitting to miRBase (http://www.mirbase.org/).
19. Direct comparison of the sequencing number for a certain miRNA among libraries is not allowed, as the sequencing amounts of each library can be different. Normalization is required prior to comparison of sequencing number among different libraries.

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