Supplementary Notes 1-4 for "Gel-free library preparation for next-generation RNA sequencing and small RNA quantification" by Ruixi Chen, Daniel Yim, Lili Liu, Bo Cao, Peter C. Dedon

Supplementary Note 1: Detailed procedures of the protocol variant 1 (V1)

It is recommended to perform a comprehensive evaluation of purity, integrity, and quantity of the input RNA samples using (i) 3% agarose gel electrophoresis, (ii) Agilent Bioanalyzer (with small RNA and Pico RNA chips), and (iii) NanoDrop Spectrophotometer and/or Qubit. All reactions and master mixes should be kept on ice, unless otherwise specified. It is recommended to transport reagents (e.g., enzymes) in cool boxes to and from -20ºC storage to preserve their shelf lives. Multiple freeze thaw cycles of library intermediates should be avoided.

1. **Dephosphorylation of RNAs**
   - 1.1 Prepare the dephosphorylation reaction in a sterile PCR tube (e.g., 200-µL or 500-µL tube) by adding up to 2 µL of the RNA sample, 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 0.5 µM internal standard, 0.5 µL of 10X T4 RNA ligase reaction buffer, 1 µL of 1 U/µL shrimp alkaline phosphatase, and sufficient RNase-free water to bring the reaction volume to 5 µL.
   - 1.2 Incubate at 37ºC for 30 min to dephosphorylate RNAs and then at 65ºC for 5 min to inactivate the enzyme and denature the RNAs. Keep samples at 4ºC for at least 10 min to prevent renaturation.

2. **Ligation of Linker 1 to the 3' end of RNAs**
   - 2.1 Prepare the Linker 1 ligation reaction in a sterile PCR tube by adding 5 µL of the dephosphorylated RNAs (products from step 1), 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 100 µM Linker 1, 3 µL of 10 mM ATP, 2.5 µL of 10X T4 RNA ligase reaction buffer, 15 µL of PEG8000 (50% solution), 2 µL of 30 U/µL T4 RNA ligase 1, and 1 µL of RNase-free water.
   - 2.2 Incubate at 25ºC for 2 h and then at 16ºC for 16 h to ligate Linker 1 to the RNAs.
   - 2.3 Column-purify the Linker-1-ligated RNAs.

     NOTE: The Oligo Clean & Concentrator Kit is used in this step. This column purification protocol applies to all subsequent column purification using the same kit.
     - 2.3.1 For sample volume < 50 µL, add RNase-free water to bring it to 50 µL.
     - 2.3.2 Add 2 volumes of the Oligo Binding Buffer (provided in the kit) to 1 volume of the sample.
     - 2.3.3 Add 8 volumes of 100% ethanol (not provided in the kit) to 1 volume of the sample.

2.3.4 Load the sample (up to 750 µL at a time) to a column placed inside a 2-mL collection tube (provided in the kit).

2.3.5 To bind RNAs to the column, centrifuge at 10,000 x g for 30 sec and discard the flow-through (i.e., the liquid in the collection tube). Place the column back into the collection tube.

2.3.6 To wash the impurities off the column, add 750 µL of the DNA Wash Buffer (provided in the kit) to the column. Centrifuge at 10,000 x g for 30 sec and discard the flow-through. Place the column back into the collection tube.

2.3.7 Centrifuge at the maximum speed (e.g., 16,000 x g for a benchtop microcentrifuge) for 1 additional minute to remove residual DNA Wash Buffer.

2.3.8 To elute the RNAs, carefully move the column to a sterile 1.5-mL tube and add RNase-free water to the column. Use a greater volume than needed when eluting the RNAs to account for potential volume loss during the elution process. For instance, if 15 µL is required for the following step, add 17 µL of RNase-free water for elution. Centrifuge at 10,000 x g for 30 sec.

3 Ligation of Linker 1 to the 3' end of RNAs

3.1 Prepare a stock solution of 1 M 2-ketoglutarate by dissolving 1.4611 g of 2-ketoglutarate (146.11 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.2 Prepare a stock solution of 0.5 M L-ascorbic acid by dissolving 0.88 g of L-ascorbic acid (176.12 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.3 Prepare a stock solution of 0.25 M ammonium ferrous sulfate hexahydrate by dissolving 0.9835 g of ammonium ferrous sulfate hexahydrate (392.14 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.4 Prepare a stock solution of 1 M HEPES by dissolving 2.383 g of HEPES (238.30 g per M) in 10 mL of RNase-free water. Adjust the pH of the solution to 8 using sodium hydroxide (NaOH) and filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.5 Prepare a 2X AlkB reaction buffer. To make 10 mL of the buffer, combine 1.5 µL of 1 M 2-ketoglutarate (made in step 3.1), 80 µL of 0.5 M L-ascorbic acid (made in step 3.2), 6 µL of 0.25 M ammonium ferrous sulfate hexahydrate (made in step 3.3), 100 µL of 10 mg/mL BSA, 1000 µL of 1 M HEPES (made in step 3.4; add last), and 8812.5 µL of RNase-free water. Filter-sterilize the buffer through a 0.2 µm syringe filter.

NOTE: The 2x AlkB reaction buffer must be prepared fresh immediately prior to each experiment due to the chemical lability of the components.

3.6 Prepare the AlkB digestion reaction in a sterile PCR tube by adding 20 µL of the Linker-1-ligated RNAs (products from step 2), 50 µL of the 2x AlkB reaction buffer (made in step 3.5), 2 µL of AlkB demethylase, 1 µL of RNase inhibitor, and 27 µL of RNase-free water.

3.7 Incubate at room temperature for 2 h to remove post-transcriptional methylations from the RNAs.

3.8 To remove AlkB from the reaction, follow the steps described below.

3.8.1 For a clean phase separation, add 50 µL of RNase-free water into the AlkB reaction, and then add 100 µL of phenol: chloroform: isoamyl alcohol 25:24:1 (pH = 5.2).

3.8.2 Shake by hand for 10 s, and then centrifuge at 16,000 x g for 10 min. Make sure that the rotor of the benchtop centrifuge is compatible with the PCR tubes. Use adaptors if needed.

3.8.3 Transfer the RNAs (i.e., the aqueous layer on top; approximately 140 µL) into a sterile 1.5 mL tube. If chloroform (i.e., the bottom layer) is mixed into the aqueous layer, centrifuge again with the same settings.

3.8.4 Add 100 µL of chloroform to the extracted RNAs to remove residual phenol. Shake by hand for 10 s, and then centrifuge at 16,000 x g for 10 min.

3.8.5 Transfer the RNAs (i.e., the aqueous layer on top; approximately 120 µL) into a sterile 1.5 mL tube.

3.8.6 Column-purify the extracted RNAs. Use a kit for DNA/RNA recovery and clean-up (see Table of Materials).

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

4 Removal of excess Linker 1

4.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 15 µL of Linker-1-ligated RNAs (products from step 3), 1 µL of 40 U/µL RNase inhibitor, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

4.2 Incubate at 30ºC for 1 h to remove the adenine at the 5’ end of Linker 1.

4.3 Add 2 µL of 30 U/µL RecJf into the deadenylation reaction.

4.4 Incubate at 37ºC for 30 min to digest excess Linker 1.

4.5 Add another 2 µL of 30 U/µL RecJf into the reaction.

4.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 1 and then at 65ºC for 20 min to denature the enzyme.

4.7 Column-purify the Linker-1-ligated RNAs.

NOTE: DyeEx 2.0 Spin Kit is used in this step, as it is effective at removing short remnants (e.g., oligonucleotides with a length of 2 to 10 bp).

4.7.1 Prepare DyeEx columns according to the manufacturer's protocol.

4.7.2 Place a column into a sterile 1.5-mL tube and load the column with 24 µL of the sample.

4.7.3 To purify the RNAs, centrifuge at 800 x g for 3 min and discard the column. The purified RNAs are in the eluent.

5 Reverse transcription (RT)

5.1 Prepare the RT primer annealing reaction in a sterile PCR tube by adding 24 µL of template RNAs (products from step 4), 1 µL of 2 µM RT primer, and 1 µL of dNTP (10 mM of each type of nucleotides).

5.2 Incubate at 80ºC for 2 min to anneal RT primers to the template RNAs, and then cool on ice immediately for 2 min.

5.3 Prepare the RT reaction by adding 6 µL of 5X PrimeScript RT reaction buffer, 1 µL of 40 U/µL RNase inhibitor, and 1 µL of 200 U/µL PrimeScript reverse transcriptase into the annealing reaction tube.

5.4 Incubate at 50ºC for 2 h to reverse transcribe the RNA templates, and then at 70ºC for 15 min to inactivate the enzyme. The RT products (i.e., RNA-cDNA hybrids) can be stored at 4ºC or -20ºC overnight.

6 RNA hydrolysis

6.1 Add 1 µL of 5 M NaOH into the RNA-cDNA hybrid (products from step 5).

6.2 Incubate at 93ºC for 3 min to hydrolyze the RNA strand of the RNA-cDNA hybrid.

6.3 Neutralize the reaction with 0.77 µL of 5 M hydrochloric acid (HCl).

NOTE: It is recommended to test the precise amount of 5 M HCl that is needed for neutralizing 1 µL of NaOH (e.g., using pH strips) in buffered conditions. After adding HCl, flick to mix, and spin down the tube. The neutralization is instant.

6.4 Column-purify the single-stranded cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

6.5 Speed-vac the purified cDNAs to <5 µL and then add RNase-free water to bring the volume back to 5 µL.

6.6 Transfer the purified cDNAs into a sterile PCR tube. The purified cDNAs can be stored at -20ºC for up to one week.

7. Ligation of Linker 2 to the 3' end of cDNAs

7.1 Prepare the Linker 2 ligation reaction in a sterile PCR tube by adding 5 µL of cDNAs (products from step 6), 1 µL of 50 µM Linker 2, 2 µL of 10X T4 DNA ligase reaction

buffer, 1 µL of 10 mM ATP, 9 µL of PEG8000 (50% solution), and 2 µL of 400 U/µL T4 DNA ligase.

7.2 Incubate at 16ºC for 16 h to ligate Linker 2 to the cDNAs.

7.3 Column-purify the Linker-2-ligated cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

8. Removal of excess Linker 2

8.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 16 µL of Linker-2-ligated cDNAs, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

8.2 Incubate at 30 °C for 1 h to remove the adenine at the 5' end of Linker 2.

8.3 Add 2 µL of RecJf into the deadenylation reaction.

8.4 Incubate at 37ºC for 30 min to digest excess Linker 2.

8.5 Add another 2 µL of RecJf into the reaction.

8.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 2 and then at 65ºC for 20 min to denature the enzyme.

9. PCR amplification of the cDNA library with sequencing adaptors

9.1 Assign PCR primers to the samples. Each sample needs a unique pair of forward and reverse primers for effective multiplexing.

9.2 Add RNase-free water to bring the sample volume to 25 µL. Save 5 µL of each sample into a sterile PCR tube as a backup in case PCR needs to be repeated.

9.3 Prepare the PCR reaction by adding 20 µL of cDNAs (products from step 8), 1 µL of 2.5 µM forward primer, 1 µL of 2.5 µM reverse primer, 25 µL of 2X DNA polymerase buffer, 2 µL of RNase-free water, and 1 µL of DNA polymerase.

9.4 Perform PCR with an initial denaturation at 94ºC for 1 min, followed by 18 cycles of denaturation (98ºC; 20 sec), annealing (58ºC; 20 sec), and extension (68ºC; 1 min).

9.5 Speed-vac the PCR products to less than 25 µL and then add RNase-free water to bring the volume back to 25 µL. Transfer 5 µL of the PCR products to a sterile tube for checking the size distribution (see step 9.6). Store the remaining 20 µL of the PCR products at -20ºC until further steps.

9.6 Check the size distribution of the cDNA libraries following PCR amplification.

9.6.1 Prepare 3% agarose gel in TAE buffer.

9.6.2 Mix 1 µL of 6X loading dye into 5 µL of PCR products (from step 9.5) and load the gel with the mixture.

9.6.3 Load 5 µL of DNA ladders into the well before the first sample and the well after the last sample. Use 50-bp and/or 100-bp DNA ladders to allow for improved size discrimination of PCR products between 150 bp and 350 bp.

9.6.4 Run the gel electrophoresis to locate the PCR products.

NOTE: The appropriate running condition may be context dependent. We have found 120 V, 400 mA, and 75 min to be sufficient for a 17.78 cm (width) × 10.16 cm (height) × 1 cm (thickness) gel slab. For smaller gel slabs, the running time may need to be reduced.

9.6.5 Place the gel in a box and fill the box with deionized (DI) water until the gel is completely immersed. Add 10 µL of ethidium bromide (EtBr) into the DI water soaking the gel. Wrap the box with foil and place it on a shaker. Stain the gel for 30 min with shaking.

9.6.6 Discard the EtBr-containing waste into a waste bottle placed in a fume hood. Rinse the gel with DI water once and discard the EtBr-containing water into the waste bottle.

9.6.7 Fill the box with DI water until the gel is completely immersed. Wrap the box with foil and place it on a shaker. Wash the gel for 10 min with shaking.

9.6.8 Discard the EtBr-containing water into the waste bottle in the fume hood.

9.6.9 Use a gel imager to visualize the bands. Acquire a high-resolution image of the gel.

10. Gel purification

10.1 Prepare 3% agarose gel in TAE buffer. Make a 1 cm thickness gel with wide combs (1 mm thickness; 5 mm width; 15 mm depth), such that each well can contain at least 25 µL of sample-loading dye mixture.

10.2 Mix 4 µL of 6X loading dye with 20 µL of PCR products (from step 9.5) and load the gel with the mixture. Leave empty lanes in between samples to minimize cross contamination during gel excision.

10.3 Load DNA ladders, run gel electrophoresis, stain and wash the gel, and take gel images as described in step 9.6.

10.4 Excise the gel blocks that contain PCR products within the target size range. To minimize contamination with primer dimers (175 bp linkers with no inserts), extract PCR products with size above 195 bp (175 bp linkers + 20 bp miRNAs).

10.5 Purify the PCR products using gel extraction.

NOTE: The QIAquick Gel Extraction Kit is used. The purification protocol is based on the manufacturer's protocol, with minor modifications for AQRNA-seq compatibility. All centrifugation steps should be conducted at 17,900 x g for 1 min using a benchtop centrifuge at room temperature, unless otherwise specified. Follow the steps described below.

10.5.1 Measure the weight of the gel blocks inside the tubes. Add 6 volumes of Buffer QG (provided in the kit) to 1 volume of gel block (1 mg gel is approximately 1 µL).

10.5.2 Incubate at 50 °C for 10 min or until complete dissolution of the gel blocks. Vortex tubes every 2 min to facilitate gel dissolution. After dissolving the gel, the mixture should resemble the color of the Buffer QG without the dissolved gel. If the color is orange or violet, add 10 µL of 3 M sodium acetate (pH = 5.0) and mix thoroughly.

10.5.3 Add 1 gel volume of isopropanol (not provided in the kit) to the mixture and mix thoroughly. Place a spin column in a 2 mL collection tube (provided in the kit).

10.5.4 To bind DNA, apply the sample (up to 750 µL each time) to the column and centrifuge. Discard the flowthrough and place the column back into the same collection tube. The maximum amount of gel per spin column is 400 mg.

10.5.5 Add 500 µL of Buffer QG to the column and centrifuge. Discard the flow-through and place the column back into the same collection tube.

10.5.6 To wash the impurities, add 750 µL of Buffer PE (provided in the kit) to the column, let the column stand for 5 min, and centrifuge. Discard the flowthrough and place the column back into the same collection tube. Centrifuge once again to remove residual wash buffer.

10.5.7 Place the column into a sterile 1.5-mL tube. To elute DNA, add 30 µL of Buffer EB (provided in the kit) to the center of the column membrane, let the column stand for 4 min, and centrifuge.

10.6 Measure the concentration of the constructed libraries.

Supplementary Note 2: Detailed procedures of the protocol variant 2 (V2)

It is recommended to perform a comprehensive evaluation of purity, integrity, and quantity of the input RNA samples using (i) 3% agarose gel electrophoresis, (ii) Agilent Bioanalyzer (with small RNA and Pico RNA chips), and (iii) NanoDrop Spectrophotometer and/or Qubit. All reactions and master mixes should be kept on ice, unless otherwise specified. It is recommended to transport reagents (e.g., enzymes) in cool boxes to and from -20ºC storage to preserve their shelf lives. Multiple freeze thaw cycles of library intermediates should be avoided.

3. Dephosphorylation of RNAs

3.1 Prepare the dephosphorylation reaction in a sterile PCR tube (e.g., 200-µL or 500-µL tube) by adding up to 2 µL of the RNA sample, 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 0.5 µM internal standard, 0.5 µL of 10X T4 RNA ligase reaction buffer, 1 µL of 1 U/µL shrimp alkaline phosphatase, and sufficient RNase-free water to bring the reaction volume to 5 µL.

3.2 Incubate at 37ºC for 30 min to dephosphorylate RNAs and then at 65ºC for 5 min to inactivate the enzyme and denature the RNAs. Keep samples at 4ºC for at least 10 min to prevent renaturation.

4. Ligation of Linker 1 to the 3' end of RNAs

4.1 Prepare the Linker 1 ligation reaction in a sterile PCR tube by adding 5 µL of the dephosphorylated RNAs (products from step 1), 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 100 µM Linker 1, 3 µL of 10 mM ATP, 2.5 µL of 10X T4 RNA ligase reaction buffer, 15 µL of PEG8000 (50% solution), 2 µL of 30 U/µL T4 RNA ligase 1, and 1 µL of RNase-free water.

4.2 Incubate at 25ºC for 2 h and then at 16ºC for 16 h to ligate Linker 1 to the RNAs.

4.3 Column-purify the Linker-1-ligated RNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. This column purification protocol applies to all subsequent column purification using the same kit.

4.3.1 For sample volume < 50 µL, add RNase-free water to bring it to 50 µL.

4.3.2 Add 2 volumes of the Oligo Binding Buffer (provided in the kit) to 1 volume of the sample.

4.3.3 Add 8 volumes of 100% ethanol (not provided in the kit) to 1 volume of the sample.

4.3.4 Load the sample (up to 750 µL at a time) to a column placed inside a 2-mL collection tube (provided in the kit).

4.3.5 To bind RNAs to the column, centrifuge at 10,000 x g for 30 sec and discard the flow-through (i.e., the liquid in the collection tube). Place the column back into the collection tube.

4.3.6 To wash the impurities off the column, add 750 µL of the DNA Wash Buffer (provided in the kit) to the column. Centrifuge at 10,000 x g for 30 sec and discard the flow-through. Place the column back into the collection tube.

4.3.7 Centrifuge at the maximum speed (e.g., 16,000 x g for a benchtop microcentrifuge) for 1 additional minute to remove residual DNA Wash Buffer.

4.3.8 To elute the RNAs, carefully move the column to a sterile 1.5-mL tube and add RNase-free water to the column. Use a greater volume than needed when eluting the RNAs to account for potential volume loss during the elution process. For instance, if 15 µL is required for the following step, add 17 µL of RNase-free water for elution. Centrifuge at 10,000 x g for 30 sec.

5. Removal of excess Linker 1

5.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 15 µL of Linker-1-ligated RNAs (products from step 2), 1 µL of 40 U/µL RNase inhibitor, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

5.2 Incubate at 30ºC for 1 h to remove the adenine at the 5’ end of Linker 1.

5.3 Add 2 µL of 30 U/µL RecJf into the deadenylation reaction.

5.4 Incubate at 37ºC for 30 min to digest excess Linker 1.

5.5 Add another 2 µL of 30 U/µL RecJf into the reaction.

5.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 1 and then at 65ºC for 20 min to denature the enzyme.

5.7 Column-purify the Linker-1-ligated RNAs.

NOTE: DyeEx 2.0 Spin Kit is used in this step, as it is effective at removing short remnants (e.g., oligonucleotides with a length of 2 to 10 bp).

3.8.1 Prepare DyeEx columns according to the manufacturer’s protocol.

3.8.2 Place a column into a sterile 1.5-mL tube and load the column with 24 µL of the sample.

3.8.3 To purify the RNAs, centrifuge at 800 x g for 3 min and discard the column. The purified RNAs are in the eluent.

6. Reverse transcription (RT)

NOTE: The following RT reaction protocol follows the manufacturer’s protocol of the SuperScript™ IV Reverse Transcriptase, with minor modifications to allow for AQRNA-seq compatibility.

6.1 Prepare the RT primer annealing reaction in a sterile PCR tube by adding 22 µL of template RNAs (products from step 3), 2 µL of 2 µM RT primer, and 2 µL of dNTP (10 mM of each type of nucleotides).

6.2 Incubate at 65ºC for 5 min to anneal RT primers to the template RNAs, and then cool on ice immediately for 2 min.

6.3 Prepare the RT reaction by adding 8 µL of 5X SuperScript™ IV RT Reaction Buffer, 2 µL of 100 mM DTT, 2 µL of 40 U/µL ribonuclease inhibitor, and 2 µL of 200 U/µL SuperScript® IV Reverse Transcriptase into the annealing reaction tube.

6.4 Incubate at 50ºC for 2 h to reverse transcribe the RNA templates, and then at 80ºC for 10 min to inactivate the enzyme. The RT products (i.e., RNA-cDNA hybrids) can be stored at 4ºC or -20ºC overnight.

7. RNA hydrolysis

7.1 Add 1.18 µL of 5 M sodium hydroxide (NaOH) into the RNA-cDNA hybrid (products from step 4).

7.2 Incubate at 93ºC for 3 min to hydrolyze the RNA strand of the RNA-cDNA hybrid.

7.3 Neutralize the reaction with 0.91 µL of 5 M hydrochloric acid (HCl).

NOTE: It is recommended to test the precise amount of 5 M HCl that is needed for neutralizing 1.18 µL of NaOH (e.g., using pH strips) in buffered conditions. After adding HCl, flick to mix, and spin down the tube. The neutralization is instant.

7.4 Column-purify the single-stranded cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

7.5 Speed-vac the purified cDNAs to <5 µL and then add RNase-free water to bring the volume back to 5 µL.

7.6 Transfer the purified cDNAs into a sterile PCR tube. The purified cDNAs can be stored at -20ºC for up to one week.

8. Ligation of Linker 2 to the 3' end of cDNAs

8.1 Prepare the Linker 2 ligation reaction in a sterile PCR tube by adding 5 µL of cDNAs (products from step 6), 1 µL of 50 µM Linker 2, 2 µL of 10X T4 DNA ligase reaction buffer, 1 µL of 10 mM ATP, 9 µL of PEG8000 (50% solution), and 2 µL of 400 U/µL T4 DNA ligase.

8.2 Incubate at 16ºC for 16 h to ligate Linker 2 to the cDNAs.

8.3 Column-purify the Linker-2-ligated cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

9. Removal of excess Linker 2

9.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 16 µL of Linker-2-ligated cDNAs, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

9.2 Incubate at 30 °C for 1 h to remove the adenine at the 5' end of Linker 2.

9.3 Add 2 µL of RecJf into the deadenylation reaction.

9.4 Incubate at 37ºC for 30 min to digest excess Linker 2.

9.5 Add another 2 µL of RecJf into the reaction.

9.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 2 and then at 65ºC for 20 min to denature the enzyme.

10. PCR amplification of the cDNA library with sequencing adaptors

10.1 Assign PCR primers to the samples. Each sample needs a unique pair of forward and reverse primers for effective multiplexing.

10.2 Add RNase-free water to bring the sample volume to 25 µL. Save 5 µL of each sample into a sterile PCR tube as a backup in case PCR needs to be repeated.

10.3 Prepare the PCR reaction by adding 20 µL of cDNAs (products from step 8), 1 µL of 2.5 µM forward primer, 1 µL of 2.5 µM reverse primer, 25 µL of 2X DNA polymerase buffer, 2 µL of RNase-free water, and 1 µL of DNA polymerase.

10.4 Perform PCR with an initial denaturation at 94ºC for 1 min, followed by 18 cycles of denaturation (98ºC; 20 sec), annealing (58ºC; 20 sec), and extension (68ºC; 1 min).

10.5 Speed-vac the PCR products to less than 25 µL and then add RNase-free water to bring the volume back to 25 µL. Transfer 5 µL of the PCR products to a sterile tube for checking the size distribution (see step 9.6). Store the remaining 20 µL of the PCR products at -20ºC until further steps.

10.6 Check the size distribution of the cDNA libraries following PCR amplification.

8.6.1 Prepare 3% agarose gel in TAE buffer.

8.6.2 Mix 1 µL of 6X loading dye into 5 µL of PCR products (from step 9.5) and load the gel with the mixture.

8.6.3 Load 5 µL of DNA ladders into the well before the first sample and the well after the last sample. Use 50-bp and/or 100-bp DNA ladders to allow for improved size discrimination of PCR products between 150 bp and 350 bp.

8.6.4 Run the gel electrophoresis to locate the PCR products.

NOTE: The appropriate running condition may be context dependent. We have found 120 V, 400 mA, and 75 min to be sufficient for a 17.78 cm (width) × 10.16 cm (height) × 1 cm (thickness) gel slab. For smaller gel slabs, the running time may need to be reduced.

8.6.5 Place the gel in a box and fill the box with deionized (DI) water until the gel is completely immersed. Add 10 µL of ethidium bromide (EtBr) into the DI water

soaking the gel. Wrap the box with foil and place it on a shaker. Stain the gel for 30 min with shaking.

8.6.6 Discard the EtBr-containing waste into a waste bottle placed in a fume hood. Rinse the gel with DI water once and discard the EtBr-containing water into the waste bottle.

8.6.7 Fill the box with DI water until the gel is completely immersed. Wrap the box with foil and place it on a shaker. Wash the gel for 10 min with shaking.

8.6.8 Discard the EtBr-containing water into the waste bottle in the fume hood.

8.6.9 Use a gel imager to visualize the bands. Acquire a high-resolution image of the gel.

11. Gel purification

11.1 Prepare 3% agarose gel in TAE buffer. Make a 1 cm thickness gel with wide combs (1 mm thickness; 5 mm width; 15 mm depth), such that each well can contain at least 25 µL of sample-loading dye mixture.

11.2 Mix 4 µL of 6X loading dye with 20 µL of PCR products (from step 9.5) and load the gel with the mixture. Leave empty lanes in between samples to minimize cross contamination during gel excision.

11.3 Load DNA ladders, run gel electrophoresis, stain and wash the gel, and take gel images as described in step 8.6.

11.4 Excise the gel blocks that contain PCR products within the target size range. To minimize contamination with primer dimers (175 bp linkers with no inserts), extract PCR products with size above 195 bp (175 bp linkers + 20 bp miRNAs).

11.5 Purify the PCR products using gel extraction.

NOTE: The QIAquick Gel Extraction Kit is used. The purification protocol is based on the manufacturer's protocol, with minor modifications for AQRNA-seq compatibility. All centrifugation steps should be conducted at 17,900 x g for 1 min using a benchtop centrifuge at room temperature, unless otherwise specified. Follow the steps described below.

9.5.1 Measure the weight of the gel blocks inside the tubes. Add 6 volumes of Buffer QG (provided in the kit) to 1 volume of gel block (1 mg gel is approximately 1 µL).

9.5.2 Incubate at 50 °C for 10 min or until complete dissolution of the gel blocks. Vortex tubes every 2 min to facilitate gel dissolution. After dissolving the gel, the mixture should resemble the color of the Buffer QG without the dissolved gel. If the color is orange or violet, add 10 µL of 3 M sodium acetate (pH = 5.0) and mix thoroughly.

9.5.3 Add 1 gel volume of isopropanol (not provided in the kit) to the mixture and mix thoroughly. Place a spin column in a 2 mL collection tube (provided in the kit).

9.5.4 To bind DNA, apply the sample (up to 750 µL each time) to the column and centrifuge. Discard the flowthrough and place the column back into the same collection tube. The maximum amount of gel per spin column is 400 mg.

9.5.5 Add 500 µL of Buffer QG to the column and centrifuge. Discard the flow-through and place the column back into the same collection tube.

9.5.6 To wash the impurities, add 750 µL of Buffer PE (provided in the kit) to the column, let the column stand for 5 min, and centrifuge. Discard the flowthrough and place the column back into the same collection tube. Centrifuge once again to remove residual wash buffer.

9.5.7 Place the column into a sterile 1.5-mL tube. To elute DNA, add 30 µL of Buffer EB (provided in the kit) to the center of the column membrane, let the column stand for 4 min, and centrifuge.

9.6 Measure the concentration of the constructed libraries.

Supplementary Note 3: Detailed procedures of the protocol variant 3 (V3)

It is recommended to perform a comprehensive evaluation of purity, integrity, and quantity of the input RNA samples using (i) 3% agarose gel electrophoresis, (ii) Agilent Bioanalyzer (with small RNA and Pico RNA chips), and (iii) NanoDrop Spectrophotometer and/or Qubit. All reactions and master mixes should be kept on ice, unless otherwise specified. It is recommended to transport reagents (e.g., enzymes) in cool boxes to and from -20ºC storage to preserve their shelf lives. Multiple freeze thaw cycles of library intermediates should be avoided.

12. Dephosphorylation of RNAs

12.1 Prepare the dephosphorylation reaction in a sterile PCR tube (e.g., 200-µL or 500-µL tube) by adding up to 2 µL of the RNA sample, 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 0.5 µM internal standard, 0.5 µL of 10X T4 RNA ligase reaction buffer, 1 µL of 1 U/µL shrimp alkaline phosphatase, and sufficient RNase-free water to bring the reaction volume to 5 µL.

12.2 Incubate at 37ºC for 30 min to dephosphorylate RNAs and then at 65ºC for 5 min to inactivate the enzyme and denature the RNAs. Keep samples at 4ºC for at least 10 min to prevent renaturation.

13. Ligation of Linker 1 to the 3' end of RNAs

13.1 Prepare the Linker 1 ligation reaction in a sterile PCR tube by adding 5 µL of the dephosphorylated RNAs (products from step 1), 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 100 µM Linker 1, 3 µL of 10 mM ATP, 2.5 µL of 10X T4 RNA ligase reaction buffer, 15 µL of PEG8000 (50% solution), 2 µL of 30 U/µL T4 RNA ligase 1, and 1 µL of RNase-free water.

13.2 Incubate at 25ºC for 2 h and then at 16ºC for 16 h to ligate Linker 1 to the RNAs.

13.3 Column-purify the Linker-1-ligated RNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. This column purification protocol applies to all subsequent column purification using the same kit.

4.3.9 For sample volume < 50 µL, add RNase-free water to bring it to 50 µL.

4.3.10 Add 2 volumes of the Oligo Binding Buffer (provided in the kit) to 1 volume of the sample.

4.3.11 Add 8 volumes of 100% ethanol (not provided in the kit) to 1 volume of the sample.

4.3.12 Load the sample (up to 750 µL at a time) to a column placed inside a 2-mL collection tube (provided in the kit).

4.3.13 To bind RNAs to the column, centrifuge at 10,000 x g for 30 sec and discard the flow-through (i.e., the liquid in the collection tube). Place the column back into the collection tube.

4.3.14 To wash the impurities off the column, add 750 µL of the DNA Wash Buffer (provided in the kit) to the column. Centrifuge at 10,000 x g for 30 sec and discard the flow-through. Place the column back into the collection tube.

4.3.15 Centrifuge at the maximum speed (e.g., 16,000 x g for a benchtop microcentrifuge) for 1 additional minute to remove residual DNA Wash Buffer.

4.3.16 To elute the RNAs, carefully move the column to a sterile 1.5-mL tube and add RNase-free water to the column. Use a greater volume than needed when eluting the RNAs to account for potential volume loss during the elution process. For instance, if 15 µL is required for the following step, add 17 µL of RNase-free water for elution. Centrifuge at 10,000 x g for 30 sec.

5 Removal of post-transcriptional methylations by AlkB demethylase

3.9 Prepare a stock solution of 1 M 2-ketoglutarate by dissolving 1.4611 g of 2-ketoglutarate (146.11 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.10 Prepare a stock solution of 0.5 M L-ascorbic acid by dissolving 0.88 g of L-ascorbic acid (176.12 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.11 Prepare a stock solution of 0.25 M ammonium ferrous sulfate hexahydrate by dissolving 0.9835 g of ammonium ferrous sulfate hexahydrate (392.14 g per M) in 10 mL of RNase-free water. Filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.12 Prepare a stock solution of 1 M HEPES by dissolving 2.383 g of HEPES (238.30 g per M) in 10 mL of RNase-free water. Adjust the pH of the solution to 8 using sodium hydroxide (NaOH) and filter-sterilize the solution through a 0.2 µm syringe filter. Aliquot the stock solution into 2 mL sterile tubes and store at -20 °C.

3.13 Prepare a 2X AlkB reaction buffer. To make 10 mL of the buffer, combine 1.5 µL of 1 M 2-ketoglutarate (made in step 3.1), 80 µL of 0.5 M L-ascorbic acid (made in step 3.2), 6 µL of 0.25 M ammonium ferrous sulfate hexahydrate (made in step 3.3), 100 µL of 10 mg/mL BSA, 1000 µL of 1 M HEPES (made in step 3.4; add last), and 8812.5 µL of RNase-free water. Filter-sterilize the buffer through a 0.2 µm syringe filter.

NOTE: The 2x AlkB reaction buffer must be prepared fresh immediately prior to each experiment due to the chemical lability of the components.

3.14 Prepare the AlkB digestion reaction in a sterile PCR tube by adding 20 µL of the Linker-1-ligated RNAs (products from step 2), 50 µL of the 2x AlkB reaction buffer

(made in step 3.5), 2 µL of AlkB demethylase, 1 µL of RNase inhibitor, and 27 µL of RNase-free water.

3.15 Incubate at room temperature for 2 h to remove post-transcriptional methylations from the RNAs.

3.16 To remove AlkB from the reaction, follow the steps described below.

3.16.1 For a clean phase separation, add 50 µL of RNase-free water into the AlkB reaction, and then add 100 µL of phenol: chloroform: isoamyl alcohol 25:24:1 (pH = 5.2).

3.16.2 Shake by hand for 10 s, and then centrifuge at 16,000 x g for 10 min. Make sure that the rotor of the benchtop centrifuge is compatible with the PCR tubes. Use adaptors if needed.

3.16.3 Transfer the RNAs (i.e., the aqueous layer on top; approximately 140 µL) into a sterile 1.5 mL tube. If chloroform (i.e., the bottom layer) is mixed into the aqueous layer, centrifuge again with the same settings.

3.16.4 Add 100 µL of chloroform to the extracted RNAs to remove residual phenol. Shake by hand for 10 s, and then centrifuge at 16,000 x g for 10 min.

3.16.5 Transfer the RNAs (i.e., the aqueous layer on top; approximately 120 µL) into a sterile 1.5 mL tube.

3.16.6 Column-purify the extracted RNAs. Use a kit for DNA/RNA recovery and clean-up (see Table of Materials).

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

6 Removal of excess Linker 1

6.7 Prepare the deadenylation reaction in a sterile PCR tube by adding 15 µL of Linker-1-ligated RNAs (products from step 3), 1 µL of 40 U/µL RNase inhibitor, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

6.8 Incubate at 30ºC for 1 h to remove the adenine at the 5’ end of Linker 1.

6.9 Add 2 µL of 30 U/µL RecJf into the deadenylation reaction.

6.10 Incubate at 37ºC for 30 min to digest excess Linker 1.

6.11 Add another 2 µL of 30 U/µL RecJf into the reaction.

6.12 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 1 and then at 65ºC for 20 min to denature the enzyme.

6.13 Column-purify the Linker-1-ligated RNAs.

NOTE: DyeEx 2.0 Spin Kit is used in this step, as it is effective at removing short remnants (e.g., oligonucleotides with a length of 2 to 10 bp).

6.13.1 Prepare DyeEx columns according to the manufacturer’s protocol.

6.13.2 Place a column into a sterile 1.5-mL tube and load the column with 24 µL of the sample.

6.13.3 To purify the RNAs, centrifuge at 800 x g for 3 min and discard the column. The purified RNAs are in the eluent.

7 Reverse transcription (RT)

7.1 Prepare the RT primer annealing reaction in a sterile PCR tube by adding 24 µL of template RNAs (products from step 4), 1 µL of 2 µM RT primer, and 1 µL of dNTP (10 mM of each type of nucleotides).

7.2 Incubate at 80ºC for 2 min to anneal RT primers to the template RNAs, and then cool on ice immediately for 2 min.

7.3 Prepare the RT reaction by adding 6 µL of 5X PrimeScript RT reaction buffer, 1 µL of 40 U/µL RNase inhibitor, and 1 µL of 200 U/µL PrimeScript reverse transcriptase into the annealing reaction tube.

7.4 Incubate at 50ºC for 2 h to reverse transcribe the RNA templates, and then at 70ºC for 15 min to inactivate the enzyme. The RT products (i.e., RNA-cDNA hybrids) can be stored at 4ºC or -20ºC overnight.

6. Exonuclease I (ExoI) treatment

NOTE: The ExoI treatment protocol follows the manufacturer's protocol, with minor modifications to allow for AQRNA-seq compatibility.

6.1 Prepare the ExoI working solution (1 U/µL) in a sterile tube freshly prior to the experiment. For 10 µL of working solution, add 0.3 µL of 3 M Tris base, 2 µL of 5X SuperScript™ IV RT Reaction Buffer, 7.2 µL of RNase-free water, and 0.5 µL of 20 U/µL ExoI.

6.2 Prepare the ExoI digestion reaction by adding 34 µL of RT products (from step 4), 1 µL of 1 U/µL ExoI working solution, 1 µL of 3 M Tris base, and 0.24 µL of 1.43 M Beta-mercaptoethanol.

6.3 Incubate at 37ºC for 15 min to digest the excess RT primers.

7. RNA hydrolysis

7.1 Add 1.07 µL of 5 M NaOH into the RNA-cDNA hybrid (products from step 6).

7.2 Incubate at 93ºC for 3 min to hydrolyze the RNA strand of the RNA-cDNA hybrid.

7.3 Neutralize the reaction with 0.82 µL of 5 M hydrochloric acid (HCl).

NOTE: It is recommended to test the precise amount of 5 M HCl that is needed for neutralizing 1.07 µL of NaOH (e.g., using pH strips) in buffered conditions. After adding HCl, flick to mix, and spin down the tube. The neutralization is instant.

7.4 Column-purify the single-stranded cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

7.5 Speed-vac the purified cDNAs to <5 µL and then add RNase-free water to bring the volume back to 5 µL.

7.6 Transfer the purified cDNAs into a sterile PCR tube. The purified cDNAs can be stored at -20ºC for up to one week.

8. Ligation of Linker 2 to the 3' end of cDNAs

8.1 Prepare the Linker 2 ligation reaction in a sterile PCR tube by adding 5 µL of cDNAs (products from step 7), 1 µL of 50 µM Linker 2, 2 µL of 10X T4 DNA ligase reaction buffer, 1 µL of 10 mM ATP, 9 µL of PEG8000 (50% solution), and 2 µL of 400 U/µL T4 DNA ligase.

8.2 Incubate at 16ºC for 16 h to ligate Linker 2 to the cDNAs.

8.3 Column-purify the Linker-2-ligated cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

9. Removal of excess Linker 2

9.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 16 µL of Linker-2-ligated cDNAs, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

9.2 Incubate at 30 °C for 1 h to remove the adenine at the 5' end of Linker 2.

9.3 Add 2 µL of RecJf into the deadenylation reaction.

9.4 Incubate at 37ºC for 30 min to digest excess Linker 2.

9.5 Add another 2 µL of RecJf into the reaction.

9.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 2 and then at 65ºC for 20 min to denature the enzyme.

10. PCR amplification of the cDNA library with sequencing adaptors

10.1 Assign PCR primers to the samples. Each sample needs a unique pair of forward and reverse primers for effective multiplexing.

10.2 Add RNase-free water to bring the sample volume to 25 µL. Save 5 µL of each sample into a sterile PCR tube as a backup in case PCR needs to be repeated.

10.3 Prepare the PCR reaction by adding 20 µL of cDNAs (products from step 8), 1 µL of 2.5 µM forward primer, 1 µL of 2.5 µM reverse primer, 25 µL of 2X DNA polymerase buffer, 2 µL of RNase-free water, and 1 µL of DNA polymerase.

10.4 Perform PCR with an initial denaturation at 94ºC for 1 min, followed by 18 cycles of denaturation (98ºC; 20 sec), annealing (58ºC; 20 sec), and extension (68ºC; 1 min).

10.5 Speed-vac the PCR products to less than 25 µL and then add RNase-free water to bring the volume back to 25 µL. Transfer 5 µL of the PCR products to a sterile tube for checking the size distribution (see step 9.6). Store the remaining 20 µL of the PCR products at -20ºC until further steps.

10.6 Check the size distribution of the cDNA libraries following PCR amplification.

10.6.1 Prepare 3% agarose gel in TAE buffer.

10.6.2 Mix 1 µL of 6X loading dye into 5 µL of PCR products (from step 9.5) and load the gel with the mixture.

10.6.3 Load 5 µL of DNA ladders into the well before the first sample and the well after the last sample. Use 50-bp and/or 100-bp DNA ladders to allow for improved size discrimination of PCR products between 150 bp and 350 bp.

10.6.4 Run the gel electrophoresis to locate the PCR products.

NOTE: The appropriate running condition may be context dependent. We have found 120 V, 400 mA, and 75 min to be sufficient for a 17.78 cm (width) × 10.16 cm (height) × 1 cm (thickness) gel slab. For smaller gel slabs, the running time may need to be reduced.

10.6.5 Place the gel in a box and fill the box with deionized (DI) water until the gel is completely immersed. Add 10 µL of ethidium bromide (EtBr) into the DI water soaking the gel. Wrap the box with foil and place it on a shaker. Stain the gel for 30 min with shaking.

10.6.6 Discard the EtBr-containing waste into a waste bottle placed in a fume hood. Rinse the gel with DI water once and discard the EtBr-containing water into the waste bottle.

10.6.7 Fill the box with DI water until the gel is completely immersed. Wrap the box with foil and place it on a shaker. Wash the gel for 10 min with shaking.

10.6.8 Discard the EtBr-containing water into the waste bottle in the fume hood.

10.6.9 Use a gel imager to visualize the bands. Acquire a high-resolution image of the gel.

11. PCR product purification

NOTE: The QIAquick PCR Purification Kit is used. The purification protocol is based on the manufacturer's protocol, with minor modifications for AQRNA-seq compatibility. All centrifugation steps should be conducted at 17,900 x g for 1 min using a benchtop centrifuge at ambient temperature, unless otherwise specified.

11.1 Add 5 volumes of Buffer PB (provided in the kit) to 1 volume of the PCR products and mix. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH = 5.0) and mix.

11.2 Place a QIAquick column in a 2-mL collection tube (provided in the kit).

11.3 To bind DNA, apply the sample (up to 750 µL each time) to the QIAquick column and centrifuge. Discard the flow-through and place the column back in the same collection tube.

11.4 To wash the impurities, add 750 µL of Buffer PE (provided in the kit) to the QIAquick column and centrifuge. Discard the flow-through and place the column back in the same collection tube.

11.5 Centrifuge the QIAquick column once again in the provided 2-mL collection tube for 1 min to remove residual wash buffer.

11.6 Place the QIAquick column into a sterile 1.5-mL tube.

11.7 To elute DNA, add 30 µL of Buffer EB (10 mM Tris·Cl, pH 8.5; provided in the kit) to the center of the QIAquick membrane, let the column stand for 1 min, and centrifuge.

11.8 Measure the concentration of the constructed libraries.

Supplementary Note 4: Detailed procedures of the protocol variant 4 (V4)

It is recommended to perform a comprehensive evaluation of purity, integrity, and quantity of the input RNA samples using (i) 3% agarose gel electrophoresis, (ii) Agilent Bioanalyzer (with small RNA and Pico RNA chips), and (iii) NanoDrop Spectrophotometer and/or Qubit. All reactions and master mixes should be kept on ice, unless otherwise specified. It is recommended to transport reagents (e.g., enzymes) in cool boxes to and from -20ºC storage to preserve their shelf lives. Multiple freeze thaw cycles of library intermediates should be avoided.

14. Dephosphorylation of RNAs

14.1 Prepare the dephosphorylation reaction in a sterile PCR tube (e.g., 200-µL or 500-µL tube) by adding up to 2 µL of the RNA sample, 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 0.5 µM internal standard, 0.5 µL of 10X T4 RNA ligase reaction buffer, 1 µL of 1 U/µL shrimp alkaline phosphatase, and sufficient RNase-free water to bring the reaction volume to 5 µL.

14.2 Incubate at 37ºC for 30 min to dephosphorylate RNAs and then at 65ºC for 5 min to inactivate the enzyme and denature the RNAs. Keep samples at 4ºC for at least 10 min to prevent renaturation.

15. Ligation of Linker 1 to the 3' end of RNAs

15.1 Prepare the Linker 1 ligation reaction in a sterile PCR tube by adding 5 µL of the dephosphorylated RNAs (products from step 1), 0.5 µL of 40 U/µL RNase inhibitor, 1 µL of 100 µM Linker 1, 3 µL of 10 mM ATP, 2.5 µL of 10X T4 RNA ligase reaction buffer, 15 µL of PEG8000 (50% solution), 2 µL of 30 U/µL T4 RNA ligase 1, and 1 µL of RNase-free water.

15.2 Incubate at 25ºC for 2 h and then at 16ºC for 16 h to ligate Linker 1 to the RNAs.

15.3 Column-purify the Linker-1-ligated RNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. This column purification protocol applies to all subsequent column purification using the same kit.

6.3.1 For sample volume < 50 µL, add RNase-free water to bring it to 50 µL.

6.3.2 Add 2 volumes of the Oligo Binding Buffer (provided in the kit) to 1 volume of the sample.

6.3.3 Add 8 volumes of 100% ethanol (not provided in the kit) to 1 volume of the sample.

6.3.4 Load the sample (up to 750 µL at a time) to a column placed inside a 2-mL collection tube (provided in the kit).

6.3.5 To bind RNAs to the column, centrifuge at 10,000 x g for 30 sec and discard the flow-through (i.e., the liquid in the collection tube). Place the column back into the collection tube.

6.3.6 To wash the impurities off the column, add 750 µL of the DNA Wash Buffer (provided in the kit) to the column. Centrifuge at 10,000 x g for 30 sec and discard the flow-through. Place the column back into the collection tube.

6.3.7 Centrifuge at the maximum speed (e.g., 16,000 x g for a benchtop microcentrifuge) for 1 additional minute to remove residual DNA Wash Buffer.

6.3.8 To elute the RNAs, carefully move the column to a sterile 1.5-mL tube and add RNase-free water to the column. Use a greater volume than needed when eluting the RNAs to account for potential volume loss during the elution process. For instance, if 15 µL is required for the following step, add 17 µL of RNase-free water for elution. Centrifuge at 10,000 x g for 30 sec.

16. Removal of excess Linker 1

16.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 15 µL of Linker-1-ligated RNAs (products from step 2), 1 µL of 40 U/µL RNase inhibitor, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

16.2 Incubate at 30ºC for 1 h to remove the adenine at the 5’ end of Linker 1.

16.3 Add 2 µL of 30 U/µL RecJf into the deadenylation reaction.

16.4 Incubate at 37ºC for 30 min to digest excess Linker 1.

16.5 Add another 2 µL of 30 U/µL RecJf into the reaction.

16.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 1 and then at 65ºC for 20 min to denature the enzyme.

16.7 Column-purify the Linker-1-ligated RNAs.

NOTE: DyeEx 2.0 Spin Kit is used in this step, as it is effective at removing short remnants (e.g., oligonucleotides with a length of 2 to 10 bp).

3.8.4 Prepare DyeEx columns according to the manufacturer’s protocol.

3.8.5 Place a column into a sterile 1.5-mL tube and load the column with 24 µL of the sample.

3.8.6 To purify the RNAs, centrifuge at 800 x g for 3 min and discard the column. The purified RNAs are in the eluent.

17. Reverse transcription (RT)

NOTE: The following RT reaction protocol follows the manufacturer’s protocol of the SuperScript™ IV Reverse Transcriptase, with minor modifications to allow for AQRNA-seq compatibility.

17.1 Prepare the RT primer annealing reaction in a sterile PCR tube by adding 22 µL of template RNAs (products from step 3), 2 µL of 2 µM RT primer, and 2 µL of dNTP (10 mM of each type of nucleotides).

17.2 Incubate at 65ºC for 5 min to anneal RT primers to the template RNAs, and then cool on ice immediately for 2 min.

17.3 Prepare the RT reaction by adding 8 µL of 5X SuperScript™ IV RT Reaction Buffer, 2 µL of 100 mM DTT, 2 µL of 40 U/µL ribonuclease inhibitor, and 2 µL of 200 U/µL SuperScript® IV Reverse Transcriptase into the annealing reaction tube.

17.4 Incubate at 50ºC for 2 h to reverse transcribe the RNA templates, and then at 80ºC for 10 min to inactivate the enzyme. The RT products (i.e., RNA-cDNA hybrids) can be stored at 4ºC or -20ºC overnight.

18. Exonuclease I (ExoI) treatment

NOTE: The ExoI treatment protocol follows the manufacturer's protocol, with minor modifications to allow for AQRNA-seq compatibility.

18.1 Prepare the ExoI working solution (1 U/µL) in a sterile tube freshly prior to the experiment. For 10 µL of working solution, add 0.3 µL of 3 M Tris base, 2 µL of 5X SuperScript™ IV RT Reaction Buffer, 7.2 µL of RNase-free water, and 0.5 µL of 20 U/µL ExoI.

18.2 Prepare the ExoI digestion reaction by adding 40 µL of RT products (from step 4), 1.2 µL of 1 U/µL ExoI working solution, 1.2 µL of 3 M Tris base, and 0.3 µL of 1.43 M Beta-mercaptoethanol.

18.3 Incubate at 37ºC for 15 min to digest the excess RT primers.

19. RNA hydrolysis

19.1 Add 1.2 µL of 5 M sodium hydroxide (NaOH) into the ExoI digestion reaction (products from step 5).

19.2 Incubate at 93ºC for 3 min to hydrolyze the RNA strand of the RNA-cDNA hybrid.

19.3 Neutralize the reaction with 0.97 µL of 5 M hydrochloric acid (HCl).

NOTE: It is recommended to test the precise amount of 5 M HCl that is needed for neutralizing 1.2 µL of NaOH (e.g., using pH strips) in buffered conditions. After adding HCl, flick to mix, and spin down the tube. The neutralization is instant.

19.4 Column-purify the single-stranded cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

19.5 Speed-vac the purified cDNAs to <5 µL and then add RNase-free water to bring the volume back to 5 µL.

19.6 Transfer the purified cDNAs into a sterile PCR tube. The purified cDNAs can be stored at -20ºC for up to one week.

20. Ligation of Linker 2 to the 3' end of cDNAs

20.1 Prepare the Linker 2 ligation reaction in a sterile PCR tube by adding 5 µL of cDNAs (products from step 6), 1 µL of 50 µM Linker 2, 2 µL of 10X T4 DNA ligase reaction buffer, 1 µL of 10 mM ATP, 9 µL of PEG8000 (50% solution), and 2 µL of 400 U/µL T4 DNA ligase.

20.2 Incubate at 16ºC for 16 h to ligate Linker 2 to the cDNAs.

20.3 Column-purify the Linker-2-ligated cDNAs.

NOTE: The Oligo Clean & Concentrator Kit is used in this step. Follow the protocol detailed in step 2.3 to perform the column purification.

21. Removal of excess Linker 2

21.1 Prepare the deadenylation reaction in a sterile PCR tube by adding 16 µL of Linker-2-ligated cDNAs, 2 µL of 10X NEBuffer™ 2, and 2 µL of 50 U/µL 5'-deadenylase.

21.2 Incubate at 30 °C for 1 h to remove the adenine at the 5' end of Linker 2.

21.3 Add 2 µL of RecJf into the deadenylation reaction.

21.4 Incubate at 37ºC for 30 min to digest excess Linker 2.

21.5 Add another 2 µL of RecJf into the reaction.

21.6 Incubate at 37ºC for 30 min to continue the digestion of excess Linker 2 and then at 65ºC for 20 min to denature the enzyme.

22. PCR amplification of the cDNA library with sequencing adaptors

22.1 Assign PCR primers to the samples. Each sample needs a unique pair of forward and reverse primers for effective multiplexing.

22.2 Add RNase-free water to bring the sample volume to 25 µL. Save 5 µL of each sample into a sterile PCR tube as a backup in case PCR needs to be repeated.

22.3 Prepare the PCR reaction by adding 20 µL of cDNAs (products from step 8), 1 µL of 2.5 µM forward primer, 1 µL of 2.5 µM reverse primer, 25 µL of 2X DNA polymerase buffer, 2 µL of RNase-free water, and 1 µL of DNA polymerase.

22.4 Perform PCR with an initial denaturation at 94ºC for 1 min, followed by 18 cycles of denaturation (98ºC; 20 sec), annealing (58ºC; 20 sec), and extension (68ºC; 1 min).

22.5 Speed-vac the PCR products to less than 25 µL and then add RNase-free water to bring the volume back to 25 µL. Transfer 5 µL of the PCR products to a sterile tube for checking the size distribution (see step 9.6). Store the remaining 20 µL of the PCR products at -20ºC until further steps.

22.6 Check the size distribution of the cDNA libraries following PCR amplification.

9.6.10 Prepare 3% agarose gel in TAE buffer.

9.6.11 Mix 1 µL of 6X loading dye into 5 µL of PCR products (from step 9.5) and load the gel with the mixture.

9.6.12 Load 5 µL of DNA ladders into the well before the first sample and the well after the last sample. Use 50-bp and/or 100-bp DNA ladders to allow for improved size discrimination of PCR products between 150 bp and 350 bp.

9.6.13 Run the gel electrophoresis to locate the PCR products.

NOTE: The appropriate running condition may be context dependent. We have found 120 V, 400 mA, and 75 min to be sufficient for a 17.78 cm (width) × 10.16 cm (height) × 1 cm (thickness) gel slab. For smaller gel slabs, the running time may need to be reduced.

9.9.1 Place the gel in a box and fill the box with deionized (DI) water until the gel is completely immersed. Add 10 µL of ethidium bromide (EtBr) into the DI water soaking the gel. Wrap the box with foil and place it on a shaker. Stain the gel for 30 min with shaking.

9.9.2 Discard the EtBr-containing waste into a waste bottle placed in a fume hood. Rinse the gel with DI water once and discard the EtBr-containing water into the waste bottle.

9.9.3 Fill the box with DI water until the gel is completely immersed. Wrap the box with foil and place it on a shaker. Wash the gel for 10 min with shaking.

9.9.4 Discard the EtBr-containing water into the waste bottle in the fume hood.

9.9.5 Use a gel imager to visualize the bands. Acquire a high-resolution image of the gel.

10 PCR product purification

NOTE: The QIAquick PCR Purification Kit is used. The purification protocol is based on the manufacturer's protocol, with minor modifications for AQRNA-seq compatibility. All centrifugation steps should be conducted at 17,900 x g for 1 min using a benchtop centrifuge at ambient temperature, unless otherwise specified.

10.7 Add 5 volumes of Buffer PB (provided in the kit) to 1 volume of the PCR products and mix. If the color of the mixture is orange or violet, add 10 µL of 3 M sodium acetate (pH = 5.0) and mix.

10.8 Place a QIAquick column in a 2-mL collection tube (provided in the kit).

10.9 To bind DNA, apply the sample (up to 750 µL each time) to the QIAquick column and centrifuge. Discard the flow-through and place the column back in the same collection tube.

10.10 To wash the impurities, add 750 µL of Buffer PE (provided in the kit) to the QIAquick column and centrifuge. Discard the flow-through and place the column back in the same collection tube.

10.11 Centrifuge the QIAquick column once again in the provided 2-mL collection tube for 1 min to remove residual wash buffer.

10.12 Place the QIAquick column into a sterile 1.5-mL tube.

10.13 To elute DNA, add 30 µL of Buffer EB (10 mM Tris·Cl, pH 8.5; provided in the kit) to the center of the QIAquick membrane, let the column stand for 1 min, and centrifuge.

10.14 Measure the concentration of the constructed libraries.