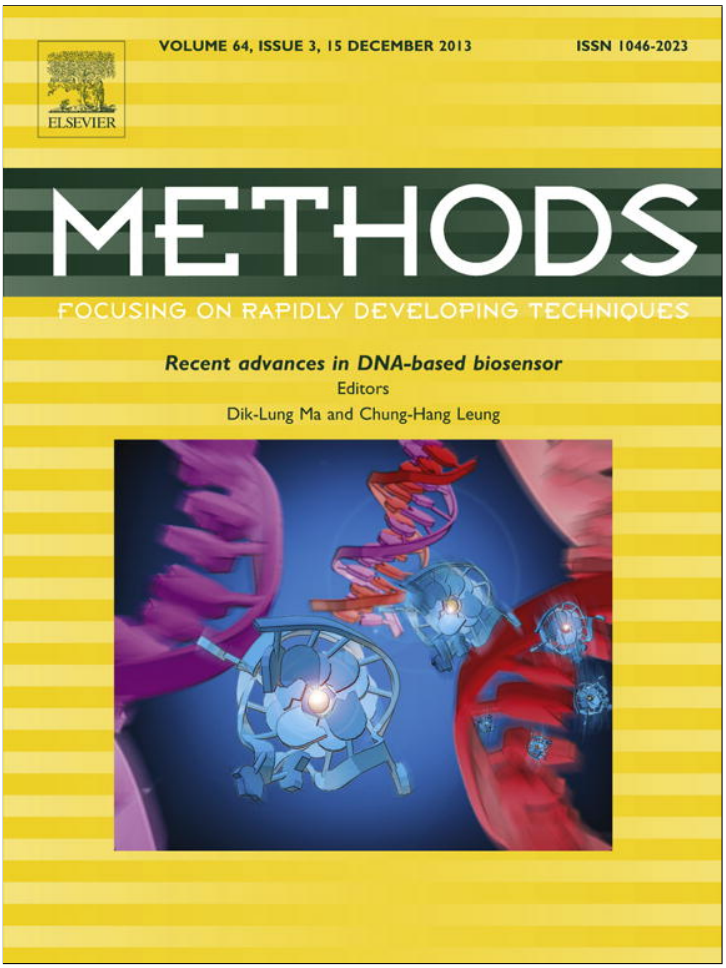

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Construction of Specific Parallel Amplification of RNA Ends (SPARE) libraries for the systematic identification of plant microRNA processing intermediates

Arnaldo L. Schapire [a,1], Nicolas G. Bologna [a,1,2], Belen Moro [a], Jixian Zhai [b], Blake C. Meyers [b], Javier F. Palatnik [a,*]

[a] IBR (Instituto de Biología Molecular y Celular de Rosario), CONICET and Facultad de Ciencias, Bioquímicas y Farmacéuticas, Universidad Nacional de Rosario, Rosario, Argentina
[b] Department of Plant & Soil Sciences, Delaware Biotechnology Institute, University of Delaware, 15 Innovation Way, Newark, DE 19711, USA

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ABSTRACT

MicroRNAs (miRNAs) are small RNAs that derive from endogenous precursors harboring foldback structures. Plant miRNA precursors are quite variable in their size and shape. Still, the miRNA processing machinery, consisting of DICER-LIKE1 (DCL1) and accessory proteins recognize structural features on the precursors to cleave them at specific places releasing the mature miRNAs. The identification of miRNA processing intermediates in plants has mostly relied on a modified 5′ RACE method, designed to detect the 5′ end of uncapped RNAs. However, this method is time consuming and is, therefore, only practical for the analysis of a handful miRNAs. Here, we present a modification of this approach in order to perform genome-wide analysis of miRNA processing intermediates. Briefly, a reverse transcription is performed with a mixture of specific primers designed against all known miRNA precursors. miRNA processing intermediates are then specifically amplified to generate a library and subjected to deep sequencing. This method, called SPARE (Specific Parallel Amplification of 5′ RNA Ends) allows the identification of processing intermediates for most of the Arabidopsis miRNAs. The results enable the determination of the DCL1 processing direction and the cleavage sites introduced by miRNA processing machinery in the precursors. The SPARE method can be easily adapted to detect miRNA-processing intermediates in other systems.

1. Introduction

MicroRNAs (miRNAs) are small RNAs of ~21 nucleotides (nt) that function as key regulators of gene expression in most eukaryotes [1,2]. MiRNAs exert their function through translational repression or post-transcriptional silencing by base-pairing to the target mRNA [3], and are critical in plant development and stress responses [4–10]. miRNA genes are transcribed by RNA polymerase II, generating a primary transcript (pri-miRNA), which is capped at the 5′ end, spliced, and 3′ polyadenylated [11,12]. In turn, pri-miRNAs harbor a stem-loop precursor formed by base-pairing between self-complementary foldback regions. As miRNAs reside in one of the arms of the miRNA precursor, the processing machinery performs consecutive staggered cleavages in the dsRNA segment of the precursor in order to release the mature miRNA, together with the opposing fragment of the precursor called miRNA* [13]. The released miRNA/miRNA* duplex contains 2 nt 3′ overhang at each end. While the miRNA becomes incorporated into an ARGONAUTE complex to exert its function, the miRNA* is usually degraded.

In *Arabidopsis thaliana* and other plants, the cuts are the product of the RNAse III DICER-LIKE1 (DCL1) enzymatic activity [14,15]. The distance between two consecutive cuts for DCL1 is ~21 nt, defining in this way the length of the mature miRNAs. In addition to DCL1, several proteins aid the processing of plant miRNA precursors. The C2H2-zinc finger protein SERRATE (SE) as well as the double-stranded RNA binding protein HYPONASTIC LEAVES1 (HYL1) also contribute to the processing of miRNA precursors [16–19]. It has been shown that HYL1 needs to be dephosphorylated for optimal activity, and this requires the interaction with C-TERMINAL DOMAIN PHOSPHATASE-LIKE 1 (CPL1) protein [20]. Furthermore, the RNA binding protein DAWDLE [21], as well as HASTY [22], TOUGH [23], NOT2 proteins [24], and STA1 [25] have also been shown to participate in the biogenesis of miRNAs in Arabidopsis.

Unlike their animal counterparts, plant miRNA precursors are highly variable in size (between 50 and 900 nucleotides) and comprise a broad range of structures and shapes [26]. Different studies

* Corresponding author.
E-mail address: palatnik@ibr.gov.ar (J.F. Palatnik).
[1] These authors contributed equally to this work.
[2] Current address: Department of Biology, Swiss Federal Institute of Technology (ETH), Zurich, Switzerland.

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have demonstrated the importance of structural motifs for the precise release of the mature miRNA. It has been shown that a stem of ~15 nt below the miRNA/miRNA* serves as a structural cue in the plant precursors that is recognized by the processing machinery [27–29]. However, this structural determinant is not present in all the miRNA precursors [27]. Hence, while most miRNA precursors are processed in a base-to-loop pathway like animal precursor biogenesis, in plants, a non-canonical biogenesis was also demonstrated where the processing of miR319 and miR159 proceeds in reverse orientation, i.e., loop-to-base processing [30,31].

Cloning and sequencing processing intermediates of miRNA precursors has been shown to be an effective tool to infer the mechanism of miRNA biogenesis that operates in a certain precursor and to infer structural determinants relevant for processing [27–30]. In these studies, the identification of processing intermediates has been mostly based on a modified 5′-rapid amplification of cDNA ends (RACE) [31,32]. Although these approaches have been proven useful to describe miRNA processing mechanisms in plants, they only allow the analysis of a handful of precursors. Here, we describe in detail the SPARE method to systematically identify processing intermediates of plant miRNAs, which has been successfully applied to analyze the processing mechanism of most of the evolutionarily conserved miRNAs and many evolutionary young miRNAs in *A. thaliana* [33]. The approach allows a genome-wide view of the processing of miRNA precursors, which is of particularly utility in plants.

1.1. Overview of the method

We referred to the method as SPARE, as it is a modification of previously described methods (e.g., PARE) to detect poly(A)+ mRNA derived fragments by deep sequencing [31,32]. The experimental protocol is depicted in Fig. 1. After ribosomal RNA depletion from total RNA, uncapped RNAs are ligated with RNA adapters at their 5′ end. The ligated RNA is used as a template for reverse transcription with a pool of precursor-specific primers containing a generic adapter tail. The resulting cDNAs consisting of miRNA processing-intermediate sequences are then amplified by PCR using general primers annealing the RNA adapter (P5) and the generic adapter tail (P7) sequences. After agarose gel purification, PCR products are subjected to deep sequencing.

Due to the relative position of the precursor-specific primers (Fig. 1), this method allows to infer the processing direction on the basis of the number of intermediates detected for a certain precursor. That is, if the precursor is processed in a base-to-loop fashion, just the first cleavage position can be detected, however, all the cleavage intermediates are expected to be found if the processing proceeds in a loop-to-base pathway (Fig. 1) [30,31].

2. Material and reagents

With the exception of materials used for PCR reactions, all reagents need to be RNase-free. In order to minimize hydrolysis, RNA samples should be stored at −70 °C or below, or kept on ice while the reactions are set up.

(1) RNA extraction:
  (a) TRIzol® reagent (Invitrogen).
  (b) Isopropanol.
  (c) 80% ethanol.
(2) RNA precipitation:
  (a) Glycogen (Invitrogen).
  (b) Sodium acetate 3 M, pH 5.2.
  (c) Absolute ethanol, 80% ethanol.
(3) DNase treatment:
  (a) RQ1 DNase 10× Reaction Buffer (Promega): 400 mM Tris–HCl (pH 8.0), 100 mM $MgSO_4$ and 10 mM $CaCl_2$.
  (b) RQ1 RNase-Free DNase (Promega. M6101).
  (c) DNasa stop solution (Promega): 20 mM EGTA (pH 8.0).
(4) Ribosomal RNA depletion:

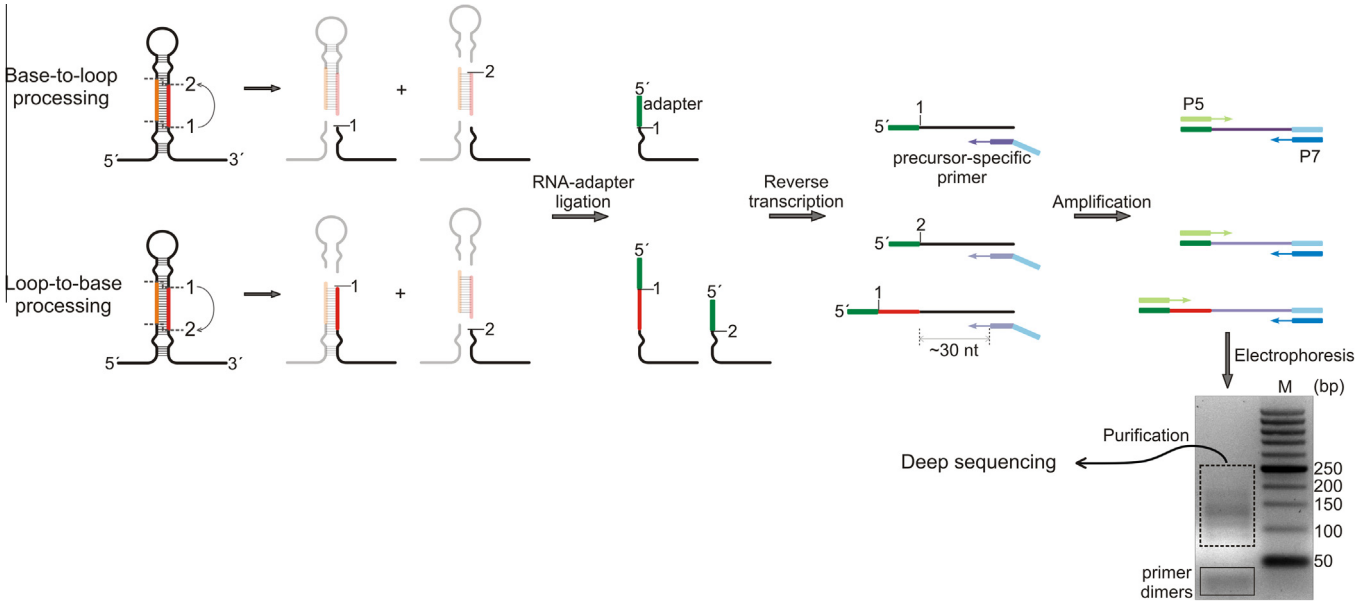

Fig. 1. Schematic representation of the SPARE method. The flowchart illustrates the detection of processing intermediates from precursors processed in opposite directions, i.e., base-to-loop and loop-to-base (numbers 1 and 2 in the precursors indicate the order and position of Dicer cuts. MiRNA/miRNA* duplexes are shown in orange and red). RNA adapters (highlighted in dark green, see Section 2.1.2) are ligated to 5′ ends of rRNA-depleted RNA. After RNA adapter ligation, cDNA is synthesized by using precursor-specific primers (precursor-specific sequences are shown in purple, and their 5′ common tail in light blue, see Section 2.1.1) that hybridize ~30 nt downstream the miRNA or miRNA* in the 3′ arm of the precursor. In the case of a base-to-loop processing, only one processing intermediate resulting from the first Dicer cut can be reverse transcribed. However, all processing intermediates can be reverse transcribed if the precursor is processed by a loop-to-base pathway. cDNA sequences corresponding to processing intermediates are then amplified by PCR using P5 (highlighted in light green) and P7 (highlighted in in dark blue) generic primers (see Section 2.1.3), and the products are purified on a 2% agarose gel (lane M, 50 bp DNA ladder). PCR products are finally analyzed by deep sequencing.

(a) RiboMinus™ Plant Kit for RNA-Seq (Invitrogen, A10838).
(b) Magna-Sep™ Magnetic Particle Separator (Invitrogen, K1585-01) or equivalent.
(c) Water baths or heat blocks.

(5) 5′ RNA-adapter ligation:
(a) T4 RNA Ligase (Fermentas, EL0021).
(b) T4 RNA Ligase 10× Reaction Buffer (Fermentas): 500 mM Tris–HCl (pH 7.5), 100 mM $MgCl_2$, 100 mM DTT, 10 mM ATP.
(c) 1 mg/ml BSA.
(d) RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777-019).

(6) Reverse transcription:
(a) 20× dNTP mix: 10 mM dATP, 10 mM dCTP, 10 mM dGTP, 10 mM dTTP.
(b) SuperScript™ III RT (Invitrogen, 18080).
(c) 0.1 M DTT (Invitrogen).
(d) 5× First-Strand Buffer (Invitrogen): 250 mM Tris–HCl (pH 8.3), 375 mM KCl, 15 mM $MgCl_2$.
(e) RNaseOUT™ Recombinant Ribonuclease Inhibitor (Invitrogen, 10777-019).
(f) RNase H (Invitrogen, 18021).

(7) PCR:
(a) TaKaRa Ex Taq™ (RR001A).
(b) dNTP Mixture (2.5 mM each) (TaKaRa).
(c) 10× Ex Taq Buffer (20 mM Mg^{2+} plus) (TaKaRa).

(8) PCR product purification
(a) Agarose (Invitrogen, 16500100).
(b) Cincuenta Marker DNA ladder (Biodynamics, B041-50).
(c) 50× TAE stock solution: 242 g Tris Base, 57.1 mL glacial acetic acid, 100 mL 0.5 M EDTA, H2O 1000 ml.
(d) 6× Agarose gel loading solution: 0.2% (w/v) Xylene cyanol FF, 0.2% (w/v) bromophenol blue, 50 mM Na2H2EDTA (pH 8.0), 60% glycerol.
(e) Wizard® SV Gel and PCR Clean-Up System (Promega, A9280).
(f) Heat block.

2.1. Oligonucleotides

2.1.1. Precursor-specific oligonucleotides

Precursor-specific primers were designed to hybridize to the 3′ arm of 105 miRNA precursors approximately 30 nt downstream the 3′ end of the miRNA or miRNA* (see Fig. 1) Other criteria used for precursor-specific primers design include: contain a C or a G base at their 3′ end, must not hybridize in introns, should not contain dinucleotide repeats, and contain a precursor-specific sequence of 20 nt.

As specified in Table 1, 64 oligonucleotides were design to detect miRNAs present in a broad range of species at least across angiosperms (named "conserved miRNAs") [23], and the remaining 41 were design to match evolutionary younger miRNAs present in fewer species more related to Arabidopsis (named "young miRNAs") [34] (Table 1).

2.1.2. Oligoribonucleotide 5́ adapter compatible with Illumina sequencing

5′ Adapter: 5′-GUUCAGAGUUCUACAGUCCGAC.

2.1.3. Generic oligonucleotides

P5 forward primer: 5′-AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA.

Underlined sequence corresponds to the Illumina sequencing primer.

P7 reverse primer: 5′-CAAGCAGAAGACGGCAACGA.

2.2. Oligonucleotides for multiplex SPARE libraries

2.2.1. Generic primers for the first PCR reaction

PCR1Fw: 5′-GTTCAGAGTTCTACAGTCCGAC.
PCR1Rv: 5′-CCTTGGCACCCGAGAATTCCA.

2.2.2. Illumina indexed primers

Illumina indexed primers are part of the Illumina RS-200-0012 TruSeq® Small RNA Sample Prep Kit.

3. Protocol

3.1. Isolation of total RNA

(1) For each tissue sample, isolate total RNA with TRIzol reagent (Invitrogen) according to the manufacturer's protocol.

3.1.1. Comment

RNA can be isolated from either flash-frozen samples stored below −70 °C, or from freshly collected tissues. We usually work with three different tissues as starting material: whole seedlings, leaves and inflorescences. This is done to increase the diversity in miRNA precursors detected, that otherwise could be missed if expressed in certain tissues or stages of development. We have used Arabidopsis wild-type plants and mutants defective in *FIERY*, which causes an increased accumulation of miRNA processing intermediates [35]. However, plant genotype, type of tissue as well as growth conditions may be chosen allowing diversity in the analyses of processing intermediates.

3.2. RNA precipitation

(2) Add RNase-free water to RNA samples to a final volume of 100 μl.
(3) Add 1 μl of 20 μg/μl glycogen, 10 μl of 3 M, pH 5.2 sodium acetate and 220 μl of absolute ethanol.
(4) Allow RNA precipitation by keeping the samples for at least 1 h at −20 °C.
(5) Centrifuge in a tabletop centrifuge at 4 °C and at maximum speed (approx. 14,000*g*) for 20 min.
(6) Discard the supernatant and wash the RNA pellets with 900 μl of 80% ethanol.
(7) Centrifuge at 4 °C and at maximum speed (approx. 14,000*g*) for 5 min.
(8) Discard the supernatant. Spin down the samples to collect residual ethanol and discard it by carefully pipetting at the bottom of the tube. Dry the RNA pellets for 5–10 min at 37 °C. Do not over dry the RNA pellet, otherwise it will be difficult to dissolve.
(9) Dissolve the RNA pellet in 10 μl of RNase-free water.

3.2.1. Comment

The RNA precipitation step at this point of the protocol is for RNA concentration purposes. Hence, and depending on the RNA yield obtained in step 1, the RNA precipitation could be avoided. Please see point 3.3 for the amount of RNA required.

3.3. DNase treatment

(10) Prepare a mix of the different RNA samples to obtain 10 μg of RNA in a final volume of 8 μl. In our approach, we mix 3.33 μg of each RNA from seedlings, leaves and inflorescences in 8 μl final volume.
(11) Add to the RNA mix 1 μl of RQ1 DNase 10× Reaction Buffer (Promega) and 1 μl of RQ1 DNase (Promega).
(12) Incubate 30 min at 37 °C.

Table 1
Sequences of precursor-specific oligonucleotides

Evolutionary conservation	Oligos	Sequences for regular SPARE libraries	Sequences for multiplexed SPARE libraries
	MIX 1		
Conserved	*MIR156a*	agcagaagacggcatacgagagattgagacatagagaac	ccttggcacccgagaattccagagattgagacatagagaac
Conserved	*MIR156b*	agcagaagacggcatacgagtctaagccaaatttgagag	ccttggcacccgagaattccagtctaagccaaatttgagag
Conserved	*MIR156c*	agcagaagacggcatacgagggaacacaattagaagaag	ccttggcacccgagaattccagggaacacaattagaagaag
Conserved	*MIR156d*	agcagaagacggcatacgacacaaaatcataactagaac	ccttggcacccgagaattccacacaaaatcataactagaac
Conserved	*MIR156h*	agcagaagacggcatacgaataacatacgctcatgacac	ccttggcacccgagaattccaataacatacgctcatgacac
Conserved	*MIR157c*	agcagaagacggcatacgatatatagatgtgaatgtgcg	ccttggcacccgagaattccatatatagatgtgaatgtgcg
Young	*MIR158a*	agcagaagacggcatacgaatttacacgctcgtagcatc	ccttggcacccgagaattccaatttacacgctcgtagcatc
Young	*MIR158b*	agcagaagacggcatacgacatatacacacgttcgtaac	ccttggcacccgagaattccacatatacacacgttcgtaac
Conserved	*MIR159b*	agcagaagacggcatacgacattcttaaaagaactaaag	ccttggcacccgagaattccacattcttaaaagaactaaag
Conserved	*MIR160a*	agcagaagacggcatacgacatcaacacaattcaattgg	ccttggcacccgagaattccacatcaacacaattcaattgg
Conserved	*MIR160b*	agcagaagacggcatacgagagacaaagtactcatatac	ccttggcacccgagaattccagagacaaagtactcatatac
Conserved	*MIR160c*	agcagaagacggcatacgaagatatttcatatttagttg	ccttggcacccgagaattccaagatatttcatatttagttg
Young	*MIR161*	agcagaagacggcatacgaccatctccttacacaatttc	ccttggcacccgagaattccaccatctccttacacaatttc
Conserved	*MIR162b*	agcagaagacggcatacgagcaatactcaaatagatatc	ccttggcacccgagaattccagcaatactcaaatagatatc
	MIX 2		
Young	*MIR163*	agcagaagacggcatacgaccatattttcaggcacaacc	ccttggcacccgagaattccaccatattttcaggcacaacc
Conserved	*MIR164b*	agcagaagacggcatacgagcgtaacacttgaaccctcg	ccttggcacccgagaattccagcgtaacacttgaaccctcg
Conserved	*MIR164c*	agcagaagacggcatacgattctgttctatccattgacg	ccttggcacccgagaattccattctgttctatccattgacg
Conserved	*MIR165a*	agcagaagacggcatacgaaattaacaaaaaaaaaatgtaac	ccttggcacccgagaattccaaattaacaaaaaaaaaatgtaac
Conserved	*MIR165b*	agcagaagacggcatacgatcaaggttttgtagtttctc	ccttggcacccgagaattccatcaaggttttgtagtttctc
Conserved	*MIR166a*	agcagaagacggcatacgaaccctaatttgaaaagaagg	ccttggcacccgagaattccaaccctaatttgaaaagaagg
Conserved	*MIR166b*	agcagaagacggcatacgaaaatggacaaatcttcttcg	ccttggcacccgagaattccaaaatggacaaatcttcttcg
Conserved	*MIR166e*	agcagaagacggcatacgaacctggagatagtttactc	ccttggcacccgagaattccaacctggagatagtttactc
Conserved	*MIR166f*	agcagaagacggcatacgatgatcggctaacgattggtc	ccttggcacccgagaattccatgatcggctaacgattggtc
Conserved	*MIR167a*	agcagaagacggcatacgaagatagagagaaaatttagg	ccttggcacccgagaattccaagatagagagaaaatttagg
Conserved	*MIR167b*	agcagaagacggcatacgatcaatcggcatgtggaatgc	ccttggcacccgagaattccatcaatcggcatgtggaatgc
Conserved	*MIR167d*	agcagaagacggcatacgactttctcatgaaatgaagtg	ccttggcacccgagaattccactttctcatgaaatgaagtg
Conserved	*MIR168a*	agcagaagacggcatacgaaatcttccagatctgatagg	ccttggcacccgagaattccaaatcttccagatctgatagg
	MIX 3		
Conserved	*MIR168b*	agcagaagacggcatacgaaattgtgtcagatctgatag	ccttggcacccgagaattccaaattgtgtcagatctgatag
Conserved	*MIR169a*	agcagaagacggcatacgagagttcttgcgatctttacc	ccttggcacccgagaattccagagttcttgcgatctttacc
Conserved	*MIR169b*	agcagaagacggcatacgaaatgaaaaggtagaatatcc	ccttggcacccgagaattccaaatgaaaaggtagaatatcc
Conserved	*MIR169d*	agcagaagacggcatacgaatataacggatagagatatc	ccttggcacccgagaattccaatataacggatagagatatc
Conserved	*MIR169e*	agcagaagacggcatacgaatgatatgtggttaggtatc	ccttggcacccgagaattccaatgatatgtggttaggtatc
Conserved	*MIR169f*	agcagaagacggcatacgatcatgactaatgtatatctg	ccttggcacccgagaattccatcatgactaatgtatatctg
Conserved	*MIR169g*	agcagaagacggcatacgatcatgatgatagatatcttg	ccttggcacccgagaattccatcatgatgatagatatcttg
Conserved	*MIR169j*	agcagaagacggcatacgaggagacagaaagaaaaacag	ccttggcacccgagaattccaggagacagaaagaaaaacag
Conserved	*MIR169l*	agcagaagacggcatacgatctagtgattcggaagacag	ccttggcacccgagaattccatctagtgattcggaagacag
Conserved	*MIR169m*	agcagaagacggcatacgaattttgaatgaaggtaaacg	ccttggcacccgagaattccaattttgaatgaaggtaaacg
Conserved	*MIR169n*	agcagaagacggcatacgatttgaagacagaaagaaaag	ccttggcacccgagaattccatttgaagacagaaagaaaag
Conserved	*MIR170*	agcagaagacggcatacgagacctaacgaggcaaacttc	ccttggcacccgagaattccagacctaacgaggcaaacttc
Conserved	*MIR171a*	agcagaagacggcatacgaaaatcgatctacaagaccac	ccttggcacccgagaattccaaaatcgatctacaagaccac
	MIX 4		
Conserved	*MIR171b*	agcagaagacggcatacgaaatgatacatacctttaagg	ccttggcacccgagaattccaaatgatacatacctttaagg
Conserved	*MIR171c*	agcagaagacggcatacgaataccttgattgatcacatc	ccttggcacccgagaattccaatacctttgattgatcacatc
Conserved	*MIR172a*	agcagaagacggcatacgaaataatctatacagagggag	ccttggcacccgagaattccaaataatctatacagagggag
Conserved	*MIR172b*	agcagaagacggcatacgatatcttcaagtttctttctc	ccttggcacccgagaattccatatcttcaagtttctttctc
Conserved	*MIR172d*	agcagaagacggcatacgataaccacatgatgaaaatgg	ccttggcacccgagaattccataaccacatgatgaaaatgg
Conserved	*MIR172e*	agcagaagacggcatacgatctcttcactagagaatgac	ccttggcacccgagaattccatctcttcactagagaatgac
Young	*MIR173*	agcagaagacggcatacgaaagatctctaacattaaatc	ccttggcacccgagaattccaaagatctctaacattaaatc
Conserved	*MIR319a*	agcagaagacggcatacgacaagcatgtttttgtgcagg	ccttggcacccgagaattccacaagcatgtttttgtgcagg
Conserved	*MIR319b*	agcagaagacggcatacgagcttgtatatatatagatataa	ccttggcacccgagaattccagcttgtatatatatagatataa
Conserved	*MIR319c*	agcagaagacggcatacgatactcatcacatataaacag	ccttggcacccgagaattccatactcatcacatataaacag
Conserved	*MIR390a*	agcagaagacggcatacgagattttaggcgttttgcctc	ccttggcacccgagaattccagattttaggcgttttgcctc
Conserved	*MIR390b*	agcagaagacggcatacgaaaacgaaggagggaatgaag	ccttggcacccgagaattccaaaacgaaggagggaatgaag
Conserved	*MIR391*	agcagaagacggcatacgaacaactgaaatacctgatc	ccttggcacccgagaattccaacaactgaaatacctgatc
	MIX 5		
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Conserved	*MIR393b*	agcagaagacggcatacgatgtctccggtcatgaaaccc	ccttggcacccgagaattccatgtctccggtcatgaaaccc
Conserved	*MIR394a*	agcagaagacggcatacgaacttgtatatagatcaagag	ccttggcacccgagaattccaacttgtatatagatcaagag
Conserved	*MIR394b*	agcagaagacggcatacgattatatatcaaatcatgagg	ccttggcacccgagaattccattatatatcaaatcatgagg

Table 1 (*continued*)

Evolutionary conservation	Oligos	Sequences for regular SPARE libraries	Sequences for multiplexed SPARE libraries
Conserved	*MIR395a*	agcagaagacggcatacgattaccatataaaatctcgtc	ccttggcacccgagaattccattaccatataaaatctcgtc
Conserved	*MIR395b*	agcagaagacggcatacgaaagaacccataaaacggctg	ccttggcacccgagaattccaaagaacccataaaacggctg
Conserved	*MIR395c*	agcagaagacggcatacgaagataatagaaaaccgcagc	ccttggcacccgagaattccaagataatagaaaaccgcagc
Conserved	*MIR396a*	agcagaagacggcatacgacgctttcatatatatgaacg	ccttggcacccgagaattccacgctttcatatatatgaacg
Conserved	*MIR396b*	agcagaagacggcatacgaaagaagaatcttgacaagtg	ccttggcacccgagaattccaaagaagaatcttgacaagtg
Conserved	*MIR397a*	agcagaagacggcatacgataaaaaacgatccgcatacc	ccttggcacccgagaattccataaaaaacgatccgcatacc
Conserved	*MIR398b*	agcagaagacggcatacgaatgagtaaaagccagccttg	ccttggcacccgagaattccaatgagtaaaagccagccttg
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Conserved	*MIR399b*	agcagaagacggcatacgatacctttgatttcttctctc	ccttggcacccgagaattccatacctttgatttcttctctc
	MIX 6		
Conserved	*MIR399c*	agcagaagacggcatacgagattgatattagcagaaccg	ccttggcacccgagaattccagattgatattagcagaaccg
Young	*MIR400*	agcagaagacggcatacgaagaagccgcaccacaagaac	ccttggcacccgagaattccaagaagccgcaccacaagaac
Young	*MIR402*	agcagaagacggcatacgattttctttgataaagttttc	ccttggcacccgagaattccattttctttgataaagttttc
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Young	*MIR447a*	agcagaagacggcatacgactctcgatatataatactac	ccttggcacccgagaattccactctcgatatataatactac
Young	*MIR447b*	agcagaagacggcatacgattctcaatatataatactac	ccttggcacccgagaattccattctcaatatataatactac
Young	*MIR472*	agcagaagacggcatacgattggactccagtagtaaccc	ccttggcacccgagaattccattggactccagtagtaaccc
Young	*MIR771*	agcagaagacggcatacgataacgatgtagagttgtaag	ccttggcacccgagaattccataacgatgtagagttgtaag
Young	*MIR773a*	agcagaagacggcatacgacaaaggcggcaacaactatg	ccttggcacccgagaattccacaaaggcggcaacaactatg
Young	*MIR775*	agcagaagacggcatacgaaagttcgaatctttatacac	ccttggcacccgagaattccaaaacgaaggagggaatgaag
Young	*MIR776*	agcagaagacggcatacgaaaggtagccatcgagtgtcg	ccttggcacccgagaattccaaaggtagccatcgagtgtcg
Young	*MIR779*	agcagaagacggcatacgacaaagcaataccgttccatg	ccttggcacccgagaattccacaaagcaataccgttccatg
	MIX 7		
Young	*MIR780*	agcagaagacggcatacgatataattatccatggaactg	ccttggcacccgagaattccatataattatccatggaactg
Young	*MIR781*	agcagaagacggcatacgaaaagacgtttacacataacc	ccttggcacccgagaattccaaaagacgtttacacataacc
Young	*MIR822*	agcagaagacggcatacgaattgacaacgaccttaagtg	ccttggcacccgagaattccaattgacaacgaccttaagtg
Young	*MIR824*	agcagaagacggcatacgatcttcacaaaatcaccattg	ccttggcacccgagaattccatcttcacaaaatcaccattg
Young	*MIR825*	agcagaagacggcatacgacaacatcacatgtgagatcc	ccttggcacccgagaattccacaacatcacatgtgagatcc
Conserved	*MIR827*	agcagaagacggcatacgaagatgctaaaaacatgatcg	ccttggcacccgagaattccaagatgctaaaaacatgatcg
Young	*MIR830*	agcagaagacggcatacgagtcgatgttcttcaaaattc	ccttggcacccgagaattccagtcgatgttcttcaaaattc
Young	*MIR833*	agcagaagacggcatacgagaggagtggtgcagcgtttc	ccttggcacccgagaattccagaggagtggtgcagcgtttc
Young	*MIR834*	agcagaagacggcatacgaatgactgcttctgacaccac	ccttggcacccgagaattccaatgactgcttctgacaccac
Young	*MIR839*	agcagaagacggcatacgatatcactcactcatgtgagc	ccttggcacccgagaattccatatcactcactcatgtgagc
Young	*MIR841*	agcagaagacggcatacgaatagcaccaagtcgaccatg	ccttggcacccgagaattccaatagcaccaagtcgaccatg
Young	*MIR846*	agcagaagacggcatacgaggtcattagtcgaatttacc	ccttggcacccgagaattccaggtcattagtcgaatttacc
Young	*MIR847*	agcagaagacggcatacgaatatctaacagatttttcac	ccttggcacccgagaattccaatatctaacagatttttcac
	MIX 8		
Young	*MIR848*	agcagaagacggcatacgaaggataaagtggttgcgacg	ccttggcacccgagaattccaaggataaagtggttgcgacg
Young	*MIR849*	agcagaagacggcatacgacaacaattaaaccagggatc	ccttggcacccgagaattccacaacaattaaaccagggatc
Young	*MIR850*	agcagaagacggcatacgatggtttcatatagttttatg	ccttggcacccgagaattccatggtttcatatagttttatg
Young	*MIR851*	agcagaagacggcatacgagggacacaagagttcacatg	ccttggcacccgagaattccagggacacaagagttcacatg
Young	*MIR853*	agcagaagacggcatacgaaatctgtttttgacaaatac	ccttggcacccgagaattccaaatctgtttttgacaaatac
Young	*MIR856*	agcagaagacggcatacgatgtgtacagatggatcatgg	ccttggcacccgagaattccatgtgtacagatggatcatgg
Young	*MIR859*	agcagaagacggcatacgaatagatagctccacaacgtc	ccttggcacccgagaattccaatagatagctccacaacgtc
Young	*MIR860*	agcagaagacggcatacgagtttctcattctttattccc	ccttggcacccgagaattccagtttctcattctttattccc
Young	*MIR862*	agcagaagacggcatacgactcgtatatacgcagtacag	ccttggcacccgagaattccactcgtatatacgcagtacag
Young	*MIR863*	agcagaagacggcatacgattgtcagatctcctcttatg	ccttggcacccgagaattccattgtcagatctcctcttatg
Young	*MIR864*	agcagaagacggcatacgaatatctctgaaatttcggac	ccttggcacccgagaattccaatatctctgaaatttcggac
Young	*MIR865*	agcagaagacggcatacgactgattttcgataaagaaac	ccttggcacccgagaattccactgattttcgataaagaaac
Young	*MIR870*	agcagaagacggcatacgaatttgacataccttggactc	ccttggcacccgagaattccaatttgacataccttggactc

(13) Stop the DNase treatment by adding 1 μl of DNase stop solution (Promega) and incubate 10 min at 65 °C.

3.3.1. Comment

30 μg of total RNA will be necessary in order to perform 8 reverse transcription reactions, which will allow the analysis of all 105 miRNA precursors presented in Table 1.

3.4. Ribosomal RNA depletion

(14) Use the DNA-free RNA (10 μg) from step 13 for rRNA depletion using the RiboMinus™ Plant Kit for RNA-Seq (Invitrogen, A10838) according to the manufacturer's protocol.
(15) Precipitate the rRNA-depleted RNA (~320 μl) by adding 3 μl of 20 μg/μl glycogen, 30 μl of 3 M pH 5.2 sodium acetate and 750 μl of absolute ethanol.

(16) Follow steps 4–8 to allow RNA precipitation and RNA pellet washing and drying.
(17) Dissolve the RNA pellet in 11 μl of RNase-free water.

3.4.1. Comment

Each RiboMinus™ purification has a limit of 10 μg of total RNA as starting material, giving a yield of ~1.5 μg of rRNA-depleted RNA (RiboMinus™ RNA). Giving that 500 ng of RNA will be needed for each RT reaction, one RiboMinus™ purification will serve for three RT reactions. Hence, the number of RT reactions must be calculated in order to perform the proper number of RiboMinus™ purifications. In the present protocol, we list 105 precursor-specific oligonucleotides (Table 1) that are used in eight multiplex RT reactions (point 3.6), therefore, three RiboMinus™ purifications are needed.

3.5. 5′ RNA-adapter ligation

(18) Add 1 μl of 200 μM 5′ adapter to each RNA sample from step 17.
(19) Incubate the tubes at 65 °C for 5 min to denature the RNA and immediately place on ice for 2 min.
(20) Each 5′ adapter ligation reaction requires 2 μl of 10× RNA Ligase Reaction Buffer, 2 μl of 1 mg/ml BSA, 2 μl of RNaseOUT and 2 μl of T4 RNA Ligase.
(21) Incubate at 37 °C for 1 h, and then inactivate the RNA Ligase by incubating at 70 °C for 15 min. RNA Ligase inactivation is critical. Residual ligase activity could lead to adapter-precursor primer ligation, resulting in high amounts of primer specific reads after deep sequencing.
(22) Precipitate and wash the adapter-ligated RNA following steps 2–8. Dissolve the RNA pellet in 16 μl of RNase-free water.

3.6. Reverse transcription (RT)

(23) Prepare eight precursor-specific primer mixes for RT reactions. Mix 13 or 14 different primers (see Table 1 for primer mix details) to obtain a final concentration of 0.05 μM for each primer.
(24) For each RT reaction, prepare the following mix: 0.5 μl of one of the precursor-specific primer mix from step 23, 6 μl (~0.5 μg) of adapter-ligated RNA from step 22, 1 μl of 20× dNTP mix and 6 μl of H_2O.
(25) Prepare an RT Mastermix containing the following components per reaction: 4 μl of 5× First-Strand Buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT and 0.5 μl of SuperScript III reverse transcriptase.
(26) Heat the mix from step 24 at 65 °C for 5 min to denature the RNA. Incubate the mix at least 1 min on ice.
(27) Add 6.5 μl of the Mastermix from step 25 to each sample and incubate for 60 min at 50 °C.
(28) Inactivate the reaction by incubating the tubes for 15 min at 70 °C.
(29) Add 1 μl (2 units) of RNase H and incubate for 30 min at 37 °C.

3.6.1. Comment

As stated above, we divided the 105 precursor-specific primers in eight groups, each one containing 13 or 14 primers. Hence, to evaluate the processing intermediates of all miRNA precursors presented in Table 1, eight RT reactions must be performed. However, it is possible to analyze a more reduced group of precursors by preparing the mix/es with the desired combination of precursor-specific primers. According to our experience, up to 20 precursor-specific primers can be used in one single multiplex RT reaction. In principle, all the precursor-specific primers might be pooled into one reaction, however, in our hands we found that primer dimers appear frequently making more complicated the following steps and diminishing the number of reads of the miRNA precursors.

3.7. PCR amplification

3.7.1. Comment

Before proceeding to cDNA libraries amplification with generic P5 and P7 primers (point 3.7.2), it is recommended to check the quality of the cDNAs by detecting the presence of precursor-specific processing intermediates. For this purpose, a nested PCR is done (Fig. 2):

(a) Select a miRNA precursor (or precursors) to be analyzed; as an example, we selected pre-miR172a. Prepare a PCR mix containing the following components (50 μl final volume): 5 μl of 10× Ex Taq Buffer, 4 μl of dNTP Mixture (2.5 mM each, TaKaRa), 1 μl of 10 μM P5 forward primer, 1 μl of 10 μM precursor-specific reverse primer (miR172a for the example shown in Fig. 2), 37.75 μl of H2O, 0.25 μl of Ex Taq polymerase and 1 μl of the corresponding cDNA (for miR172a, the cDNA prepared with primer MIX 4. See Table 1)
(b) Program the following PCR conditions: 94 °C for 3 min; 18 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s; final extension of 72 °C for 5 min. Keep 45 μl of this first PCR for further analysis on agarose gel, and 5 μl to be used as a template for the second PCR.
(c) Second PCR: Prepare a PCR mix containing the following components (50 μl final volume): 5 μl of 10× Ex Taq Buffer, 4 μl of dNTP Mixture (2.5 mM each, TaKaRa), 1 μl of 10 μM P5 forward primer, 1 μl of 10 μM nested reverse primer (for our example, pre-miR172a-nested primer: 5′-GGAAAGAATAGTCGTTGATT), 37.75 μl of H2O, 0.25 μl of Ex Taq polymerase and 1 μl of the first PCR from step b as template.
(d) Perform the second PCR as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s; final extension of 72 °C for 5 min.
(e) Resolve 45 μl products from first and second PCRs on a 2.5% agarose gel (Fig. 2). Check for the expected product size, according to the processing of the precursor under analysis, and the position of nested primer (Fig. 2). For the example shown, pre-miR172 is processed by a base-to-loop mechanism, thus, leading to one detectable processing intermediate (see Fig. 1). Alternatively as an additional check, PCR products may be gel purified and cloned using a kit such as Zero Blunt TOPO PCR cloning kit (Invitrogen) for low throughput sequencing.

3.7.2. cDNA libraries amplification

(30) Prepare a PCR Mastermix containing the following components per reaction (100 μl final volume per reaction): 10 μl of 10× Ex Taq Buffer, 8 μl of dNTP Mixture (2.5 mM each, TaKaRa), 2 μl of 10 μM P5 forward primer, 2 μl of 10 μM P7 reverse primer, 75.5 μl of H2O and 0.5 μl of Ex Taq polymerase.
(31) Aliquot 2 μl of each cDNA from point 3.6 in PCR tubes and add 98 μl of the Mastermix from step 30 to each tube. Also prepare a no-template control PCR reaction with H_2O instead of cDNA to check for DNA contamination.
(32) Program the following cycle conditions: 94 °C for 3 min; 94 °C for 20 s, 60 °C for 30 s, 72 °C for 20 s. Remove 15 μl aliquots every other cycle following cycle number 10

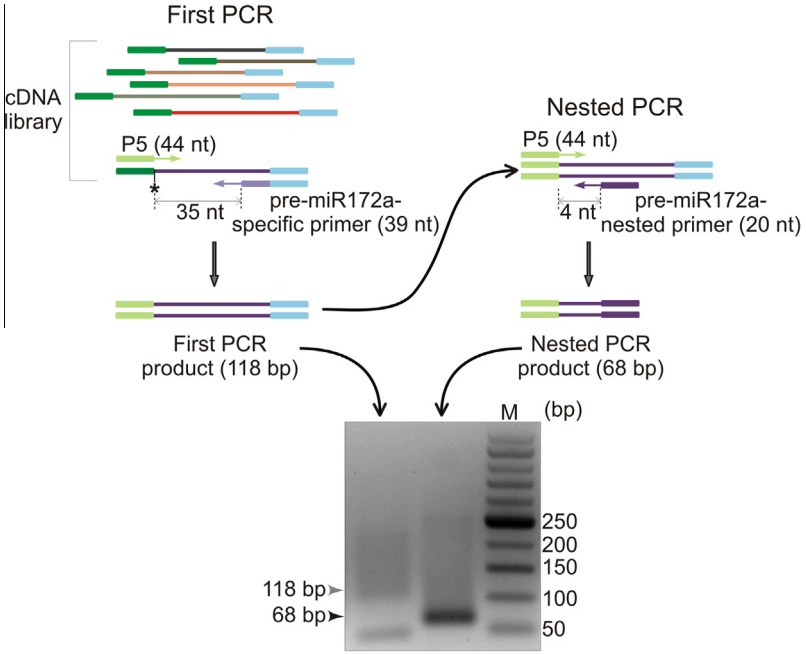

Fig. 2. Detection of precursor-specific processing intermediates by nested PCR. By using as a template the cDNA library, the first PCR is done with P5 forward generic primer (in light green, see Section 2.1.3) and a precursor-specific primer (in this case we have chosen *MIR172a*, see Table 1, Mix 4). The first PCR product is then used as a template for the second nested PCR, using P5 forward primer and a nested oligonucleotide specific for the precursor under study (pre-miR172a-nested primer, shown in dark purple, see Section 3.7.1 step c). The products from both PCRs are resolved on a 2.5% agarose gel. The asterisk indicates the position of the DCL1 cleavage, where the adapter was ligated. Arrowheads on the gel indicate the expected sizes for PCR products of the first PCR (grey arrowhead) and nested PCR (black arrowhead). Lane M on the gel, 50 bp DNA ladder. Adapters are shown in dark green (see Section 2.1.2). The common 5′ sequence corresponding to all precursor-specific primers is shown in light blue.

(by putting the cycler on hold at the end of the 72 °C step) to determine the necessary number of cycles for amplifying the cDNA libraries. According to our experience, it is not necessary to amplify for more than 18 cycles.

(33) Resolve the PCR products on a 2.5% agarose gel. These products should appear as several faint bands with a difference of about 21 bp in size between them due to the sequential Dicer activity during precursor processing (see Fig. 1).

(34) Perform a 100 µl PCR according to steps 30, 31 and 32 with the previously determined optimal cycle number.

3.8. PCR products purification

(35) Add 20 µl of 6× agarose gel loading solution to each 100 µl PCR samples from step 34, and resolve samples on a 2% agarose gel.

(36) For each PCR product cut out a block of agarose containing bands ranging from ~70 bp to ~250 bp and transfer them (by making gel slices) to pre-weighed 1.5 ml tubes and weight them again.

(37) Elute the DNA from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Recover the DNA in 20 µl of nuclease-free water.

(38) The DNA samples (eight different samples if analyzing the 105 precursors presented in the present protocol) are then pooled together to be subjected to Illumina SBS sequencing. Please see comment 4.2.4.

4. Construction of multiplexed SPARE libraries

The SPARE protocol may result in a useful data set for a global comparison of processing intermediates of miRNA precursors under different biological conditions. Hence, if it is desirable to construct more than one SPARE library, we present an extended protocol that allows multiplexed sequencing, and therefore permitting the simultaneous sequencing of different libraries. The multiplexing strategy, depicted in Fig. 3, introduces to the protocol described in point 3 one additional step of PCR for the addition of the index sequence to the library.

4.1. Comment

Precursor-specific primers for indexed SPARE libraries must contain at their 5′ end a sequence that allows the hybridization of Illumina indexed primers (see Table 1). It is important to note that this 5′sequence differs from that of precursor-specific primers used for the SPARE library protocol presented in point 3. Therefore, special attention must be taken when choosing the SPARE strategy to order the proper set of precursor-specific primers.

As stated above, the single PCR step from the SPARE protocol presented in point 3 (see Fig. 1), has been replaced by two rounds of PCR. This has been done because we have observed high levels of primer dimer and nonspecific PCR products when performing just a single PCR with P5 and the long Illumina indexed primers after cDNA synthesis. Conversely, a strong specific amplification of the cDNA library has been observed by using short primers for the first PCR. Then, using this first PCR product as a template, a few PCR cycles with P5 primer and an Illumina indexed primer are enough for "tagging" the DNA library with the index sequence.

4.2. Protocol for the construction of multiplexed SPARE libraries

(39) Follow steps 1 to 29 from point 3, for RNA isolation, RNA precipitation, DNAse treatment, ribosomal RNA depletion, 5′ RNA-adapter ligation and reverse transcription. At this point, per library constructed, a total of eight cDNA samples are obtained if the 105 precursors presented in the present protocol are analyzed (see comment 3.6.1).

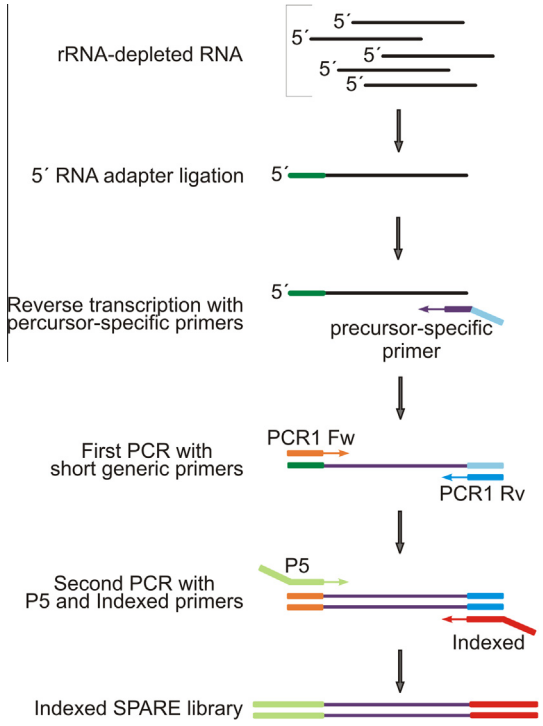

Fig. 3. SPARE protocol for multiple libraries. After RNA adapter ligation to rRNA-depleted RNA, cDNA is synthesized by using precursor-specific primers. Then, two rounds of PCR are done. The first PCR is performed with short (~ 20 nt) generic primers: PCR1Fw shown in orange and PCR1Rv shown in blue (see Section 2.2.1). This short primers hybridize to the adapter sequence (highlighted in dark green) and to the 5′ tail sequence present in precursor-specific primers (highlighted in light blue). Using as a template the first PCR, P5 (highlighted in light green, see Section 2.1.3) and indexed primers (highlighted in red, see Section 2.2.2) are used for a second PCR, leading to indexed DNA products.

4.2.1. First PCR for cDNA libraries amplification

(40) Prepare a PCR Mastermix containing the following components per reaction (25 µl final volume per reaction): 2.5 µl of 10× Ex Taq Buffer, 2 µl of dNTP Mixture (2.5 mM each, TaKaRa), 1,25 µl of 10 µM PCR1Fw primer, 1.25 µl of 10 µM PCR1Rv primer, 16.9 µl of H2O and 0.1 µl of Ex Taq polymerase.

(41) Aliquot 1 µl of each cDNA from step 39 in PCR tubes and add 24 µl of the Mastermix from step 40 to each tube. Also prepare a no-template control PCR reaction with H_2O instead of cDNA to check for DNA contamination.

(42) Perform the first PCR as follows: 94 °C for 3 min; 30 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s; and a final extension of 72 °C for 5 min. Use 5 µl of this PCR product to make a 1/10 dilution that will serve as a template for the second PCR (step 44).

4.2.2. Second PCR with P5 and indexed primers

(43) Prepare a PCR Mastermix containing the following components per reaction (100 µl final volume per reaction): 10 µl of 10× Ex Taq Buffer, 8 µl of dNTP Mixture (2.5 mM each, TaKaRa), 2.5 µl of 10 µM P5 primer, 72.5 µl of H2O and 0.5 µl of Ex Taq polymerase.

(44) Aliquot 4 µl of each first PCR diluted product from step 42 in PCR tubes. Add to each tube 2.5 µl of a 1/10 dilution of an Illumina indexed primer. Make sure to use one different indexed primer for each different SPARE library. Finally, add 96 µl of the Mastermix from step 43 to each tube.

(45) Perform the second PCR as follows: 94 °C for 3 min; 7 cycles of 94 °C for 30 s, 55 °C for 30 s and 72 °C for 50 s; and a final extension of 72 °C for 5 min.

4.2.3. PCR products purification

(46) Add 20 µl of 6× agarose gel loading solution to each 100 µl PCR samples from step 42, and resolve samples on a 2% agarose gel.

(47) For each PCR product cut out a block of agarose containing bands ranging from ~110 bp to ~290 bp and transfer them (by making gel slices) to pre-weighed 1.5 ml tubes and weight them again.

(48) Elute the DNA from the gel using the Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. Recover the DNA in 20 µl of nuclease-free water.

(49) For each indexed SPARE library prepared, 8 different DNA samples are obtained if the 105 precursors presented in Table 1 are analyzed. The 8 DNA samples corresponding to one indexed SPARE library can be mixed, and libraries are then ready to be subjected to Illumina multiplexed sequencing.

4.2.4. Comment

The SPARE library sequencing was done by Illumina GA II for regular SPARE presented in point 3, and Hi-Seq 2000 and Hi-Seq 2500 for multiplexed libraries presented in point 4, with single-end sequencing of 50 nt. For multiplexed sequencing, up to 24 indexed libraries can be sequenced simultaneously.

5. Concluding remarks

In general, miRNA processing intermediates in plants are hard to detect in RNA blots, probably due to their low abundance, while other approaches such as the modified 5′ RACE PCR are time-consuming. Based on the advantages of next generation sequencing, we described a protocol to systematically evaluate miRNA precursor intermediates in Arabidopsis. DCL1 cleavage positions on a certain precursor can be precisely identified, allowing the deduction of the processing direction as well as the accuracy of the processing machinery. We also present an extended protocol for multiplexed sequencing of different libraries. The method may also be useful to evaluate the global miRNA processing in different biological conditions.

6. Troubleshooting

- High smear background is seen in gel after second PCR (point 4.2.2): use ExTaq polymerase for all PCR reactions. Set up this second PCR using as a template different dilutions from the first PCR product (step 42).
- High amounts of primer reads are obtained after deep sequencing: inactivate RNA ligase as stated in step 21. Reduce the concentration of precursor-specific primer mixes in step 23.
- Low or no reads from certain precursors: poor quality of the starting RNA. Evaluate tissue, stage of development, or plant growth conditions in which the precursor under study is expressed. Test the detection of precursor-specific intermediates by modified 5′ RACE PCR (see Fig. 2).

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