GENERATING A cDNA LIBRARY FROM A SMALL RNA SAMPLE

Purpose: To identify microRNA and/or siRNA genes active in a tissue (e.g., seed coat parenchyma layer) or an organ (e.g., a seed).

Reference:

1. RNAqueous-Micro Kit Procedure of LCM (attachment, Ambion website)
2. Small RNA v.1.5 Sample Prep Guide 15002615B (Released August 2009; attachment; Illumina website,)
3. Lu et al. (2007) Construction of Small RNA cDNA Libraries for Deep Sequencing. Methods 43: 110-117
4. Sambrook et al. (1989) pp. 13.47-13.48

STRATEGY

I. ISOLATION OF TOTAL RNA FROM A TISSUE CAPTURED BY LCM
II. ENRICHMENT OF SMALL RNA FROM THE TOTAL RNA SAMPLE
III. MAKING A cDNA LIBRARY FROM ISOLATED SMALL RNA
IV. SEQUENCING OF THE cDNA LIBRARY USING AN ILLUMINA SEQUENCER

ATTENTION:

1. The lysis buffer of the RNAqueous-Micro kit (Ambion) is SAME as that of the mirVANA miRNA Isolation kit (Ambion).
2. It is optional to isolate total RNA and then enrich for small RNA. In other words, small RNA can be isolated from the LCM sample using the mirVANA miRNA Isolation kit.
3. Due to high level of self-ligation of version 1.0 SRA adapter, a version 1.5 3' SRA adapter was developed. Although this new version adapter should not ligate to itself, a small level of self-ligation occurred.

ISOLATION OF TOTAL RNA FROM A TISSUE CAPTURED BY LCM

1. If the **total volume** of the **Lysis Solution including captured sample** is **less** than **100µL**, bring the volume to 100µL with Lysis Solution (Lot# ; Date of received:).
2. Mix well by vortexing and centrifuge for 10 seconds for bringing down the liquid to the bottom of the tube.
3. Incubate for **30 min** at **42°C** (an air-incubator or a heat-block). NOTE: It is okay to incubate up to 1 hour. *This mixture is called **LYSATE***.
4. Vortex briefly to mix and centrifuge for 10 seconds for bringing down the liquid to the bottom of the tube.
5. Prewet the Micro Filter Cartridge Assembly with Lysis Solution
   a. Pipet **30 µL** of **Lysis Solution** to the center of the filter
   b. Allow it to soak for **at least 5 minutes**.
   c. Centrifuge the prewetted filter for **~30 seconds** at **top speed** to remove liquid. If the lysate solution is NOT available yet, keep the Micro Filter Cartridge Assembly at room temperature.
6. Add **3µL** of **LCM Additive** (Lot#: ; Received Date:) (or **miRNA homogenate**; *it may be a 3M sodium acetate because it has a smell of acetic acid*) to the lysate.
   a. mix well by briefly vortexing.
   b. Centrifuge for 10-15 seconds to collect the fluid at the bottom of the tube.
7. To the **lysate and additive mix**, add **a certain volume** of **100% ethanol** to recover either **all RNA** or only large RNA species. *The amount of ethanol added to the lysate mixture determines what size of RNA will be captured on the filter. Instructions are provided below for recovering only RNA species larger than ~75nt, or for recovering **both large and small RNA species**, including tRNAs and microRNAs.*
   - (**Preferred**) To recover ***large and small*** RNA species:
     Add **1.25 volumes** (**129µL**) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing. (Yields of total RNA using this method may vary from those obtained using the above method.)

- To recover ***only large*** RNA species:
  Add **0.5 volume** (**52µL**) of 100% ethanol to the lysate mixture, and mix by pipetting up and down or by gently vortexing.

8. Load the lysate/ethanol mixture (**up to 150µL**) onto a Micro Filter Cartridge Assembly and close the cap.
9. Centrifuge for 1 minute at **maximum speed** or until all of the mixture has passed through the filter.
   ***IMPORTANT:* SAVE the flow-through solution in a 1.5-mL RNase-free tube just in case a mistake would have been made. After you are sure that total RNA was isolated from the tissue, you can discard this solution**.
   NOTE: Longer centrifugation times may be needed to filter the lysate from tissue samples. For lysate/ethanol mixtures >150 µL, load and filter the first 150 µL, then repeat with additional aliquots until the entire sample has passed through the filter. The Collection Tube has a capacity of ~700 µL when assembled with a Micro Filter Cartridge; if more than 150 µL of lysate/ethanol mixture is filtered, empty the Collection Tube before proceeding.
   ***The RNA is now bound to the filter in the Micro Filter Cartridge*.**
10. Wash the filter with **180 µL Wash Solution1** (Lot#: ; Received Date:). *NOTE: Make sure to add **10.5 mL** of **100% ethanol** to the **bottle labeled Wash Soln 1 Concentrate**. Mix well. Place a **check mark** in the empty box on the label to indicate that the ethanol has been added.*
    a. Open the Micro Filter Cartridge, pipet **180 µL** of **Wash Solution1** (working solution mixed with ethanol) to the filter and close the cap.
    b. Centrifuge for **~10 seconds** to pass the solution through the filter.
11. Wash the filter with **180 µL Wash Solution2/3** (Lot#: ; Received Date:), **TWICE**
    *NOTE: Make sure to add **22.4 mL** of **100% ethanol** to the **bottle labeled Wash 2/3 Concentrate**. Mix well. Place a **check mark** in the empty box on the label to indicate that the ethanol has been added.*
    a. Open the Micro Filter Cartridge, add **180 µL** of **Wash Solution2/3** (working

solution mixed with ethanol) to the filter and close the cap.

b. Centrifuge for ~10 seconds to pass the solution through the filter.
c. Repeat with a **second** **180 µL** of **Wash Solution2/3**.

12. Discard the flow-through and centrifuge the filter for 1 minute at max speed
    a. Open the Micro Filter Cartridge assembly,
    b. remove the filter cartridge from the Collection Tube, and
    c. pour out the flow-through.
    d. Replace the Micro Filter Cartridge into the same Collection Tube, close the cap
    e. centrifuge at maximum speed for 1min to remove residual fluid and dry the filter.
13. Elute the RNA into a Micro Elution Tube with 5–10µL preheated Elution Solution TWICE. *NOTE: You can heat **100 µL** of **Elution Solution** in a **200-µL RNase-free tube** to **95°C** using a **thermocycler**.*
    a. Label a **Micro Elution Tube** (1.5 mL tubes provided with the kit) and transfer the Micro Filter Cartridge into it.
    b. Apply **5–10 µL** of preheated Elution Solution to the center of the filter.
    c. Close the cap and store the assembly for 1 minute at room temperature.
    d. Centrifuge the assembly for ~30 seconds to elute the RNA from the filter.
    NOTE: Tension from the hinge of the Micro Elution Tube can occasionally cause the cap to pop off during the elution spin. To minimize the chance of this happening, bend the cap hinge back and forth several times, then press the cap securely onto the Micro Filter Cartridge.
    e. Repeat with a **second** 5–10 µL aliquot of preheated Elution Solution, collecting the eluate in the same Micro Elution Tube.

NOTE: The exact volume of Elution Solution used is not critical and may be increased if desired. In general, ~75–85% of the RNA will be recovered from samples derived from up to ~100,000 cells (or 3mg tissue) using 2x5µL of Elution Solution, and ≥85% will be recovered using 2x10µL of Elution Solution. A larger volume of Elution Solution may be required for thorough elution of the RNA from larger samples, especially tissue samples >3 mg.

ENRICHMENT OF SMALL RNA USING A mirVANA miRNA ISOLATION KIT

Reference: mirVana miRNA Isolation kit instruction manual (Applied Biosystems/Ambion, AM1560, AM1561)

Purpose: To enrich small RNAs including tRNAs, miRNA, siRNA, and 5S rRNA, but eliminate large ribosomal RNA samples (25S, 23S, 18S, 16S) from the total RNA sample, for a cDNA library construction.

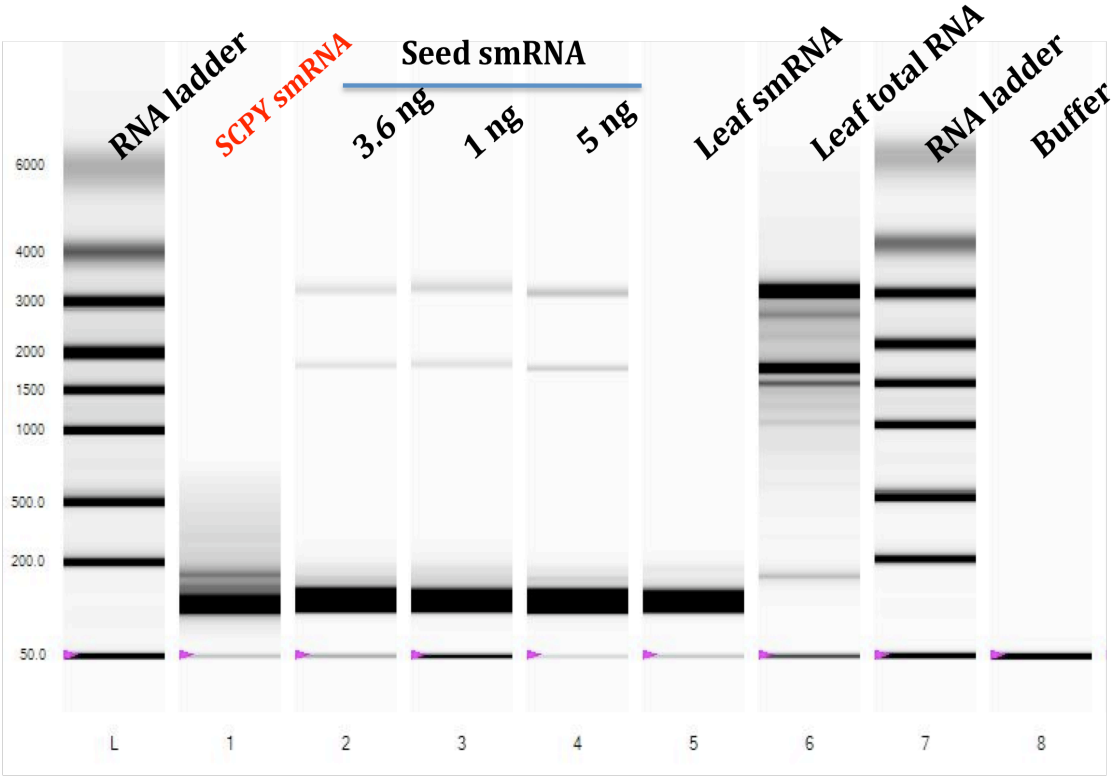
PROCEDURE

1. Reconstitute the RNA solution as an aqueous phase of RNA extraction as following:
   a. Bring the **total volume** to **100 µL** with **Lysis Solution**
   b. Add **1/10 volume** of **homogenate additive** to the RNA/lysis mixture. Mix well by pipetting up and down for 10-25 times.
   *NOTE: The total volume of the mixture is **110 µL**.*
2. Pipet 1/3 volume (**37 µL**) of 100% ethanol solution at room temperature to the aqueous phase (e.g. add 100 µL of 100% ethanol to 300 µL of aqueous phase). Mix the contents well by vortexing or inverting the tubes several times.
3. For each sample, place each Filter cartridge into each of two Collection tubes.
4. Pipet up to 700 µL of the **lysate/ethanol mixture** onto the Filter cartridge.
   NOTE: If the mixture volume is greater than 700 µL, apply the mixture to the same Filter cartridge after the centrifugation.
5. Centrifuge the cartridge and tube at **10,000 rpm** for 15 seconds.
   NOTE: Spinning >10,000 rpm may damage the filter.
6. Collect the filtrate (or flow-through) and transfer to a new 1.5-mL RNase-free tube.
7. Determine the recovery volume.
8. Add 2/3 volume of 100% ethanol at room temperature to the filtrate (e.g. 400 µL of filtrate, add 266 µL of 100% ethanol). Mix the content thoroughly by vortexing for 5 seconds.

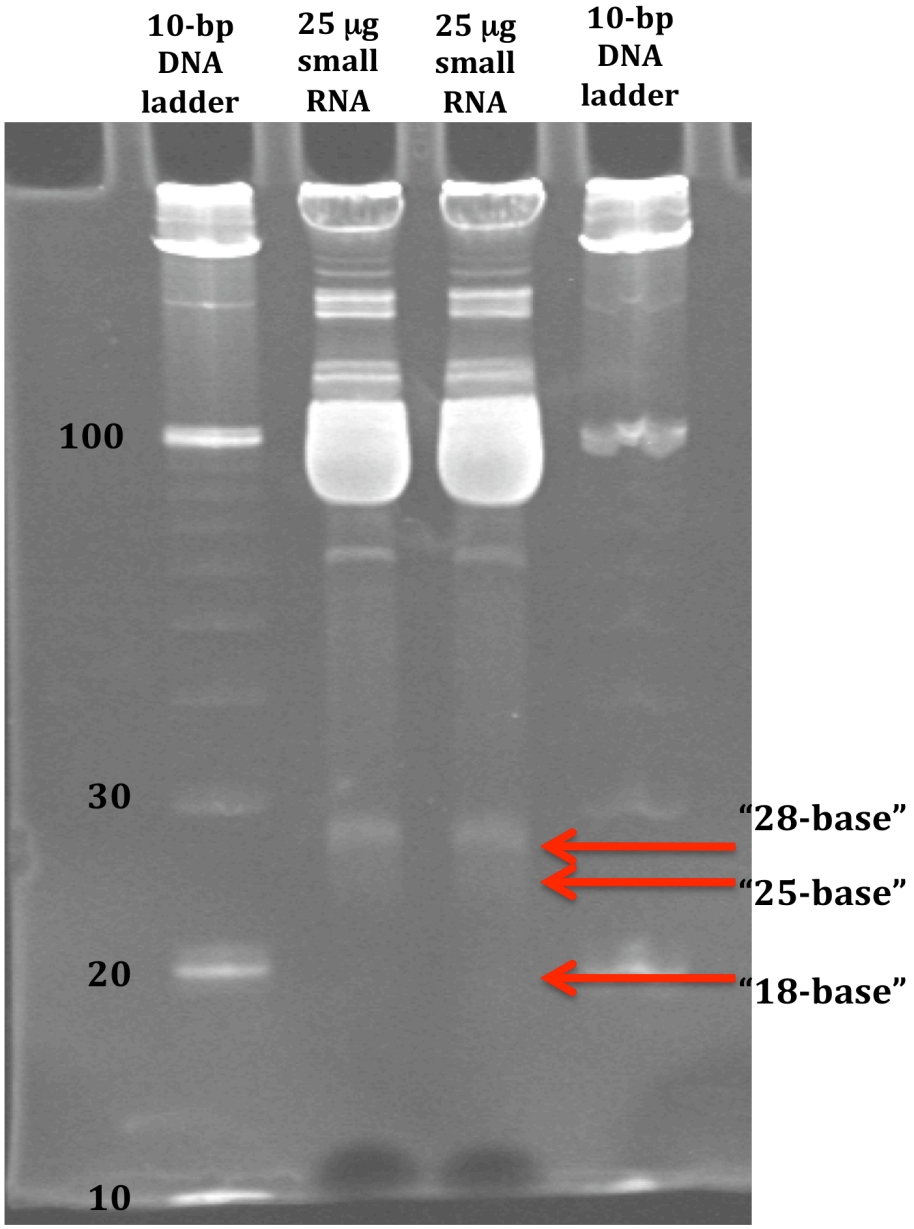
9. For each sample, place a new Filter cartridge into one of the Collection tube.
10. Pipet 700 μL of the lysate/ethanol mixture onto the second Filter cartridge. If the mixture volume is greater than 700 μL, apply the mixture to the same Filter cartridge after the centrifugation.
11. Centrifuge the cartridge and tube at 10,000 rpm for 15 seconds. Note: Spinning >10,000 rpm may damage the filter. Centrifugation of 10 seconds is sufficient.
12. Discard the flow-through by pipetting.
13. Wash the filter with 700 μL of miRNA Wash solution 1. Centrifuge for 5-10 seconds at 10,000 rpm.
14. Discard the flow-through by pipetting.
15. Wash the filter with 500 μL of miRNA Wash solution 2/3. Centrifuge for 5-10 seconds at 10,000 rpm.
16. Discard the flow-through by pipetting.
17. Spin the filter for 1 minute at 10,000 rpm to remove trace amount of ethanol from the filter.
18. Transfer the filter to a new Collection tube.
19. Heat the Elution solution or RNase-free water in 0.2-mL PCR tubes to 95°C using a thermocycler. Note: EDTA may inhibit downstream processes.
20. Pipet 100 μL of 95°C Elution solution (0.1 mM EDTA) or (preferable) RNase-free water to the center of the filter.
21. (Option) Incubate for 1 minute.
22. Centrifuge the filter/tube at 13,000 rpm for 30 seconds to elute small RNA from the filter.
23. Determine the volume of eluate (small RNAs). Keep the tube on ice.
24. Determine RNA concentration using a Nanodrop UV spectrophotometer.
25. Determine the total amount of RNA.
26. Analyze RNA solution on either a capillary gel electrophoresis system which requires at least 100 pg of RNA (Experion – BioRad – see an example #1 below) or a denaturing 15% polyacrylamide gel for a better resolution, but requires at least 50 μg of enriched small RNA (see an example # 2 below). <u>*Note:*</u> *To observe*

small RNA bands of less than 60 bases on the acrylamide gel, I loaded 50 µg of small RNA solution on the gel; but, I had a difficult time to see the small RNA bands on the UV box. So, I used the 10-bp ladder and a ruler as the guide to excise the bands.

Example #1: Quality of small RNA isolated from LCM SCPY and whole-mount seeds determined by using a capillary gel electrophoresis Experion system



Example #1: Quality of small RNA isolated from whole-mount seeds determined by using an acrylamide gel electrophoresis and stained with ethidium bromide



cDNA Library Construction of smRNA Isolated from LCM Sample Using Illumina's Small RNA v1.5 Sample Preparation Kit

*Note: The original Illumina protocol is for **1-5 µg** of total RNA sample. According to Anhthu's data, **enriched small RNA is ~20% of total RNA**; thus, the original protocol was modified for 50 ng or less small RNA so that self-ligation of adapters can be minimized. (December 14, 2009; Updated Feb. 3, 2010)*

Objective:

Background Information:

Summary of Results:

PROCEDURE

LIGATION OF ADAPTERS TO SMALL RNA

1. Dilute **1 µL** of **10x v1.5 SRA 3' adapter (10 µM)** with **9 µL** of **nuclease-free water** (**10-fold dilution**). Keep the diluted adapter solution **on ice**.
2. Dilute **1 µL** of **SRA RT primer** (**100 µM**) with **4 µL** of **nuclease-free water** (**5-fold dilution**). Keep the diluted adapter solution **on ice**.
   Note: I talked with Illumina tech support (Jim Gordon) whether it was a typo in the protocol because it was too early to prepare for reverse transcription reaction. Instead, this should be a dilution of SRA 5' adapter (5 µM). Jim said that according to Illumina Research and Development the dilution of SRA RT primer was correct. I doubted that Jim was correct because in later step there was NO indication of diluting the SRA 5' adapter solution. To me, it was logical to use the same amount of adapters (3' and 5') in a ligation reaction. (Anhthu Bui)
3. (Modification by Anhthu Bui) Dilute **1 µL** of **SRA 5' adapter** (**5 µM**) with **4 µL** of **nuclease-free water** (**5-fold dilution**). Keep the diluted adapter solution on ice.
4. Make a **100 mM $MgCl_2$** solution by diluting **100 µL** of **1M $MgCl_2$** by **10-fold** with **900 µL** of nuclease-free water. Mix. Spin briefly.
5. Preheat the PCR thermocycler to 22°C and a heat block to 70°C.
6. Prepare an enriched small RNA solution for the ligation reaction. *Note: If the volume of RNA solution is greater than 5.0 µL, then pipet the volume of a such amount (e.g. 50 ng) into a 1.5-mL RNase-free microcentrifuge tube and concentrate RNA in a RNase-free speed-vac for 5-30 minutes, depending the starting volume.*
7. Determine and record the volume of the concentrated RNA solution.
8. Ligate enriched small RNA with **diluted v1.5 SRA 3' adapter** in 200-µL nuclease-free PCR tubes

	Original	Modified
Enriched small RNA		
1X Diluted v1.5 SRA 3' adapter (1 μM)	1.0 μL	**0.5 μL**
Nuclease-free water		
Total volume	**6.0 μL**	**6.0 μL**

- Mix the contents by flicking the tube. Spin briefly.
- Heat to **70°C** for **2 min**. Quench **on ice** for **2 min**.
- Add **3.8 μL of T4 RNA ligase 2 mix** to the sample (see table below). Mix well. Spin briefly.

	Original	Modified
10X T4 RNA ligase 2, truncated reaction buffer (NEB)	1.0 μL	1.0 μL
100 mM $MgCl_2$	0.8 μL	0.8 μL
Nuclease-free water	0.0 μL	**0.2 μL**
T4 RNA ligase 2, truncated (NEB)	1.5 μL	**0.5 μL**
RNaseOut/RNase Inhibitor	0.5 μL	0.5 μL
Total volume of Ligase 2 Mix	**3.8 μL**	**3.0 μL**

- Incubate at **22°C** for **1 hour** in the PCR thermocycler.

9. With **5 min. remaining** of the ligation of SRA 3' adapter, prepare the **SRA 5' adapter** by heating the diluted SRA 5' adapter to **70°C** for **2 min**. Quench **on ice** for **2 min**.
10. Add to the ligation mixture from step 7 above the following components:

	Original	Modified
10 mM ATP	1.0 μL	1.0 μL
Diluted SRA 5' adapter	1.0 μL	**0.5 μL**
Nuclease-free water	0.0 μL	**1.0 μL**
T4 RNA ligase	1.0 μL	**0.5 μL**
Total Volume of Ligase Mix	**3.0 μL**	**3.0 μL**

- If work with a master mix, aliquot **3 μL of the master mix** to each tube.
- Mix well. Spin briefly.

11. Incubate in the thermocycler at **22°C** for **1 hour**.

REVERSE TRANSCRIPTION

1. Preheat the PCR thermocycler or the heat block to **70°C**.
2. Combine in **nuclease-free 200 µL PCR** tubes. *Note: Because a little amount of enriched small RNA was used, it is best to convert all ligated smRNAs into cDNAs.*

	Original	Modified
5' & 3' Ligated smRNA	4.0 µL	**12.0 µL**
1/5 Diluted SRA RT primer	1.0 µL	**1.0 µL**
Total volume of smRNA-RT mix	**5.0 µL**	**13.0 µL**

   - Mix contents in tube(s). Spin briefly.

3. Heat the tubes of mixture to **70°C** for **2 min**. Quench tubes on **ice** for **2 min**.
4. Dilute 25 mM dNTP mix to **12.5 mM dNTP mix**

	1 Rxn	Mmix (x)
Nuclease-free water	0.5 µL	
25 mM dNTP mix	0.5 µL	
Total volume of dNTP mix	**1.0 µL**	

5. Preheat the PCR thermocycler to **48°C**.
6. Prepare a RT mix for **10 µL-RT** (original) or **20 µL-RT** (modified) reactions.

	Original	Modified
5x First strand buffer	2.0 µL	4.0 µL
12.5 mM dNTP mix	0.5 µL	**0.5µL**
100 mM DTT	1.0 µL	**2.0 µL**
RNase Out/RNase Inhibitor	0.5 µL	0.5 µL
Total volume of buffer-dNTP mix	**4.0 µL**	**7.0 µL**

   - Mix the contents in the tube well. Spin briefly.

7. Add **7 µL** of the **RT mix** to each tube containing ligated sRNA sample. Mix by pipetting up and down 5 times. Spin tubes briefly.
8. Heat the tubes to **48°C** for **3 min** with a profile **"44 degree1hr"** on the thermocyler.
9. Add **1 µL** of **superscript II-reverse transcriptase** to each tube. Mix well by pipetting up and down >5 times. Spin tube briefly (if necessary).
10. Incubate tubes of mixture in the thermocycler at **44°C** for **1 hour**.

PCR AMPLIFICATION

1. Prepare a master mix for multiple samples

	Original	**Modified**
Sterile water	27.0 μL	28.0 μL
5X Phusion HF buffer	10.0 μL	10.0 μL
Primer GX1 (25 μM)	1.0 μL	0.5 μL
Primer GX2 (25 μM)	1.0 μL	0.5 μL
25 mM dNTP mix	0.5 μL	0.5 μL
Phusion DNA polymerase (2 U/μL)	0.5 μL	0.5 μL
Total volume of PCR mix	**40.0 μL**	**40.0 μL**

   - Mix the contents well. Spin briefly.

2. Add **40 μL** of the master mix to each RT tube with **10 μL** of first strand cDNA solution.
3. Carry out PCR amplification with the following profile:

   98°C, 30 sec.; **12 cycles** of 98°C, 10 sec./60°C, 30 sec./72°C, 15 sec.; 72°C, 10 min.; 4°C, indefinite

PURIFICATION OF PCR PRODUCT (cDNA)

1. Prepare a 7.5% polyacrylamide gel (see page)
2. Prepare samples for loading on the gel

	25-bp Ladder	10-bp Ladder	RT-PCR		
DNA sample	2 μL	2 μL	50 μL		
TE buffer	8 μL	8 μL	0 μL		
DNA Loading dye (Illumina)	2 μL	2 μL	10 μL		

   - Mix the mixture well. Spin briefly.

3. Load samples on the gel

4. Run the gel at 200 volts for ~90 minutes or until the second dye (xylene cyanol light green) near the bottom of the gel. In this case, the 50-bp band of the 25-bp DNA ladder is at the bottom of the gel.
5. Turn off the power supply.
6. Pour ~100 mL 1X TBE buffer in the upper reservoir of the gel electrophoresis into a plastic container. Pipet **10 µL** of **10 mg/mL ethidium bromide** to the 1X TBE buffer. Rock the container gently to dissipate the ethidium bromide.
7. Disassemble the gel plates, gently remove the gel and place it in the TBE-EtBr solution.
8. Stain the gel for 5-10 min.
9. Destain the gel in distilled water for 5 min.
10. Take picture(s) of the gel.
11. Determine the 93-bp and 100-bp DNA fragments.
12. Label 1.5 mL microcentrifuge tubes according to samples.
13. Excise the gel piece containing 93-bp and 100-bp DNA fragments and place it in the appropriate tube.
14. Use a sterie 21-gauge needle to poke **2-3 holes** at the bottom of 0.5 mL microfuge tube.
15. Place the **0.5 mL microfuge tube** in either 2-mL round bottom and lidless tube (preferable from Plant RNeasy kit) or 1.5-mL microfuge tube.
16. Transfer the gel piece from 1.5-mL tube to the 0.5 mL tube.
17. Spin the **tube assembly** at **13,200 rpm** for **2 min**. Note: Gel piece is macerated into fine powder.
18. If use 2-mL tube, transfer the gel powder to the appropriate labeled 1.5-mL microfuge tube.
19. Dilute **10x gel elution buffer** by **10-fold** with nuclease-free water to get **1X gel elution buffer**.
20. Pipet **100 µL** of **1X gel elution buffer** to each tube of **gel powder**.
21. Elute DNA off gel powder by placing the tube on a rotator at room temperature for 2 hours or overnight.

22. After two hours of elution, label a **spin-x column** (supplied with the kit) with sample name.
23. Pipet **DNA elution buffer** to the membrane of the labeled spin-x column.
24. Spin the tube at **13,200 rpm** for **2 min**.
25. Remove the spin column.
26. Determine the volume of **eluate** (DNA solution) with **P100 filter tip**.
27. Precipitate DNA with 1 μL of glycogen, 1/10 volume of 3M NaOAc, and 325 μL of ice-chilled 100% ethanol. Mix by brief vortexing. Note: if **room temperature 100% ethanol solution is used**, **freeze** the mixture at **-80°C** for **30 min**.
28. Spin at **13,200 rpm at room temperature** for **20 min**.
29. Carefully, pipet off the supernatant and save it in another 1.5-mL microfuge tube (just in case you do not see a pellet or suck up the pellet)
30. Wash the pellet with **500 μL of 70% room temperature ethanol solution**. Spin at room temperature for **5 min**.
31. Carefully, pipet off the supernatant.
32. Spin the tube for **30 seconds**. Pipet off the residual ethanol solution.
33. Dry the pellet in a speedvac for **5 min**.
34. Resuspend the pellet in **10-20 μL** of **TE, pH 8.0 buffer**.
35. Determine **DNA concentration** using **picogreen** and **fluorometer**.

	DNA conc. (pg/μL)	**Total Volume (μL)**	**Total Amount of DNA (pg)**

36. Store DNA solution at **4°C** until submitting to the **Solexa sequencing**.

PURIFICATION OF cDNAs FROM A POLYACRYLAMIDE GEL

References:

1. Adapted from the protocol written by Lu et al. (2007) Construction of Small RNA cDNA Libraries for Deep Sequencing. Methods 43: 110-117.
2. Sambrook et al (1989) pp. 13.47-13.48
3. Fermentas. com for 2X RNA Loading dye

REAGENTS AND BUFFERS

40% Acrylamide Stock

	25 mL
Acrylamide (ultra pure)	9.5 g
N,N'-Methylene bisacrylamide	0.5 g
DEPC'd water	15 mL

- Weigh out acrylamide and bisacrylamide (see chart above) and put them in an RNase-free 50-mL beaker.
- Warm the beaker of mixture in a 37^{o}C water bath to dissolve acrylamide granules. The volume is almost 24 mL.
- Bring the final volume to 25 mL with DEPC'd water.
- Filter the acrylamide mixture through a 0.2 micron filtering unit.
- Store the filtered mixture in a dark glass bottle. Note: If the bottle is clear, wrap it with a piece of aluminum foil.
- Store the stock at 4^{o}C until needed.

10X TBE Stock

	250 mL
Tris Base (MW= 121)	27 g

Boric Acid	13.75 g
0.5 M EDTA (pH 8.0)	10 mL
DEPC'd water	200 mL

- Pour in an RNase-free 400-mL beaker with an RNase-free stir bar 200 mL of DEPC'd water.
- Weigh out Tris Base and pour it in the beaker.
- Stir the mixture at a moderate speed.
- Weigh out Boric acid and pour it in the beaker.
- Pipet 10 mL of RNase-free 0.5 M EDTA buffer into the mixture.
- Stir the mixture at a moderate speed until all granules dissolved.
- Transfer the mixture in a 250-mL RNase-free glass graduated cylinder.
- Bring the final volume to 250 mL with DEPC'd water.
- Filter the mixture through a 0.2 micron filtering unit.
- Store the bottle of 10X TBE stock at room temperature.

10% Ammonium Per Sulfate (APS)

	1 mL
Ammonium persulfate	0.1g
DEPC'd water	0.99 mL

- Label a 1.5 mL RNase-free microcentrifuge tube as "10% APS" and "Date".
- Weigh out 0.1 g of ammonium persulfate (APS).
- Transfer APS granules into the microfuge tube.
- Pipet 0.99 mL of DEPC'd water into the tube.
- Cap the tube and mix the contents by vortexing.
- Spin the tube briefly.
- Store the tube of 10% APS at 4^{o}C until needed. *Note: The stock is good for one week at 4^{o}C*.

2X RNA Loading Bufer (Fermentas.com)

	10 mL	**Stock Conc.**
95% formamide (Ultra pure, Invitrogen)	9.5 mL	100%
0.025% SDS	25 μL	10%
0.025% Bromophenol blue (BPB)	2.5 mg	
0.025% Xylene cyanol (XC)*	2.5 mg	
0.5 mM EDTA (pH 8.0)	100 μL	0.5 M
DEPC'd water	375 μL	

- Prepare in a 14-mL RNase-free centrifuge tube.
- Mix the contents well.
- Aliquot into 1 mL solution.
- Store the aliquots at -20oC until use.

Note: *Xylene cyanol masks bands at 40 bases on a 15% acrylamide gel. So, it can be left out of the loading buffer.

PROCEDURE

1. Assemble the gel cast for the PROTEAN gel system (Bio-Rad) as following:
   - Microwave **50 mL** of a **1% agarose solution in DEPC'd water** for sealing the gel plates. Cool it in the 55-60^{o}C water bath until needed.
   - Wipe the glass plates (8 x 10 cm), gel comb and spacers for a 1.5-mm thick gel with freshly prepared 0.05% DEPC-treated water.
   - Rinse the PROTEAN gel system (Bio-Rad) with freshly prepared 0.05% DEPC-treated water. Wipe off the excess water with kimwipes.
   - Assemble the gel plates with gel spacers. Seal the bottom and sides of the gel cast by pipetting the 1% agarose solution using a P-1000 PCR pipet tip.
   - Allow the agarose solution solidified for a few minutes.
2. Prepare **20 mL** of a **15% polyacrylamide/urea gel** in a 125-mL Erlenmeyer flask as following:
   - Weigh out **9.6 g** of **ultra pure Urea** (Invitrogen) and put it into the flask
   - Pipet **7.5 mL** of **40% acrylamide:bis-acrylamide solution**
   - Pipet **2.0 mL** of **10X TBE buffer**
   - Pipet **3.3 mL** of **DEPC'd water**
   - Swirl the flask with the mixture in a 37^{o}C water bath for a few minutes until urea granules completely dissolved
   - Filter the polyacrylamide/urea mixture through a nitrocellulose filtering disc (0.45 μm) using a 10 cc syringe into another 125-mL Erlenmyer flask.
   - Pipet **120 μL** of **10% APS** (Ammonium PerSulfate). Swirl the flask gently to mix the contents.
   - Pipet **9.2 μL** of **TEMED** (Bio-Rad). Swirl the flask gently to mix the contents.
   - Immediately, pour the polyacrylamide/urea gel mixture to the assembled gel plate.
   - Insert the **1.5-mm gel comb**.
   - Allow the polyacrylamide to set for 30 minutes.

3. Prepare 600 mL of 1X TBE solution with 60 mL of 10X TBE buffer and 540 mL of DEPC'd water.
4. Pour about 200 mL of 1X TBE solution to the reservoir of the gel system.
5. Once the polyacrylamide set, carefully remove the gel comb.
6. Assemble the gel cast system to the electrode part of the Protean gel system.
7. Set the whole set into the reservoir. Make sure that the bottom of the gel plate is completely submerged in the 1X TBE solution.
8. Pour ~100 mL of 1X TBE solution to the electrode/gel cast part.
9. Rinse all wells with 1X TBE solution using a PCR P-1000 tip.
10. Put the lid with electrode cords on. Note: make sure the RED dot on the electrode assembly part connecting to the RED electrode cord of the lid.
11. Pre-run the gel at 200 volts for 15-30 minutes.
12. Meanwhile, prepare tubes of 10-bp DNA ladder (Invitrogen) and small RNA samples as following:
    - 2 tubes of 10-bp DNA ladder: 3 μL (1 μg/μL) + 3 μL of 2X RNA loading buffer with Bromophenol Blue (BPB)
    - Small RNA sample: 15 μL (1.67 μg/μL) + 15 μL of 2X RNA loading buffer
    - Incubate the tubes at 70°C for 5 minutes. Quench on ice for at least 2 minutes. Spin tubes briefly.
13. After at least 15 minutes of gel pre-run, turn off the power supply and remove the lid of the electrophoresis system.
14. Rinse all wells as previously done in step 9.
15. Pipet 6 μL of denatured 10-bp DNA ladder solution to well #1.
16. Pipet 30 μL of denatured small RNA solution to well #2.
17. Repeat step 16 for other small RNA sample(s) in adjacent well(s).
18. Pipet 6 μL of denatured 10-bp DNA ladder solution to the last well.
19. Put the lid on the electrophoresis system.
20. Run the gel at 200 volts for 50-55 minutes. Note: Do not run the gel at this voltage for more than 55 minutes because the BPB dye and the 10-base band will come off the gel.

21. After 50-55 minutes of gel electrophoresis, turn off the power supply. Disassemble the gel cast.
22. Prepare 100 mL of 1 μg/mL ethidium bromide (EtBr) in 1X TBE solution in an RNase-free plastic container.
23. Stain the polyacrylamide gel in the EtBr solution in dark for 5 minutes.
24. Rinse the gel with ~100 mL of 1X TBE solution for 2 minutes.
25. Take pictures of the gel using the gel doc system (Bio-Rad) as following:
    - Clean the surface of the UV box by wiping with double-distilled water.
    - Put a new piece of plastic wrap on the UV box. Make sure no bubbles are present.
    - Carefully, place the destained gel on the UV box.
    - Wipe the fluorescent ruler with freshly prepared DEPC-treated water.
    - Place the ruler next to the gel. Note: The fluorescence of the ruler is lots stronger than the fluorescence from the EtBr-stained RNA bands. We need to to take pictures of the gel with and without the ruler.
    - Take a picture of the gel with the ruler with short exposure such that the ruler's lines are not overexposed. Save the file. Print the picture.
    - Remove the ruler, take a picture of the gel with longer exposure such that all small RNA bands are clearly observed. Save the file. Print the picture.
    - Cut the ruler portion from the gel picture and paste it to the gel picture without the ruler.
    - Mark the positions of the desired RNA bands (miRNA and siRNA corresponding to the 20-b and 30-b DNA ladder) on the composite gel picture. Notice the marks on the ruler. So, the gel pieces corresponding to the marks on the ruler can be excised because the RNA bands are not quite observable to our eyes.
26. Excise the desired small RNA bands as following:
    - (Option) use another UV box to observe the bands before cutting. Note: the bands are not quite observable under the UV light due to their amount.
    - Label RNase-free tubes according to small RNA bands.

- Transfer the plastic wrap with the gel and the ruler to the bench.
- Excise each of the desired bands according to its position corresponding to the marks on the ruler.
- Put the excised gel pieces to appropriate labeled tubes.
- Take a picture of the excised gel on the plastic wrap as a record of correct small RNA bands excised. Save the file. Print the picture.

27. Elute small RNA molecules from excised gel pieces in 0.3 M NaCl (RNase-free) at room temperature for at least 4 hours as following:
    - Crush the gel piece in the tube to powder with a blue RNase-free plastic pestle. Note: It takes about one minute or so to crush the gel piece into powder. Use an RNase-free P-20 pipet tip to brush powder off the pestle into the tube.
    - Repeat crushing for other gel pieces.
    - Spin the tubes for 5 seconds to bring down all powder to the bottom of the tubes.
    - Estimate the volume (in μL) of the powder using the graduated marks on the tube. Record the volume in the table below.
    - Determine 2 volumes of 0.3M NaCl for each gel piece. Record the volume in the table below.

Volume of gel powder				
Volume of 0.3M NaCl				

    - Flick each tube to loosen the powder in the NaCl solution.
    - Spin the tubes for 2 seconds to bring down all powder from the wall to the bottom of the tubes.
    - Put all tubes on a rotator (Dynal).
    - Adjust the rotation speed to ~50 rpm.

- Allow the elution to occur for at least 4 hours at room temperature. Note: For convenience and best recovery, elute RNA molecules from the gel pieces for overnight.
- Store the tubes at 4°C until purification step. Note: if the RNA tubes are stored at 4°C, warm the solutions at room temperature for 5 minutes or so before preceeding with purification and precipitation steps below.

28. Spin RNA tubes briefly to bring down mixture to the bottom of the tubes.
29. Label Qiagen Shredder columns according to RNA samples.
30. Pipet the RNA solution to the labeled shredder column.
31. Spin the columns in their collection tubes at 10,000 rpm for 15 seconds.
32. Determine the volume of RNA solution (or the flow-through) using RNase-free pipet tip (P-200 or P-1000 depending on the volume). Record the volumes on the table below.

Volume of RNA				
2.5 volumes of 100% EtOH				
Volume of 15 μg/μL Glycoblue				

33. Precipitate RNA in the flow-through solution with 1 μL of 15 μg/μL Glycoblue (Ambion) and 2.5 volumes of 100% ethanol solution. Mix the contents well. Freeze the tubes of RNA mixture at -20°C for overnight or at -80°C for at least 2 hours.
34. Spin the tubes at 4°C for 30 minutes.
35. Carefully, pipet off the supernatant and save the supernatant in a new set of tubes
36. Wash each pellet by pipetting 1 mL of 80% ethanol solution at room temperature to each tube. Spin the tubes at room temperature for 5 minutes.
37. Carefully pipet off the ethanol solution.
38. Spin the tubes again for 30 seconds.

39. Pipet off the residual ethanol solution using a P-20 PCR tip.
40. Air-dry the pellets at room temperature for 5-10 minutes.
41. Dissolve each pellet in 10 μL of DEPC'd water. Keep tubes of RNA solutions on ice.
42. Determine RNA concentration using Ribogreen. *<u>Note:</u> use 1 μL of RNA solution*.
43. Calculate the total amount of RNA recovered from gel piece.
44. Store RNA solutions in the -80^{o}C freezer until needed.