Chapter 6

Viral Small RNA Cloning and Sequencing

Valérie Gausson and Maria-Carla Saleh

Abstract

At the current rate of technological progress, high-throughput sequencing of nucleic acids has become a commodity. These techniques are perfectly suitable for viral small RNAs sequencing and contribute to the understanding of many aspects of virus biology in the context of host–pathogen interaction. However, the generation of high quality data is still an issue and the preparation of small RNAs libraries that accurately reflect the viral siRNAs in the sample remains a challenge. In this chapter we describe how to clone and sequence libraries of viral small RNAs from infected insect samples (mosquito, drosophilidae, insect-derived cell lines).

Key words: Small RNAs, Deep sequencing, Illumina, Library preparation, RNA ligation

1. Introduction

Virus-derived small RNAs (vsiRNAs) were first identified in plants as a result of the antiviral RNAi host response (1). The dicing of the viral RNA into siRNAs was later shown in *Drosophila* (2) and the nematode *C. elegans* (3). These vsiRNAs reflect the activation of the antiviral immune system in the infected species.

Because vsiRNAs arise from replicative intermediates or structured genome sequences of viruses, their characterization greatly contributes to the understanding of many aspects of virus biology in the context of host–pathogen interaction. The abundance of vsiRNAs reflects the levels of viral replication and gene expression. The polarity of the vsiRNAs (they can correspond to the (+) strand or the (−) strand of the virus) reflects the source of dsRNA. vsiRNAs can be derived from the dicing of dsRNA in replication intermediates, in structured regions, or in convergent viral transcripts. Their mapping on the reference viral genome reflects

Ronald P. van Rij (ed.), *Antiviral RNAi: Concepts, Methods, and Applications*, Methods in Molecular Biology, vol. 721,
DOI 10.1007/978-1-61779-037-9_6,

structured and exposed regions and allows the identification of new viral variants with deletion/insertions compared to the reference. Analysis of the vsiRNAs size is indicative of the RNAi pathway involved in their biogenesis. In *Drosophila*, the main vsiRNAs are 21 nts and a product of Dcr2 (2), whereas in plants they are 21 nts when produced from DCL-4 and 22 nts when produced by DCL-2 (4). In addition, in *Drosophila* vsiRNAs may exhibit 3′ O-methylation which is the signature of their loading in the Ago2 RISC complex (5, 6). Finally, the *de novo* assembly of vsiRNAs can be used for the discovery of new virus species (7).

Several techniques have been developed for massive sequencing of nucleic acids and they are perfectly suitable for small RNAs sequencing, including vsiRNAs. These techniques include pyrosequencing (454–Roche), sequencing-by-synthesis (Solexa – Illumina), and sequencing by ligation (SOLID – Applied Biosystems). Next generation sequencing involves direct sequencing of single DNA or even RNA molecules (Helicos BioScience) (8).

To successfully apply these techniques, one faces two steps: the preparation of small RNAs libraries that accurately reflect the vsiRNAs in the sample and the management of a considerable amount of sequencing data. Here, we describe the method we use to clone and sequence small RNAs using Illumina technology. Our protocol is adapted from Pffefer (9). The main steps are summarized in Fig. 1. Briefly, total RNA from infected samples is purified, and the small RNA fraction is recovered and ligated at the 5′ and 3′ ends to RNA adapters. The ligated RNA is then reverse-transcribed and specifically amplified with DNA primers complementary to the primers spotted on the flowcell provided by Illumina. Once the sequence data have been collected, they can be analyzed using the bioinformatic pipeline described in Chap. 7.

2. Material

2.1. Total RNAs Isolation from Infected Samples (Mosquito, Drosophilidae, Insect-Derived Cell Lines)

1. TRIzol reagent (Invitrogen, Carlsbad, CA).
2. Chloroform.
3. Isopropyl alcohol.
4. Nuclease-free 75% ethanol conserved at –20°C.
5. Nuclease-free water.
6. Deionized formamide.
7. RNaseZap RNase decontamination solution (Ambion, Austin, TX).
8. Pellet pestle (Sigma, St. Louis, MO).

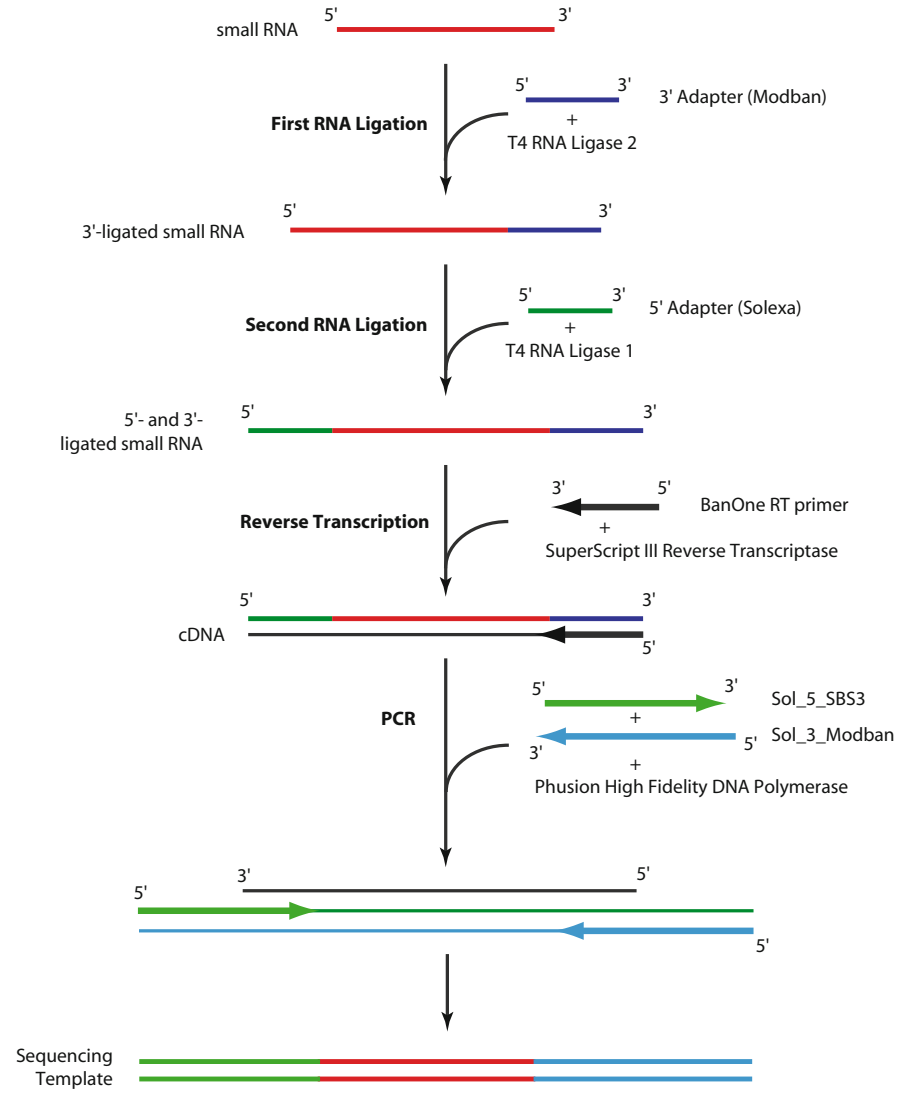

Fig. 1. Schematic representation of the small RNA library preparation (see text for details).

2.2. Preparation of Radiolabeled Size Markers

1. 100 µM of 19-nt oligoribonucleotide: 5′-rCrGrUrArCrGrCrGrGrGrUrUrUrArArArCrGrA-3′. The RNA oligonucleotides (see item 1–3) are used as size markers and contain the PmeI GTTT/AAAC restriction site (underlined) (see Note 1).
2. 100 µM of 24-nt oligoribonucleotide: 5′-rCrGrUrArCrGrCrGrGrArArUrArGrUrUrUrArArArCrUrGrU-3′.
3. 100 µM of 33-nt oligoribonucleotide: 5′-rCrGrUrArCrGrCrGrGrGrArArUrArGrUrUrUrArArArCrUrGrUrArGrUrGrCrUrGrArU-3′.
4. [γ-^{32}P] ATP (6,000 Ci/mmol, 10 mCi/mL).

5. T4 Polynucleotide kinase (T4 PNK) (10 U/μL).
6. Polynucleotide kinase (PNK) buffer (10×).
7. Nuclease-free water.
8. 1 M EDTA, pH 8.
9. Gel loading II buffer (Ambion, Austin, TX).
10. 30% Acrylamide/bisacrylamide (37.5:1).
11. Urea.
12. Tetramethylethylenediamine (TEMED).
13. 10% (w/v) Ammonium persulfate. Store in aliquots at –20°C.
14. 10× Tris/borate/EDTA buffer (TBE): 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0, diluted in water.
15. 5 M NaCl.
16. Absolute ethanol.
17. Thermomixer (Eppendorf, Hamburg, Germany).

2.3. Isolation of ~19–24nt RNAs

1. Gel loading II buffer (Ambion, Austin, TX).
2. Nuclease-free water.
3. 30% Acrylamide/bisacrylamide (37.5:1).
4. Urea.
5. TEMED.
6. 10% (w/v) Ammonium persulfate. Store in aliquots at –20°C.
7. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0, diluted in water.
8. 5 M NaCl.
9. Absolute ethanol.
10. Thermomixer (Eppendorf, Hamburg, Germany).

2.4. Ligation of the 3′ Adapter

1. 10× ATP-free ligation buffer: 100 mM $MgCl_2$, 100 mM dithiothreitol (DTT), 500 mM Tris–HCl, pH 7.6, 1 mg/mL acetylated BSA (Ambion, Austin, TX).
2. DMSO.
3. 5 nM of Modban adapter 5′-rAppCTGTAGGCACCATCAAT /3ddC/-3′ (IDT, San Jose, CA) (miRNA cloning linker 1, reference 60910274). This oligonucleotide is provided lyophilized and is ready for use in cloning. Resuspend at a concentration of 100 μM in water and store at –80°C (see Note 2).
4. T4 RNA ligase 2 truncated (200 U/μL) (New England Bioloabs, Ipswich, MA).

5. Gel loading II Buffer (Ambion, Austin, TX).
6. 30% Acrylamide/bisacrylamide (37.5:1).
7. Urea.
8. TEMED.
9. 10% (w/v) Ammonium persulfate. Store in aliquots at –20°C.
10. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0, diluted in water.
11. 5 M NaCl.
12. Absolute ethanol.
13. Nuclease-free water.
14. Thermomixer (Eppendorf, Hamburg, Germany).

2.5. Preparation of Radiolabeled Decade Marker

1. Decade marker (100 ng/μL) (Ambion, Austin, TX).
2. Nuclease-free water.
3. Kinase reaction buffer (10×) (Ambion, Austin, TX).
4. [γ-^{32}P] ATP (6,000 Ci/mmol, 10 mCi/mL).
5. T4 polynucleotide kinase (10 U/μL) (Ambion, Austin, TX).
6. Cleavage reagent (10×) (Ambion, Austin, TX).
7. Gel loading II Buffer (Ambion, Austin, TX).

2.6. Ligation of the 5′ Adapter

1. 10× ATP-containing ligation buffer: 2 mM ATP, 100 mM $MgCl_2$, 100 mM DTT, 500 mM Tris–HCl, pH 7.6, 1 mg/mL acetylated BSA (Ambion, Austin, TX).
2. DMSO.
3. 100 μM of Solexa adapter, HPLC purified: 5′-rArCrArCrUr C r U r U r U r C r C r C r U r A r C r A r C r G r A r C r G r C rUrCrUrUrCrCrGrArUrC-3′.
4. T4 RNA Ligase 1 (20 U/μL) (New England Biolabs, Ipswich, MA).
5. Gel loading II buffer (Ambion, Austin, TX).
6. 30% Acrylamide/bisacrylamide (37.5:1).
7. Urea.
8. TEMED.
9. 10% (w/v) ammonium persulfate. Store in aliquots at –20°C.
10. 10× TBE buffer: 890 mM Tris base, 890 mM boric acid, 20 mM EDTA, pH 8.0, diluted in water.
11. 5 M NaCl.
12. Absolute ethanol.
13. Nuclease-free water.
14. Thermomixer (Eppendorf, Hamburg, Germany).

2.7. Reverse Transcription

1. 100 µM RT primer BanOne, HPLC purified: 5′-ATTGATGGTGCCTACAG-3′.
2. 5× First-Strand Buffer: 250 mM Tris–HCl, pH 8.3, 375 mM KCl, 15 mM $MgCl_2$ (Invitrogen, Carlsbad, CA).
3. 20 mM (each) dNTPs Mix (dATP, dCTP, dGTP, dTTP).
4. 100 mM DTT.
5. SuperScript III Reverse Transcriptase (200 U/µL) (Invitrogen, Carlsbad, CA).
6. Thermocycler.

2.8. PCR Amplification of the Library

1. 5× Phusion HF buffer (Finnzymes, Espoo, Finland).
2. 20 mM (each) dNTPs Mix (dATP, dCTP, dGTP, dTTP).
3. 100 µM Primer Sol_5_SBS3, standard desalted: 5′-AATG ATACGGCGACCACCGAACACTCTTTCCCTAC ACGACG-3′.
4. 100 µM Primer Sol_3_Modban, HPLC purified: 5′-CAA-GCAGAAGACGGCATACGATTGATGGTGCCTACAG-3′.
5. Phusion high-fidelity DNA polymerase (2 U/µL) (Finnzymes, Espoo, Finland).
6. Nuclease-free water.
7. NuSieve GTG agarose (Cambrex, East Rutherford, NJ).
8. 50× Tris/acetate/EDTA (TAE) buffer: 2 M Tris base, 1 M acetic acid, 50 mM EDTA, pH 8.3, diluted in water.
9. Ethidium bromide.
10. Tracklt 25 bp DNA ladder (0.5 µg/µL) (Invitrogen, Carlsbad, CA).
11. Thermocycler.

2.9. PmeI Digestion of Size Markers

1. 5 M NaCl.
2. Phenol/chloroform/isoamylic alcohol, pH 8.
3. 3 M NaAc, pH 5.5.
4. Absolute ethanol.
5. Nuclease-free water.
6. PmeI (10 U/µL) (New England Bioloabs, Ipswich, MA).
7. NEBuffer 4 (10×): 500 mM KAc, 200 mM Tris–acetate, 100 mM MgAc, 10 mM DTT, pH 7.9 (New England Bioloabs, Ipswich, MA).
8. BSA (10 mg/mL).
9. Nuclease-free water.
10. 5× Nondenaturing loading dye: 0.025% bromophenol blue, 0.025% xylene cyanol, 0.125% orange G, 50% Glycerol, 10 mM Tris–HCl, pH 7.5, 50 mM EDTA.

2.10. Gel Purification of Libraries

1. NuSieve GTG agarose (Cambrex, East Rutherford, NJ).
2. Ethidium bromide.
3. Tracklt 25 bp DNA ladder (0.5 μg/μL) (Invitrogen, Carlsbad, CA).
4. 5 M NaCl.
5. Phenol (warmed to 70°C before use).
6. Phenol/chloroform/isoamylic alcohol (25:24:1), pH 8.
7. Chloroform.
8. 70% Ethanol.
9. Nuclease-free water.

3. Methods

For all steps, use nonsticky low-binding tubes to prevent material loss.

3.1. Total RNAs Isolation

TRIzol Reagent combines acidic phenol and guanidinium thiocyanate to lyse cells, inactivate RNases, and remove lipids. Manipulate this toxic solution with gloves and in a chemical fume hood. Remember to work in an RNase-free area (cleaned with RNase Zap) and keep samples on ice as much as possible.

1. Harvest five to ten insects in a clear polypropylene tube and euthanize them by flash freezing (submerge the tube in liquid nitrogen or in a dry ice/ethanol mixture). If you are working with cells, pellet 10^7 cells by gentle centrifugation (1,300 × *g* 5 min at RT) and wash them in 1× PBS.
2. Add 200 μL TRIzol Reagent and process the sample with a Kontes pellet pestle until a homogenized tissue sample is obtained. Complete with 800 μL TRIzol Reagent and incubate 5 min at room temperature.
3. Proceed to phase separation by adding 200 μL chloroform, vortex for 1 min, and centrifuge at 12,000 × *g* for 15 min at 4°C. In order to improve RNA purity, it is preferable to sacrifice a little amount of the aqueous phase close to the interface.
4. To precipitate RNA, transfer the aqueous phase to a new prechilled tube and add 400 μL isopropyl alcohol before centrifugation at 12,000 × *g* for 15 min at 4°C.
5. Remove the supernatant and wash the RNA pellet with 500 μL chilled 75% ethanol. Mix by vortexing and centrifuge at 7,500 × *g* for 5 min at 4°C.
6. Air-dry the RNA pellet by leaving the sample open under the extractor hood and resuspend it by pipetting up and down in

deionized formamide so that final RNA concentration is 1 µg/µL. RNA can be stored at this step in nuclease-free water at –20 or –70°C. This is necessary if beta-elimination and/or 2S ribosomal RNA depletion is required.

3.2. Preparation of Radiolabeled Size Markers

3.2.1. Radiolabeling of RNAs Oligonucleotides

1. For each marker, mix in a clear polypropylene low-binding tube:
   (a) 1 µL of 10 µM marker.
   (b) 5 µL of [γ-^{32}P] ATP (6,000 Ci/mmol, 10 mCi/mL).
   (c) 0.2 µL of 10 U/µL T4 PNK.
   (d) 2 µL of 10× PNK buffer.
   (e) Up to 20 µL with nuclease-free water.
2. Incubate 30 min in water bath at 37°C, in radioactive room.
3. Stop the reaction by adding 20 µL of 30 mM EDTA, pH 8.
4. Add 1 volume of Gel loading II Buffer, incubate 2 min at 95°C, and quickly chill on ice.

3.2.2. Gel Purification of Radiolabeled Size Markers

1. Prepare a 20×20 cm gel of 15% acrylamide/bisacrylamide (37.5:1), 7 M urea, 0.5× TBE, with spacers of 0.8 mm and a 15-well comb.
2. Prerun the gel in 0.5× TBE for 30 min at 30 W. Check that your generator can deliver this power.
3. Wash carefully the wells with a needle and a syringe.
4. Load samples and load markers in outer wells. Be aware of leaving empty wells between samples.
5. Run your gel for 45 min at 15 W (750 V). Remember that the 19 nt size marker migrates with the bromophenol blue.
6. After migration, remove the upper glass plate with a spatula, cover the gel with saran wrap, place an autoradiography ruler for orientation, and expose on film for 2 min.
7. Pierce the film at the position corresponding to the markers.
8. Place the film on top of the gel using the autoradiography ruler to position.
9. Cut the size markers from the gel with a clean scalpel, using the pierced film to indicate their position.
10. Place the gel slice in a clear polypropylene low-binding tube with 300 µL of 0.3 M NaCl.
11. Elute overnight under agitation at 17°C.
12. Next day, briefly spin down the tubes and recover the supernatant avoiding polyacrylamide debris.
13. Precipitate with 3 volumes of 100% ethanol for 1 h at –20°C.
14. Centrifuge at 16,000×*g* for 10 min.

15. Dry out excess ethanol.
16. Dissolve in 20 µL of nuclease-free water.

3.3. Isolation of ~19–24nt RNAs

1. Prepare a size marker mix with 3 µL of each radiolabeled marker and 100 µL of gel loading blue II.
2. In a polypropylene low-binding tube, mix:
   (a) 30 µL RNA prepared at Subheading 3.1 (30 µg).
   (b) 3 µL of size marker mix from Subheading 3.3, step 1.
   (c) 33 µL gel loading blue II.
3. Denature for 30 s at 95°C and quick chill on ice.
4. Prepare a 20×20 cm gel of 15% acrylamide/bisacrylamide (37.5:1), 7 M urea, 0.5× TBE, with spacers of 1.5 mm and a 15-well comb.
5. Prerun the gel on 0.5× TBE for 30 min at 30 W.
6. Wash the wells carefully with a needle and syringe.
7. Dilute 6 µL of size marker mix prepared in Subheading 3.3, step 1 in a final volume of 40 µL and load 20 µL in the first and last lane of the gel.
8. Load samples. Be sure to leave empty wells between samples.
9. Run the gel at 30 W until the bromophenol blue reaches two third of the gel.
10. After migration, remove the upper glass plate with a spatula, cover the gel with saran wrap, and place a autoradiography ruler for orientation and expose on phosphorimager screen for 1 h (see Note 3).
11. Replace the print from the phosphorimager in top of the gel using the autoradiography ruler to position.
12. Cut the samples corresponding to the 19–24 nt position using the size markers as a guide.
13. Cut out the markers as well from the first and last gel lanes.
14. Put the gel slice in a polypropylene low-binding tube with 300 µL of 0.3 M NaCl.
15. Elute overnight under agitation at 17°C.
16. Next day, briefly spin down the tubes and recover the supernatant.
17. Precipitate with 3 volumes of 100% ethanol for 1 h at –20°C.
18. Centrifuge at 16,000×*g* for 10 min.
19. Dry out excess ethanol.

3.4. Ligation of the 3′ Adapter

1. Dissolve small RNAs and eluted markers mix in:
   (a) 10 µL RNase-free water.
   (b) 2 µL 10× ATP-free ligation buffer.
   (c) 6 µL 50% DMSO (diluted in water).
   (d) 1 µL 50 µM of Modban adapter.
2. Denature for 30 s at 95°C and quickly chill on ice.
3. Add 1 µL (200 U/µL) of truncated T4 RNA ligase 2.
4. Incubate 1 h at 37°C.
5. Stop the reaction by adding 20 µL of gel loading II buffer.
6. Prepare a 20×20 cm gel of 15% acrylamide/bisacrylamide (37.5/1), 7 M urea, 0.5× TBE, with spacers of 0.8 mm and a 15-well comb.
7. Prerun the gel on 0.5× TBE for 30 min at 30 W.
8. Wash the wells carefully with a needle and syringe.
9. Load samples. Be sure to leave empty wells between samples. Load nonligated and ligated markers at the most external wells.
10. Run the gel at 30 W until the bromophenol blue reaches two third of the gel.
11. After migration, remove the upper glass plate with a spatula, cover the gel with saran wrap, and place a autoradiography ruler for orientation and expose on phosphorimager screen for ~2.5 h.
12. Replace the print from the phosphorimager on top of the gel using the autoradiography ruler to position.
13. Cut samples in the lanes between the 37–42 nt positions using the ligated size markers as a guide. Ligation of Modban adapter to the 19 and 24 nt markers gives rise to 37 and 42 species, respectively. Remember that nonligated products will also be detectable on gel.
14. Cut out the ligated markers as well from the first and last gel lanes.
15. Put the gel slice in a polypropylene tube with 300 µL 0.3 M NaCl.
16. Elute overnight under agitation at 17°C.
17. Next day, briefly spin down the tubes and recover the supernatant.
18. Precipitate with 3 volumes of 100% ethanol for 1 h at −20°C.
19. Centrifuge at 16,000×*g* for 10 min.
20. Dry out excess ethanol.

3.5. Preparation of Radiolabeled Decade Marker

1. In a clear polypropylene low-binding tube, mix:
   (a) 1 µL Decade marker.
   (b) 6 µL Nuclease-free water.
   (c) 1 µL 10× Kinase reaction buffer.
   (d) 1 µL [γ-^{32}P] ATP (6,000 Ci/mmol, 10 mCi/mL).
   (e) 1 µL T4 PNK.
2. Incubate 1 h at 37°C.
3. Add 8 µL of nuclease-free water and 2 µL of 10× cleavage reagent.
4. Incubate 5 min at room temperature and stop the reaction by adding 20 µL of gel loading II buffer. Store at –20°C. The radiolabeled Decade marker can be used for 15 days according to ^{32}P half-life (14.3 days).

3.6. Ligation of the 5′ Adapter

1. Dissolve 3′-ligated small RNAs and markers in:
   (a) 10 µL RNase-free water.
   (b) 2 µL 10× ATP-containing ligation buffer.
   (c) 6 µL 50% DMSO (diluted in water).
   (d) 1 µL 50 µM 5′ Solexa adapter.
2. Denature for 30 s at 95°C and quickly chill on ice.
3. Add 1 µL of 20 U/µL T4 RNA ligase 1.
4. Incubate 1 h at 37°C.
5. Stop the reaction by adding 20 µL of gel loading II buffer.
6. Prepare a 20×20 cm gel of 15% acrylamide/bisacrylamide (37.5:1), 7 M urea, 0.5× TBE, with spacers of 0.8 mm and a 15-well comb.
7. Prerun the gel on 0.5× TBE for 30 min at 15 W.
8. Wash the wells carefully with a needle and syringe.
9. Load samples. Be sure to leave empty wells between samples.
10. Run the gel at 30 W until bromophenol blue is completely out of the gel.
11. After migration, remove the upper glass plate with a spatula, cover the gel with saran wrap, place an autoradiography ruler for orientation, and expose on phosphorimager screen for 3 h.
12. Replace the print from the phosphorimager on top of the gel using the autoradiography ruler to position.
13. Cut samples in the lanes between the 69 and 74 nt positions using the ligated size markers as a guide (ligation of 5′ Solexa adapter to the 37 and 42 nt markers gives rise to 69 and 74 nt long species, respectively, as well as nonligated products).

14. Put the gel slice in a clear polypropylene low-binding tube with 300 µL of 0.3 M NaCl and 1 µL of 100 µM RT primer BanOne.
15. Elute overnight under agitation at 17°C.
16. Next day, briefly spin down the tubes and recover the supernatant.
17. Precipitate with 3 volumes of 100% ethanol for 1 h at –20°C.
18. Centrifuge at 16,000 × *g* for 10 min.
19. Dry out excess ethanol.
20. Dissolve in 10 µL of nuclease-free water.

3.7. Reverse Transcription

1. In a PCR tube, mix:
   (a) 5 µL of ligated RNA samples.
   (b) 4 µL 5× First-strand buffer.
   (c) 2 µL 20 mM dNTPs mix.
   (d) 1 µL 100 mM DTT.
   (e) Up to 18 µL with nuclease-free water.
2. Incubate 3 min at 50°C.
3. Split each sample into two PCR tubes of 9 µL.
4. In one tube, add 1 µL of 200 U/µL superscript III reverse transcriptase. Use the other tube as a negative control for reverse transcription activity (RT control, see Note 4).
5. Incubate 1 h at 50°C, then 15 min at 70°C to inactivate the enzyme.

3.8. PCR Amplification of the Library

1. In a PCR tube, mix:
   (a) 5 µL of cDNA or RT control.
   (b) 20 µL 5× Phusion HF buffer.
   (c) 1 µL 20 mM dNTPs mix.
   (d) 1 µL 100 µM Primer Sol_5_SBS3.
   (e) 1 µL 100 µM Primer Sol_3_Modban.
   (f) 1 µL Phusion DNA polymerase (2 U).
   (g) Up to 100 µL with nuclease-free water.
2. Run in thermocyler:
   (a) 94°C for 2 min.
   (b) 5 cycles: 94°C 15 s, 54°C 30 s, 72°C 30 s.
   (c) 17 cycles: 94°C 15 s, 60°C 30 s, 72°C 30 s.
   (d) 72°C for 7 min.
   (e) Maintaining at 4°C.

3. Run 5 µL of the PCR reaction in a 3% NuSieve GTG agarose gel in 0.5× TAE.
4. Check for one band between 108 and 113 bp (see Note 5).

3.9. PmeI Digestion of Size Markers

1. In a polypropylene low-binding tube, add to the 95 µL remaining of the PCR reaction:
   (a) 6 µL 5 M NaCl.
   (b) 100 µL Phenol/chloroform/isoamylic alcohol, pH 8.
2. Vortex.
3. Centrifuge at 16,000 × *g* for 5 min at 4°C.
4. Recover the aqueous phase.
5. Add 1/10 volume 3 M NaAc, pH 4.5 and 3 volumes of 100% ethanol.
6. Precipitate at −20°C for at least 3 h.
7. Centrifuge at 16,000 × *g* for 10 min.
8. Remove supernatant.
9. Dry out excess ethanol.
10. Dissolve the pellet in PmeI digestion mixture (see Note 1):
    (a) 23.7 µL of nuclease-free water.
    (b) 3 µL of 10× NEBuffer 4.
    (c) 0.3 µL of 10 mg/mL BSA.
    (d) 3 µL of 10 U/µL PmeI enzyme.
11. Incubate 2 h at 37°C.
12. After the digestion, add to each tube:
    (a) 200 µL Nuclease-free water.
    (b) 230 µL Phenol/chloroform/isoamylic alcohol, pH 8.
13. Vortex.
14. Centrifuge at 16,000 × *g* for 10 min at 4°C.
15. Recover the aqueous phase, add 1/10 volume 3 M NaAc, pH 4.5, and precipitate overnight at −20°C in 3 volumes of 100% ethanol.
16. Centrifuge at 16,000 × *g* for 10 min at 4°C.
17. Discard the supernatant.
18. Dry and dissolve the pellet in 14.4 µL of nuclease-free water and 1.6 µL of nondenaturing loading dye.

3.10. Gel Purification of Libraries

1. Prepare a 3% NuSieve GTG agarose gel (20 × 20 cm) in 0.5× TAE with 100 ng/mL ethidium bromide. Use a comb that will accommodate total sample volume (16 µL).
2. Load the samples and a DNA ladder (for example, 25 bp DNA ladder Invitrogen).

3. Migrate at 100 V. Let the bromophenol blue progressing at least 10 cm.
4. Cut the band of interest (108–113 bp). Remember that the upper band is the insert library, whereas the lower band corresponds to amplification of self-ligated adapters.
5. Weigh the gel slice and distribute 250 mg of gel into polypropylene tubes.
6. Add 2 volumes of 0.4 M NaCl. Melt the gel slice at 70°C, mixing every 2 min.
7. Add 1 volume of warm phenol, pH 8 (see Notes 6 and 7).
8. Vortex 30 s.
9. Centrifuge 5 min at 16,000 × *g* at room temperature.
10. Recover the aqueous phase.
11. Add 1 volume of phenol/chloroform/isoamylic alcohol, pH 8.
12. Vortex 30 s.
13. Centrifuge 5 min at 16,000 × *g* at room temperature.
14. Recover the aqueous phase.
15. Add 1 volume of chloroform.
16. Vortex 30 s.
17. Centrifuge 5 min at 16,000 × *g* at room temperature.
18. Recover the aqueous phase.
19. Add 1/10 3 M NaAc, pH 4.5 and 3 volume of 100% ethanol.
20. Incubate overnight at –20°C.
21. Centrifuge 10 min 16,000 × *g* at 4°C.
22. Wash with 700 µL of 70% ethanol.
23. Centrifuge 10 min 16,000 × *g* at 4°C.
24. Remove the supernatant.
25. Dry excess ethanol.
26. Dissolve in 20 µL of nuclease-free water.

At this step, your library is ready to be sent for sequencing. Some companies or core facilities may ask you to determine the concentration of your library. Use accurate technologies such as fluorometry or microfluidics-based platforms for DNA.

4. Notes

1. PmeI is an octanucleotide-recognizing rare-cutting restriction endonuclease. Size markers oligonucleotides containing the PmeI restriction site can be eliminated from the final

DNA sequencing template by PmeI digestion. Failure to remove the size markers from the final cDNA library will result in a sequence dataset that is predominated by size marker sequences.

2. The Modban adapter is a fully activated 5′ adenylated oligonucleotide and is a substrate for T4 RNA ligase in the absence of ATP. The use of this adenylated adapter improves the cloning efficiency of small RNAs, which have a 5′-phosphate and will circularize if adapters are attached using T4 RNA ligase in presence of ATP. For more details, see refs. (10) and (11).
3. Use of the phosphorimager represents a quicker and quantitative method of visualizing radioactive gels. The phosphorimager screen needs to be exposed only for one tenth of the time required for exposure to film. Because the amount of radiolabeled size markers present in your sample is subject to successive loss at each purification step, exposing your gel to a phosphorimager screen allows a sensitive gain of time. It would take an overnight exposure to get the equivalent signal intensity on film.
4. As PCR cannot discriminate between cDNA targets synthesized by reverse transcription and genomic DNA contamination, the RT control tube detects DNA contamination in the RNA preparation.
5. Adapters can self-ligate, which generates chimeric sequences. As the relative concentration of adapter primers increases, the formation of adapters dimers is promoted. If these dimers are not removed, they will ultimately be sequenced along with the intended template, wasting the capacity of the flowcell. At this stage of the library preparation, an extra lower band corresponding to self-ligated adapters can be observed. Be careful to prevent transferring the lower band when excising the library from the gel.
6. The use of warm phenol prevents agarose from solidifying by getting cold during the purification steps.
7. Commercial DNA gel extraction and clean-up kits do not have an exact size cut-off. The cloned library and the self-ligated adapters are too close in size to be adequately discriminated using a commercial kit.

Acknowledgments

The authors would like to thank Christophe Antoniewski for insight and discussion into deep sequencing, Marco Vignuzzi for comments on the manuscript, Nicolas Vodovar for helping with figures, and Ronald van Rij for being such a great and patient editor.

This work was financially supported by the Agence Nationale de la Recherche (ANR-09-JCJC-0045-01) and the European Research Council (FP7/2007-2013 ERC 242703) to MCS.

References

1. Hamilton, A. J., and Baulcombe, D. C. (1999) A species of small antisense RNA in posttranscriptional gene silencing in plants, *Science* ***286***, 950–952.
2. Li, H., Li, W. X., and Ding, S. W. (2002) Induction and suppression of RNA silencing by an animal virus, *Science* ***296***, 1319–1321.
3. Lu, R., Maduro, M., Li, F., Li, H. W., Broitman-Maduro, G., Li, W. X., and Ding, S. W. (2005) Animal virus replication and RNAi-mediated antiviral silencing in *Caenorhabditis elegans*, *Nature* ***436***, 1040–1043.
4. Deleris, A., Gallego-Bartolome, J., Bao, J., Kasschau, K. D., Carrington, J. C., and Voinnet, O. (2006) Hierarchical action and inhibition of plant Dicer-like proteins in antiviral defense, *Science* ***313***, 68–71.
5. Horwich, M. D., Li, C., Matranga, C., Vagin, V., Farley, G., Wang, P., and Zamore, P. D. (2007) The *Drosophila* RNA methyltransferase, DmHen1, modifies germline piRNAs and single-stranded siRNAs in RISC, *Curr Biol* ***17***, 1265–1272.
6. Flynt, A., Liu, N., Martin, R., and Lai, E. C. (2009) Dicing of viral replication intermediates during silencing of latent *Drosophila* viruses, *Proc Natl Acad Sci U S A* ***106***, 5270–5275.
7. Wu, Q., Luo, Y., Lu, R., Lau, N., Lai, E. C., Li, W. X., and Ding, S. W. (2010) Virus discovery by deep sequencing and assembly of virus-derived small silencing RNAs, *Proc Natl Acad Sci U S A* ***107***, 1606–1611.
8. Anon. (2010) Human genome at ten: The sequence explosion, *Nature* 464, 670–671.
9. Pfeffer, S. (2007) Identification of virally encoded microRNAs, *Methods Enzymol* ***427***, 51–63.
10. England, T. E., Gumport, R. I., and Uhlenbeck, O. C. (1977) Dinucleoside pyrophosphate are substrates for T4-induced RNA ligase, *Proc Natl Acad Sci U S A* ***74***, 4839–4842.
11. Ho, C. K., Wang, L. K., Lima, C. D., and Shuman, S. (2004) Structure and mechanism of RNA ligase, *Structure* ***12***, 327–339.