Cloning Small RNAs for Sequencing with 454 Technology

Protocol provided by Dr. Greg Hannon, Cold Spring Harbor Laboratory

1. RNA preparation

1. Total RNA is isolated from tissue or cells with TRIZOL® followed by two extractions with phenol chloroform, precipitation with isopropanol, and an ethanol wash.
2. RNA is then solubilized in water and the concentration is determined (should be at least 3 μg/μl)

2. Separation of small RNAs.

1. Prepare a 1.5 mm thick, 15% PAA urea gel with a 10-well comb. Spike radio-labelled 19mer and 24mer RNA oligos into the RNA sample, and load 50 μg of total RNA per well with 2x sample buffer in about 30-40 μl total volume. Load radiolabelled decade marker (Ambion) in flanking lanes. Run the gel until the lower dye reaches about 2/3 of gel.
2. Implant three hot gel slices and wrap the gel in Saran™ wrap. Alternatively stain the gel with SYBR Gold® (only in cases where the amount of small RNAs is sufficient to allow direct visualization). Expose the gel to a phosphoimager screen. Cut out gel slices containing the RNAs of desired size with clean scalpel. Re-expose the gel to make sure that correct sizes were excised.
3. Place the gel slices into pre-weighed siliconized Eppendorf tubes, weigh the slices and crush them with a rounded 1 ml pipette tip.
4. Add four- to six-fold volume of 0.4 M NaCl and freeze in dry ice. Thaw and shake overnight at room temperature.

3. Precipitation of small RNAs

1. Next day, spin the gel slices through a microcolumn (Nanosep® 100 filter) and collect the clean eluate.
2. Precipitate the small RNA by adding 1 μl of glycogen (20 μg) and 3 volumes of absolute ethanol. Put at -20ºC for 4h. Spin for 30 min and remove the supernatant. Re-spin for 10 seconds and completely remove all liquid. Do not wash in 70% ethanol. Air dry the pellet for 5 min and dissolve in water (13 μl per sample).

4. First ligation:

1. Set up the following 20 μl ligation reaction:
   - 13 μl RNA
   - 2 μl 10X ligase buffer without ATP
   - 3 μl DMSO
   - 1 μl 3’ adaptor Modban 100 μM
   - 1 μl ligase*

* it is best to use a mutant version of T4 RNA ligase that you prepare yourself. The reference for this protein is : Ho et al., (2004) Structure 12:327-339.

10X T4 homemade ATP-free RNA ligase buffer:
500 mM Tris-HCl pH 7.5, 100 mM $MgCl_2$, 100 mM DTT, 600µg/ml BSA

Modban : AMP-5'p-5'p/CTGTAGGCACCATCAATdi-deoxyC- 3'
IDT (Integrated DNA Technologies,)– Product name: miRNA cloning linker No. 1

2. Incubate at room temp for 1 to 2 hours.

3. Add 20 µl of 2x loading dye, heat to 65ºC for a few minutes and load 40 µl of each sample per lane (10-well comb) on a 15% PAA urea gel,1.5 mm thick. Load decade markers in flanking lanes.

4. Run the gel until the lower dye reaches about 2/3, implant hot gel slices and expose for 10 min. Excise the correct bands and elute as above, overnight.

5. Second ligation

1. Precipitate the ligation products as above and dissolve them each in 13 µl of water.

2. Set up the following 20 µl ligation reaction:
   13 µl 3' ligation product
   2 µl 10X T4 RNA ligase buffer (Ambion)
   2 µl DMSO
   1 µl 100 µM RNA 5' linker oligonucleotide,
   "Nelson's linker" 5'ATCGTrArGrGrCrArCrCrUrGrArArA 3'
   2 µl T4 RNA ligase (Ambion)

   Incubate at 37ºC for 1 hour.

3. Add 100 µl of 10 mM Tris (pH 8.0), 1 mM EDTA, 0.4 M NH_4Acetate; Phenol extract once; Chloroform extract once.
4. Add 20 µg of glycogen; add 3 volumes (300 µl) of 100% ethanol; chill to –20ºC (at least 3 hours); and centrifuge at 12,000 x *g* for 30 minutes at 4ºC. Re-centrifuge for 10 seconds and remove all liquid. Allow the pellet to (almost) dry, and dissolve in 7 µl of water.

6. Reverse transcription

1. Set up the following annealing reaction:
   6.3 µl RNA ligation product
   4.2 µl 5 µM BanOne: 5'-ATTGATGGTGCCTACAG-3'

   10.5 µl total

Incubate at 72°C for 2 min.
Spin at 20°C for 1 min.
Cool on ice for 2 min.

2. Add the following (to make 18.9 µl total):
   8.4 µl "RT Mix" Stock of RT Mix; 30 µl 5X first strand buffer
   15 µl 20 mM DTT
   15 µl dNTPs (10 mM each)
3. Split the sample into two tubes, 9 µl in each tube:

   + RT : add 1 µl (200 U) of RNase H$^-$ RT (Invitrogen Superscript™).
   - RT : add 1 µl water.

4. Incubate at 42°C for 1 hour.

7. PCR amplification of cDNA

1. Set up the following 100 µl PCR reaction:
   2 µl first-strand cDNA, or "minus RT control" (from above)
   74 µl water
   10 µl 10X PCR Buffer w/ 15 mM $MgCl_2$
   10 µl dNTP Mix (2 mM each)
   1 µl 100 µM 5' PCR primer, BanTwo: 5'-ATCGTAGGCACCTGAAA-3'
   1 µl 100 µM 3' PCR primer, BanOne: 5'-ATTGATGGTGCCTACAG-3'
   2 µl Taq polymerase (Roche)

   Mix contents by gently flicking the tube.
   Centrifuge briefly to collect the contents at the bottom of the tube.
   Cap the tube, and place it in a preheated (95°C) thermal cycler.

2. Run the following PCR program:
   Step 1: 96°C 1 minute
   Step 2: 96°C 10 sec.
   Step 3: 50°C 60 sec
   Step 4: 72°C 20 sec
   Step 5: 26 cycles to Step 2
   Step 6: 72°C 3 minutes
   Step 7: 10°C indefinitely

8. Gel purification of cDNA

1. Prepare a 4% MetaPhor® Agarose gel (Cambrex) – Follow the protocol carefully for preparation of the gel.

2. Load the cDNA on the gel, alongside 4 µl of the 10 bp DNA ladder (Promega) and the rest of the "minus RT control". Run the gel at 100 volts for 1 hour.

3. View the DNA fragments in the gel under illumination with a long wave UV source in a dark room. Mark the position of the PCR product (about 70 nt), and excise it using a clean scalpel.

4. Transfer the gel slice into a pre-weighed 1.5 ml reaction tube. If the gel slice weighs more than 250 mg, split the agarose band into two tubes. Add at least 1 volume (v/w) of 0.4 M NaCl to obtain a final volume of 500 µl. Incubate the tube for 10 min at 70°C to melt the gel slices and add 500 µl of 70°C pre-heated buffered water-saturated phenol (pH 7.8). Vortex the solutions vigorously and immediately separate the phases for 5 min at maximum speed in a tabletop centrifuge adjusted to room temperature. The agarose should accumulate at the interphase. Collect the aqueous upper phase and extract the aqueous phase once again with 65^{o}C phenol.

5. For each 400 µl of eluate, add 20 µg of glycogen and 1 ml of ethanol. Chill at –20^{o}C for at least one hour and recover the precipitate by centrifugation. Wash the pellet with 70% ethanol, and dissolve it in 30-50 µl of TE buffer. *Do not allow the pellet to dry completely*, as this can cause the DNA to denature, and since it is a heterogeneous population of sequences, complementary strands may not reanneal properly. *Do not resuspend the pellet in water but in buffer for the same reason.*

Note : Alternatively the use of Qiaex II Gel Extraction Kit (Qiagen) is possible.

9. 454 sequencing

We start from the previous gel-purified PCR product.

1. Set up the following 100 µl PCR reaction:
   - 1 µl purified PCR product
   - 74 µl water
   - 10 µl 10X PCR Buffer w/ 15 mM $MgCl_2$
   - 10 µl dNTP Mix (2 mM each)
   - 1 µl 20 µM 5' 454 PCR primer, 5’-GCCTCCCTCGCGCCATCAGATCGTAGGCACCTGAAA-3’
   - 1 µl 20 µM 3' 454 PCR primer, 5’-GCCTTGCCAGCCCGCTCAGATTGATGGTGCCTACAG-3’
   - 2 µl Taq polymerase (Roche)

   Mix contents by gently flicking the tube.
   Centrifuge briefly to collect the contents at the bottom of the tube.

2. Run the following PCR program:
   - Step 1: 96^{o}C 1 minute
   - Step 2: 96^{o}C 10 sec.
   - Step 3: 50^{o}C 60 sec
   - Step 4: 72^{o}C 20 sec
   - Step 5: 10-15 cycles to Step 2

Step 6: 72°C 3 minutes
Step 7: 10°C indefinitely

3. Gel purify using a 2% MetaPhor® Agarose gel (or a 2% regular agarose gel) and a Qiagen gel purification kit (either Qiaex II or QIAquick)

4. Check the concentration by nanodrop A260 measurement and the quality by running 1 µl of the purified PCR product on a 2% gel. The DNA quality for 454 Sequencing has to be as good as possible.

References:

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