Small RNA library prep protocol

Note: This protocol was used in Fang and Bartel, *MolCell*, 2015.

The main changes from the 2013 protocol are:

1) The use of degenerate adapters in both ligations (4 N's in the 3' adapter and 8 N's in the 5' adapter). These degenerate nucleotides at the ligation ends of the adapters help reduce ligation biases of the T4 RNA ligases, allowing for a more complete and quantitative profiling of the small RNA pool.
2) The use of PEG in both ligations to improve ligation efficiency
3) The use of RNL2 truncated KQ (NEB) in the 3' ligation to reduce unintended ligations during the 3' ligation
4) RT using SuperScript III
5) PCR using KAPA

A. Make hot markers (18 and 30nt or 32nt) with 32P-gamma-ATP

1. Kinase 5' end of marker RNAs:

10 µM 18nt RNA marker	0.5 µl (5 pmol)
10 µM 30nt RNA marker	0.5 µl (5 pmol)
10x PNK buffer	1 µl
32P-gamma-ATP	1 µl
Nuclease-free H2O	6.5 µl
PNK	0.5 µl
Total volume	*10 µl*

2. Incubate for 1 hr at 37C
3. Bring up to 60 µl with nuclease-free H2O and remove excess unincorporated ATP with Micro Bio-Spin P-30 column (Bio-Rad) If need to limit the amount of marker, use fresh ATP and purify away hot marker on 15% urea gel (+phosphate should run ½ nt faster than untreated. See previous protocols).
4. Geiger for total counts. Use ~10K counts per sample.

A'. OPTIONAL: Prepare spike-in miRNA standards

Adding small RNA oligonucleotides to your total RNA samples can help when trying to make quantitative comparisons between samples. Non-conserved small RNAs from other species can be used (e.g. using dme miR-14 and xtr miR-427 to spike into a human or mouse sample), or a designed sequence of the appropriate length.

From Sean: dme miR-14 and xtr miR-427 at 20 µM

1. Dilute spike in RNAs to 0.1 µM, then dilute a further 1:100 in yeast total RNA (200 ng/µl) to avoid losing RNA to the tube. Combine 1 nM standards 1:1 to generate standard mix, final concentration of each spike-in should

be ~0.5 fmol/µl (aka 0.5 nM). For 5 µg total RNA, add 1µl of each spike-in to have spike-in at ~1/100th of total miRNA pool.

B. Size select for small RNAs

1. Pour 0.75 mm, 20 cm long, 15% urea polyacrylamide gel.
2. Mix ~5 to 10 µg total RNA with 10–20K counts of marker and bring up to 5 µl with water. Add 5 µl 2x Ambion formamide loading buffer (store aliquots at -20C), denature at ~80C for 5 min then load on gel in every other lane (i.e., max 6 samples per gel using 14 well comb).
3. Run at 5 W for 15 min, 12 W for 40 min until bromophenol blue is ~2/3rd of the way down the gel. The goal is to separate small RNAs from other RNAs but also not to have too big a gel slice.
4. Size select by cutting from 18–30 nt, inclusive of the markers.

Gel purification:

5. Macerate the excised gel slice as follows: place gel slice inside 0.5 mL microcentrifuge tube that contains a hole at the bottom created by heating 22-gauge needle with a flame and then poking the hot needle through the bottom of the tube (can also just push a needle through without heating though it is somewhat harder). Place the 0.5 mL tube inside a 1.5 mL microcentrifuge tube and spin the slice through the hole into the 1.5 mL tube at 10,000 g for 5 min.
6. Add 420 µl 0.3 M NaCl. Vortex and elute the RNA in shaking heat block at 50C for 1 h or 70C for 10–15 min or overnight at 4C
7. Cut the tip off a P1000 pipet tip to create a larger opening, transfer the gel-eluate slurry to a spin-x filter basket placed inside a G tube (don't use tube that Spin-X filter comes with). Spin the gel-eluate slurry through a Spin-X filter into the G tube at 16,000 g for 2 min.
8. Add 2–4 µl linear acrylamide, mix, and then add 1 mL 100% ethanol, mix well, and precipitate at -20C for >2.5h or at -80C for >45 min.
9. Spin the ethanol precipitation at max speed for 30 min at 4C.
10. Remove the supernatant and air-dry at room temp for ~5 min.
11. Resuspend the pellet in 4.5 µl nuclease-free H2O (or 10 µl if saving ½ of sample).

C. 3' adapter ligation and gel purification

1. Ligate pre-adenylated 3' adapter (Stephen's tail-seq 3' adapter, 4Ns) to small RNAs:

Resuspended RNA	4.5 µl
100 µM pre-adenylated 3'adapter	1 µl
10X T4 RNA ligase buffer (no ATP)	1 µl
PEG 50% (warm to 37C to pipet)	2 µl
T4 RNA ligase 2 KQ mutant (NEB)	1 µl
Superasin	0.5 µl (optional)
Total volume	*10 µl*

2. Incubate at 22C overnight (2.5 hrs is also fine).
3. Pour 0.75 mm, 20 cm long, 10% urea polyacrylamide gel.

4. Stop reactions by adding 10 µl 2x Ambion formamide loading buffer. Denature at ~80C for 5-10 min.
5. Run gel at 5W for 15 min, 12W for ~40 min.
6. Gel purify as above and resuspend final pellet in 3.5 µl nuclease-free H2O. Markers should now be 44 and 56/61 nt (+26). Expect a ligation efficiency of > 70%. An experienced person with this protocol routinely gets > 90% ligation efficiency at this step.

D. 5' adapter ligation and gel purification

1. Ligate 5' adapter (Solexa stock, 8 Ns) to small RNAs:

Resuspended RNA	3.5 µl
100 µM 5' adapter (Solexa stock)	1.76 µl
10X T4 RNA ligase buffer, no ATP	1 µl
PEG 50% (warm to 37C to pipet)	2 µl
T4 RNA ligase I (NEB)	1 µl
10 mM ATP	0.24 µl
Superasin	0.5 µl (optional)
Total volume	*10 µl*

2. Incubate at 22C overnight (~18 hrs).
3. Pour 0.75 mm, 20 cm long, 8% urea polyacrylamide gel.
4. Stop reactions by adding 10 µl 2x Ambion formamide loading buffer. Denature at ~80C for 5-10 min.
5. Run gel at 5W for 15 min, 12W for ~35 min.
6. Gel purify as above, markers should be 78 and 90/96 nt (+34). Expect a ligation efficiency of > 60%. An experienced person with this protocol routinely gets > 90% ligation efficiency at this step.
7. When ready to proceed, spin down and resuspend final pellet in 5 ul nuclease-free H2O. If desired, some fraction of the sample can be saved at this step and stored at –20 C.

E. Reverse transcribe small RNA pool ligated with both 5' and 3' adapter then hydrolyze RNA:

1. Hybridize RT primer:

Resuspended RNA	5 µl
100 µM P7 Universal RT primer	1 µl
dNTPs (10 mM)	1.6 µl
H2O	5.4 µl
Total volume	*13* µl

2. Incubate at 65C for 5 min then snap cool on ice for > 1 min and spin down.
3. Add RT master mix:

5x FS buffer	6.4 µl
0.1 M DTT	1.6 µl
Superasin	1.6 µl
H2O	9.4 µl
Total volume	*19 µl*

4. Remove 3 µl from each sample for no RT control. Then add 1 µl SSIII to each sample. Incubate RT+ and RT– at 50C for 1 hr.
5. Hydrolyze RNA by adding 5 or 0.5 µl 1 M NaOH to RT+ and RT– reactions, respectively, and incubate for 10 min at 90C. Place on ice for 1 min to cool.
6. Neutralize base hydrolysis by adding 25 or 2.5 µl 1 M HEPES pH 7.0 to RT+ and RT– reactions, respectively.
7. Bring the RT– reaction up to 60 µl with nuclease-free H2O and desalt both over Micro Bio-Spin P-30 column.

F. PCR and gel purification

1. Set up test PCR using 2 µl of RT product as follows:

2x Kapa HF buffer	25 µl
small RNA P5 primer (5 µM)	3 µl
H2O	17 µl
Total volume	*45* µl
RT product	2 µl
P7 Index primer (5 µM)	3 µl

2. Amplify cDNA with the following program, removing 10 ul every 3 cycles starting at cycle 9:

95C for 3 min
98C for 20 s
60C for 15 s } x 21 cycles
72C for 15 s
72C for 5 min
Hold at 10C

3. Run on 1.8–2.0% agarose gel to verify product (~145–165 bp) and determine cycle #. For 5 µg total RNA starting material, I have generally needed 8–10 cycles of PCR.
4. Set up actual PCR using 19 µl of RT product in 50µl total volume, perform the PCR with 3 fewer cycles than were necessary to observe the amplified product of the test PCR. After PCR, transfer to eppendorf tubes and EtOH precipitate (pellet large enough that don't need to add linear acrylamide).
5. Purify via 90% formamide 8% acrylamide gel. To make gel, mix:

Formamide	90 mL
10 mL 10X TBE	10 mL
Bis	0.4 g
Acrylamide	7.6 g

then polymerize with 4x recommended TEMED and APS. Heat samples and 0.3 µg 10bp DNA ladder in 2x Formamide loading buffer at 85C for 10 min. Run in 1X TBE at 20W for 45-60 min.

6. Stain with Sybr Gold diluted 1:5000 in 1X TBE. Cut from 145bp–165bp and elute at 4C (or room temp) overnight in 0.3 M NaCl. Then EtOH precipitate. Resuspend in 30 µl 10 mM Tris and submit 10-15 µl to WIGTC for sequencing.

Note: Wenwen used AMPure beads to purify the PCR product following manufacturer's instructions. To 50 µl PCR reaction add 90 µl beads.

G. Oligos

3' adapter (26nt) (aliquots in my RNA oligo2 box):
Ordering: /5Phos/NNNNAGATCGGAAGAGCACACGTCT/3ddC/
Sequence: AppNNNNAGATCGGAAGAGCACACGTCTddC

5' adapter (34nt, Truseq small RNA 5’ adapter RA5 + 8Ns, in Solexa Stocks Box):
GUUCAGAGUUCUACAGUCCGACGAUCNNNNNNNN

P7 Universal RT primer (#546):
AGACGTGTGCTCTTCCGATCT

Small RNA P5 primer (Truseq small RNA primer RP1 aka #547):
AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGA

P7.X:
CAAGCAGAAGACGGCATACGAGAT**BBBBBB**GTGACTGGAGTTCAGACGTGTGCTCTTCCG

Highlighted is flow cell binding, underlined is P7 sequencing primer binding, bold is barcode, magenta is complementarity to 5’ adapter; last 4 nt of P7 are not present in my P7 primers but are in 3’ adapter

H. Products (where X are small RNAs)

Pre-RT:

5’GUUCAGAGUUCUACAGUCCGACGAUCNNNNNNNN**XXXXXX**NNNNAGATCGGAAGAGCACACGTCT 3’

Post-RT:

5’AGACGTGTGCTCTTCCGATCTNNNN**XXXXXX**NNNNNNNNGATCGTCGGACTGTAGAACTCTGAAC 3’

Final construct (PCR with Small RNA P5 primer and indexed P7 primer):

5’AATGATACGGCGACCACCGAGATCTACACGTTCAGAGTTCTACAGTCCGACGATCNNNNNNNN**XXXXXX**NNNNAGATCGGAAGAGCACACGTCTGAACTCCAGTCAC**BBBBBB**ATCTCGTATGCCGTCTTCTGCTTG 3’

Final construct with miRNA insert is ~151-155 nt (mostly ~20–24nt miRNAs)