High sensitivity miRNA library preparation protocol by TailorMix miRNA Sample Preparation Kit (Version 2) T

Karen Yip

Abstract

ThI high sensitivity miRNA library generation for the Illumina sequencing platform. Our enhanced reagent kit enables the discovery and profiling of small RNAs from a variety of sources including FFPE, exosome, serum, and whole blood. The TailorMix workflow is designed for ease of use, enabling library preparation a single day.

Features:

- **User Friendly Workflow:** Libraries can be prepared in a single day.
- **No Additional Reagents Necessary:** All reaction enzymes and buffers are provided.
- **Stress Free Gel Purification:** TailorCut Gel Extraction Tool Set is included for easy gel excision and purification.
- **Easy to Use:** Reagents are supplied as ready-to-use mixtures which improves consistency and reproducibility.
- **Ultra High Sensitivity:** Prepare miRNA libraries from any source with as little as 10ng of total RNA input**.**

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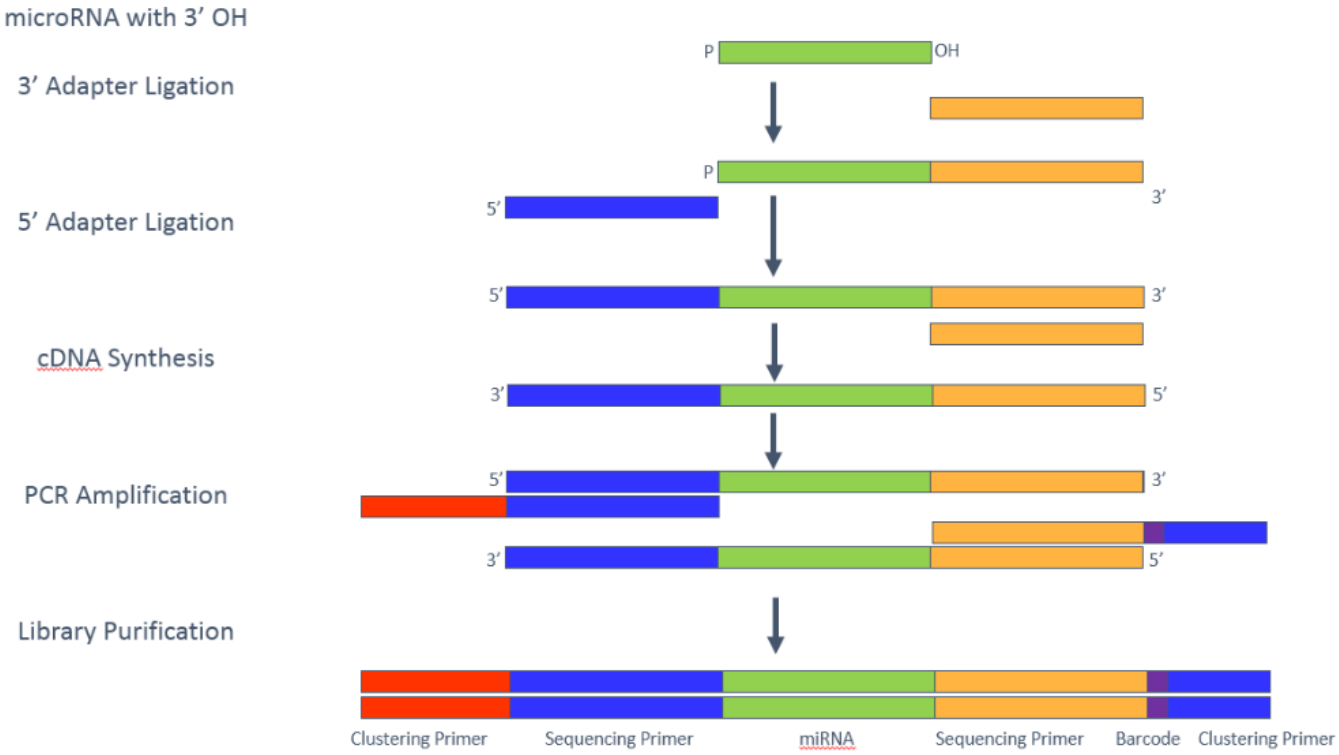
Guidelines

Figure 1 TailorMix miRNA Sample Preparation V2 Overview



Best Practices

- Always wear gloves and use sterile technique.
- Set up reactions using sterile non-stick nuclease-free tubes.
- Place samples and reagents on ice or on chilled cooler block at all times and avoid extended pauses.
- Reagents should be prepared using RNAse-free components
- Prepare an extra 10% mixture when running multiple samples.
- Avoid repeated freeze/thaw cycles.

RNA Input

This protocol has been optimized using 10 - 100 ng of purified high quality human kidney total RNA as input. Because miRNA populations vary among different tissue types and species, the use of total RNA from other tissue or species may require optimization. You may also use isolated miRNA as the starting material.

Sample Pooling Guidelines

The TailorMix MiRNA Sample Preparation kit is capable of multiplexing up to 96 samples into a single lane of an Illumina flow cell. While processing multiple samples in parallel, use a unique index primer for each sample at the PCR step. Samples can be pooled before or after the library purification step.

Citations & References:

Niu, Jinzhi, et al. "In vivo study of Dicer-2-mediated immune response of the small interfering RNA pathway upon systemic infections of virulent and avirulent viruses in Bombus terrestris." *Insect biochemistry and molecular biology* 70 (2016): 127-137.

Materials

✓ TailorMix miRNA Sample Preparation Kit V2 TM302 by Contributed by users

Protocol

Step 1.

3' Adapter Ligation

1. Thaw Mix C300 from -20°C storage. Allow it to equilibrate to room temperature for a minimum of 30 minutes before use.

2. Pre-heat the thermal cycler to 70°C and pre-heat another thermal cycler to 25°C if available.

3. Denature the RNA Sample by assembling the following components in a sterile 200 µL PCR tube on ice:

Reagent	Volume (µL)
RNA Sample	6
Mix A300	2
Total	**8**

4. Gently pipette mix thoroughly and incubate at 70°C for 1 minute and then place the tube on ice.

5. Set up the following 3’ Adapter Ligation reaction on ice:

Reagent	Volume (µL)
Denatured RNA mix from step 4	8
Mix B300	2
Mix C300	6.5
Total	**16.5**

Note: Mix C300 is a highly viscous reagent. Handle with care and pipette slowly to ensure the correct amount of Mix C300 is dispensed for each reaction.

6. Gently pipette mix thoroughly and incubate at 25°C for 1 hour.

Step 2.

Ligation Product Clean Up

1. Vortex the RNA Purification Beads (RPB) until they are evenly resuspended.

2. Prepare 80% ethanol for wash steps.

3. Mix 30 µL of RPB with each sample. Gently pipette mix thoroughly and incubate at room temperature for 15 minutes.

4. Place the sample tube on the magnetic stand at room temperature for 5 minutes.

5. Remove and discard 40 µL of the supernatant.

6. Keep sample tube on the magnetic stand. Gently add 100 µL of 80% ethanol into each sample tube without disrupting the beads. Incubate at room temperature for 30 seconds.

7. Remove and discard 95 µL of the supernatant.

8. Repeat steps 12 and 13 once. Remove and discard all residual supernatant after the second 80% ethanol wash.

9. Air dry sample tube at room temperature.

Note: Beads are dried within 5 to 15 minutes at room temperature. Proceed to Step 16 when beads begin to turn light brown in color. Sample recovery may be affected if beads are over-dried and appear powdery.

10. Remove sample tube from the magnetic stand. Resuspend the dried RPB in 8.5 µL of nuclease free water. Incubate resuspension at room temperature for 2 minutes.

11. Place the sample tube on the magnetic stand at room temperature for 5 minutes.

12. Transfer 7 µL of the supernatant into a fresh 200 µL PCR tube.

Step 3.

5’ Adapter Ligation

1. Set up the following 5’ Adapter Ligation reaction on ice:

Reagent	Volume (µL)
3’ Adapter Ligated RNA from step 18	7
Mix D300	3
Mix E300	2
Total	**12**

2. Gently pipette mix thoroughly and incubate at 25°C for 1 hour and then place the tube on ice.

Step 4.

cDNA Synthesis

1. Pre-heat the thermal cycler to 50°C.

2. Set up the following cDNA Synthesis reaction on ice.

Reagent	Volume (µL)
3’ and 5’ Adapter Ligated RNA from step 20	12
Mix F300	2
Mix G300	1
Total	**15**

3. Gently pipette mix thoroughly and incubate at 50°C for 1 hour and then place the tube on ice.

Step 5.

PCR Amplification

Note: This protocol has been optimized using 10 - 100 ng of purified high quality human kidney total RNA as input. Because miRNA populations vary among different tissue types and species, the use of total RNA from other tissue or species may require optimization. An alternative PCR protocol is available for increasing libraries from low yield samples. See the Appendix A for details.

1. Set up the following PCR reaction in a fresh sterile 200 µl PCR tube on ice:

Reagent	Volume (µL)
cDNA from step 23	5
Mix H300	18
PCR Primer	1
Index Primer*	1
Total	**25**

*Only one of the Index primers is used for each sample.

2. Gently pipette mix thoroughly and amplify the samples in the thermal cycler using the following PCR cycling conditions:

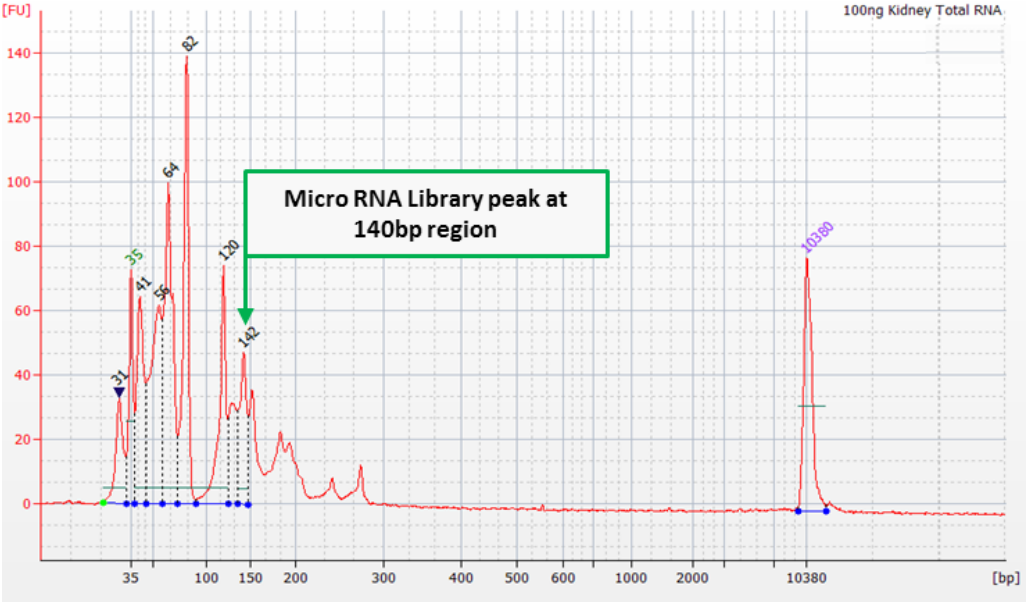
- 98°C for 30 seconds
- 15 cycles of:
    1. 98°C for 5 seconds
    2. 60°C for 15 seconds
- 72°C for 1 minute
- 72°C for 5 minutes
- Hold at 4°C

3. PCR yield can be monitored by running an Agilent BioAnalyzer High Sensitivity DNA assay using a dilution of 1 µL of PCR product and 9 µL of nuclease-free water. A typical result shows a distinct peak at approximately 140bp (Figure 2).

Note: See Appendix B for a more detail description of BioAnalyzer High Sensitivity DNA assay profile of the PCR products.

Figure 2 BioAnalyzer High Sensitivity DNA assay of PCR Product

from a Human Kidney Tissue Total RNA Sample



Step 6.

Library Purification

1. Determine the volume of TBE buffer needed and dilute 5X TBE Buffer to 1X for use in gel electrophoresis.

2. Assemble the gel electrophoresis apparatus.

3. Mix 2 µL of Custom Ladder with 2 µL of Hi-Density TBE Sample Buffer.

4. (Optional) Mix 1 µL of 100bp DNA ladder with 1 µL of Hi-Density TBE Sample Buffer.

5. Add 2.5 µL of Hi-Density TBE Sample Buffer to 25 µL of PCR product and pipet mix thoroughly.

6. Load 25 µL of the PCR product-Sample Buffer mix into one well in the middle of the 8% PAGE gel. Refer to Figure 3 for an example.

7. Load 2 µL of the custom ladder and dye mix into the neighboring wells of the PCR products.

Note: Always bracketing each PCR product lane with two custom ladder lanes to ensure precise excision of the miRNA band.

8. (Optional) Load 2 µL of the 100bp DNA ladder and dye mix into a separate well.

9. Run the gel for 65 minutes at 145V and immediately remove the gel from the apparatus.

Note: Performance of electrophoresis apparatus varies. Optimization of the setting may be needed for sufficient band separation.

Step 7.

Recover Purified Library

1. Prepare TE buffer with 0.1% Tween-20.

Reagent	Volume (µL)
TE buffer	9,990
Tween-20	10
Total	**10,000**

2. Open the gel cassette and stain with 1µg/mL ethidium bromide solution according to the manufacturer's instructions.

3. Place the gel on a UV Transilluminator and observe the banding pattern (Figure 3).

4. **(Alternative)** Stain gel with Sybr Gold according to the manufacturer's instructions and observe the banding pattern on a Dark Reader Transilluminator.

5. Place the gel breaker tube into a sterile 1.5mL microcentrifuge tube.

6. The 140bp band represents the highest concentration of micro RNA library. To excise the 140bp band, align the center of the gel cutter tool with the 140 bp band of the custom ladder (Figure 4). Press down firmly into the gel and excise the gel fragment.

Note: The 150 bp band represents a combination of micro RNA and other small RNA species (see Appendix B, Q10 if a strong 150bp band is observed). To include the 150bp band in the extraction, align the bottom of the gel cutter tool with the 140bp band of the custom ladder (Figure 5). Press down firmly into the gel and excise the gel fragment in between the two custom ladder markers.

See Appendix B for a more detail description of the gel bands.

Figure 3 Micro RNA Library PCR products on 8% TBE gel

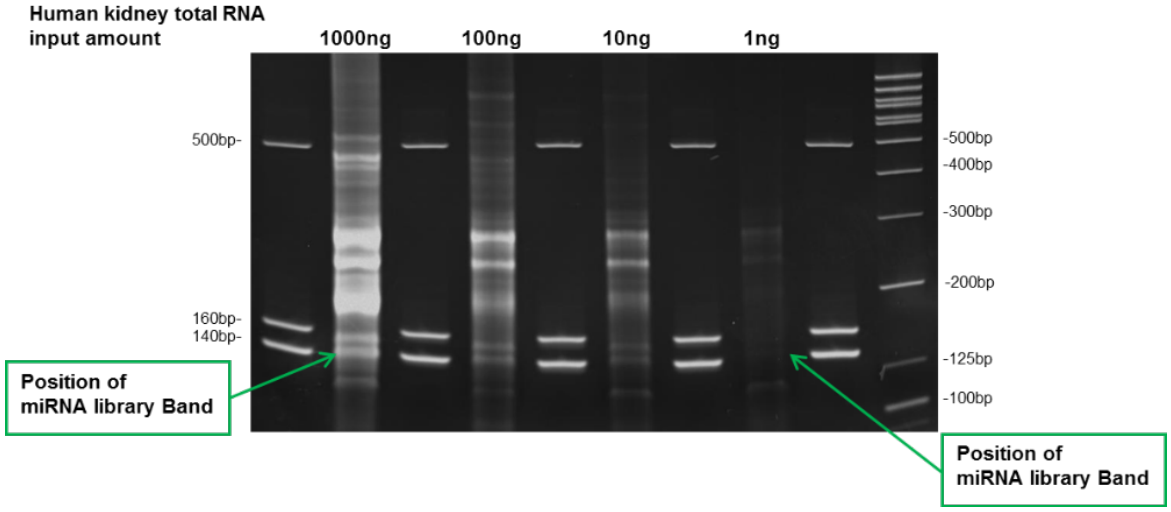


Figure 4 Close up of gel cutting position for miRNA libraries

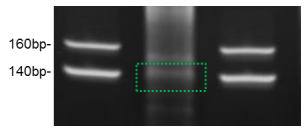
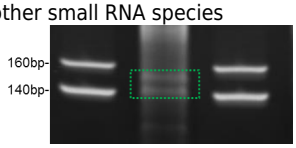


Figure 5 Close up of gel cutting position for isolating miRNA and other small RNA species



7. Insert the gel cutter tool containing the gel slice into the gel breaker tube.

8. Pulse-spin the gel cutter and gel breaker assembly in a minifuge. Make sure the gel slice is collected in the gel breaker tube. Remove gel cutter from the assembly and discard.

9. Add 30 µL of TE buffer with 0.1% Tween-20 to the gel breaker tube containing the gel slice.

10. Centrifuge the gel breaker assembly in a bench top centrifuge at maximum speed (approximately 13,000x G) for two minutes at room temperature. Ensure that all of the gel has moved through the holes into the collection tube.

11. Elute the micro RNA library by shaking the tube at 600 rpm at room temperature overnight.

Note: Do NOT heat up gel buffer mix.

12. To collect the micro RNA library, spin the gel mix at maximum speed (approximately 13,000x G) for 2 minutes.

13. With a P10 pipette, gently transfer 20-25 µl eluate from gel mix to a fresh 1.5ml tube.

Step 8.

Library Validation

1. Use of an Agilent Technologies 2100 Bioanalyzer is recommended as a quality control analysis of your sample library. Use 1 µL of resuspended construct from step 47 on a High Sensitivity DNA chip to check the size, purity and concentration of the sample.

Note: Remnant of gel loading dye may appear as a high molecular weight peak on the Bioanalyzer profile. Presence of the dye would not affect performance of the micro RNA library during the sequencing run.

Note: The BioAnalyzer High Sensitivity DNA assay has a 10% deviation on sizing accuracy.

Figure 6 BioAnalyzer High Sensitivity DNA assay of Gel Purified Library from Human Kidney Tissue Total RNA Sample