In vitro analysis of pri-miRNA processing by Drosha-DGCR8 complex (Narry Kim's lab)

1-1. Preparation of radiolabeled pri-miRNA transcript

The RNA substrate for a cropping reaction can be prepared by *in vitro* transcription. The DNA segment containing pri-miRNA sequences can be amplified from genomic DNA by PCR. The PCR product is then inserted into a plasmid containing the T7 promoter (or SP6 promoter). The resulting plasmid should be linearized at the downstream of the pri-miRNA sequences. Alternatively, the PCR product can be used directly for *in vitro* transcription. In this case, the forward PCR primer should contain the promoter sequences (usually the T7 promoter) to drive transcription directly from the PCR product.

When designing the PCR primers, one has to decide which region around the miRNA should be included to achieve efficient processing. In general, all the cis-acting element(s) for processing are located close to the stem-loop [8, 3230 32, 3334]. By the rule of thumb, we amplify a genomic segment containing a given miRNA stem-loop plus the surrounding sequences ~100 bp from each side of the stem-loop. This should normally include all the cis-acting element(s) recognized by the Drosha-DGCR8 complex.

Materials

1. Template plasmid (1 μg/μl) or PCR product (30-100 ng/μl); The plasmid must be

linearized at a restriction site located downstream of the pri-miRNA sequences. The PCR products are subcloned into a cloning vector containing the T7 or SP6 promoter (e.g. pGEM-T-easy, Promega). It is also possible to use the PCR product directly as the template in transcription reaction if the T7 promoter sequence is included in the forward PCR primer. For efficient transcription, the promoter sequences should be followed by at least two consecutive G's.

2. NTP mixture (10mM ATP, 10mM GTP, 10mM CTP, 1mM UTP)

3. α-^{32}P-UTP (20μCi/μl, 800 mCi/mmole)

4. T7 or SP6 RNA polymerase

5. 10X transcription buffer (Use the buffer provided with the polymerase)

6. RNase inhibiter (RNasin or equivalent, 40U/μl)

7. RNase-free water

8. Hoeffer gel apparatus (SE600, 18 X 16cm) or equivalent, plates, combs (0.75mm, 10 wells), spacers (0.75mm), and a power supply

9. TBE stock solution (5X): Dissolve 54g of Tris base, 27.5g of boric acid and 20 ml of 0.5 M EDTA, pH 8.0 in distilled water to make 1 L.

10. 6% Urea-polyacrylamide stock solution: Mix 57g acrylamide, 3g Bis-acrylamide, 100ml of 5X TBE stock solution (final 0.5X) and 420.42g urea (final 7M) in distilled water to make 1 L. When preparing urea-polyacrylamide stock solution, dissolve urea in water at 60℃ because this reaction is an endothermic reaction.

11. 20% ammonium persulfate (APS) solution

12. *N,N,N',N'*-tetramethylethylenediamine (TEMED)

13. Phenol:chloroform, pH 4.5

14. 3M sodium acetate, pH 5.5

15. Glycogen (5mg/ml)

16. TE buffer: 10 mM Tris-HCl, pH 7.5 and 1 mM EDTA

17. RNA elution buffer: 0.3 M sodium acetate, pH 5.5 and 2% SDS

18. RNA loading buffer: 95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5mM EDTA and 0.025% SDS

19. Ethanol: 100% and 75%

20. Microcentrifuge

21. Microcentrifuge with cooling

22. Thermoblocks at 37°C/95°C

23. Kodak X-AR5 autoradiography films

Procedure

1. Mix the following at room temperature (RT): 1 μl of template DNA, 2 μl of 10X transcription buffer, 2 μl of NTP mixture (10mM ATP, 10mM GTP, 10mM CTP, 1mM UTP), 0.5 μl of RNase inhibiter (40u/μl), 1.5 μl of α-^{32}P-UTP (20μCi/μl, 800 mCi/mmole), 1 μl of RNA polymerase (T7 or SP6) and 12 μl of RNase-free water. Adjust the total volume to 20 μl.

2. Incubate at 37°C for 3 hr.

3. Add 220 μl of TE buffer to the above reaction mixture.

4. Add 240 μl of phenol:chloroform and vortex for 30 sec. Centrifuge for 5 min at RT and take the upper layer.

5. Add 160 μl of 5M ammonium acetate, 1 μl of glycogen, 1 ml of 100% ethanol. Mix and leave the tube at –80°C for at least 20 min (or 5 min in dry ice-methanol mix, or overnight at -20°C).

6. Centrifuge at full speed (at least 12,000 rpm) at 4°C for 15 min.

7. Remove the supernatant carefully so as not to disturb the RNA pellet.

8. Wash the pellet with 500 μl of 75% ethanol, centrifuge at 12,000 rpm at 4°C for 5 min and decant the ethanol. Quick-spin the tube and carefully remove the residual alcohol.

9. Air-dry the pellet for ~5 min (be careful not to over-dry).

10. Resuspend the pellet in 20 μl of RNA loading buffer. Boil this RNA sample at 95°C for 5 min.

11. Assemble a gel cast.

12. Mix 20 ml of 6% urea-polyacrylamide stock solution, 100 μl of 20% APS and 20 μl of TEMED. Pour this mixture into the gel cast immediately and insert a comb as fast as possible because the gel solidifies in a few minutes.

13. Pre-run at 350V for at least 60 min. Running buffer is 0.5X TBE.

14. Load the RNA sample (step 10) on 6% urea-polyacrylamide gel and run at 350V until bromophenol blue reaches the bottom of the gel.

15. Dissemble the gel cast and remove one of the glass plates from the gel. Wrap the gel with plastic wrap and place an X-ray film on the gel for 30-60 sec. Make sure to mark the position and orientation of the gel on the film so that the gel can be aligned with the film once the film is developed. The radiolabeled transcript will appear as a strong band on the developed film.

16. Align the film with the gel and cut out the gel slice containing the labeled transcript. Put the gel slice in 350 μl of RNA elution buffer.

17. Incubate overnight at 42°C.

18. Transfer the supernatant (about 300 μl) to a fresh tube.

19. Add 100 μl of RNA elution buffer to the gel slice and vortex. Remove the supernatant and add it to the previous supernatant.

20. To the supernatant, add 1 μl of glycogen and 1 ml of 100% ethanol.

21. Mix and place the tube at –80°C for at least 20 min (or 5 min in dry ice-methanol mix, or overnight at -20°C).

22. Spin at full speed at 4°C for 15 min.

23. Wash the pellet with 500 μl of 75% ethanol, centrifuge at 12,000 rpm at 4°C for 5 min and decant the ethanol. Quick-spin the tube and carefully remove the residual alcohol.

24. Air-dry the pellet. Be careful not to over-dry the pellet.

25. Count c.p.m. and resuspend in RNase-free TE buffer.

1-2. Preparation of the Microprocessor

Materials

1. Mammalian expression plasmids containing human Drosha fused with FLAG epitope at its C-terminus (Drosha-FLAG) or DGCR8 fused with FLAG epitope at its N-terminus (FLAG-DGCR8)

2. HEK293T cells, culture medium (DMEM supplemented with 10% FBS), culture equipments, transfection reagent (Calcium phosphate method is the most economic and efficient choice for this experiment.)

3. Phosphate-buffered saline (PBS): Dissolve 8 g NaCl, 0.2 g KCl, 1.44g $Na_2HPO_4{\cdot}2H_2O$, 0.2 g KH_2PO_4 in water. Check pH (should be 7.2), set volume to 1 L, and autoclave

4. Lysis buffer: 20 mM Tris-HCl (pH 8.0), 100 mM KCl and 0.2 mM EDTA

5. Anti-FLAG antibody conjugated agarose (Anti-FLAG M2 Affinity Gel Freezer-Safe, Sigma)

6. Sonicator (Sonics, VC130)

Procedure

Preparation of HEK293T whole cell extract

1. Grow HEK293T cells on 100mm dish to 95% confluency.

2. Remove the media and rinse the cells with 5 ml of ice-cold PBS.

3. Add 1 ml of ice-cold PBS, collect the cells by scrapping, and transfer to an eppendorf tube.

4. Centrifuge at 6000 rpm at 4°C for 5 min.

5. Decant the PBS and resuspend the cell pellet in 500 μl of lysis buffer.

6. Sonicate ten times (for 5 sec each) with short intervals at 30% amplitude.

7. Centrifuge at 12000 rpm at 4°C for 15 min.

8. Transfer the supernatant to a fresh tube. This is the ‘HEK293T whole cell extract’.

Immunoprecipitation of the Microprocessor

1. Prepare ‘HEK293T whole cell extract’ as described above using HEK293T cells transfected with the Drosha-FLAG or FLAG-DGCR8 expression plasmid. For more efficient processing activity, Drosha-FLAG and V5-DGCR8 are co-transfected into cells.

2. Take ~10μl (bead volume) of anti-FLAG antibody conjugated beads and wash the beads by adding 1ml of lysis buffer, mixing, centrifugation, and decanting the

supernatant. Repeat washing once.

3. Add 500μl of HEK293T whole cell extract to the washed beads.

4. Rotate the tube at 4°C for 1 hr.

5. Centrifuge at 8000rpm at 4°C for 30 sec.

6. Remove the supernatant, add 1ml of lysis buffer, and wash the beads by inverting the tube 6-7 times.

7. Centrifuge at 8000rpm at 4°C for 30 sec.

8. Repeat steps 6 and 7 four times. Change the tube for the last washing.

9. Carefully remove the residual buffer so that the residual volume becomes approximately 15 μl. Keep the tube on ice until the immobilized Microprocessor complex is used for assay. (We do not freeze or store the immunoprecipitate for more than 3 hours. Basically we prepare the immunoprecipitate freshly for each assay.)

1-3. *In vitro* pri-miRNA processing assay

Materials

1. Radiolabeled pri-miRNA transcript

2. HEK293T whole cell extract or the agarose beads holding the immunoprecipitated Microprocessor

3. 10X reaction buffer (64mM $MgCl_2$); RNase III family proteins require magnesium ion for catalysis. The optimal concentration of magnesium chloride for Drosha is 6.4 mM. The processing efficiency is not significantly reduced down to 3.2 mM, but below 3.2 mM, the processing efficiency decreases gradually with a reduced amount of magnesium in the reaction mixture.

4. RNase inhibitor (RNasin or equivalent, 40 U/μl)

5. Hoeffer gel apparatus (18 X 16cm) or equivalent, combs (0.75mm, 15 wells), spacers (0.75mm), and a power supply

6. Urea-polyacrylamide stock solution (12.5%): Dissolve 118.75g acrylamide, 6.25g Bis-acrylamide, 100ml 5X TBE (final 0.5X) and 420.42g Urea (final 7M) in water to make 1 L.

7. Ammonium persulfate (APS) solution: 20% dissolved in water

8. TEMED

9. RNA elution buffer: 0.3M sodium acetate, pH 5.5 and 2% SDS

10. Phenol:chloroform, pH 4.5

11. 3M sodium acetate solution, pH 5.5

12. RNA loading buffer: 95% deionized formamide, 0.025% bromophenol blue, 0.025% xylene cyanol, 5mM EDTA and 0.025% SDS

13. Thermoblock at 37°C/95°C

14. Kodak X-AR5 autoradiography films

15. Autoradiography cassettes with intensifying screens

Procedure

1. Mix the following; 3 μl of 10X reaction buffer (final 6.4mM), 3 μl of radiolabeled pri-miRNA (10^4~10^5 cpm), 0.75 μl of RNase inhibitor (final 1U/μl), 8.25 μl of RNase free water, and 15 μl of HEK293T whole extract or immunoprecipitate (immobilized on the beads).

2. Incubate at 37℃ for 90 min.

3. Add 170 μl of RNA elution buffer to the reaction mixture.

4. Add 200 μl of phenol:chloroform and vortex for 30 sec.

5. Centrifuge for 5 min at RT and take the upper layer.

6. Add 20 μl of 3M sodium acetate, 1 μl of glycogen, and 1ml of 100% ethanol. Mix and leave at -80℃ for at least 20 min. (or 5 min in dry ice-methanol mix, or overnight at -20℃)

7. Centrifuge at maximum speed at 4℃ for 15 min.

8. Remove the supernatant carefully so as not to disturb the RNA pellet. Wash the pellet with 500 μl of 75% ethanol.

9. Air-dry the pellet for ~5 min (be careful not to over-dry).

10. Resuspend the pellet in 15 μl of RNA loading buffer.

11. Prepare 12.5% urea-polyacrylamide gel as described above (See 1-1. Preparation of radiolabeled pri-miRNA transcript, *procedure*, steps 11-13).

12. Heat the RNA sample at 95℃ for 5 min. Load 7.5 μl of RNA samples on the gel and run at 350V~390V until bromophenol blue reaches the bottom of the gel.

13. Expose the gel on an X-ray film overnight at -80℃ and develop this film. The product of processing will appear as a band of 60-75 nt depending on the substrate used in the assay. For instance, pre-let-7a is 72 nt in length (Fig. 3B). Additional bands may appear, which usually correspond to the flanking sequences around the cleavage sites. Contamination by other nuclease(s), nonspecific chemical cleavage reaction, or abortive processing can also result in unexpected cleavage products (Fig. 3B).

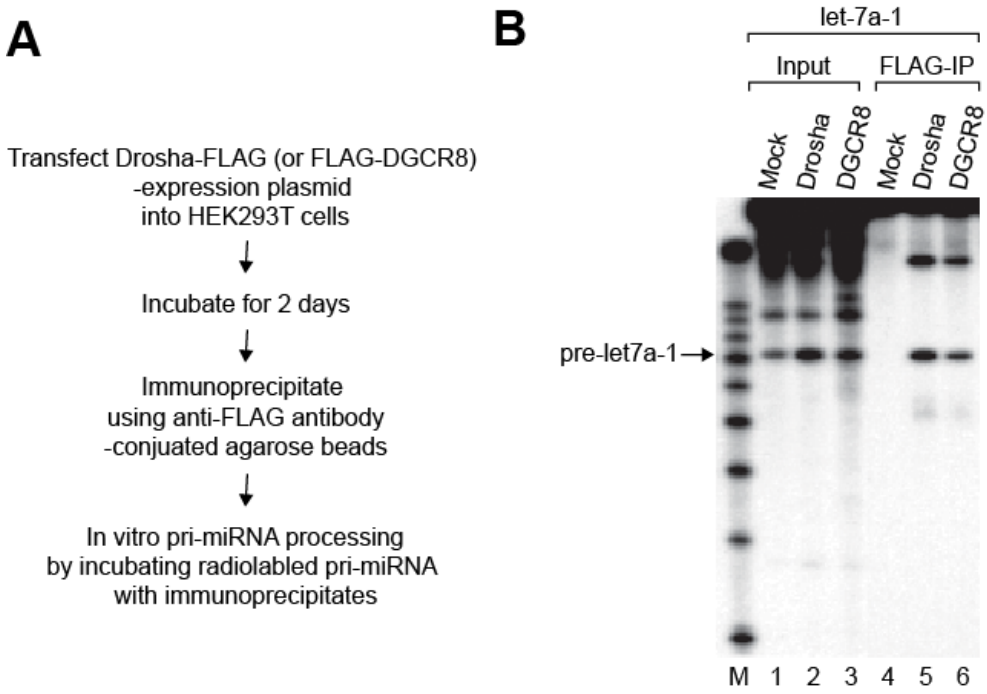

Figure 3. *In vitro* pri-miRNA processing assay. (A) Experimental scheme. (B) Typical results from processing assay. Pri-let-7a-1 was incubated with HEK293T whole cell extract (input) or immunoprecitates (FLAG-IP). HEK293T cells had been transfected with pCK (Mock), pCK-Drosha-FLAG (Drosha) or pCK-FLAG-DGCR8 (DGCR8). Pre-let-7a-1 is produced when pri-miRNA is incubated with Drosha or DGCR8 immunoprecipitate or whole cell extract.