Preparation of cDNA Library for dRNA-seq

Feng Li[1, 2*] and Barbara Baker[1, 2*]

[1]Department of Plant and Microbial Biology, Plant Gene Expression Center, University of California, Berkeley, USA

[2]Agricultural Research Services (USDA-ARS), United States Department of Agriculture, Albany, USA

*For correspondence: chdlifeng@mail.hzau.edu.cn; bbaker@berkeley.edu

[Abstract] microRNAs (miRNAs) are ubiquitous regulators of gene expression in eukaryotic organisms, which guide Argonaute proteins (AGO) to cleave target mRNA or inhibit its translation based on sequence complementarity. In plants, miRNA directed cleavage occurs on the target mRNA at about 10 to 11 nucleotide (nt) up stream to the site where the 5' end of miRNA binds. Sequencing of the miRNA directed cleavage products (degradome) is widely employed as a way to both verify bioinformatic predictions of miRNA mediated regulation and identify novel targets of known miRNAs. Here we describe a protocol for preparation of degradome RNA complementary DNA library for high-through-put sequencing (dRNA-seq) using Illumina GA II sequencing platform, which is currently most popular and cost-effective. Using this protocol we successfully generated three dRNA-seq libraries using three solanaceae plants, including tobacco, tomato and potato. Although this protocol was developed with single-plexed adapter, it should be able to generate multiplexed libraries by replacing the 3' adapter with multiplexing compatible 3' adapter and replacing the PCR primer with indexed primers.

Materials and Reagents

1. RNeasy Plant Mini Kit (QIAGEN, catalog number: 74903)
2. OligodT Dynabeads (Life Technologies, Invitrogen™, catalog number: 610-02)
3. SeaKem LE Agrose (Lonza, catalog number: 50004)
4. Illumina sRNA-seq 3' adapter (Illumina, catalog number: 1000596)
5. RNase free water (Life Technologies, Invitrogen™, catalog number: 10977-023)
6. RNeasy Micro Kit (QIAGEN, catalog number: 74004)
7. Antarctic phosphatase (New England Biolabs, catalog number: M0289S)
8. RNase OUT(Life Technologies, Invitrogen™, catalog number: 10777-019)
9. T4 RNA Ligase 1 (New England Biolabs, catalog number: M0204S)
10. Illumina sRNA-seq RT primer (Illumina, catalog number: 1000597)
11. Illumina sRNA-seq 5' adapter (Illumina , catalog number: 1000595)

12. Illumina sRNA-seq PCR primer (Illumina, catalog number: 1000591, 1000592)
13. Gel purification kit (QIAGEN, catalog number: 28704)
14. dNTP (New England Biolabs, catalog number: N0447S)
15. SuperScript II RT(Life Technologies, Invitrogen™, catalog number: 18064-022)
16. Zero Blunt® PCR Cloning Kit (Life Technologies, Invitrogen™, catalog number: K2700-40)
17. Agrose gel (Lonza)

Equipment

1. PCR Thermal Cycler
2. Illumina GA II sequencing system
3. Pipette (20 µl, 200 µl, 1,000 µl)
4. Magnetic bar

Procedure

1. Isolation of high molecular weight RNA (with length > 200 bp) from plant tissue using RNeasy Plant Mini Kit according to manufacturer's protocol (according to the manufacturer's protocol, about 60 µg high molecular weight RNA can be obtained from 100 mg tobacco leaf tissue).
2. Purification of polyA RNA from 10 µg of total RNA using OligodT Dynabeads according to manufacturer's protocol and elute the polyA RNA in 15 µl RNase free water (a thermal cycler and a magnetic bar are used in this step).
3. Ligate sRNA 5' adapter:
   Purified mRNA 14 µl
   sRNA 5' adapter (10 µM) 2 µl (Illumina sRNA-seq 5' adapter)
   10x T4 RNA Ligase buffer 2 µl (* If ATP is not included, add ATP to 1 mM final)
   T4 RNA Ligase I (10 U/µl) 1.5 µl
   RNase OUT (40 U/µl) 0.5 µl
   20 °C, 6 h.
4. Dynabeads purification and elute in 15 µl RNase free water according to manufacturer's protocol.
5. RNA fragmentation
   Fragmentation buffer 1.6 µl (100 mM $ZnCl_2$, 100 Mm Tris-HCl, pH7.0).
   Ligated mRNA 14.4 µl.
   70 °C 2.5 min.

Purify fragmented RNA using RNeasy Micro Kit and elute RNA in 17 µl RNase free water after purification.

6. Phosphotase treatment to remove 3' phosphate resulted from fragmentation:

   Fragmented RNA 16 µl
   10x phosphatase buffer 2 µl
   Antarctic phosphatase 1 µl
   RNase OUT (40 U/µl) 1 µl
   37 °C, 30 min
   4 °C hold

   Purify RNA by RNeasy Micro Kit and elute in 15 µl RNase free water.

7. Ligate sRNA 3' adaptor

   Purified RNA from step 6 14.5 µl
   10x RNA Ligase buffer 2 µl (* if ATP is not included, add ATP to 1 mM final)
   RNA Ligase 1 (10 U/µl) 2 µl
   RNase OUT (40 U/µl) 1 µl
   RNA adapter 3' 0.5 µl (10 µM, Illumina sRNA-seq 3' adapter)
   20 °C, 4 h

8. Reverse transcriptation

   Prepare the following mix, heat at 70 °C for 2 min and place on ice.

   Adapter ligated RNA 4 µl
   SRA RT primer 0.5 µl (Illumina sRNA-seq RT primer)
   50 mM dNTP 0.5 µl

   Prepare the following mix and add to the above reaction:

   5x first strand buffer 2 µl
   100 mM DTT 2 µl
   RNase OUT (40 U/µl) 0.25 µl
   48 °C for 3 min then add:
   SuperScript II RT 0.75 µl
   44 °C for 60 min

9. PCR amplification

   Prepare the following mix and add to RT reaction:

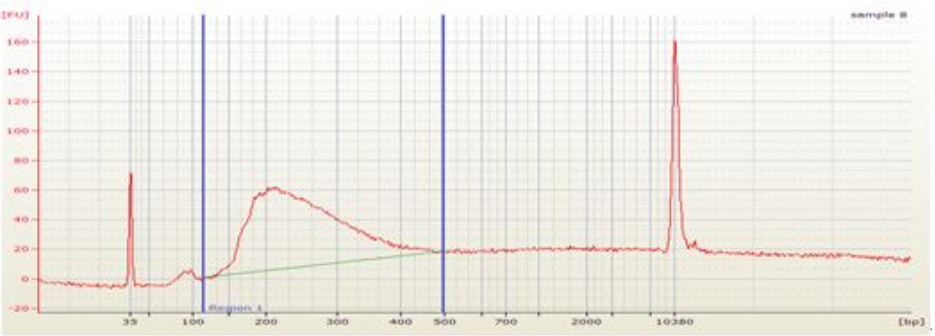
   Phusion HF 2x mix 25 µl
   Primer GX1 1 µl (Illumina sRNA-seq PCR primer)
   Primer GX2 1 µl (Illumina sRNA-seq PCR primer)
   Nuclease-free water 13 µl

   Run the following protocol:

   a. 98 °C 30 sec

b. 30-35 cycles of:
   98 °C 10 sec
   60 °C 30 sec
   72 °C 15 sec
c. 72 °C 10 min
d. 4 °C hold

10. Run the PCR product through a 1.5% Agrose gel, cut a smear region between 150 bp and 250 bp and purify by Gel purification kit and elute in 25 µl elution buffer.
11. Check the library quality by bio-analyzer High sensitivity DNA assay to check the size distribution (one µl sample is used in this step and a smear region centered around 250 bp is expected from the bio-analyzer electrophoresis profile, see Figure 1).



From [bp]	To [bp]	Corr. Area	% of Total	Average Size [bp]	Size distr. in CV [%]	Conc. [pg/µl]	Molarity [pmol/l]
115	497	1,083.6	100	252	25.1	952.47	6,198.5

Figure 1. Bioanalyzer analysis of dRNA-seq library. The upper part is the electrophoresis graph from the bio-analyzer run and the peak region between the two blue lines represents the purified dRNA constructs. Table below the electrophoresis graph shows the analysis of the peak region by the bio-analyzer 2100 software.

12. Use Zero Blunt® PCR Cloning Kit to clone the library and sequence of a few clones to verify the presence of inserts derived from plant transcripts.
13. Sequence the library using small RNA sequencing run on an Illumina GA II sequencing system.

Acknowledgments

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1. Li, F., Orban, R. and Baker, B. (2012). SoMART: a web server for plant miRNA, tasiRNA and target gene analysis. *Plant J* 70(5): 891-901.
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