

## Structure Analysis of MicroRNA Precursors

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### Summary

MicroRNA biogenesis occurs in several steps from their precursors having irregular hairpin structures. The highly variable architecture of these stem-and-loop structures, which have terminal loops of various sizes and diverse structure destabilizing motifs present in their stem sections, may strongly influence the process of microRNA liberation. In order to better understand this process, more details regarding its structural basis are required. A substantial part of this information may be derived from the structure analysis of microRNA precursor using biochemical methods. Here we show how the analysis with the use of various nucleases and metal ions is performed. The presented protocols include the design of DNA template-phage promoter fusions to generate natural precursor ends, and the tests performed to check the sequence and structure homogeneity of the in vitro transcripts prior to probing their structures.

**Key Words:** miRNA; miRNA precursor; structure analysis.

### 1. Introduction

MicroRNAs (miRNAs) are a large family of short 20- to 25-nucleotide (nt) long single-stranded noncoding RNAs identified in many eukaryotes, from nematodes to humans (1,2). They trigger the translational inhibition of target messenger RNAs by binding to their 3'-untranslated region (3,4). Genes encoding miRNA contribute more than 1% to the total gene content of the investigated organisms (2,5), making this regulatory mechanism more common than previously thought. Specific miRNAs were shown to be engaged in the regulation of apoptosis and cell proliferation in *Drosophila*, neuronal asymmetry in *Caenorhabditis elegans*, leaf and flower development in plants, hematopoietic differentiation in humans, and the control of human cell development (6,7). The biological function of a great majority of miRNAs remains unknown.

The primary transcripts of the miRNA genes (pri-miRNAs) are generated by RNA polymerase II (8) as unclustered monocistronic or clustered polycistronic RNAs, which may be several hundred nucleotides long. The pri-miRNAs, which typically form a

stem and loop structure, are processed in the nucleus to shorter, approx 60-nt, hairpin precursor (pre)-miRNAs by the ribonuclease, Drosha (9), which acts together with the double-stranded (dsRNA)-binding protein, DiGeorge syndrome critical region gene 8, in a complex termed Microprocessor (Fig. 1A) (10). Drosha leaves a 2-nt overhang at the 3' end of pre-miRNA and defines one end of the mature miRNA strand. After the overhang recognition, pre-miRNAs are exported from the nucleus by Exportin-5 (11) and further processed to miRNAs by a cytoplasmic ribonuclease, Dicer (12,13).

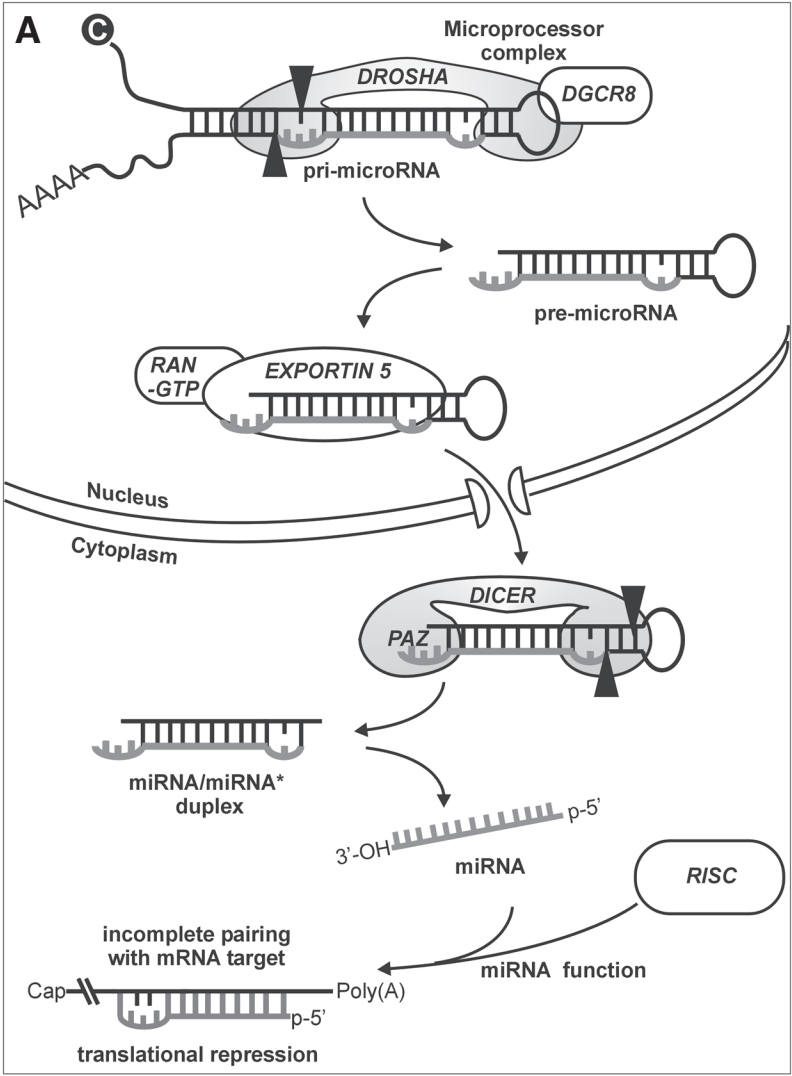
Dicer is composed of several functional domains: the Piwi-Argonaute-Zwille (PAZ) domain, which is used for high-affinity binding to the 3' overhanging nucleotides of pre-miRNA, the helicase domain, the DUF283 subunit, the dsRNA binding domain, and two ribonuclease (RNase) III catalytic domains (Fig. 1B) that form the intramolecular dimer during pre-miRNA cleavage (14). The pre-miRNA is processed by Dicer symmetrically, as proposed for the siRNA (15,16). Usually, only the miRNA strand of the excised duplex intermediate is stabilized by the successful entering of the RNA-induced silencing complex (RISC) (17,18). The miRNA\* strand and the remainder of the precursor is degraded (Fig. 1A).

The process of excision of the miRNA-miRNA\* duplex is controlled by various structure-destabilizing elements present in the precursor hairpin (19), and the existence of sequence determinants or antideterminants of the Dicer cleavage has also been proposed (20). The pre-miRNAs are predicted to form irregular hairpin structures containing various base mismatches, internal loops, and bulges (21). However, these predicted structures need to be experimentally verified to serve as a reliable basis for the analysis of structural aspects of miRNA biogenesis. The necessity of the experimental structure analysis was documented in a recent study, in which differences were shown to exist between the majority of predicted and experimentally established precursor structures (22).

The steps of experimental procedure used for the structure probing of miRNA precursors are shown in the diagram in Fig. 2. In brief, they include RNA synthesis by in

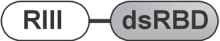
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Fig. 1. (*Opposite page*) (A) The mechanism of microRNA (miRNA) biogenesis (13); after miRNA is produced by the activity of two class III ribonucleases, Drosha and Dicer, and selected by the RNA-induced silencing complex (RISC). After the transcription by RNA polymerase II, the primary transcript of the miRNA gene (pri-miRNA) is cleaved in the nucleus by Drosha, forming a heterodimeric complex (named Microprocessor), with the double-stranded RNA (dsRNA)-binding protein, DiGeorge syndrome critical region 8 (DGCR8). Drosha generates the miRNA precursor (pre-miRNA), which binds Exportin-5 and is exported to the cytoplasm. In the cytoplasm, Dicer binds to the pre-miRNA and generates a duplex intermediate comprising the active miRNA (indicated in gray color) and inactive miRNA\* strands. The cleavage sites of the nucleases Drosha and Dicer are indicated by black triangles. After the preferential duplex unwinding at the miRNA 5' end, the active strand is selectively incorporated into RISC by the miRNA-specific RISC assembly. The activated miRNA-RISC complex directs either translational repression or degradation of the targeted messenger RNA, depending on the degree of sequence complementarity. (B) A schematic illustration of the domain structure of human Drosha and Dicer proteins. RIII, ribonuclease III catalytic domain; dsRBD, dsRNA-binding domain. The "x" symbol indicates the inactive ribonuclease center in one of the RIII Dicer domains.



**B**

Class I - RNase III E.coli (226aa)



Class II- Drosha (1374aa)



Class III- Dicer (1912aa)



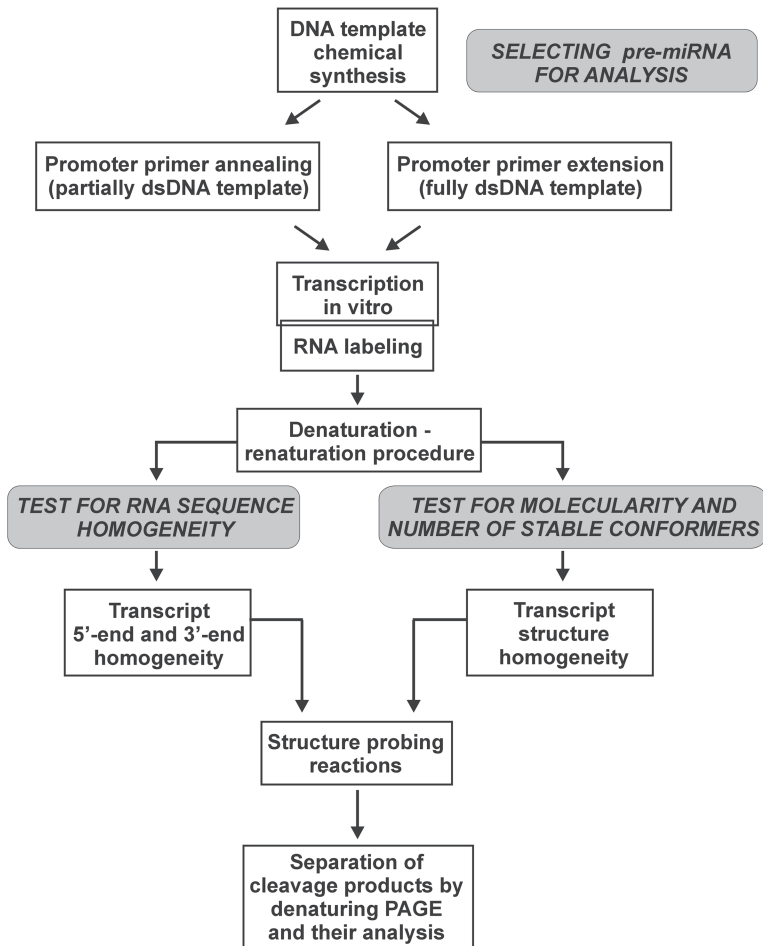


Fig. 2. Steps involved in the structure analysis of microRNA precursors.

vitro transcription, necessary to ensure the required RNA sequence devoid of 5' end (23) and 3' end (24) heterogeneities. After RNA labeling, the tests for transcript sequence and structure homogeneity are performed, and the RNA is subjected to structure-probing reactions.

Human miRNA precursors contain the 5'-monophosphate and 3'-OH termini. They may be obtained by in vitro transcription from chemically synthesized DNA templates containing the phage RNA polymerase promoter sequence. The pre-miRNAs have various sequences at their 5' end, beginning either with G, C, A, or U, and different strategies may be used to obtain these transcripts. One strategy is to introduce a ribozyme structure that will liberate the required precursor sequence from the extended transcript (14). Another possible method is to introduce a short initiator oligoribonucleotide homologous to the precursor 5' end to the in vitro transcription mixture (25). The most

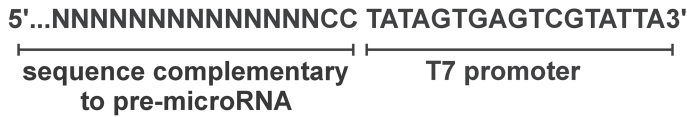
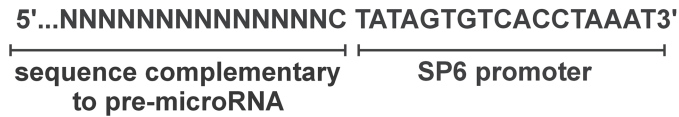
**For T7 Polymerase****miRNA precursor starting with GG at the 5'-end****For SP6 Polymerase****miRNA precursor starting with G at the 5'-end****miRNA precursor starting with A at the 5'-end**

Fig. 3. Phage RNA polymerase promoter sequences enabling the generation of the required transcript ends by in vitro transcription.

simple approach, which is described in this chapter, takes advantage of the alternative T7 and SP6 RNA polymerase promoters (26,27). They provide correct 5' end sequences for a number of miRNA precursors, whose sequences start, for example, with a single G or A residue (Fig. 3).

The chemical and enzymatic probing of nucleotide accessibility (28,29) can be used to rapidly obtain reliable structure information, taking advantage of the distinct specificity of different chemical reagents and nucleases. Ribonucleases T1 and T2 and nuclease S1 recognize the single-stranded regions of RNA (30,31), and they map the terminal loops of the precursor hairpins as well (22). The double-stranded helical regions are cleaved by ribonuclease V1. This small, 15-kDa protein from cobra venom (32), which accommodates only one strand of a helix at its recognition site, requires a helical sugar phosphate backbone conformation for its activity (33). The minimum V1 recognition site is usually six nucleotides, three at each side of the cleavage site, and their conformation may strongly influence the cleavage efficiency (33). Ribonuclease V1, showing some preference for recognizing simple sequence repeats, reveals the distribution of a specific, more regular helical conformation along the miRNA precursor hairpin (22).

The nucleases map small internal loops, bulges, and mismatches poorly. On the contrary, nearly all of these stem-structure irregularities are well-recognized by metal ions:  $Mg^{2+}$  (34,35),  $Mn^{2+}$  (36),  $Ca^{2+}$  (37), and  $Pb^{2+}$  ions (38–40). The advantageous feature

of metal ions is that their hydrates are much smaller than nucleases, penetrate folded RNAs more easily, and reveal more details of the analyzed structures (41). In addition, metal ions are sensitive to differences in the phosphodiester bond flexibility, in accordance with the proposed mechanism of RNA cleavage by lead ions (42). In brief, the reaction begins with the activation of the ribose 2'-OH group by the metal ion hydroxide, and the attack of the 2'-O-nucleophile on the adjacent phosphorus atom, which requires the conformational flexibility of the sugar phosphate backbone (43). The  $Mg^{2+}$  and  $Ca^{2+}$  ions both prefer coordination to oxygen ligands, but differ in their ionic radii and coordination geometry. The  $Mg^{2+}$  and  $Ca^{2+}$  ions distinguish between paired and unpaired nucleotides most precisely (44,45). The reactive phosphodiester bonds mapped by the  $Mg^{2+}$  and  $Ca^{2+}$  ions often overlap each other. Generally, the  $Ca^{2+}$  ions induce cleavages at more sites and map the great majority of internucleotide bonds present in numerous bulges and internal loops. This is in accordance with the flexible geometry and variable coordination number of the  $Ca^{2+}$  aquacation in solution (45). The  $Pb^{2+}$  and  $Mn^{2+}$  ions cleaved more internucleotide bonds than the  $Ca^{2+}$  and  $Mg^{2+}$  ions in the miRNA precursors. They also mapped the phosphodiester bonds of several paired nucleotides located in the neighborhood of bulges or internal loops (22).

## 2. Materials

### 2.1. DNA Templates for In Vitro Transcription

1. DNA oligomers obtained by chemical synthesis were purchased from commercial sources.
2. T7 promoter primer, 5'TAATACGACTCACTATAGG.
3. SP6 promoter primer, 5'ATTTAGGTGACACTATA.
4. 10% polyacrylamide gel (acrylamide:bis acrylamide ratio, 19:1) buffered with 1X TBE (89 mM Tris, 89 mM boric acid, and 20 mM ethylenediaminetetraacetic acid [EDTA], pH 8.0).
5. *Taq* DNA polymerase 5 U/ $\mu$ L in storage buffer A (Promega, Madison, WI).
6. Set of 2.5 mM deoxyribonucleoside triphosphates (dNTPs) (Promega).
7. 2X TE buffer: 20 mM Tris-HCl, 2 mM EDTA.
8. 10X TBE buffer: 890 mM Tris-HCl, 890 mM boric acid, and 20 mM EDTA.
9. Stains-All dye reagent (Serva, Heidelberg, Germany).
10. Microcon YM30 microcentrifuge tubes (Millipore, Billerica, MA).

### 2.2. In Vitro Transcription

1. 200 U/ $\mu$ L T7 RNA polymerase (Epicentre, Madison, WI) and 200 U/ $\mu$ L SP6 RNA polymerase (Fermentas, Burlington).
2. Set of 5 mM NTPs (Roche, Mannheim), 10 mM guanosine, and 10 mM adenosine.
3. 40 U/ $\mu$ L RNaseOUT recombinant ribonuclease inhibitor (Invitrogen, Carlsbad, CA).
4. Urea, EDTA, and dyes (UED) buffer: 7.5 M urea and 20 mM EDTA with dyes (bromophenol blue and xylene cyanol FF; Fluka, Buchs, Switzerland).
5. Elution buffer: 0.3 M sodium acetate, pH 5.2, 0.5 mM EDTA, and 0.1% sodium dodecylsulfate.

### 2.3. RNA-Labeling Reagents

1. 4500 Ci/mmol [ $\gamma$ - $^{32}$ P] adenosine triphosphate (ATP) (MP Biomedicals, Irvine, CA).
2. 3000 Ci/mmol [5'- $^{32}$ P] cytidine-3', 5'-biphosphate (pCp) (Perkin Elmer, Wellesley, MA).

3. 3000 Ci/mmol [ $\alpha$ - $^{32}$ P] cytidine triphosphate (CTP) (MP Biomedicals).
4. 10 U/ $\mu$ L T4 polynucleotide kinase (Epicenter).
5. 20 U/ $\mu$ L T4 RNA ligase (Fermentas).
6. 2 U/ $\mu$ L alkaline phosphatase, calf intestinal (Pharmacia, Rockville, MD).

### **2.4. Testing RNA Sequence and Structure Homogeneity**

1. Formamide buffer: 99% formamide, 0.5 mM MgCl<sub>2</sub>.
2. 2X T2 buffer: 20 mM sodium citrate, pH 5.0, and 7 M urea.
3. 0.2 U/ $\mu$ L T1 ribonuclease (USB, Cleveland, OH).
4. Structure-probing buffer A: 12 mM Tris-HCl, pH 7.2, 48 mM NaCl, and 1.2 mM MgCl<sub>2</sub>.
5. Structure-probing buffer B: 12 mM Tris-HCl, pH 8.5 or 9.0, and 48 mM NaCl.
6. 140 U/ $\mu$ L nuclease S1 (Amersham Biosciences, Piscataway, NJ), 100 U/ $\mu$ L ribonuclease T1 (USB), 20 U/ $\mu$ L T2 (Invitrogen), 0.1 U/ $\mu$ L V1 (Ambion, Austin, TX).
7. Ion solutions: 30 mM MgCl<sub>2</sub>, 30 mM CaCl<sub>2</sub>, 30 mM Pb(CH<sub>3</sub>COO)<sub>2</sub>.

## **3. Methods**

### **3.1. RNA Structure Prediction Procedure and Setting Variables**

RNA secondary structure prediction was performed using the mfold program, version 3.1. This program is designed to determine the optimal and suboptimal secondary structures of RNA calculated for a 1 M NaCl solution at 37°C, and to count free energy contributions for various secondary structure motifs. The structure prediction is usually performed with the suboptimality parameter set at 10%. At this setting, the program shows all of the structures that have a free energy of formation of up to 10% higher than the optimal structure.

### **3.2. DNA Template/Promoter Purification**

1. Dilute the DNA oligomer, which has a sequence of the antisense strand of the miRNA precursor, with the attached phage promoter sequence (*see Note 1*) to a concentration of 100 pmol/ $\mu$ L with RNase- and deoxyribonuclease-free water.
2. Add 20  $\mu$ L of UED buffer to 20  $\mu$ L of the DNA oligomer solution in **Subheading 3.2.**, incubate the sample at 95°C for 1 min, and immediately load the sample on the pre-electrophoresed 10% (acrylamide:bis acrylamide ratio, 19:1) polyacrylamide gel (420  $\times$  340  $\times$  1 mm), buffered with 1X TBE. Perform the electrophoresis at 600 V/40 mA for 10 min and then at 1200 V/90 mA for 3 h.
3. Stain the gel with 0.1% Stains-All dye (Serva) at room temperature for 30 min.
4. Cut the band corresponding to the desired DNA template and elute the DNA to 500  $\mu$ L of elution buffer at 4°C overnight.
5. Precipitate the DNA with three volumes of EtOH and resuspend the pellet in 20  $\mu$ L of water. Store at -20°C.

### **3.3. Preparation of DNA Templates for In Vitro Transcription (see Note 2)**

#### **3.3.1. Annealing Procedure**

1. Mix 8  $\mu$ L (600 pmol) of the purified template oligomer and 8  $\mu$ L (600 pmol) of T7 or SP6 promoter primer with 16  $\mu$ L of 2X TE buffer.
2. Incubate the sample at 90°C for 5 min and cool the sample slowly to 37°C for 10 min.

### 3.3.2. Primer Extension Procedure

1. Mix 2  $\mu\text{L}$  (final concentration, 200 pmol) of purified template oligomer, 5  $\mu\text{L}$  (1 mmol) of T7 or SP6 promoter primer, and 8  $\mu\text{L}$  of dNTPs (200  $\mu\text{M}$  each) with 10  $\mu\text{L}$  of standard 1X polymerase chain reaction (PCR) buffer and 0.5  $\mu\text{L}$  (0.5 U) of *Taq* DNA polymerase in a 100- $\mu\text{L}$  reaction.
2. Perform the primer extension: 50 cycles at 94°C for 15 s and 45°C for 15 s.
3. Purify the dsDNA product using Microcon YM30 centrifugal filter devices, according to the manufacturer's (Millipore) instructions, and dilute the DNA template to 60  $\mu\text{L}$  with water.

### 3.4. In Vitro Transcription With Phage Polymerases

The pre-miRNA transcripts can be prepared by in vitro transcription with T7 or SP6 RNA polymerases.

1. In a 50- $\mu\text{L}$  volume at room temperature, mix the 40 pmol of DNA template (12  $\mu\text{L}$  of DNA after the primer extension procedure or 2  $\mu\text{L}$  of DNA after phage promoter primer annealing), 50  $\mu\text{M}$  of rNTPs, 30  $\mu\text{L}$  of 10 mM guanosine (preheated to 65°C for 3 min) or adenosine solution (*see Note 3*), 10  $\mu\text{L}$  of 1X standard transcription buffer, 40 U of ribonuclease inhibitor, RNase Out, and 400 U of T7 or SP6 RNA polymerase.
2. Incubate the reaction mixture at 37°C for 1.5 h.
3. After EtOH precipitation at -20°C overnight, resuspend the transcript in 20  $\mu\text{L}$  of water and add 20  $\mu\text{L}$  of UED buffer.
4. Purify the in vitro transcription product in a denaturing 10% polyacrylamide gel, excise the product band, elute the RNA from the gel with RNA elution buffer, and precipitate with EtOH as described in **Subheading 3.2**. Resuspend the pellet in a 10  $\mu\text{L}$  of RNase-free water. Store at -80°C.

### 3.5. RNA-Labeling Procedures

#### 3.5.1. 5' End Labeling

1. Mix 10  $\mu\text{L}$  of purified pre-miRNA transcript (~1 nmol) with 3  $\mu\text{L}$  of 4500 Ci/mmol [ $\gamma$ -<sup>32</sup>P] ATP, 1.5  $\mu\text{L}$  of 10X T4 polynucleotide kinase buffer, and 1  $\mu\text{L}$  of T4 polynucleotide kinase.
2. Incubate the mixture at 37°C for 10 min.
3. Stop the labeling reaction by adding an equal volume of UED buffer.
4. Purify the labeled RNA in 10% polyacrylamide gel, as described for DNA template purification in **Subheading 3.2**. After electrophoresis, attach the labeling markers to the gel and place the gel in the autoradiography cassette with the X-ray film for approx 3 min. After autoradiography, correctly orient the film with respect to the markers and cut out the single gel band corresponding to the desired RNA.
5. Elute the RNA with approx 500  $\mu\text{L}$  of the elution buffer at 4°C overnight. Precipitate the RNA with EtOH, and resuspend the pellet in approx 20  $\mu\text{L}$  of RNase-free water.

#### 3.5.2. 3' End Labeling

1. Mix 10  $\mu\text{L}$  of purified pre-miRNA transcript with 5  $\mu\text{L}$  of 3000 Ci/mmol [5'-<sup>32</sup>P] pCp, 2  $\mu\text{L}$  of reaction buffer, 2  $\mu\text{L}$  of 10 mM ATP, and 2  $\mu\text{L}$  of T4 RNA ligase in a volume of 20  $\mu\text{L}$ .
2. Incubate the reaction mixture at 4°C for 16 h.
3. Optionally (*see Note 4*): add 2  $\mu\text{L}$  of 10X alkaline phosphatase buffer and 1  $\mu\text{L}$  of alkaline phosphatase and incubate at 37°C for 20 min.
4. Purify the labeled RNA as described in **Subheading 3.4**.



### 3.5.3. Internal Labeling

1. For internal labeling, the pre-miRNA RNA is synthesized by in vitro transcription, as described in **Subheading 3.4.**, with the exception that the set of NTPs (5 mM ATP, 5 mM GTP, 5 mM UTP, and 0.5 mM CTP) is supplemented with 5  $\mu$ L of 3000 Ci/mmol [ $\alpha$ - $^{32}$ P] CTP.
2. Incubate the reaction mixture at 37°C for 1.5 h.
3. Purify the labeled RNA as described in **Subheading 3.4.**

### 3.6. 5' End and 3' End Sequence Homogeneity Testing (see Note 5)

1. The limited T1 ribonuclease ladder generated from the 5' end-labeled transcript of a discrete length is visually inspected for the presence of extra fragments not predicted from the correct nucleotide sequence. These extra fragments are usually one nucleotide longer than the expected T1 fragments. The presence of such extra bands in addition to those expected from the correct sequence indicates the 5' end heterogeneity. It indicates that some of the transcripts are longer by one nucleotide at the 5' end and shorter by one nucleotide at the 3' end (**Fig. 3**).

### 3.7. Structure Homogeneity and Molecularity Testing (see Note 6)

1. Incubate approx 5 pmol (30,000 cpm) of the 5' end-labeled transcript in a structure-probing solution (buffer A: 10 mM Tris-HCl, pH 7.2, 40 mM NaCl, and 1 mM MgCl<sub>2</sub>) at 90°C for 1 min, followed by slow cooling to 37°C.
2. Mix with an equal volume of the same buffer containing 7% sucrose and dyes.
3. Load the samples onto a 10% nondenaturing polyacrylamide gel (dimensions, 150  $\times$  140  $\times$  1 mm; acrylamide:bis acrylamide ratio, 29:1) buffered with the structure-probing buffer. Perform electrophoresis at 100 V at a controlled temperature of 37°C, with a buffer circulation at 2 L/h.
4. Dry the gel and visualize the RNA by autoradiography.

### 3.8. Structure Probing With Nucleases (see Note 7)

1. Subject the  $^{32}$ P-end-labeled transcript to a denaturation and renaturation procedure before structure probing. For a single reaction, in an 8- $\mu$ L volume, mix the  $^{32}$ P-end-labeled RNA (~10 pmol; 60,000 cpm) and structure-probing buffer A to obtain a final concentration of 12 mM Tris-HCl, pH 7.2, 48 mM NaCl, and 1.2 mM MgCl<sub>2</sub>. Heat the sample at 90°C for 1 min and leave the reaction mixture in a thermal block for slow (~5 min) cooling to 37°C.
2. Add 2  $\mu$ L of nuclease water solution at one of three different concentrations. Typically used concentrations are: T1 (0.5, 1.0, and 1.5 U/ $\mu$ L), T2 (0.15, 0.2, and 0.25 U/ $\mu$ L), V1 (0.15, 0.2, and 0.25 U/mL), and nuclease S1 (1.5, 3.0, and 6.0 U/ $\mu$ L) (see **Note 8**). Incubate at 37°C for 10 min.
3. Stop the reaction by adding 10  $\mu$ L of 7.5 M urea and 20 mM EDTA with dyes.

### 3.9. Structure Probing With Metal Ions

1. Before structure probing, subject the  $^{32}$ P-end-labeled transcript to a denaturation and renaturation procedure. For single reaction with Pb<sup>2+</sup> ions, in an 8- $\mu$ L volume, mix the  $^{32}$ P-end-labeled RNA solution (~10 pmol; 60,000 cpm) and structure-probing buffer A to obtain a final concentration of 12 mM Tris-HCl, pH 7.2, 48 mM NaCl, and 1.2 mM MgCl<sub>2</sub>. For a single reaction with Mn<sup>2+</sup>, Mg<sup>2+</sup>, or Ca<sup>2+</sup> ions, in an 8- $\mu$ L volume, mix the  $^{32}$ P-end-labeled RNA (~10 pmol; 60,000 cpm) and structure-probing buffer B to obtain a concentration of 12 mM Tris-HCl, pH 8.5 or 9.0, and 48 mM NaCl.

2. Heat the sample at 90°C for 1 min followed by slow cooling to 37°C.
3. Add 2  $\mu\text{L}$  of the metal ions solution at the appropriate concentrations. Use the  $\text{Pb}(\text{CH}_3\text{COO})_2$  solution at 0.5, 1.0, and 2.0 mM; the  $\text{MgCl}_2$  solution at 3, 10, and 15 mM; the  $\text{CaCl}_2$  solution at 3, 15, and 30 mM; or the  $\text{MnCl}_2$  solution at 3, 10, and 15 mM.
4. At 37°C, incubate the RNA with  $\text{Pb}^{2+}$  ions for 10 min, with  $\text{Mn}^{2+}$  ions for 2 h, and with  $\text{Mg}^{2+}$  or  $\text{Ca}^{2+}$  ions for 16 h.
5. Stop the reaction by adding 10  $\mu\text{L}$  of solution containing 7.5 M urea and 20 mM EDTA with dyes.

### 3.10. Gel Electrophoresis and Autoradiography

1. Denature the sample briefly by a 30-s incubation at 90°C before loading the gel.
2. Analyze the cleavage products by electrophoresis in a 15% polyacrylamide gel (dimensions 420  $\times$  340  $\times$  0.4 mm) (acrylamide:bis acrylamide ratio, 19:1) buffered with 1X TBE under denaturing conditions.
3. Generate the alkaline hydrolysis ladder by incubating 2  $\mu\text{L}$  of the end-labeled RNA (~10 pmol) with 9  $\mu\text{L}$  of formamide containing 0.5 mM  $\text{MgCl}_2$  at 100°C for 10 min. Stop the reaction by adding 9  $\mu\text{L}$  of UED buffer.
4. Generate the T1 ladder by incubating 2  $\mu\text{L}$  of the end-labeled RNA (~10 pmol) with 3  $\mu\text{L}$  of semidenaturing buffer (10 mM sodium citrate, pH 5.0, and 3.5 M urea) for 20 s at 100°C. Place the tube with the reaction mixture on ice immediately and keep it on ice for 5 min. Add 1  $\mu\text{L}$  of T1 ribonuclease (1 U/ $\mu\text{L}$ ), and incubate at 55°C for 12 min. Stop the reaction by adding 14  $\mu\text{L}$  of UED buffer.
5. Typical structure analysis with a single probe requires six gel lanes: the incubation control line (without a probe), three lanes of reactions with a probe used at different concentrations, a lane of alkaline hydrolysis ladder, and a lane of T1 ladder. Typically, 3  $\mu\text{L}$  of each sample is loaded into each gel well.
6. Run the electrophoresis at 40 mA/1500 V for approx 2 h.
7. Transfer the gel onto Whatman 3MM paper, cover with plastic wrap, and subject to autoradiography at -80°C with an intensifying screen.

### 3.11. Phosphor Imaging and Quantitative Analysis of Cleavages

1. Place the dried gel into the PhosphorImager cassette for 16 h.
2. Scan the screen on a Typhoon (Molecular Dynamics) laser densitometer operating at 633 nm.
3. Analyze the peak heights obtained from gel bands representing the relative cleavage efficiency at the corresponding sites, as shown in (46–48).

## 4. Notes

1. The in vitro transcription of the desired pre-miRNA is performed from the DNA template obtained by chemical synthesis, which offers the DNA template designed to contain the antisense miRNA precursor sequence extended by the phage promoter sequence, so that the end of the latter defines the 5' end of the former (Fig. 3).
2. If using synthetic DNA templates for in vitro transcription, at least the promoter portion of the template has to be double-stranded. For such templates, the promoter primer annealing procedure is performed to obtain a double-stranded promoter region. For GC-rich templates with high propensities to form secondary structures, we recommend performing primer extension to obtain a fully double-stranded template. The disadvantage of using the primer-extension procedure is the increased risk of introducing 3' end heterogeneity into the DNA template.

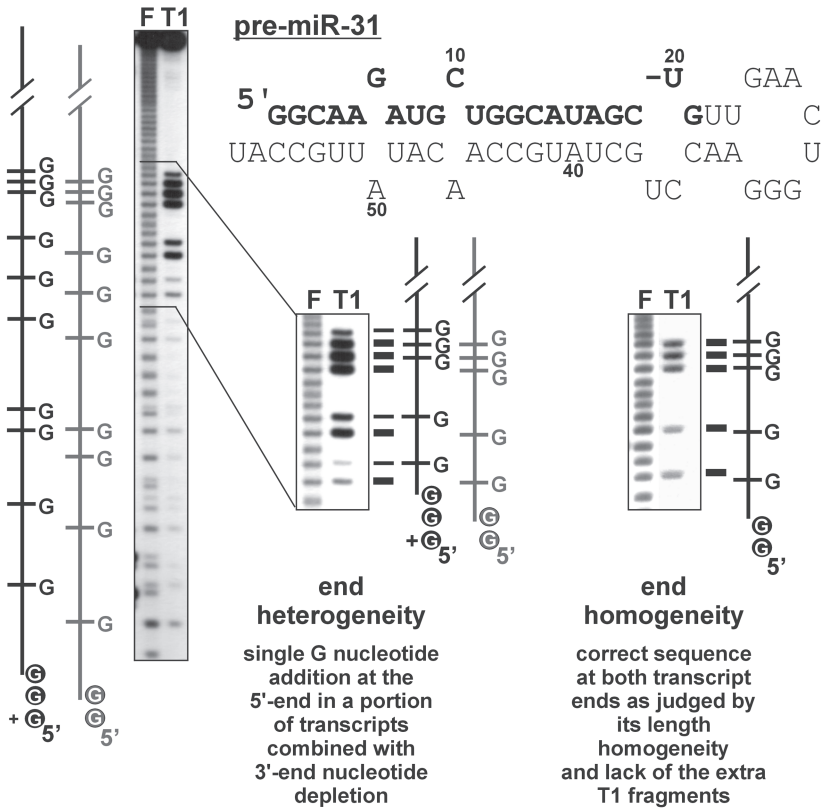


Fig. 4. Examples of end sequence heterogeneity in the 5' end-labeled microRNA (miRNA) precursor transcript. The predicted secondary structure of miR-31 precursor (pre-miR-31) with a boldface miRNA sequence is shown. RNA cleavages by T1 ribonuclease (T1) are performed in semidenaturing conditions for mapping all G residues. The vertical lines with positions of G residues indicated represent various compositions of T1 fragments that are observed in the presented autoradiograms, derived from precursors with a 5' end heterogeneity (left) and a homogeneous sequence (right). F, formamide ladder.

3. Guanosine or adenosine is added to the reaction mixture at the appropriate concentrations to introduce them at the 5' end in a high proportion of transcripts. This makes the 5' end dephosphorylation step unnecessary before its labeling. The guanosine concentration in the water solution used is the highest possible concentration and is determined by the nucleoside solubility in water. Adenosine may be used at the same or higher concentration for SP6 transcription beginning with A.
4. If using the labeled pre-miRNA transcript in the reaction with Dicer, it is necessary to produce the pre-miRNA that contains the specific ends: 5'-monophosphate and 3'-OH.
5. Because of the tendency of different RNA polymerases to add extra nucleotides to the ends of transcripts (usually  $n + 1$ ) that are not encoded by the template, some pre-miRNAs may contain extra nucleotides at their 5' (Fig. 4) and/or 3' ends. This sequence heterogeneity, which is highly undesired in detailed structural studies, can be suppressed by reducing the

length of consecutive mononucleotide tracts (G or A) at the 3' end of the RNA polymerase promoter. With relatively short pre-miRNAs (~60 nt), it is easy to cut out single bands representing discrete sequences from the gel. The strongest band usually corresponds to the correct sequence. Alternatively, transcripts labeled at their 5' or 3' end may be exhaustively digested with nuclease as described in (23) and (37) and labeled fragments with either homogeneous or heterogeneous lengths are visualized in a 20% polyacrylamide gel, as described in **Subheading 3.10**.

6. A test for structure homogeneity is strongly recommended before the structure probing of pre-miRNA. Some RNAs may form two or more stable conformers or dimers, which can be detected in nondenaturing polyacrylamide gels. If such a gel shows one stable conformer, its structure can be relatively easily established. In case of the coexistence of two or more stable conformers, the single conformers may be isolated from the native gel, and their structures probed separately. Alternatively, structure probing may be performed on a mixture of conformers, which are then separated in native gel, as described earlier (49,50).
7. The failure of nucleases, and, in some cases, of metal ions, to detect small symmetrical internal loops and mismatches when they are surrounded by stable double-helical regions, may indicate that these motifs form noncanonical base pairs, which do not distort the duplex structure significantly.
8. Optimize the probe concentration with respect to every transcript to assure less than 10% RNA fragmentation and to minimize the possible contributions from secondary cleavages. To distinguish between the primary and secondary cuts, the structure-probing experiments with RNA labeled independently at the 5' end and 3' end may also be performed.

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