Chapter 6

Small RNA Library Cloning Procedure for Deep Sequencing of Specific Endogenous siRNA Classes in *Caenorhabditis elegans*

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Abstract

In recent years, distinct classes of small RNAs ranging in size from ~21 to 26 nucleotides have been discovered and shown to play important roles in a wide array of cellular functions. Because of the abundance of these small RNAs, library preparation from an RNA sample followed by deep sequencing provides the identity and quantity of a particular class of small RNAs. In this chapter we describe a detailed protocol for preparing small RNA libraries for deep sequencing on the Illumina platform from the nematode *C. elegans*.

Key words Endogenous siRNAs, *C. elegans*, RNAi, 26G-siRNA, 22G-siRNA, Small RNA library cloning

1 Introduction

Since the description of the first regulatory noncoding RNA cloned from the nematode *C. elegans* two decades ago, the field of small RNAs (sRNAs) has been implicated in a myriad of developmental and gene regulation processes in eukaryotes spanning from yeast to humans [1]. In animals, three major types of small RNAs have been identified: microRNAs (miRNAs), PIWI-interacting RNAs (piRNAs), and endogenous short interfering RNAs (endo-siRNAs) (Fig. 1). MicroRNAs are generated by processing single-stranded RNA hairpin loops into ~21–22 nucleotides sRNAs through the action of two RNase III-like enzymes, Dicer and Drosha, and RNA-binding protein Pasha/DGCR8. MicroRNAs suppress translation and promote degradation of their target mRNAs by imperfectly base-pairing to their 3′ untranslated regions [2]. Both miRNAs and piRNAs are generated from single-stranded RNA precursors transcribed from genomic regions, although their biogenesis and respective function differ significantly. While the 5′ ends of both miRNAs and piRNAs have a 5′ monophosphate, their

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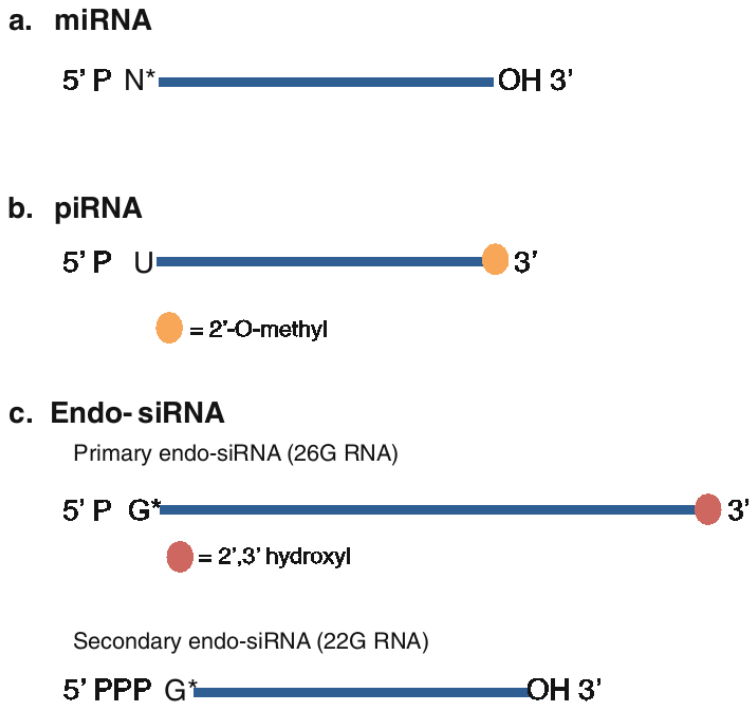

Fig. 1 The three major classes of sRNAs in *C. elegans*. (**a**) miRNAs (~21–22 nucleotides long) are Dicer-dependent and thus have a 5′ monophosphate and a 3′ hydroxyl modifications. Most (>70 %) miRNAs start with a uridine at the 5′ end (indicated as N*). The ~30 % of the remaining miRNAs start with an adenosine, cytidine, or guanosine. (**b**) 21U-piRNAs exhibit a 5′ monophosphate uracil and a 2′-methyl moiety at the 3′ end. (**c**) Endo-siRNAs are categorized as primary endo-siRNAs (26G-siRNAs) and secondary endo-siRNAs (22G-siRNAs). The lesser abundant 26G-siRNAs are Dicer-dependent and have a 5′ monophosphate guanosine and a 2′,3′ hydroxyl group at the 3′ end. The more abundant 22G-siRNAs are Dicer-independent, RdRP-dependent, and have a 5′ triphosphate guanosine and a 3′ hydroxyl group. The use of TAP (*see* Subheading 3.1) allows for the conversion of 5′ triphosphate to a monophosphate group required for sRNA library cloning following this protocol. G* indicates that over 90 % of endo-siRNAs have a guanosine at the 5′ end. The remaining ~10 % of endo-siRNAs can start with an adenosine, cytidine, or uridine

3′ ends differ significantly with miRNAs having a 3′ OH and piRNAs possessing a 2′-O-methyl moiety (Fig. 1). Unlike miRNAs, production of piRNAs is Dicer-independent and involves a "ping pong" amplification mechanism. All *C. elegans* piRNAs bind to the PIWI-containing clade of Argonautes (AGOs) and possess a 5′ uracil (Fig. 1); however, the length of piRNAs varies from 21 nt in *C. elegans* (also referred to as 21U-RNAs) to ~24–30 nt in *Drosophila*, zebrafish, and mammals. Although the original function of piRNAs was characterized as transposon silencing in the germ line, recent identified functions in *C. elegans* include regulation of chromatin structure and transgenerational cellular memory of "self" versus "nonself" [3–5].

Of the three major classes of sRNAs in *C. elegans* adults, endo-siRNAs are the most abundant and variable class. Endo-siRNAs range from ~21 to 26 nucleotides in length and are most often antisense to coding regions of transposons and endogenous genes, although endo-siRNAs mapping to intergenic regions such as the X-cluster have been identified. One method of characterizing this class of sRNAs is by their biogenesis. Primary endo-siRNAs are generated from double-stranded RNA processed by Dicer, which results in a siRNA containing a characteristic 5′ monophosphate and 2′,3′ hydroxy terminus (Fig. 1). Primary endo-siRNAs are of low abundance, ~26 nucleotides in length, and possess a 5′ guanosine (26G-siRNAs) (Fig. 1). Alternatively, secondary endo-siRNAs are highly abundant, are ~22 nucleotides in length, and possess a 5′ guanosine (22G-siRNAs). In contrast to 26G-siRNAs, the biogenesis of 22G-siRNAs is through the action of RNA-dependent RNA polymerases (RdRPs) and is Dicer-independent, resulting in a 5′ triphosphate cap (Fig. 1) [6, 7]. While the biogenesis of secondary siRNAs in worms and plants is dependent on RdRPs, no RdRPs have been identified in flies and mammals, suggestive of an RdRP-independent biogenesis mechanism [2]. Although evidence suggests that secondary endo-siRNA biogenesis in *C. elegans* is stimulated by the primary 26G-endo-siRNAs, the mechanisms regulating this process remain unclear [8].

The use of exogenous dsRNA to "knockdown" expression of a target gene locus through RNAi has become routine practice in many model organisms; however, the function of endogenous siRNAs is less understood. The *C. elegans* genome contains 26 AGO genes that play a role in the regulation of gene expression and chromatin state. The large number of *C. elegans* AGOs, a majority of which bind endo-siRNAs, allows for functional specificity within worms based on endo-siRNA biogenesis, tissue specificity, and site of action [9, 10]. For example, NRDE-3 AGO is expressed in somatic tissue, binds to 22G-siRNAs, and targets genomic loci in the nucleus to regulate chromatin state. Alternatively, ALG-3/4 AGOs are expressed in sperm, bind 26G-endo-siRNAs and target sperm-enriched mRNAs to regulate thermotolerant male fertility [11–14]. In recent years with the prevalence of deep-sequencing technologies, much progress has been made identifying potential targets of individual AGO proteins by sequencing AGO-bound siRNAs or by identifying genes with a reduction in siRNA abundance antisense to their coding sequences in mutant backgrounds [11, 12, 14–17]. However, efforts to correlate endo-siRNA abundance with changes in target gene expression have only been moderately successful, suggesting that endogenous RNAi pathways may have more complex gene regulatory functions than the exogenous RNAi pathways [11, 12, 16–18].

In this chapter, we present a general protocol for preparing sRNA libraries from *C. elegans* for deep sequencing on the Illumina

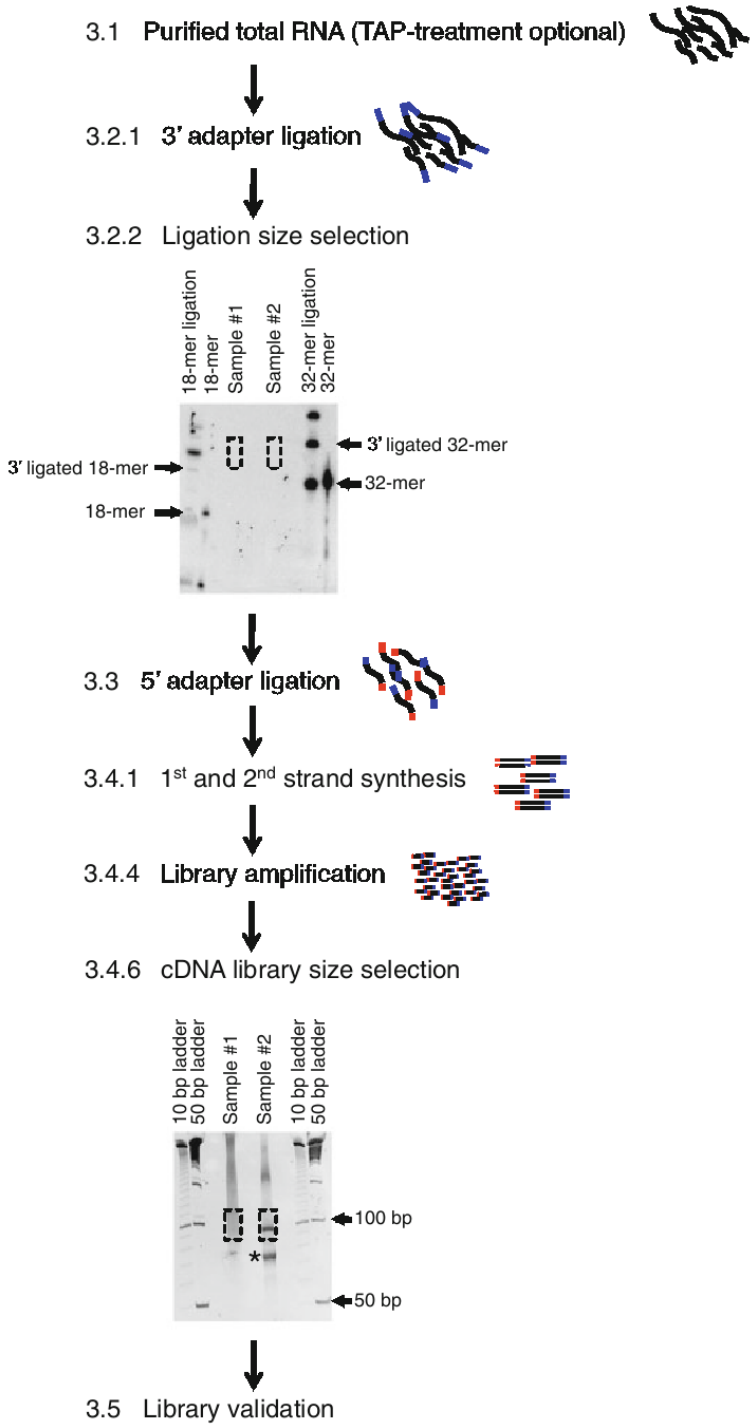

Fig. 2 Flowchart of sRNA library synthesis. This flowchart illustrates the steps of sRNA library synthesis outlined in this chapter. The numbers indicate which step in the protocol is being illustrated. Illustrative examples of polyacrylamide gels used for sRNA library size selection after completion of (**a**) Subheading 3.2 3′ adapter ligation and (**b**) Subheading 3.4 reverse transcription and library amplification steps are shown. The *dotted lines* indicate the regions of the gels that were extracted. *Asterisk* indicates the primer dimer artifact described in the text

platform (Fig. 2) (modified from ref. 19). This process requires purification of small RNAs from total RNA and ligation of adapter oligos to the 5′ and 3′ ends of the sRNAs. The ligation reactions are sensitive to the 5′ phosphate status of the sRNAs, allowing for enrichment of either primary endo-siRNAs (5′ monophosphate) or secondary endo-siRNAs (5′ triphosphate), if desired (Fig. 1). An optional enzymatic step using Tobacco Acid Phosphatase (TAP) hydrolyzes the phosphoric acid anhydride bonds in the 5′ triphosphorylated ends of 22G-endo-siRNAs (*see* Subheading 3.1), leaving them with a 5′ monophosphate that can be substrates for the ligation of the 5′ adapter oligo. Finally, we describe reverse transcription, amplification, and validation steps to prepare the sRNA libraries for deep sequencing. The protocol described here differs from the endo-siRNA sequencing protocols used for other metazoans. For instance, in *Drosophila melanogaster*, endo-siRNAs bear 5′-monophosphorylated and 2′-O-methylated 3′ ends. This feature is used to select against other sRNAs (e.g., miRNAs which are not modified at the 3′ end) by treating a total sRNA sample with $NaIO_4$ followed by β-elimination, resulting in the blockage of adapter ligation to sRNAs with a 2′,3′ hydroxyl termini and thus excluding them from being cloned [20]. In the future, endo-siRNA sequencing and analysis from specific tissue types, developmental stages, and environmental conditions will likely be required to fully elucidate the complex regulatory mechanisms of endogenous RNAi pathways.

2 Materials

Take proper precautions to avoid RNase contamination throughout the protocol, including using baked glassware and DEPC or Milli-Q-treated water to make RNase-free solutions.

2.1 Preparation of RNA

Trizol reagent (Invitrogen).

Tobacco acid pyrophosphatase (TAP) (Epicentre) (optional).

3 M Sodium acetate (NaOAc).

100 % Ethanol.

2.2 3′ Adapter Ligations and Purification of Samples

Denaturing and native polyacrylamide gel electrophoresis supplies (such as SequaGel).

5× Ligation buffer: 250 mM 2-[4[(2-hydroxyethyl)piperazin-1-yl] ethanesulfonic acid (HEPES) pH 8.3, 50 mM magnesium chloride ($MgCl_2$), 16.5 mM dithiothreitol (DTT), 50 μg/mL bovine serum albumin (BSA), and 41.5 % glycerol. Store at −20 °C.

T4 single-stranded RNA ligase enzyme.

RNase Inhibitor (optional).

PhosphorImager and exposure screens.

0.3 M Sodium chloride (NaCl).

Glycogen.

3′ adapter (for Hi-Seq 2000).

pCGTCGTATGCCGTCTTCTGCTTGT/3AmMO/.

This is adapter is phosphorylated at the 5′ end and contains a /3AmMO/ amino modifier at the 3′ end.

2.3 5′ Adapter Ligation of Experimental Samples

5′ adapters with barcode (for Hi-Seq 2000).

Barcode CAA: gttcagagttctacagtccgacgatcNNN**CAAAA**.

Barcode ACC: gttcagagttctacagtccgacgatcNNN**ACCAA**.

Barcode GUU: gttcagagttctacagtccgacgatcNNN**GUUAA**.

Barcode UGG: gttcagagttctacagtccgacgatcNNN**UGGAA**.

DNA bases are in lowercase, N is a random DNA base, and RNA bases are in uppercase bold with the unique barcode underlined.

5 mM Adenosine triphosphate (ATP).

2.4 Reverse Transcription and Library Amplification

5′ PCR primer (18.206): CAAGCAGAAGACGGCATA.

3′ PCR primer (44.45): AATGATACGGCGACCACCGACAG GTTCAGAGTTCTACAGTCCGA.

Reverse transcription kit with enzyme and buffer (such as SuperScript III, Invitrogen).

2 mM dNTPs.

High fidelity DNA polymerase enzyme with buffer (such as Phusion, New England Biolabs).

10 and 50 bp DNA ladders.

1× Tris–Borate–EDTA (TBE) buffer.

Ethidium bromide (EtBr).

10 mM Tris pH 8.

1× glycerol loading dye.

2.5 Library Validation (Optional)

Blunt-end or TA cloning kit.

2.6 Radioactive Labeling of RNA Oligo Size Markers

18mer RNA oligo: AGCGUGUAGGGAUCCAAA.

32mer RNA oligo: GGCAUUAACGCGGCCGCUCUACAAUA GUGA.

Polynucleotide kinase (PNK) enzyme with buffer.

^{32}P γ–Adenosine triphosphate (^{32}P γ–ATP).

2.7 3′ Adapter Synthesis

100 μM of 3′ adapter.

5′ DNA Adenylation Kit (New England Biolabs): 10× DNA Adenylation Buffer, 1 mM ATP, and Mth RNA Ligase.

3 Methods

Carry out all procedures at room temperature unless otherwise specified.

3.1 Preparation of RNA

1. Before beginning library preparation, isolate high-quality total RNA from *C. elegans* samples (*see* **Note 1**). If capture of all types of *C. elegans* endo-siRNAs is desired (*see* Subheading 1), proceed to treatment of the total RNA with tobacco acid pyrophosphatase (TAP) below. If cloning of Dicer-generated primary endo-siRNAs only is desired, proceed to Subheading 3.2.
2. TAP enzyme converts the 5′ triphosphate cap that is present on RdRP generated endo-siRNAs to a 5′ monophosphate that is compatible with the 5′ ligation procedure described here. Manufacturer's instructions should be followed for RNA treatment in a volume <100 μL (*see* **Note 2**).
3. Add water to the stopped-TAP treatment reaction for a total volume of 100 μL (*see* **Note 3**). Ethanol-precipitate treated RNA by first adding 10 μL 3 M sodium acetate and then 250 μL of 100 % ethanol, mixing well after addition of each reagent. Incubate the sample at −20 °C for at least 30 min, and then spin the sample in a microcentrifuge at the maximum speed for 10 min at 4 °C. You should be able to visualize a clear to white pellet. Remove supernatant and allow RNA pellet to dry for ~5 min. Resuspend RNA in 9 μL of water.

3.2 3′ Adapter Ligation and Purification of Samples

1. Assemble separate 3′ adapter ligation reactions with experimental samples and radiolabeled 18-mer and 32-mer RNA oligos (*see* Subheading 3.6 for generation of labeled size oligos and Subheading 3.7 for generation of 3′ adapters). For experimental samples, add 9 μL of total RNA (2–3 μg/μL), 1 μL of 100 μM 3′ adapter, 4 μL 5× ligation buffer (no ATP), 2 μL T4 single-stranded RNA Ligase, 1 μL RNase Inhibitor (optional), and H_2O up to 20 μL. For reactions with RNA marker oligos, modify components above to add the 18-mer or 32-mer oligos and adjust water accordingly. Incubate reaction at 18 °C for 2–4 h.
2. Gel-purify the adapter ligation reaction products on a 15 % polyacrylamide gel. Run lanes of unligated size markers in addition to 3′ adapter ligated size markers (*see* **Note 4** and Fig. 2). Run the gel at 30 W for ~1 h. Disassemble gel apparatus and adhere gel to plastic wrap. Expose the gel to a PhosphorImager

screen for ~15–30 min. Scan the PhosphorImager screen and print the image at 100 % zoom to act as a template for gel extraction. Place the plastic wrap and gel on top of the gel image. Using the gel image as a guide, cut out the gel region corresponding to the size range between 3′ adapter ligated 18-mer and 3′ adapter ligated 32-mer and put into separate 1.5 mL microcentrifuge tubes, using a new razor blade for each sample (*see* **Note 5** and Fig. 2). Also cut out the gel regions corresponding to the 3′ adapter ligated radiolabeled markers.

3. Elute RNA from gel slices overnight using 500 μL of 0.3 M NaCl per 1.5 mL microcentrifuge tube with shaking or rotation at 4 °C. Move the supernatant to a new microcentrifuge tube taking care to avoid the gel slices. Add 40 μg of glycogen and 1 mL of 100 % EtOH. Mix the reagents well and incubate at −20 °C for >1 h. Spin the samples in a microcentrifuge at maximum speed for 20 min at 4 °C. A whitish pellet should be visible. Carefully remove supernatant and air-dry pellet for ~5 min. Resuspend the RNA in 10 μL of H_2O. For the 18-mer and 32-mer ligations, resuspend the RNA pellets in 20 μL of H_2O.

3.3 5′ Adapter Ligation of Experimental Samples

Next-generation sequencing technology allows for the generation of several hundred million short DNA reads in a single run. The remarkable depth in the number of reads allows for the analysis of different experimental samples in parallel simply by the introduction of a unique identifier or barcode in the 5′ adapter that is ligated to the sRNAs during the cloning procedure.

1. To proceed with the 5′ adapter ligations, assemble the following reaction for each experimental sample and radiolabeled marker: 10 μL of purified 3′ ligation product, 4 μL 100 μM 5′ adapter, 4 μL 5× T4 RNA ligase buffer, 1 μL 5 mM ATP, and 1 μL T4 single-stranded RNA ligase. Incubate the reaction at 22 °C overnight.
2. Check for the completion of 5′ adapter ligation reaction by examining the ligation products of radiolabeled size markers on a 15 % polyacrylamide gel. Few side products are generated during this ligation reaction. Include the remaining 10 μL of 3′ adapter ligated products in separate lanes to verify the shift to a larger product in the 5′ and 3′ adapter ligated products. (*Optional*: You can also gel-purify the experimental sample ligations at this step if desired.) Run the gel at 30 W for ~1 h. If the 5′ adapter ligation reaction was successful, then ethanol-precipitate (or gel-extract) ligated experimental RNA products with glycogen. Resuspend pellet in 10 μL H_2O (*see* Subheading 3.2, **step 3**).

3.4 Reverse Transcription and Library Amplification

1. On ice, assemble a first strand reverse transcription reaction for each experimental sample. For best results, incubate reactions in a thermocycler. For each reaction, add in order: 10 μL purified 5′, 3′ adapter-ligated RNA sample, 1 μL 100 μM 5′ PCR primer, and 5 μL RNase-free H_2O. Heat the reaction to 65 °C for 5 min and cool on ice. Centrifuge the reactions to collect condensation before proceeding.
2. On ice, add the following reagents in order to the cooled reactions: 6 μL 5× first strand buffer, 6 μL 2 mM dNTPs, 2 μL 100 mM DTT, and 1 μL reverse transcriptase enzyme. Incubate the reaction at 50 °C for 1 h, 85 °C for 15 min, then cool to 4 °C (*see* **Note 6**).
3. After reverse transcription reaction is completed, immediately proceed to second strand extension. On ice, assemble a PCR reaction with the following reagents: 5 μL of first strand reverse transcription reaction from Subheading 3.4, **step 2**, 10 μL 5× PCR buffer, 6 μL 2 mM dNTPs, 2 μL 150 nM 3′ PCR primer, 25 μL RNase-free H_2O, and 1 μL high fidelity DNA polymerase. Perform second strand extension under the following conditions: 98 °C for 3 min, 5 cycles of 94 °C for 45 s, 60 °C for 45 s, and 72 °C for 45 s, then a final extension of 72 °C for 10 min. Cool the reaction to 10 °C in the thermocycler or on ice.
4. Next, add the following additional reagents to each reaction for library amplification: 10 μL 5× PCR buffer, 6 μL 2 mM dNTPs, 0.5 μL 25 μM 3′ PCR primer, 0.5 μL 25 μM 5′ PCR primer, 25 μL H_2O, and 1 μL high fidelity DNA polymerase. Perform library amplification under the following conditions: 98 °C for 3 min, 15–20 cycles of 94 °C for 30 s, 60 °C for 30 s, and 72 °C for 15 s, then a final extension of 72 °C for 10 min. Cool samples to 10 °C in the thermocycler or on ice.
5. Precipitate cDNA by adding 40 μg glycogen, 10 μL 3 M NaOAc, and 200 μL 100 % EtOH, mixing after addition of each reagent. Incubate the samples at −20 °C for >1 h, then spin at maximum speed in a microcentrifuge for 20 min at 4 °C. Remove supernatant and air-dry pellet for ~5 min. Resuspend cDNA in 15 μL of 1× glycerol loading dye.
6. Gel-extract the cDNA library using a 10 % native polyacrylamide gel. Load the samples near 10 and 50 bp DNA ladders, leaving empty lanes adjacent to the experimental samples to prevent contamination during gel extraction (Fig. 2). Run the gel in 1× TBE buffer at constant 25 W until the dye front reaches the bottom of the gel. Stain the gel with 4 μg/mL EtBr in 0.5× TBE buffer. Drain the EtBr stain from the gel and image on a gel documentation system. Using the gel image as a guide, cut out the gel pieces corresponding to 85–110 bp and place in separate microcentrifuge tubes (*see* **Note 7**).

7. Elute the cDNA library from the gel slices with 3 M NaCl overnight at room temperature (*see* Subheading 3.2, **step 3**). Ethanol-precipitate the cDNA with glycogen and resuspend in 15 μL of 10 mM Tris pH 8. Quantitate the concentrations of small RNA libraries. The desired concentration is >20 ng/μL with a total >200 ng DNA.

3.5 Library Validation (Optional)

1. Following the instructions of the blunt-end or TA ligation cloning kit, clone 1 μL of purified cDNA library sample for further validation. Purify the plasmid from 12 to 24 bacterial colonies per library samples and analyze by Sanger sequencing.
2. Use NCBI BLAST to determine sequence contents of plasmids. Libraries should contain clones of desired sRNAs to warrant sequencing.

3.6 Radioactive Labeling of RNA Oligo Size Markers

1. Assemble a separate polynucleotide kinase (PNK) reaction for each RNA oligo size marker: 1 μL of 10 μM RNA oligo (32mer or 18mer), 2 μL of 10× PNK buffer, 14 μL of RNase-free H_2O, 2 μL ^{32}P γ-ATP, and 1 μL T4 PNK (10 U/μL). Incubate reactions for 1 h at 37 °C.
2. Purify radiolabeled RNA oligos using a 15 % polyacrylamide gel. Run the gel at a constant 25 W in 0.5× TBE buffer until bromophenol blue dye front migrates to 1 in. from the bottom to eliminate excess radioactive ATP. Gel-extract and elute labeled RNA oligos (*see* Subheading 3.2, **step 3**). Resuspend the gel-purified RNA oligos in 20 μL H_2O.

3.7 3′ Adapter Synthesis

1. The 3′ adapter used for the preparation of sRNA libraries is a modified DNA oligonucleotide containing a 5′,5′-adenylpyrophosphoryl moiety and a blocking group (e.g., amine or dideoxynucleotides) at the 3′ end. The use of pre-adenylated oligodeoxynucleotides in the 3′ adapter ligation step allows the adapter to be ligated to the sRNAs by T4 RNA ligase in the absence of ATP, thus preventing the sRNA from self-ligating or concatamerizing. While these adenylated DNA oligos can be purchased from a vendor, synthesizing them as described below can be more cost-effective.
2. Adenylate the 3′ adapter following the instructions of the 5′ DNA Adenylation Kit: 100 μM of 3′ adapter, 2 μL of 10× DNA Adenylation Buffer, 2 μL of 1 mM ATP, 2 μL of Mth RNA Ligase (100 pmol), and RNase-free water to a volume of 20 μL.
3. Incubate the reaction at 65 °C for 1 h. Inactivate the enzyme by incubating at 85 °C for 5 min.

4 Notes

1. Trizol reagent (Invitrogen) works well to isolate high-quality RNA from *C. elegans* samples. The 260–280 absorbance ratio should be between 1.8 and 2.0 for high-quality RNA samples.
2. Starting with 20–30 μg of total RNA is ideal for small RNA gel extraction, and should be treated by TAP enzyme if the capture of all endo-siRNAs is desired.
3. Purification of the RNA by phenol–chloroform extraction is not necessary.
4. During this ligation step, side products are generated from ligations of just the radiolabeled oligo markers to themselves. Run unligated markers in separate lanes from the 3′ adapter ligated oligos to assist in size selection of RNA from the experimental samples. The side products will migrate faster than the unligated marker, or will appear too large (two markers concatamerizing).
5. Elution of RNA from the gel can be facilitated by cutting the larger slice into smaller pieces. However, make sure that the slices remain large enough so can be easily avoided while pipetting.
6. Instructions for the reverse transcription and PCR reactions described in this chapter are based on the optimized protocol for Superscript III reverse transcriptase (Invitrogen) and Phusion DNA polymerase (New England Biolabs). If using different enzymes, follow the manufacturer's instructions for the reactions.
7. If the starting material from the reverse transcription reaction is low, often a primer-dimer artifact (~70 bp long) will amplify and dominate the reaction, which can be identified during the gel purification step (Fig. 2). If this occurs, gel-purify the desired products between 85 and 110 bp, and use half of the resuspended sample for further PCR reamplification starting from Subheading 3.4, **step 4**.

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