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Small RNA Cloning

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Abstract

The next generation sequencing technologies have made the exhaustive sequencing of all small RNA species within a small RNA library possible. Thus, comparative studies on changes in small RNA transcriptome between two biological samples represent a powerful means of revealing the functions of small RNAs in physiological and pathophysiological processes. Here, we describe a small RNA cloning method that can be used to generate small RNA cDNA libraries for both conventional and next generation (deep) sequencing. It can also be used for detection and quantitative analyses of small RNAs using PCR.

Keywords

small RNAs; miRNAs; piRNAs; cloning; deep sequencing

1. Introduction

In addition to mRNA/protein-encoding genes, the genomes of almost all species encode abundant small non-coding RNAs including microRNAs (miRNAs) [1–4], Piwi-interacting RNAs (piRNAs) [5–8], repeat-associated siRNAs (RasiRNAs) [9–12], 21U endo-siRNAs [13], and small nucleolar RNAs (snoRNAs) [14]. Although functional studies on these small RNAs are just beginning, findings to date have already suggested that these small RNAs are involved in regulating a wide variety of functions including: genome stability, epigenetic programming, transcription, translation, and individual protein functions [15–18]. The state-of-the-art next generation sequencing technologies have made the exhaustive sequencing of all small RNA species within a small RNA library possible [19]. Thus, comparative studies by analyzing small RNA transcriptome between two samples would be a powerful way of

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¹The cloning method can be used for manual sequencing and 454 sequencing.

²This cloning method identifies small RNAs such as miRNAs [20–22], snoRNAs [12,20,22], tRNAs [12,20,22]) and rRNAs [12,21,22]) by manual sequencing and by 454 sequencing (unpublished data).

³Manual sequencing can be also used for checking if small RNA cDNA libraries are properly constructed, or for prescreening if particular small RNA molecules are present in your cDNA library before massive, costly, and time-consuming 454 sequencing.

⁵For 454 sequencing, either Primer A or Primer B can be used. However, Primer A is recommended because there is a poly 'A' tail (25 As) at the end of a small RNA on the Primer B side (see Fig. 1).

⁶A pair of Primer A and a small RNA specific reverse primer, or a small RNA specific forward primer and Primer B can be used for checking if sequencing templates are properly constructed by PCR.

⁷Since our cloning method uses two degenerate nucleotides (VN) at the 3' end to make small RNA cDNA library (see miRT in Table 1), the two nucleotides in sequencing may not be reliable.

⁸In cloning miRNAs, our cloning method preferentially amplifies mature miRNAs over precursor miRNAs (pre-miRNAs). pre-miRNAs may be eliminated during cDNA synthesis due to a hairpin-loop structure. To clone pre-miRNAs, RT reactions should be performed at high temperature using a high-temperature stable reverse transcriptase. In addition, the sequencing reaction should be optimized with DMSO (5%) and 1.0 M of betain at an increased denaturation and annealing temperature.

investigating the relations between small RNA expression and physiological/pathophysiological conditions. The generation of a small RNA library is an essential step for either the identification of novel small RNA species, or comparative studies using next generation sequencing technologies. Here we described a method for generating small RNA cDNA libraries. In this method (Fig. 1), small RNA fractions isolated from tissues or cells are first polyadenylated using RNA polymerase. A RNA oligo linker (Table 1) is then added to the 5' ends of the polyadenylated small RNAs followed by reverse transcription using an oligo with a linked sequence in its 5' end and polyT in its 3' end. The resultant small RNA cDNAs are then amplified with a pair of the 5' linker and the 3' linker primers (Table 1). The cDNAs can be cloned in a TA cloning vector for manual sequencing (a conventional sequencing). Alternatively, the adaptor sequences for 454 sequencing (a large-scale parallel pyrosequencing) can be incorporated at the ends of the small RNA cDNAs by a second round of PCR. The small RNA cDNA library generated using this method can be used for both manual sequencing and 454 sequencing analyses.

2. Materials

2.1. Isolation of Small RNAs

mirVana miRNA isolation kit (Ambion), Phosphate buffered saline (PBS) buffer (SIGMA-ALDRICH)

2.2. Polyadenylation

Poly(A) Polymerase (Ambion)

2.3. Purification

mirVana Probe & Marker kit (Ambion)

2.4. RNA Ligation

RNA T4 ligase (NEB), mi5-RNA (see Table 1)

2.5. Purification

mirVana Probe & Marker kit (Ambion)

2.6. Reverse Transcription

Superscript III First-Strand synthesis System for RT-PCR (Invitrogen), miRT (see Table 1)

2.7. PCR

AmpliTaq Gold PCR Master Mix (Applied Biosystems), miP1 and miP1r (see Table 1), agarose (Fisher Scientific), 100 bp DNA ladder (Promega)

2.8. Cloning

2.8.1. Gel Elution—QIAquick Gel Extraction Kit (QIAGEN)

2.8.2. TA Cloning (Optional for Manual Sequencing)—pcDNA3.1/V5-His TOPO TA Cloning Kit (Invitrogen)

2.8.3. Transformation (Optional for Manual Sequencing)—One Shot Kit (Invitrogen), pre-poured LB agar ampicillin plates (SIGMA-ALDRICH), Parafilm (Pechiney Plastic Packaging Company)

2.8.4. Colony-Directed PCR (Optional for Manual Sequencing)—TE buffer (Affymetrix/USB), GoTaq Green Master Mix (Promega), pcDNA and BGHr (see Table 1), agarose (Fisher Scientific), 100 bp DNA ladder (Promega)

2.8.5. PCR Purification (Optional for Manual Sequencing)—QIAquick PCR Purification Kit (QIAGEN), agarose (Fisher Scientific), 100 bp DNA ladder (Promega)

2.8.6. Manual Sequencing—pcDNA or BGHr (see Table 1)

2.8.7. Sequencing Template Preparation (Optional for 454 Sequencing)—Advantage GC 2 PCR Kit (ClonTech), Fusion-A, Fusion-B (see Table 1)

2.8.8. PCR Purification (Optional for 454 Sequencing)—QIAquick PCR Purification Kit (QIAGEN), agarose (Fisher Scientific), 100 bp DNA ladder (Promega)

2.8.9. 454 Sequencing—Primer A (see Table 1)

3. Methods

3.1. Isolation of Small RNAs

1. Harvest tissue (≥ 250 mg) or cells in a 1.7 ml tube with 500 μ l of cold PBS.
2. Centrifuge at $\sim 5,000$ rpm for 2 min at room temperature (RT).
3. Remove PBS as much as possible.
 - * For cells, remove PBS carefully without breaking the pellet, leave ~ 100 μ l of PBS, and resuspend cells by tapping gently.
4. Add 300–600 μ l of Lysis/Binding buffer (10 volumes per tissue mass) on ice.
 - * When you start with frozen tissue or cells, immediately add Lysis/Binding buffer (10 volumes per tissue mass) on ice.
5. Cut tissue into small pieces using scissors, and grind it using a homogenizer.
 - * For cells, skip this step.
6. Vortex for 40 sec to mix.
7. Add 1/10 volume of miRNA Homogenate Additive on ice, mix well by vortexing.
8. Leave the mixture on ice for 10 min.
 - * For tissue, mix it every 2 min.
9. Add an equal volume (330–660 μ l) of Acid-Phenol: Chloroform
 - * Be sure to withdraw from the bottom phase (the upper phase is an aqueous buffer).
10. Mix thoroughly by inverting the tubes several times.
11. Centrifuge at 10,000 rpm for 5 min at RT.
12. Recover the aqueous phase carefully without disrupting the lower phase, and transfer it to a fresh tube.
13. Measure the volume using a scale (1g = ~ 1 ml) and note it.
14. Add 1/3 volume of 100% ethanol at RT to the recovered aqueous phase.

15. Mix thoroughly by inverting the tubes several times.
16. Transfer up to 700 μ l of the mixture into a Filter Cartridge within a Collection Tube.
 - * Label the filter as 'total RNA'
 - * When you have >700 μ l of the mixture, apply it in successive application to the same filter.
17. Centrifuge at 10,000 rpm for 15 sec at RT.
18. Collect the filtrate (the flow-through).
 - * Save the Cartridge for total RNA isolation (go to #24)
19. Add 2/3 volume of 100% ethanol at RT to the flow-through.
20. Mix thoroughly by inverting the tubes several times.
21. Transfer up to 700 μ l of the mixture into a new Filter Cartridge.
 - * Label the filter as 'small RNA'
 - * When you have >700 μ l of the filtrate mixture, apply it in successive application to the same filter.
22. Centrifuge at 10,000 rpm for 15 sec at RT.
23. Discard the flow-through, and repeat until all of the filtrate mixture is through the filter. Reuse the collection tube for the following washing steps.
24. Apply 700 μ l of miRNA Wash Solution 1 (working solution mixed with ethanol) to the filter.
25. Centrifuge at 10,000 rpm for 15 sec at RT.
26. Discard the flow-through.
27. Apply 500 μ l of miRNA Wash Solution 2/3 (working solution mixed with ethanol) to the filter.
28. Centrifuge at 10,000 rpm for 15 sec at RT.
29. Discard the flow-through and repeat #27.
30. Centrifuge at 12,000 rpm for 1 min at RT.
31. Transfer the filter to a new Collection Tube
32. Apply 100 μ l of pre-heated (95 °C) Elution Solution or RNase-free water to the center of the filter, and close the cap.
 - * Aliquot a desired amount of Elution Solution into a 1.7 ml tube, heat it on a heat block at 95 °C for ~15 min. Open the cap carefully because it might be splash due to pressure build up.
33. Let the filter tube stand for 1 min at RT.
34. Centrifuge at 12,000 rpm for 1 min at RT.
35. Measure total RNA and small RNA concentration using NanoDrop or another spectrophotometer.
36. Store it at -80 °C until used.

3.2. Polyadenylation

1. Set up a reaction mixture with a total volume of 50 μ l in a 0.5 ml tube containing 100 ng-2 μ g of small RNAs, 10 μ l of 5 $\times$ E-PAP Buffer, 5 μ l of 25 mM $MnCl_2$, 5 μ l of 10 mM ATP, 1 μ l (2 U) of *E. coli* Poly(A) Polymerase I and RNase-free water (up to 50 μ l).
* When you have a low concentration of small RNAs, increase the total volume. 5 $\times$ E-PAP Buffer, 25 mM $MnCl_2$, and 10 mM ATP should be increased accordingly.
2. Mix well and spin the tube briefly.
3. Incubate for 1 hr at 37 $^{\circ}$ C.

3.3. Purification

1. Add an equal volume (50 μ l) of Acid-Phenol: Chloroform to the polyadenylation reaction mixture. * When you have ≥ 50 μ l of the mixture, increase Acid-Phenol: Chloroform accordingly.
2. Mix thoroughly by tapping the tube.
3. Centrifuge at 10,000 rpm for 5 min at RT.
4. Recover the aqueous phase carefully without disrupting the lower phase, and transfer it to a fresh tube.
5. Add 12 volumes (600 μ l) of Binding/Washing buffer to the aqueous phase.
* When you have ≥ 50 μ l of the aqueous phase, increase Binding/Washing buffer accordingly.
6. Transfer up to 460 μ l of the mixture into a Purification Cartridge within a Collection Tube.
7. Centrifuge at 10,000 rpm for 15 sec at RT.
8. Discard the filtrate (the flow-through), and repeat until all of the mixture is through the cartridge. Reuse the collection tube.
9. Apply 300 μ l of Binding/Washing buffer to the cartridge.
10. Centrifuge at 12,000 rpm for 1 min at RT.
11. Transfer the cartridge to a new collection tube.
12. Apply 25 μ l of pre-heated (95 $^{\circ}$ C) Elution Solution to the center of the filter, and close the cap.
* Aliquot a desired amount of Elution Solution into a 1.7 ml tube, heat it on a heat block at 95 $^{\circ}$ C for ~15 min. Open the cap carefully because it might be splash due to pressure build up.
13. Let the filter tube stand for 1 min at RT.
14. Centrifuge at 12,000 rpm for 1 min at RT.
15. Repeat steps #12–14 with a second aliquot of 25 μ l of pre-heated (95 $^{\circ}$ C) Elution Solution.
16. Measure polyadenylated (tailed) RNA concentration using NanoDrop or another spectrophotometer.
17. Store it at -80 $^{\circ}$ C until used.

* After polyadenylation, RNA concentration should increase up to 5–10 times of the starting concentration.

3.4. RNA Ligation

1. Set up a reaction mixture with a total volume of 50 μ l in a 0.5 ml tube containing 1–2 μ g of tailed RNAs, 3 μ l (3 μ g) of mi5-RNA, 5 μ l of 1 $\times$ T4 RNA Ligase 1 Reaction Buffer, 1 μ l (20 U) of T4 RNA Ligase (ssRNA Ligase) and RNase-free water (up to 50 μ l).

* When you have a low concentration of tailed RNAs, increase the total volume. 1 $\times$ T4 RNA Ligase 1 Reaction Buffer should be accordingly increased.
2. Mix well and spin the tube briefly.
3. Incubate for 30 min at 37 °C and then for 15 min at 65 °C for inactivation of the Ligase.

3.5. Purification

Ligated RNAs should be purified as described in 3.3 *Purification* above.

3.6. Reverse Transcription

1. Mix 2 μ g of ligated RNAs, 1 μ l (1 μ g) of miRT, and RNase-free water (up to 21 μ l) in a PCR tube.
2. Incubate for 10 min at 65 °C and for 5 min at 4 °C.
3. Add 1 μ l of 10 mM dNTP mix, 1 μ l of RNaseOUT, 4 μ l of 10 $\times$ RT buffer, 4 μ l of 0.1 M DTT, 8 μ l of 25 mM MgCl₂, and 1 μ l of SuperScript III Reverse Transcriptase to the mixture.

* When you have a low concentration of ligated RNAs, increase the total volume. 10 $\times$ RT buffer, 0.1 M DTT, and 25 mM MgCl₂ should be increased accordingly.
4. Mix well and spin the tube briefly.
5. Incubate for 60 min at 50 °C and for 5 min at 85 °C for inactivation of the reaction.
6. Add 1 μ l of RNase H to the mixture.
7. Incubate for 20 min at 37 °C.
8. Add 60 μ l of nuclease-free water.

3.7. PCR

1. Set up a reaction mixture with a total volume of 25 μ l in a PCR tube containing 1 μ l of small RNA cDNAs, 1 μ l (10 pm) of miP1, 1 μ l (10 pm) of miP1r, 12.5 μ l of AmpliTaq Gold PCR Master Mix and 9.5 μ l of nuclease-free water.
2. Mix well and spin the tube briefly.
3. Start PCR with conditions: [at 95 °C for 10 min, then 40 cycles (at 95 °C for 15 sec, and at 60 °C for 1 min)].
4. Run 2 μ l of PCR products along with a 100 bp DNA ladder on a 2% agarose gel.

* PCR products should be ~120–200 bp depending on small RNA species (e.g. ~120–130 bp for miRNAs and piRNAs).

3.8. Cloning

3.8.1. Gel Elution

1. Excise the DNA fragments of ~120–130 bp on a 2% gel.
2. Add 6 volumes of Buffer QG.
3. Incubate at 50 °C for 10 min. Mix the tube every 2 min during incubation.
4. Add 1 volume of isopropanol and mix.
5. Pass the sample through a QIAquick spin column in a collection tube.
6. Discard flow-through.
7. Add 0.5 ml of Buffer QG.
8. Centrifuge at 12,000 rpm for 1 min.
9. Discard flow-through.
10. Add 0.75 ml of Buffer PE.
11. Centrifuge at 12,000 rpm for 1 min.
12. Discard flow-through and centrifuge at 13,000 rpm for additional 1 min.
13. Place the column into a new collection tube.
14. Add 30–50 µl of EB buffer to the center of the column and let it stand for 1 min.
15. Centrifuge at 12,000 rpm for 1 min.
16. Measure eluted DNA concentration using NanoDrop or another spectrophotometer.
17. Store it at –20 °C until used.

Proceed to 3.8.2 for Manual Sequencing or Go to 3.8.7 for 454 Sequencing.

3.8.2. TA Cloning (Optional for Manual sequencing)

1. Mix 0.5–4 µl (50–100 ng) of eluted DNA, 1 µl of diluted salt, and Sterile Water to yield a final volume of 5 µl, and add 1 µl of TOPO vector.
* When you clone several samples, you can use 0.5 µl of TOPO vector with half the final volume in order to save costs.
2. Place the reaction mixture on ice for 5 min at RT.
3. Proceed to Transformation or store the TOPO Cloning reaction at –20 °C overnight.

3.8.3. Transformation (Optional for Manual Sequencing)

1. Add 1.5 µl of the TOPO Cloning reaction into a 0.1 cm cuvette containing 25 µl of electrocompetent (EC) cells (One Shot TOP 10 or other EC cells).
2. Electroporate the sample using your own protocol and electroporator.
3. Immediately add 600 µl of RT SOC medium.
4. Transfer the solution to 15 ml Falcon tube.
5. Shake at 200 rpm for 1 hour at 37 °C.
6. Spread 10–100 µl of transformed cells on pre-warmed LB agar plates containing ampicillin (100 µg/ml) (LB-amp plates). Plate at least two different volumes to

ensure that at least one plate will have well-spaced colonies. Store remaining cells at 4 °C for additional plating.

7. Incubate plates at 37 °C overnight until colonies are grown.
8. Spread an optimal amount of stored transformed cells (500–1000 colonies/plate) on LB-amp plates.
9. Incubate plates at 37 °C overnight until colonies are grown.
10. Proceed to Colony-directed PCR or save plates sealed with Parafilm at 4 °C.

3.8.4. Colony-Directed PCR (Optional for Manual Sequencing)

1. Pick up a single colony using a 10 µl tip on a 10 µl pipette and resuspend it in 10 µl TE buffer in a PCR tube.
* Take 5 µl of TE buffer with the pipette, pipette the buffer out gently onto a colony and pipette it up in order to pick it up. Do not pick up too many cells.
2. Add 1 µl (5 pm) of pcDNA, 1 µl (5 pm) of BGHr, 12.5 µl of GoTaq Green Master Mix (2×) to the cell PCR tube as well as Nuclease-Free water to yield a final volume of 25 µl.
3. Mix well and spin the tube briefly.
4. Start PCR with the conditions: [3 min at 95 °C, then 40 cycles (for 30 sec at 95 °C, 30 sec at 55 °C, and 1 min at 72 °C)].
5. Run 2 µl of the PCR products along with a 100 bp DNA ladder on a 2% agarose gel.
* PCR products with no insert and with no small RNA are 319 bp and 419 bp, respectively. Therefore, a PCR product with small RNA insert should be >439 bp.
6. Proceed to PCR purification with PCR product samples >439 bp

3.8.5. PCR Purification (Optional for Manual Sequencing)

1. Add 5 volumes (115 µl) of Buffer PBI to the PCR sample and mix well.
2. Pass the sample through a QIAquick spin column in a collection tube.
3. Centrifuge at 12,000 rpm for 1 min.
4. Discard flow-through.
5. Add 0.75 ml of Buffer PE.
6. Centrifuge at 12,000 rpm for 1 min.
7. Discard flow-through and centrifuge at 13,000 rpm for an additional 1 min.
8. Place the column into a new collection tube.
9. Add 30–50 µl of EB buffer to the center of the column and let it stand for 1 min.
10. Centrifuge at 12,000 rpm for 1 min.
11. Measure the eluted DNA concentration using NanoDrop or another spectrophotometer.
12. Run 2 µl of the PCR products along with a 100 bp DNA ladder on a 2% agarose gel to double check the size and amount.

13. Proceed to Sequencing or store it at -20°C until used.

3.8.6. Manual Sequencing

1. Set up a sequence sample with a total volume of 5–10 μl containing 40 ng of a PCR product and 2 pm of pcDNA or BGHR.
2. Submit samples for sequencing

3.8.7. Sequencing Template Preparation (Optional for 454 Sequencing)

1. Set up a reaction mixture with a total volume of 25 μl in a PCR tube containing 5 μl of small RNA cDNAs (50–100 ng), 2 μl (20 pm) of Fusion-A*, 2 μl (20 pm) of Fusion-B, 5 μl of GC Melt (5M), 0.5 μl of dNTP mix (10 mM each), 5 μl of GC 2 PCR Buffer, 0.5 μl of Advantage-GC 2 Polymerase Mix (50 $\times$) and 5 μl of PCR-Grade H_2O .

* In order to sequence multiple samples, each sample should be amplified with Fusion-A* containing two different nucleotide tags (see Table 1 and Notes 4).

2. Mix well and spin the tube briefly.
3. Start PCR with the conditions: [3 min at 94°C , 2 cycles (at 94°C for 30 sec, at 62°C for 30 sec and at 68°C for 1 min), 3 cycles (at 94°C for 30 sec, at 59°C for 30 sec and at 68°C for 1 min), 20 cycles (at 94°C for 30 sec, at 56°C for 30 sec and at 68°C for 1 min), and at 68°C for 3 min].

4. Run 2 μl of the PCR products along with a 100 bp DNA ladder on a 2% agarose gel.

* Major PCR products should be ~160–220 bp.

3.8.8. PCR Purification (Optional for 454 Sequencing)

1. Sequencing template PCR products should be purified as described in **3.8.5 PCR Purification** above.
2. Measure the eluted DNA concentration using NanoDrop or another spectrophotometer.
3. Run 2 μl of the PCR products along with a 100 bp DNA ladder on a 2% agarose gel to double check the size and amount (save the gel image).
4. Proceed to 454 sequencing or store it at -20°C until used.

3.8.9. 454 Sequencing

1. Set up a sequence sample with a total volume of 5–10 μl containing 10–50 ng of a purified PCR product and 2 pm of Primer A (the sequencing company will add this primer).
2. Submit samples and the gel image for sequencing

⁴Since 454 sequencing is massive (capable for 400–600 million base pairs with 400–500 base pair read lengths) and expensive, multiple samples can be mixed and sequenced together. In this case, you should design Fusion-A* with a different combination of the two nucleotide tags for each sample (see Table 1) to amplify each sequencing template. After sequencing, reads should be sorted by each nucleotide tag to trace the source of the samples.

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Fig. 1.

Overview of cloning strategy. Small RNAs isolated from tissues or cells are polyadenylated and ligated with a 5' RNA adapter mi5-RNA. Reverse transcription is performed using a RT primer miRT. Small RNAs are amplified by PCR using a pair of primers miP1 and miP1r from the small RNA cDNA library. For conventional (manual) sequencing, the PCR products are cloned into a TA cloning vector, transformed into bacteria, and amplified with a pair of primers, pcDNA and BGHr, by colony-directed PCR. The amplicons are sequenced with either pcDNA or BGHr, and analyzed. For 454 sequencing, a sequencing template is amplified with a pair of primers Fusion-A* and Fusion-B by a second PCR. The amplicons are sequenced with Primer A and then analyzed.

Table 1

Oligonucleotides used

Name	Sequence (5' to 3')	Quality	Usage
mi5-RNA	GACUGGAGCACGAGGACACUGACAUGGACUGAAGGAGUAGAAA	RNase free HPLC	RNA ligation
miRT	CGAATTCTAGAGCTCGAGGCAGGCGACATGTTTTTTTTTTTTTTTTTTTTTVN ¹	RNase free HPLC	Reverse transcription
miP1	CGAATTCTAGAGCTCGAGGCAGG	Regular desalting	PCR
miP1r	GACTGGAGCACGAGGACACTGA	Regular desalting	PCR
pcDNA	GCAGAGCTCTCTGGCTAAC	Regular desalting	Colony PCR/sequencing
BGHr	CTAGAAGGCACAGTCGAGG	Regular desalting	Colony PCR/sequencing
Fusion-A*	GCCTCCCTCGCGCCATCAGATCTGACCTCGTGCTCCTGTGACT ²	PAGE purification	454 sequencing template preparation
Fusion-B	GCCTTGCCAGCCCGCTCAGGCTTAAGATCTCGAGCTCCGTCC	PAGE purification	454 sequencing template preparation
Primer A	GCCTCCCTCGCGCCATCAG	Regular desalting	454 sequencing
Primer B	GCCTTGCCAGCCCGCTCAG	Regular desalting	454 sequencing

¹ V = A, C, or G; N = A, C, G, or T.

² AT are two tag nucleotides, which can be used for sample tags in sequencing multiple samples.