Gel Purification of miRNA from Total RNA

A. Nuclease-free Solutions and Reagents Needed

- 15% denaturing acrylamide gel [1X TBE, 7 M urea, 15% acrylamide (19:1 acryl:bis-acryl)]
  Since this gel is used to separate the ~21-mer mature miRNA species from RNA that is significantly longer, the resolution does not need to be optimal. We recommend using a 0.75 mm spacer, and a comb appropriate for the sample volume.

for 15ml	Component
6.3 g	Urea (high quality, e.g. Ambion cat #9900)
1.5 ml	10X TBE
5.6 ml	40% Acrylamide (19 acryl:1 bis-acryl, e.g. Ambion cat #9022)
to 15 ml	distilled deionized water
Stir at room temperature until the urea is completely dissolved, then add:	
75 µl	10% ammonium persulfate
15 µl	TEMED

  Mix briefly after adding the last two ingredients, which will catalyze polymerization, then pour the gel into a prepared mold immediately.
- 10X TBE: Tris-Borate-EDTA Buffer; Ambion Cat #9862–9864
- Ambion Gel Loading Buffer II for denaturing PAGE (Cat #8546G, 8547)
- Linear Acrylamide: Ambion Cat #9520
- 1M NaCl: Dilute Ambion's 5 M NaCl Cat #9759 with high quality nuclease-free water to 1 M.
- 100% ethanol: ACS grade or higher quality
- 80% ethanol: Make 80% ethanol from high quality nuclease-free water and ACS grade (or better) 100% ethanol.
- Ambion MEGAclear™ Kit Cat #1908

B. Electrophoretic Separation of miRNA from Total RNA

1. Prepare a 15% denaturing gel and pre-run it for 10–15 min

a. Using standard procedures, prepare a 15% denaturing acrylamide gel [1X TBE, 7 M urea, 15% acrylamide (19:1 acryl:bis-acryl)].

b. Warm the gel to ~50°C by running it for 10–15 minutes in 1X TBE running buffer.

2. Mix 5–50 µg of total RNA with an equal volume of Gel Loading Buffer II and heat for 5 min at 95°C

a. Add an equal volume of Gel Loading Buffer II to each RNA sample.

b. Heat to 95°C for 5 min to denature the RNA, then place the tube in ice.

3. Load the gel and run until the leading dye travels about 4–5 cm down the gel

a. Rinse the wells of the gel with 1X TBE and load sample.

b. Run the gel until the bromophenol blue dye front (the leading dye) migrates about 4–5 cm down the gel.

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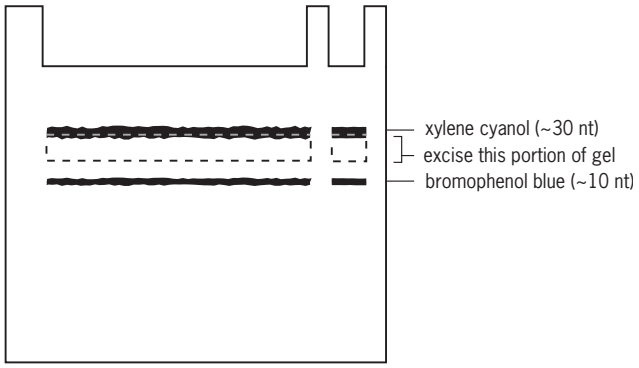
C. Elution of miRNA from the Gel Slice

1. Recover the gel slice that contains miRNA, add 10 ml 1 M NaCl, crush gel, and incubate overnight at 4°C with rocking

a. Excise a band from the gel starting just below the center of the xylene cyanol band, and extending to three quarters of the way to the bromophenol blue band (see Figure 1). Place the gel piece in a 15 ml conical tube.

Figure 1. Excising the Gel Band with the miRNA Fraction.



b. Add 10 ml nuclease-free 1 M NaCl to the tube with the gel slice.

c. Crush the gel slice with a syringe plunger until the pieces are as small as possible (≤1 mm).

d. Soak the crushed gel slice overnight with rocking at 4°C.

2. Centrifuge 5 min at 2000 X g and transfer the supernatant to a 50 ml tube

Centrifuge the crushed gel mixture for 5 min at 2000 x g. Decant the 1 M NaCl into a sterile 50 ml screw cap tube.

This solution contains most of the miRNA; store it at 4°C while you complete the elution.

3. Re-elute with 2 ml more 1 M NaCl for 1 hr at room temp with rocking

a. Add 2 ml 1 M NaCl to the crushed gel slice and incubate for 1 hour at room temp with rocking..

b. Centrifuge for 5 min at 2000 X g and pool the supernatant with the first supernatant (from step 2).

D. miRNA Clean-Up

IMPORTANT

Before starting the miRNA clean-up, preheat the Elution Solution (from the MEGAclear Kit) to 95°C.

1. Add the following to each miRNA sample, and pass the mixture through a MEGAclear Filter Cartridge

a. Place a MEGAclear Filter Cartridge in a 5 ml syringe barrel, and mount the assembly on a vacuum manifold.

b. Add linear acrylamide and ethanol to each sample, vortexing after each addition to mix thoroughly:

Amount	Component
1.5 µl	Linear Acrylamide (5 mg/ml)
to 60%	100% ethanol (typically 18 ml)

c. Pipet the RNA mixture from the previous step into the MEGAclear Filter Cartridge. Vacuum pressure will pull the solution through the filer.

2. Wash with 4 ml of 80% ethanol

With the vacuum still on, slowly pipet 4 ml of 80% ethanol into the column, allowing the vacuum to pull the solution through the filter.

3. Using a centrifuge, wash with 500 µl of 80% ethanol, then dry the filter

a. Place the MEGAclear Filter Cartridge in a Collection Tube, and pipet 500 µl of 80% ethanol into the column.

b. Centrifuge for 1 min at 5,000 x g and empty the Collection Tube.

c. Centrifuge again at 10,000 x g to remove excess ethanol.

d. Move the MEGAclear Filter Cartridge to a fresh Collection Tube.

4. Elute the miRNA in 2 x 50 µl 95°C Elution Solution and dry the miRNA to completion

a. Apply 50 µl heated (95°C) Elution Solution (from the MEGAclear Kit) to the MEGAclear Filter Cartridge, and leave at room temp for 2 min.

b. Centrifuge for 1 min at 10,000 x g. Purified miRNA will be centrifuged into the Collection Tube.

c. Repeat the elution with a second 50 µl aliquot of heated Elution Solution, centrifuging purified miRNA into the same tube.

d. If you isolated miRNA from more total RNA than is needed for a single *mir*Vana miRNA Labeling Kit reaction, split the eluted miRNA into single reaction-sized aliquots. (Use miRNA obtained from 5–50 µg of total RNA per *mir*Vana miRNA Labeling Kit reaction.)

e. Place miRNA sample in a vacuum concentrator and dry completely.