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Identification and Expression Analysis of Small RNAs During Development

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Summary

RNA interference (RNAi) is a sequence-specific gene regulatory mechanism in which the specificity is determined by small RNAs. Three major classes of endogenous small RNAs, namely microRNAs (miRNAs), small interfering RNAs (siRNAs), and piwi-interacting RNAs (piRNAs/gsRNAs), have been characterized in vertebrates. The miRNAs are mainly involved in development and differentiation and alter gene expression through translational repression or mRNA cleavage. The siRNAs, in contrast, mainly defend against molecular parasites including viruses, transposons, and transgenes. We reported on the expression profile of miRNAs during Xenopus development using a combination of cloning and Northern blot analysis of stage-specific small RNAs. The expression of most miRNAs appeared to be regulated, and some were only expressed at specific stages of development. We also reported on small RNAs specifically expressed during gametogenesis in the mouse. The study revealed the existence of retrotransposon-derived siRNAs in oocytes and a novel class of small RNA (piRNAs/gsRNAs) in testes. In this chapter, we describe methods of low molecular weight RNA preparation, small RNA cloning, annotation of small RNAs, and analysis of expression during development.

Key Words: miRNA; siRNA; rasiRNA; piRNA; RNAi; development; small RNA.

1. Introduction

miRNAs—the best-characterized endogenous small RNAs in eukaryotes—have been identified in diverse plants and animals and are mainly involved in development and differentiation. They are generated by processing of the miRNA precursors (pre-miRNAs) by Dicer (*see* **Fig. 1** in **Chapter 4**) and regulate gene expression through translational repression or cleavage of

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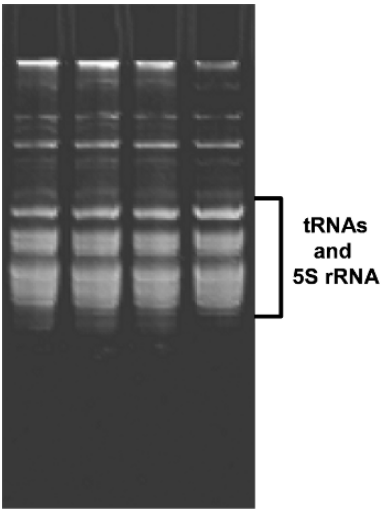

Fig. 1. Low molecular weight RNA obtained from mouse testes. Each ~2 g of low molecular weight RNA was analyzed by 15% PAGE and stained with EtBr. The tRNA and 5S rRNA bands are visible, and depletion of high molecular weight RNA is confirmed.

the target mRNA ***(1)***. The siRNAs are generated from long double-stranded RNA (dsRNA) by Dicer cleavage and are mainly involved in defense against molecular parasites including viruses, transposons, and transgenes through RNAi ***(2)***. Numerous studies have revealed the importance of miRNAs and other small RNAs in animal and plant development. In *Caenorhabditis elegans*, for instance, lin-4 and let-7 miRNAs are expressed at distinct stages of development and regulate the timing of larval developmental ***(3,4)***. In vertebrates, posttranscriptional regulation of HOX gene expression ***(5)*** and clearance of maternal mRNAs are mediated by miRNAs ***(6)***. Mutations in Dicer cause developmental arrest in zebrafish ***(7)*** and early developmental arrest with abnormal gene expression in mice ***(8)***. Lastly, our laboratory has unraveled specific expression of individual miRNAs and other small RNAs during Xenopus development ***(9)*** and mouse gametogenesis ***(10)***.

To study small RNAs that are involved in development, we first needed to determine their identity and expression in each developmental stage. Recombinant cloning and sequencing of small RNAs indeed enabled us to discover unidentified novel small RNAs. Since cloning results are not quantitative due to the multiple steps of the small RNA cloning procedure, it seemed necessary to confirm the expression by Northern blotting or RNA protection assay. To analyze spatial expression, *in situ* hybridization using modified synthetic

oligonucleotides has been developed ***(11)***. An alternative approach is to use the sensor assay ***(7,12)***, in which the sensor encodes a reporter gene carrying a 3′-UTR with a sequence complementary to the small RNA such that co-expression of small RNA and sensor mRNA leads to repression of reporter gene expression. A transgenic or mRNA injection approach can be used for *in vivo* reporter assay. A reporter construct with cloned 3′-UTR of the predicted target gene is used to validate the silencing of the target gene ***(13)***. In complementary experiments, one can deplete the endogenous miRNA using 2′-O-methyl antisense oligoribonucleotides. Together, these methods serve to unravel the biological function of individual miRNAs and identification of their cognate target mRNAs ***(14)***.

2. Materials

2.1. Low Molecular Weight RNA Preparation and Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE)

1. Fresh embryos of specific stages or tissues. Samples that are quick-frozen after isolation using liquid nitrogen are also usable.
2. Guanidium thiocyanate-phenol-based RNA extraction reagents such as TRIzol (Invitrogen, Carlsbad, CA).
3. Polyethylene glycol (PEG) solution (*see* **Note 1**): 13% (w/v) polyethylene glycol 6000 or 8000, 1.6 *M* NaCl. Autoclave and store at room temperature.
4. 100% isopropanol, 80% ethanol. Store at –20 °C.
5. TBE (20X): 1.78 *M* Tris base, 1.78 *M* boric acid, 40 m*M* EDTA, pH 8.0. Autoclave and store at room temperature.
6. RNase-free water.
7. Formamide loading buffer: 0.5 mL of 20X TBE, 10 mg of bromphenol blue, 10 mg of xylene cyanol, 10 mL of formamide. Store at 4 °C.
8. Forty percent acrylamide/bis solution (38:2). Store at 4 °C.
9. Ammonium persulfate: Prepare 10% solution in water and freeze in single-use (200-µL) aliquots. Store at –20 °C.
10. N,N,N’,N’-Tetramethyl-ethylenediamine (TEMED).
11. Urea (high quality, such as Sigma U5378).
12. Ethidium bromide (EtBr) solution in water (~2 µg/mL) for staining.

2.2. Cloning of miRNAs

1. 5′ DNA/RNA linker (5′-TACTGAATTCGCACGACTCACTaaa-3′), 3′ DNA/RNA linker (5′-P-uuuAACCGCATCCTCGAATTC Amin-3′), 24-nt RNA size marker (5′-uagcuucaagguucaggagaaguu-3′), 21-nt RNA size marker (5′-ucggaccaggcuucaucccc-3′), PCR primers, sense (5′-GTCTGAGGCACC-GCACGACTCACTAAA-3′), and antisense (5′-TCAGGAGGTGCCGAGG-ATGCGGTTAAA-3′). RNA is represented in lowercase letters and DNA in uppercase.

2. T4 RNA ligase 10 U/μL (Takara, NEB), 10X T4 RNA ligase buffer, 0.1 % BSA, DMSO.
3. 3.0 *M* Sodium acetate, pH 5.6. Autoclave and store at room temperature.
4. Elution buffer: 0.5 *M* of ammonium acetate, 1 m*M* of EDTA, adjust pH to 8.0. Autoclave and store at room temperature.
5. Glycogen, 20 mg/mL (Roche).
6. Reverse transcriptase, 200 U/μL (Superscript II, Superscript III, M-MLV reverse transcriptase, Invitrogen).
7. ExTaq DNA polymerase, 10 U/μL (Takara).
8. *Ban*I restriction enzyme, 20 U/μL (NEB).
9. T4 DNA Ligase, 400 U/μL (NEB).
10. Agarose.
11. Phenol, pH 8.0.
12. Chloroform.
13. 100% ethanol, 80% ethanol. Store at −20 ºC.
14. 10-bp DNA ladder marker (Wako).
15. TOPO-TA cloning kit (Invitrogen).
16. LB agar plates with ampicillin (100 μg/μL).
17. 5-Bromo-4-cholo-3-indolyl-β-D-galactosidase (X-gal).
18. LB with ampicillin (100 μg/μL).
19. Reagents for plasmid miniprep.
20. Reagents and/or facility for sequencing.

2.3. Northern Blot Analysis

1. SSC (20X): 3 *M* NaCl, 0.3 *M* trisodium citrate; adjust pH to 7.0 using 1 *N* HCl.
2. Low-stringency wash buffer: SSC (2X), 0.1% SDS.
3. Hybond XL membrane (GE Healthcare).
4. Oligo DNA probes, which are complementary to small RNAs.
5. T4 polynucleotide kinase (NEB).
5. [γ-^{32}P]ATP, ∼6,000 Ci/mmol (GE Healthcare).
6. PerfectHyb Plus hybridization solution (Sigma).
7. Sephadex G-25 or G-50 (GE Healthcare).
8. Stripping buffer: 10 m*M* of Tris-Cl (pH 7.4), 0.2 % SDS.

3. Methods

For small RNA cloning and Northern blotting, generally as much as 50 μg of total RNA are required as starting material. On the other hand, loading a large amount of RNA on acrylamide gel results in low-resolution and blurred bands. Therefore, enrichment of low molecular weight RNA using a PEG solution or an ion-exchange column is recommended. However, if the initial amount of total RNA is small (e.g., less than 50 μg), enrichment should be avoided. We

have succeeded in small RNA cloning using as little as 1.2 μg of total RNA from mouse oocytes.

Protocols of small RNA cloning were first developed in Tuschl's laboratory ***(15)***. Since this protocol needed radioisotope for labeling of small RNAs, we modified the protocol to avoid the use of radioactivity. Small RNAs of desired size range are isolated using polyacrylamide gel electrophoresis and then ligated to 3′ and 5′ linkers. After reverse transcription and PCR amplification, DNA fragments are concatemerized and ligated into sequencing vectors. Currently, a convenient kit (Small RNA Cloning Kit) is available from Takara; its use may save time and money. Once clones are sequenced, we annotate them by using public databases. It should be noted that the most abundant sequences are often breakdown products of functional RNAs such as rRNAs and tRNAs; therefore, it is important to minimize degradation of the RNA during isolation.

Since the small RNA population consists of roughly 20–30-nt long molecules, the hybridization temperature for Northern blotting needs to be relatively low. A temperature of 40–50 ºC is generally optimal for annealing of oligonucleotide probes. Use of longer transcribed riboprobes or random primed probes at this temperature, however, can result in cross-hybridization to abundant RNA species such as rRNAs. As much as 5 μg of low molecular weight RNA are required for detection of the majority of the abundantly expressed miRNAs. Thus, stripping and reprobing of the membrane are recommended when RNAs from rare samples are blotted.

3.1. Low Molecular Weight RNA Preparation and Urea-Polyacrylamide Gel Electrophoresis (Urea-PAGE)

Extract total RNA from embryos at defined developmental stages (the wash step using 70–80% ethanol after alcohol precipitation of RNA is not needed; *see* **Note 2**) and dissolve in water to a final concentration of ~2 μg/μL. Add an equal amount of PEG solution to it and mix thoroughly by vortexing. Store the tube on ice for 1 h to precipitate large molecular weight RNA.

Centrifuge the tube at 12,000 x g for 10 min at 4 ºC. Transfer the supernatant to a fresh tube and add an equal amount of ice-cold isopropanol. Mix thoroughly by vortexing and store the tube at –20 ºC for 1 h to precipitate low molecular weight RNA. Centrifuge again at 12,000 x g for 30 min at 4 ºC. Remove the supernatant and briefly centrifuge to collect the residual liquid at the bottom of the tube. Remove the residual liquid and keep the tube open at room temperature for 10 min for drying.

Dissolve the low molecular weight RNA in water at a final concentration of ~2 μg/μL (~1/10th of the total RNA). The concentration of low molecular

weight RNA is estimated by absorbance at 260 nm. A solution with an A_{260} of 1 contains ~40 μg/μL of low molecular weight RNA.

Prepare equipment for PAGE (minigel, 10 × 10 × 0.1 cm, 10-mL gel volume). To prepare the 15% urea-polyacrylamide gel, mix the following reagents: 0.5 mL of 20X TBE, 3.75 mL of 40% acrylamide (19:1) solution, 4.2 g of urea. Add water to a final volume of 10 mL, and stir the mixture at room temperature (if not dissolved, warm the mixture) on magnetic stirrer until the urea dissolves. Add 50 μL of 10% ammonium persulfate and 10 μL of TEMED, and then mix and pour into the gel apparatus. The gel should polymerize within ~30 min. Add an equal volume of loading buffer to 2 μg of low molecular weight RNA. Incubate the mixture at 65 ºC for 15 min to denature the RNA and then put on ice for at least 1 min. Once the gel has polymerized, carefully remove the comb and wash the well using water. Set the gel plate to the apparatus. Add 0.5X TBE to the upper and lower chambers of the gel unit and pre-run for 5 min at 100 V. Turn off the power supply and wash the wells to remove the accumulated urea. Load the samples in wells and start electrophoresis at a constant voltage of 100 V. When the bromophenol blue (BPB, the faster migrating dye; the slower one is xylene cyanol) reaches the bottom of the gel, turn off the power supply and stain the gel with EtBr solution for 20 min. If the quality of the low molecular weight RNA is good, clear bands of tRNAs and 5S rRNA will appear at 70–120 nt (the bands can be seen above the xylene cyanol dye). A representative result is shown in **Fig. 1**.

3.2. Cloning of miRNAs

Load the low molecular weight RNA (50 μg) in two center wells of a minigel. Load 3′-linker (5 μg) (*see* **Note 3**) and RNA size markers (21 and 24 nt, 0.5 μg each) in the other three lanes. To avoid contamination of the sample with the RNA size markers, leave at least one well empty between the sample and the markers. Electrophorese at 100 V until the BPB dye reaches the bottom of the gel.

Stain the gel with EtBr solution for 20 min and then visualize under the UV light. Excise the gel fragment in the sample lane in the 21–24-nt size range (based on RNA markers) and put in a 1.5-mL tube. Bands of 3′-linker, 21-nt marker, and 24-nt marker are also cut and put into separate tubes (i.e., a total of four tubes: sample, 3′-linker, 21-nt RNA marker, and 24-nt RNA marker). Crush the gel fragments in the tubes by using pipette tips, and then add elution buffer (50–200 μL) to each tube to completely soak the fragments. Incubate at 55 ºC for 1 h to elute the nucleic acids.

Transfer the supernatants to fresh tubes and centrifuge at 15,000 rpm for 5 min to remove the debris. Transfer the supernatants to fresh tubes again, measure the volume in the 3′-linker tube, and divide equally into the other

three tubes (sample, 21-nt RNA marker, and 24-nt RNA marker) (*see* **Note 2**). Measure the volume in each tube and add 1/10th volume of 3 *M* sodium acetate (pH 5.6), 1 µL of glycogen (as carrier) and 3 vol of ice-cold 100% ethanol to each tube. Store the tubes at –80 ºC for at least 1 h to precipitate the nucleic acids. Centrifuge for 30 min at 15,000 rpm at 4 ºC. Remove the supernatant and briefly centrifuge to collect the residual liquid at the bottom of the tubes. Remove the residual liquid, and keep the tubes open at room temperature for 10 min for drying.

Add 6.4 µL of water to each tube and dissolve the nucleic acids. Spin down briefly, add 1 µL of DMSO, 0.6 µL of BSA, 1 µL of 10X buffer, and 1 µL of T4 RNA ligase to each tube, and incubate at 16 ºC for 3 h. Add 10 µL of loading buffer to each tube and incubate at 65 ºC for 15 min. Load the small RNA-3′-linker (*see* **Note 3**) in a center well of a 12% urea-polyacrylamide gel. Load 24-nt RNA marker-3′-linker, 21-nt RNA marker-3′-linker, and 5′-linker (5 µg) in the three other wells on the same gel. To avoid contamination of the RNA marker-3′-linkers to small RNA-3′-linker well, leave a few wells empty between them. Electrophorese at 100 V until the BPB dye reaches the bottom of the gel. Stain gel with EtBr solution for 20 min and visualize under UV light.

To isolate the RNA, excise the gel of small RNA-3′-linker lane according to RNA marker (21- and 24-nt)-3′-linker and put into a tube. Bands of 5′-linker, 21-nt RNA marker-3′-linker, and 24-nt RNA marker-3′-linker are also cut and put into separate tubes (a total of four tubes: small RNA-3′-linker, 5′-linker, 21-nt RNA marker-3′-linker, and 24-nt RNA marker-3′-linker) (*see* **Note 2**). As before, crush the gel fragments, add elution buffer (50–200 µL to each tube), and incubate at 55 ºC for 1 h to elute the nucleic acids. Transfer the supernatant to fresh tubes, centrifuge at 15,000 rpm for 5 min, and transfer the supernatant to fresh tubes again. Measure the volume in the 5′-linker tube, and divide equally into the three other tubes (small RNA-3′-linker, 21-nt RNA marker-3′-linker, and 24-nt RNA marker-3′-linker). Measure the amount of solutions in each tube and add 1/10th of the amount of 3 M sodium acetate (pH 5.6), 1 µL of glycogen as carrier, and 3 vol of ice-cold 100% ethanol in each tube. Store the tubes at –80 ºC for at least 1 h to precipitate the nucleic acids. Centrifuge for 30 min at 15,000 rpm at 4 ºC. Remove the supernatant and briefly centrifuge to collect the residual liquid at the bottom of the tubes. Remove the residual liquid and keep the tube open at room temperature for 10 min for drying. Add 6.4 µL of water in the tubes and dissolve the nucleic acids. Spin down briefly and add 1 µL of DMSO, 0.6 µL of BSA, 1 µL of 10X buffer, and 1 µL of T4 RNA ligase in each tube, and incubate the tubes at 16 ºC for 3 h.

For analysis, add 10 µL of loading buffer to each tube and incubate at 65 ºC for 15 min. Load the 5′-linker-small RNA-3′-linker in a center well of a 12%

urea-polyacrylamide gel. Load 5′-linker-24-nt RNA marker-3′-linker and 5′-linker-21-nt RNA marker-3′-linker in the other two wells on the same gel. To avoid contamination of the 5′-linker-RNA marker-3′-linkers to 5′-linker-small RNA-3′-linker well, leave a few wells empty between them. Electrophorese as before and locate the bands by EtBr staining.

Isolate the RNA again, essentially as described before. In brief, cut the gel of the 5′-linker-small RNA-3′-linker lane according to the 5′-linker-RNA marker (21- and 24-nt)-3′-linker and put the gel fragment in a tube. Crush the fragment and soak in elution buffer (50–200 µL). Incubate at 55 ºC for 1 h to elute the nucleic acids. Transfer the supernatant to a fresh tube and centrifuge at 15,000 rpm for 5 min to remove the debris. Transfer the supernatant to a fresh tube again. Measure the amount of solution and add 1/10th of the amount of 3 M sodium acetate (pH 5.6), 1 µL of antisense PCR primer (10 µM) for RT reaction (*see* **Note 2**), 1 µL of glycogen as carrier, and 3 vol of ice-cold 100% ethanol. Store the tube at –80 ºC for at least 1 h to precipitate the nucleic acids. Centrifuge for 30 min at 15,000 rpm at 4 ºC. Remove the supernatant and briefly centrifuge to collect the residual liquid at the bottom of the tube. Remove the residual liquid and keep the tube open at room temperature for 10 min to dry.

Reverse-transcribe 5′-linker-small RNA-3′-linker using standard reverse transcriptase (20-µL scale). Use 1 µL of the RT reaction as a template and a listed primer set for the first PCR. Perform 23 cycles of PCR (50-µL volume) using Taq DNA polymerase. Load 5 µL of the PCR product onto 12% nondenaturing polyacrylamide gel along with 10-bp size marker. The size of the PCR product should be approximately 75 nt. When the 55-nt band (ligation product of 3′ and 5′ linker without a small RNA insert) is observed, cut the 75-nt band and isolate the DNA to perform the first PCR again.

Use 1 µL of the first PCR solution as a template and a listed primer set for the second PCR. Perform 10 cycles of PCR (500-µL volume) using Taq DNA polymerase. Load 5 µL of the PCR product onto a 12 % nondenaturing polyacrylamide gel with 10-bp size marker to confirm the 75-nt band. If the 75-nt band is not observed, perform three or four more cycles.

Perform phenol extraction followed by a chloroform extraction. Add 1/10th of the amount of 3 *M* of sodium acetate (pH 5.6) and 3 vol of 100% ethanol and store at –20 ºC for 1 h to precipitate. Centrifuge for 10 min at 15,000 rpm at 4 ºC. Remove the supernatant and briefly centrifuge to collect the residual liquid at the bottom of the tube. Remove the liquid, air-dry the pellet at room temperature, and dissolve in 190 µL of BanI buffer. Add 10 µL of BanI, and incubate overnight at 37 ºC. Load 2 µL of digested DNA onto a 12% nondenaturing polyacrylamide gel with 10-bp size marker to confirm digestion (a 60–65-nt band is observed). Perform phenol–chloroform extraction and ethanol precipitation exactly as described above, and dissolve the dry pellet

in 45 µL of T4 DNA ligase buffer containing 300 pmol each of sense and antisense PCR primers. Incubate for 10 min at 60 ºC and store on ice to anneal the BanI-digested short fragment and excess PCR primers. Add 5 µL of T4 DNA ligase, and incubate overnight at 22 ºC. Load ligated DNA on 2% agarose gel with 100-bp DNA marker, stain with EtBr, and visualize under UV light. Cut the DNA band between 500–1,000 bp according to DNA size marker. Crush the gel fragment in the tube, and then add 5 vol of phenol. Mix well and store at –80 ºC for 1 h. Centrifuge at 15,000 rpm for 10 min at room temperature. Transfer the aqueous phase to a fresh tube and extract with chloroform. Add 1/10th of the amount of 3 *M* of sodium acetate (pH 5.6) and 3 vol of 100% ethanol, and store at –20 ºC for 1 h to precipitate. Centrifuge for 10 min at 15,000 rpm at 4 ºC. Remove the supernatant as before, and dissolve the pellet in a small volume of water. Add dNTP, PCR buffer, and Taq polymerase, incubate at 72 ºC for 30 min to tail the end of concatemers, and insert into the T-vector according to the manufacturer's instructions. Finally, sequence the plasmid vectors with concatemers (**Fig. 1**).

3.3. Annotation of Small RNAs

The small RNA fractions contain diverse classes of RNAs: degradation products of functional RNAs (tRNAs, rRNAs, snRNAs, snoRNAs, and mRNAs) and functional small RNAs (miRNAs, siRNAs, rasiRNAs, and piRNAs). In some model organisms, annotation of the genomic sequence is complete or nearing completion, but this remains to be done for many other genomes. Optimal methods to annotate small RNAs may differ between species and also depend on the number of sequences to be analyzed. When the number of sequences is large, bioinformatic analysis is recommended. Here, we introduce an essentially manual method that was used to annotate small RNAs from the mouse germline.

First, search small RNA sequences by NCBI blastn by choosing nr database (http://www.ncbi.nlm.nih.gov/) and by miRNA registry (http://microrna.sanger.ac.uk/sequences/index.shtml). When an annotation is not found in the first round of search, 200 bp of genomic sequence encompassing the small RNA are subjected to the next rounds of the search. To determine whether the small RNA is a tRNA breakdown product, the tRNAscan-SE program (http://www.genetics.wustl.edu/eddy/tRNAscan-SE/) is used. To determine whether the small RNA is rasiRNA, the Repeat Masker program (http://www.repeatmasker.org/) is used. To determine if it is a novel miRNA, the MFOLD 3.2 program (http://www.bioinfo.rpi.edu/applications/mfold/) is used (a guideline to the annotation of novel miRNA has been described) ***(16)***.

3.4. Northern Blot Analysis

Load the low molecular weight RNA (5–30 μg) in a 15% urea-polyacrylamide gel (2-mm thick gel; *see* **Note 2**). Electrophorese at 100 V until BPB reaches the bottom of the gel. In the meantime, cut the Hybond XL membrane and eight sheets of filter paper to a size that is just larger than the gel. Submerge the membrane and the filter papers in 0.5X TBE buffer. Disconnect the gel unit from the power supply and disassemble. Lay the gel on top of four sheets of wet filter paper, and then lay the membrane on top of the gel. Lay the other four sheets of wet filter paper on the membrane, ensuring that no bubbles are trapped in the resulting sandwich. Load the sandwich onto the transfer cassette (the wet type is better; *see* **Note 3**), and place it in the transfer tank such that the nitrocellulose membrane is between the gel and the anode. Transfer at 100 mA for 2 h.

Following transfer, rinse the membrane in 2X SSC for 10 min on a rocking platform and then link the RNA to membrane using a UV cross-linker or trans-illuminator. The membrane can be stored at least for one month at room temperature. For prehybridization, put the membrane in a hybridization bottle and add 10 mL of PerfectHyb hybridization buffer. Put the bottle in the hybridizer and rotate for at least 1 h at 40 ºC.

For end-labeling of the oligo DNA probe that is complementary to the small RNA, mix the following reagents: 1 μL of 10X PNK buffer, 1 μL of oligo DNA solution (10 μM), 1 μL of PNK, and 7 μL of [γ-^{32}P] ATP, and then incubate for 30 min at 37 ºC. To prepare the size-exclusion chromatography column, plug the bottom of a sterile 1-mL syringe with sterile glass wool. Put the syringe into the 15-mL tube and fill with a slurry of G-25 or G-50 Sepharose. Centrifuge for 5 min at 3,000 rpm, and then put the syringe into a new 15-mL tube. Add 90 μL of water to the tube containing the radiolabeled oligo and load onto the column. Centrifuge for 5 min at 3,000 rpm and collect the eluted fraction (containing the radiolabeled oligo) in a fresh tube. Measure the radioactivity by liquid scintillation counting to confirm labeling. Add the labeled oligo directly to the prehybridization bottle and hybridize for 20 h at 40 ºC.

Following hybridization, discard the solution and wash the membrane with 15 mL of low-stringency buffer at 50 ºC for 5 min. Repeat the wash three more times using 15 mL of low-stringency buffer at 50 ºC for 15 min. Remove the membrane from the bottle, cover with Saran Wrap, put into the imaging plate (IP) cassette, and expose for 1 or 3 days according to the signal intensity. Detect the signals in a phosphorimager.

An example of the results is shown in **Fig. 2**. Once the result of an miRNA expression profile has been obtained, the membrane is stripped of the signal and then reprobed with a probe that recognizes other small RNA or tRNA (to confirm equal loading; *see* **Note 4**). Incubate the membrane with 20 mL of the

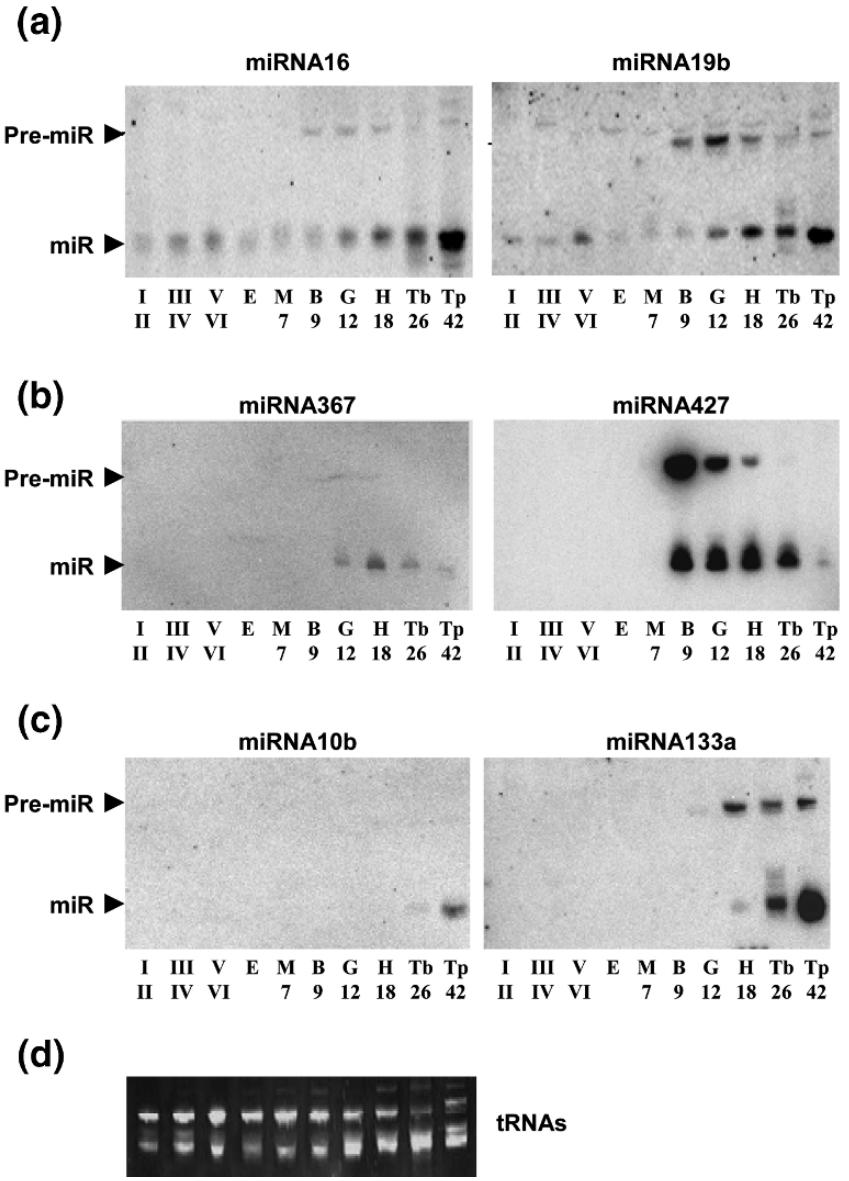

Fig. 2. Expression profile of Xenopus miRNAs by Northern blot analysis. Roman numerals represent developmental stages at oocyte according to Dumont. E = ovulated egg; M = morula (stage 7); B = blastula (stage 9); G = gastrula (stage 12); N = neurula (stage 18); Tb = tailbud (stage 26); Tp = tadpole (stage 42). (a) miRNAs that are generally expressed. (b) miRNAs that are detectable at defined stages. (c) miRNAs

stripping buffer for 30 min at 70 ºC and then wash once in 10 mL of 2X SSC at room temperature. The membrane is now ready to be prehybridized.

4. Notes

1. Electrophoresis of sugar-containing RNA results in a blurred band. To isolate low molecular weight RNA from samples that contain abundant sugars, the PEG precipitation method is not recommended, because it is unable to remove the sugars. The ion-exchange column method can be used instead ***(17)***.
2. Since short RNAs are to some extent soluble to 70–80% ethanol, a wash step is not recommended at any time. Linker oligonucleotides purchased from commercial sources sometimes contain concatenated species that are double the length of the desired ones. Because the length of the linker is 25 nt and near that of small RNAs, contamination of the concatenate results in the cloning of the linker sequence. Thus, purification of the 25-nt species using PAGE is required. The RNA linker and RT-PCR primer serve as carriers. The use of thick gel (2 mm) results in sharper and clearer bands.
3. The transfer efficiency of small RNA in the semidry-type blotting apparatus is comparable to that in the wet-type apparatus. However, the wet-type apparatus transfers larger RNA species (such as pre-miRNA) more efficiently.
4. To confirm equal loading of RNAs among developmental stages, analyze one-tenth to one-fifth of the Northern blot amount on a 15% urea gel and stain the gel with EtBr to visualize 5S rRNA and tRNAs.

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References

1. Bartel, D. P. (2004). MicroRNAs: Genomics, biogenesis, mechanism, and function. *Cell* **116,** 281–297.
2. Sijen, T., and Plasterk, R. H. (2003). Transposon silencing in the *Caenorhabditis elegans* germ line by natural RNAi. *Nature* **426,** 310–314.
3. Reinhart, B. J., Slack, F. J., Basson, M., et al. (2000). The 21-nucleotide let-7 RNA regulates developmental timing in *Caenorhabditis elegans*. *Nature* **403,** 901–906.

Fig. 2. (*Continued*) that are detectable from a certain stage after MBT and continue to be detectable until tadpole stage. (d) One-tenth of the amount of RNA (500 ng) was loaded on each lane and stained with EtBr. The tRNAs serve as loading controls. [Reproduced from **Ref.** ***(9)*** with permission from Elsevier Science.]

4. Ambros, V. (2003). MicroRNA pathways in flies and worms: Growth, death, fat, stress, and timing. *Cell* **113,** 673–676.
5. Yekta, S., Shih, I. H., and Bartel, D. P. (2004). MicroRNA-directed cleavage of HOXB8 mRNA. *Science* **304,** 594–596.
6. Giraldez, A. J., Mishima, Y., Rihel, J., et al. (2006). Zebrafish MiR-430 promotes deadenylation and clearance of maternal mRNAs. *Science* **312,** 75–79.
7. Giraldez, A. J., Cinalli, R. M., Glasner, M. E., et al. (2005). MicroRNAs regulate brain morphogenesis in zebrafish. *Science* **308,** 833–838.
8. Bernstein, E., Kim, S. Y., Carmell, M. A., et al. (2003). Dicer is essential for mouse development. *Nat. Genet*. **35,** 215–217.
9. Watanabe, T., Takeda, A., Mise, K., et al. (2005). Stage-specific expression of microRNAs during Xenopus development. *FEBS Lett*. **579,** 318–324.
10. Watanabe, T., Takeda, A., Tsukiyama, T., et al. (2006). Identification and characterization of two novel classes of small RNAs in the mouse germline: Retrotransposon-derived siRNAs in oocytes and germline small RNAs in testes. *Genes Dev*. **20,** 1732–1743.
11. Valoczi, A., Hornyik, C., Varga, N., Burgyan, J., Kauppinen, S., and Havelda, Z. (2004). Sensitive and specific detection of microRNAs by Northern blot analysis using LNA-modified oligonucleotide probes. *Nucleic Acids Res*. **32,** e175.
12. Mansfield, J. H., Harfe, B. D., Nissen, R., et al. (2004). MicroRNA-responsive "sensor" transgenes uncover Hox-like and other developmentally regulated patterns of vertebrate microRNA expression. *Nat. Genet*. **36,** 1079–1083.
13. Lai, E. C., Tam, B., and Rubin, G. M. (2005). Pervasive regulation of *Drosophila* Notch target genes by GY-box-, Brd-box-, and K-box-class microRNAs. *Genes Dev*. **19,** 1067–1080.
14. Leaman, D., Chen, P. Y., Fak, J., et al. (2005). Antisense-mediated depletion reveals essential and specific functions of microRNAs in *Drosophila* development. *Cell* **121,** 1097–1108.
15. Elbashir, S. M., Lendeckel, W., and Tuschl, T. (2001). RNA interference is mediated by 21- and 22-nucleotide RNAs. *Genes Dev*. **15,** 188–200.
16. Ambros, V., Bartel, B., Bartel, D. P., et al. (2003). A uniform system for microRNA annotation. *RNA* **9,** 277–279.
17. Kurihara, Y., and Watanabe, Y. (2004). Arabidopsis micro-RNA biogenesis through Dicer-like 1 protein functions. *Proc. Natl. Acad. Sci. USA* **101,** 12753–12758.