Preparation of small-RNA libraries for Solexa sequencing

Modified from Solexa sequencing protocol by Shujun Luo
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FLOWCHART:

1. 5'-end label 18-mer and 30-mer RNA markers separately with ^{32}P γ-ATP. Gel purify: electrophorese on denaturing gel; visualize by phosphorimaging; cut out gel slices containing markers; elute, precipitate and resuspend. Combine markers such that activity of each is about equal.	Extract total RNA from sample.

2. Combine total RNA with radiolabeled RNA markers and gel purify as above, cutting out the bands containing the labeled markers and everything in-between.

3. Ligate Adenylated 3' Adapter to the 3' ends of gel-purified small RNA pool. Gel purify.

4. Ligate 5' Adapter to the 5' ends of RNA pool. Gel purify.

5. Reverse transcribe small RNA pool with RT/PCR 5' Primer. PCR amplify resulting cDNA pool with 3 cycles using 3' PCR Primer, then 15-18 cycles with 3' and 5' PCR Primers.

6. Ethanol precipitate DNA and electrophorese on formamide gel alongside a 10 bp DNA marker. Stain with SYBR Gold. Cut and elute 85-105 nt gel piece. Ethanol precipitate and resuspend DNA for sequencing.

DETAILED PROTOCOL:

Note: when working with RNA, all reagents and equipment must be RNAse-free.

1. Kinase 5' end of Marker RNAs with ^{32}P-gamma-ATP

Kinase 18-mer and 30-mer RNA markers separately to very high specific activity. Keep the markers separate until the final step.

	Amount
10 μM RNA marker	2 μL
10X PNK buffer	2 μL
^{32}P γ-ATP (6000 ci/mmol, 150 mCi/mL)	2 μL
dH_2O	13 μL
PNK	1 μL

- Incubate 1 hour at 37°C.
- (Optional: Before gel-purifying, add 5 μL H_2O for total of 25 μL reaction volume and spin through a Micro Bio-Spin P-30 column (Bio-Rad) to remove excess, unincorporated ATP.)
- **Gel purification:**
  - add 2X urea loading buffer to each marker. Heat to 80°C for 5 min and run on a 10% denaturing polyacrylamide gel until bromophenol blue (BB) dye is ~1 inch from bottom of gel.
  - Dismantle gel apparatus and separate plates. Leave the gel on one of the glass plates. Cover the gel with clear plastic film and visualize it by exposing to phosphorimager plate. Develop image. Align a printed image of the gel under the actual gel on the glass plate. Cut out the gel pieces containing marker bands and put into 1.5 mL Eppendorf tubes. (Note: to assist in aligning gel to picture, use a pipette tip with a small amount of hot dye to prick gel at several spots. Expose gel to plate and develop picture, then align the dots of dye in the gel to the dots of signal on the picture.)
  - Elute RNA: add 400 μL of 0.3 M NaCl to the gel slices and rotate the tubes at 4°C overnight.
  - Precipitate RNA: remove the supernatant and add 2.5 volumes of 100% ethanol and 1 uL GlycoBlue (Ambion) to it; mix well. Incubate at -20°C for 2 hours.
  - Spin samples at max speed (21,000 x g) for 30 min at 4°C in a microcentrifuge. Carefully remove all supernatant and resuspend each pellet in 10-30 μL dH_2O.
- To combine markers: measure the activity of each marker separately and combine the two markers so that CPM of each are approximately equal.

2. Purify small RNA from total RNA

- Add purified marker to 10 μg total RNA. Adding approximately 50,000 total counts of combined marker is sufficient to observe marker signal throughout all the steps of the protocol, but you may vary this amount as necessary.

- Gel purify the RNA running between these markers by cutting bands from the gel that just include the labeled markers and everything in-between. Elute and precipitate as before, but increase the elution volume to 800 of 0.3 M NaCl to improve elution efficiency from the large gel slice and then split for precipitation.

3. Adenylated 3' Adaptor ligation and purification

Resuspend RNA pellet following size selection in H_2O:

	Volume
Purified 18-30nt RNA	5 µL
100 µM Adenylated 3' Adaptor	1 µL
10X Ligation Buffer	1 µL
dH_2O	2 µL
T4 RNA Ligase 1 (NEB)	1 µL
Total reaction volume (µL)	10 µL

- Incubate at 22ºC for 2.5 hours. Stop reactions by adding 2X urea loading buffer.
- Gel-purify ligated product (39–51 nt) on 10% gel as above. Elute and precipitate as before, but increase the elution volume to 800 of 0.3 M NaCl to improve elution efficiency from the large gel slice and then split for precipitation.

4. 5' Adaptor ligation and purification

Resuspend RNA pellet following 3' ligation in H_2O

	Amount
Purified 3' Ligation product	4.8 µL
100 µM 5' Adaptor	2.6 µL
10X Ligation Buffer	1 µL
T4 RNA Ligase 1 (NEB)	1 µL
4 mM ATP	0.6 µL
Total reaction volume (µL)	10 µL

- Incubate at 22ºC for 18 hours. Stop reaction by adding 2X urea loading buffer.
- Gel-purify ligated product (65–77 nt) on 10% gel. Elute and precipitate as before, but increase the elution volume to 800 of 0.3 M NaCl to improve elution efficiency from the large gel slice and then split for precipitation.

5. RT-PCR and Splicing by Overlap Extension by PCR (SOE-PCR) of small RNAs with adaptors

Resuspend RNA pellet following 5' ligation in H_2O:

	Amount
Purified 5' ligation product	5 µL
100 uM RT-Primer (5' PCR primer)	1 µL
dH_2O	9.6 µL
Total reaction volume (µL)	15.6 µL

- Heat to 65°C for 10 min, snap cool and then spin down.
- Add following in order:

Reagent	Amount
5X first strand buffer (Invitrogen)	6.4 µL
10X dNTPs (2 mM)	7 µL
DTT (0.1 M)	3 µL

- Heat to 48°C for 3 min, snap cool then spin down.
- Remove 3 µL for a RT-minus control.
- Add 1 µL of Superscript II RT (Invitrogen) (200 U/µL) to RT-plus reaction and incubate at 44°C for 1 hour.
- Add 5 or 0.5 µL of 1 M NaOH to the RT-plus and RT-minus reactions, respectively, and incubate for 10 min at 90ºC.
- Neutralize the base hydrolysis reaction by adding 25 or 2.5 µL of 1 M HEPES pH 7.0 to the RT-plus and RT-minus reactions, respectively, bring the RT-minus reaction up to 60 µL with $H_2O$and spin both through Micro Bio-Spin P-30 column.
- Set up SOE-PCR. (Note: You can use less RT reaction and increase PCR cycles.):

	RT sample	RT-minus sample
RT Reaction	4 µL	0
RT-minus control	0	4 µL
5X PCR Buffer	10 µL	10 µL
2 mM dNTP (10x)	6.3 µL	6.3 µL
150 nM 3' PCR primer	1 µL	1 µL
Phusion (NEB)	0.5 µL	0.5 µL
dH_2O	27.2 µL	27.2 uL

- Perform 3 cycles of PCR (to let small RNAs extend before amplification):

98°C	30 sec	
94°C	30 sec	3 Cycles
60°C	30 sec	
72°C	15 sec	
72°C	10 min	

- To each sample add:

0.5 µL	25 µM 5' PCR primer
0.5 µL	25 µM 3' PCR primer

- Perform 10-18 cycles of PCR (perform the number of cycles necessary to see an amplified product on the following gel):

98°C	30 sec	
94°C	30 sec	10-18 Cycles of PCR
60°C	30 sec	
72°C	15 sec	
72°C	10 min	

- Ethanol precipitate & resuspend in 15 µL 1X formamide loading buffer.
- Resuspend 0.2 µL 10bp DNA marker (1.0 µg/µL) in 13 µL 1x formamide buffer.

- Heat samples and marker for 10 min at 85°C and gel-purify on 90% formamide, 8% acrylamide gel.
- Stain with SYBR Gold (Invitrogen) (1 μL/50 mL 1X TBE). Cut and elute smear running at ~85-105nt gel piece. (RT-minus sample will run at ~40-50bp.)
- Ethanol precipitate as above, but do not add glycogen during final purification. Wash 1 X with 70% ethanol, then air dry pellet for 5 minutes.
- Resuspend in 20 μL of 10 mM Tris and submit sample for sequencing.

SOLUTIONS AND REAGENTS:

Oligos (PAGE purified)

18mer marker RNA
18.113R: AGCGUGUAGGGAUCCAAA

30mer marker RNA
30.139: GGCAUUAACGCGGCCGCUCUACAAUAGUGA

3' Adaptor (before adenylation)
21.340x: pTCGTATGCCGTCTTCTGCTTGddC

Adenylated 3' adapter
21.340x: AppTCGTATGCCGTCTTCTGCTTGddC

5' Adaptor
26.71: GUUCAGAGUUCUACAGUCCGACGAUC

RT-primer and 5'PCR primer
18.206: CAAGCAGAAGACGGCATA

3'PCR primer
44.45: AATGATACGGCGACCACCGACAGGTTCAGAGTTCTACAGTCCGA

Final Construct

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CAAGCAGAAG ACGGCATACG ANNNNNNNNN NNNNNNNNNNNNNGAT CGTCGGACTG TAGAACTCTG AACCTGTCGG TGGTCGCCGT ATCATT
GTTCGTCTTC TGCCGTATGC TNNNNNNNNN NNNNNNNNNNNNNCTA GCAGCCTGAC ATCTTGAGAC TTGGACAGCC ACCAGCGGCA TAGTAA
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Denaturing Acrylamide gel

Adapted from PAGE Northerns for MicroRNAs Protocol, Updated April, 2007, on the Bartel Lab website.

For 1 gel: 19.5cm x 16cm x 0.8mm

UreaGel-SequaGel-System (National Diagnostics):

	10%
Concentrate	12 mL
Diluent	15 mL
Buffer	3 mL

Add:

100 µl 10% fresh APS
30 µl TEMED
Mix and pour immediately.

Notes: Clean glass plates thoroughly. (Optional, silanize plates to prevent gel from sticking.) Mix gel solution and pour immediately. Let polymerize for at least 30 min. Be careful when pulling out combs, want very flat wells. Assemble gel onto the gel rig. (Optional, assemble with an aluminum plate backing. The aluminum insures even heat distribution during run and gives you more uniform mobility among the lanes.) After samples are loaded, run gel at 5W to allow small RNAs to enter gel matrix. After about 20 min, raise to 12W.

2X Urea Loading Buffer

Adapted from Schultes, Dec. 1999.
Notes: Work entirely RNase-free: do not use glass containers; use only weigh boats from middle of stack and only plastic containers from 0.22 µm filter apparatus and plastic sterile bottles.

A. Make 1 L of stop solution:
1. Prepare 500 mL of 500 mM EDTA stock at pH 8.0 (see Maniatis 3 B.11).
    a. To 93.1 g EDTA, add 10 g NaOH pellets; put into 500 mL sterile bottle.
    b. Add RNase-free H_2O to 500 mL.
    c. Mix on orbital shaker until dissolved (overnight).
    d. Filter.

2. Dissolve urea:
    a. Add 480 g urea to 1000 mL filter bottle.
    b. Add RNase-free H_2O to 900 mL.
    c. Mix on orbital shaker until dissolved (overnight).

3. Mix solutions:
    a. Add 50 mL of 500 mM EDTA stock solution to dissolved urea.
    b. Top-off to 1000 mL with RNase-free H_2O.
    c. Filter.

B. Aliquot 50 mL of stop solution; to each 50 mL aliquot add:
    0.0125 g Xylene cyanol (XC)
    0.0125 g Bromophenol blue (BB)

Final mix:
8 M urea, 25 mM EDTA, 0.025% (w/v) each XC, BB.

10x RNA ligation buffer

500 mM Tris-HCl (pH 7.8), 100 mM $MgCl_2$, 100 mM DTT.
Store at -20°C.
Thaw and mix completely before use to get entirely into solution.

90% Formamide, 8% Acrylamide gel (1.5 mm thick)

Wendy Johnston, Sept. 2007

- Use 7.5 in. x 7.5 in. plates and 20-well comb. (Each lane is 0.5 cm wide.)
- In beaker, combine 10 mL 10X TBE, 400 mg BIS acrylamide, 90 mL formamide, and 7.6 g acrylamide. Stir with magnet on stir plate until solution is dissolved.
- Sterile suction filter to remove any particles and oxygen.
- Store in light blocking bottle at 4°C.
- To 50 ml (enough for 2 gels), add 500 µL APS, 150 µL TEMED. Mix vigorously.
- Polymerization is very slow; best to let it polymerize O/N at RT.

2X Formamide loading dye

95% formamide, 18 mM EDTA, 0.025% (w/v) XC, 0.025% (w/v) BB, 0.025% (w/v) SDS.

Adenylated 3' adapter

***Warning*: read carefully all MSDS before working with organic solvents.**

1. Synthesizing ImpA

- Rinse 2 beakers in acetonitrile and air dry.
- Make two mixtures:
  Mixture A:
  174 mg AMP (FW347.2) (0.5 mmol)
  15 mL Dimethylformamide
  Mixture B:
  262 mg Triphenylphosphine (FW 262.3) (1 mmol)
  220 mg 2,2'-dipyridyldisulfide (FW 220.3) (1 mmol)
  170 mg Imidazole (FW 68.08) (2.5 mmol)
  0.90 mL Triethylamine (FW 101.2, d=0.726)
  15 mL Dimethylformamide
- Add Mixture A slowly into Mixture B while stirring until precipitates dissolve.
- Stir for 1-1.5 hr at RT with cover over beaker.
- Make Precipitation Mixture:
  1.1 g $NaClO_4$ (FW 122.4) (9 mmol)
  225 mL Acetone
  115 mL Anhydrous ethyl ether
- Add Mixture A+B dropwise to Precipitation Mixture.
- Remove solvent phase down to ~60 mL.
- Transfer precipitates to 50 mL conical bottom Corex or Teflon centrifuge tubes, rinse with acetone, centrifuge at 5000 rpm (3000g in ss34 rotor) for 10 min and pour off acetone. Repeat rinse 3 times.
- Do a final rinse with just ether, and spin down for 20 min.
- Dry overnight in a vacuum vessel between 22.5-45°C.
- Store at -20°C.

2. Adenylation of 3' adaptor

- Set up reaction:

Reagents	Stock conc.	Amount	Final conc.
ImpA (FW 423)		9 mg in 420 μL dH_2O	50 mM
$MgCl_2$	2 M	7 μL	25 mM
3' adaptor	1.3 mM	80 μL	0.2 mM

- Incubate at 50°C for 3 hrs.
- Gel purify on 20% gel.