Protocol for cloning bacterial sRNAs for 454 sequencing[1,2,3]

Jane M. Liu, Ph.D., Camilli Lab, HHMI, Tufts University School of Medicine

Please note that the following protocol (and the volumes used) is approximately what I used when preparing the samples submitted to 454 Dec 2007. Thus, these are only suggested volumes and methods. In addition, the tRNA depletion step is in the process of being further optimized.

1. **Total RNA preparation and small RNA enrichment**
   a. Isolate total RNA from bacteria using hot-phenol, precipitation with isopropanol, and an ethanol wash. [See separate protocol: "Total RNA Prep (for 454)"]
   b. Run out 1-2 μg total RNA on agarose gel to ensure high quality RNA
   c. Run out 500 μg total RNA on TBE-Urea PAGE gel
      i. Use Century and/or Decade Markers (Ambion), and control oligos (e.g. 18-, 37-, 50-mers) as "markers" in separate lanes.
      ii. 200V, 30 minutes (approximate; depends on fraction you want to isolate)
      iii. Stain gel for 30 minutes with SYBR Gold (Invitrogen)
      iv. Visualize gel and cut out gel slices containing RNA of interest (e.g. 10-200 nt)
          Note: I would cut out 10-60 nt RNA from one 15% gel and 60-200 nt RNA from another gel run on a 10% gel
      v. Crush up gel slices and elute RNA
          1. Option 1: overnight elution in 4-6 fold volume 0.4M NaCL. Next day, spin gel slices in microcolumn (Nanosep 0.2 μm), EtOH ppt with 2.5 vol 100% EtOH and and 20 μg glycogen. Resuspend in ~20 μL DEPC-H_2O
          2. Option 2: IDT suggests using DTR cartridges from Edge Biosystems, which I have started to use (does not require overnight step).

          I have not yet directly compared Options 1 and 2

2. **First 3' Cloning linker ligation**
   a. Small RNAs were ligated with the pre-activated 3' cloning linker (Linker 1, IDT, 5'-rAppCTGTAGGCACCATCAAT/3ddC/-3'), using T4 RNA Ligase.
      i. Use 5x T4 Ligase Buffer (no ATP)
          250 mM Hepes pH 8.3
          50 mM MgCl2
          16.5 mM DTT
          50 μg/mL BSA
          41.5% glycerol
      ii. Use 1U/μL T4 Ligase (Promega, FPLC pure, diluted in 1x Ligase Buffer)
      iii. Set up 10 μL reactions, 2 hours, RT
          I always save half of the RNA from the previous step at –80°, just in case....
   b. Linkered RNAs were recovered through PAGE purification as above.
   c. Prior to EtOH ppt, add 1000 pmol of "oligo mix" to the RNA. Ppt as above.
   d. Resuspend samples with 5 μL H2O and 5 μL 2x CRH buffer
          100 mM TRIS-HCl pH 7.8

[1] Pak, J. and Fire, A. (2007) Distinct populations of primary and secondary effectors during RNAi in C. elegans. *Science* **315**:241-244.
[2] Integrated DNA Technologies (2007) microRNA Cloning Kit Technical Manual.
[3] Lau, N., Lim, L.P., Wienstein, E.G. and Bartel, D.P. (2001) An abundant class of tiny RNAs with probable regulatory roles in Caenorhabditis elegans. *Science* **204**: 858-862.

600 mM Kcl
20 mM MgCl2
20 mM DTT

3. **tRNA Depletion**
    a. Heat samples to 65 °C, 5 min. Slow cool (0.1°/s) to 37 °C. Add 1 μL RNaseH (NEB), 37 °C, 30 min.
    b. Repeat step 3a.
    c. Depleted RNAs were recovered through PAGE purification as above

4. **Reverse transcription reaction**
    a. Using RT/REV primer, the linkered-RNA was reverse transcribed, then treated with an exonuclease to remove unused deoxynucleotides and the primer.
    b. The reaction was terminated by phenol:chloroform treatment and ethanol precipitation.

These steps were directly from the IDT miRCAT protocol

5. **Second 3' cloning linker ligation**
    a. cDNAs were ligated with the pre-activated 3' cloning linker (Linker 2, IDT, 5'-rAppCACTCGGGCACCAAGGA/3ddC/-3'), using T4 RNA ligase as in step 2 above.
    b. Linkered cDNA was recovered through PAGE purification as above

6. **PCR amplification**
    a. PAGE purified material was amplified with Taq polymerase with the following primers:
    Primer A: 5'-GCCTCCCTCGCGCCATCAGGATTGATGGTGCCTACAG-3'
    Primer B: 5'-GCCTTGCCAGCCCGCTCAGGTCCTTGGTGCCCGAGTG-3'
    b. The cDNA libraries were isolated through native PAGE purification.
    c. After recovery of the DNA by the crush-and-soak method and ethanol precipitation, the DNA was resuspended with TE buffer (pH 7.5).
    d. DNA was quantified using a Nanodrop and analyzed on a BioAnalyzer DNA 1000 LabChip. One should see a broad peak (or peaks) over the desired size range.
    e. Approximately 150 ng (in 10-14 μL) of each sample was submitted to 454 Life Sciences. Sequencing should be done with 454 Primer B to get forward reads of the original sRNA sequences.

Materials:

Decade Marker	Ambion	AM7778
Century Marker	Ambion	AM7140
Nonstick 1.5 mL eppis	Ambion	AM12450
DTR Gel Filtration Cartridges	Edge Biosystems	42453
SYBR Gold	Invitrogen	S11494
SuperScript III RT	Invitrogen	18080093 (2000 U)
RNaseH	NEB	M0297S (250 U, 5U/μL)
T4 RNA Ligase	Promega	M1051 (500 U, 10U/μL)
Nanosep 100K	VWR	29300-612 (pack of 24)
Nanosep .2 μm	VWR	29300-638 (pack of 24)

Primers:

Linker 1, IDT, 5'-rAppCTGTAGGCACCATCAAT/3ddC/-3'

Linker 2, IDT, 5'-rAppCACTCGGGCACCAAGGA/3ddC/-3'

RT/REV primer 5'-GATTGATGGTGCCTACAG

Primer A: 5'-GCCTCCCTCGCGCCATCAGGATTGATGGTGCCTACAG-3'

Primer B: 5'-GCCTTGCCAGCCCGCTCAGGTCCTTGGTGCCCGAGTG-3'

"Oligo mix": mix of ~30 29-mers that are complementary to the 3' ends of Vc tRNA, and several regions of the Vc 5S rRNA