Gel-Free miRNA Illumina Library Preparation Protocol by TailorMix Gel-Free miRNA Sample Preparation Kit

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Abstract

This is a magnetic bead-based protocol enables the generation of high quality Illumina miRNA libraries without the need of PAGE-gel size selection.

Our protocol enables the discovery and profiling of miRNAs from various organisms and tissues via the Illumina sequencing platform. The unique TailorMix reagents and workflow have been developed for simplicity and reproducibility without sacrificing quality or yield.

Features

- No need for PAGE-gel size selection
    - Final miRNA libraries are cleaned up with magnetic bead-based protocol.
- Low input requirement
    - Compatible for as little as 150ng total RNA input
- User friendly workflow
    - Libraries can be prepared in a single day with less than one hour of hands on time.
- Comprehensive sample prep kit
    - Most components are supplied as ready-to-use mixtures which improves consistency and reproducibility.

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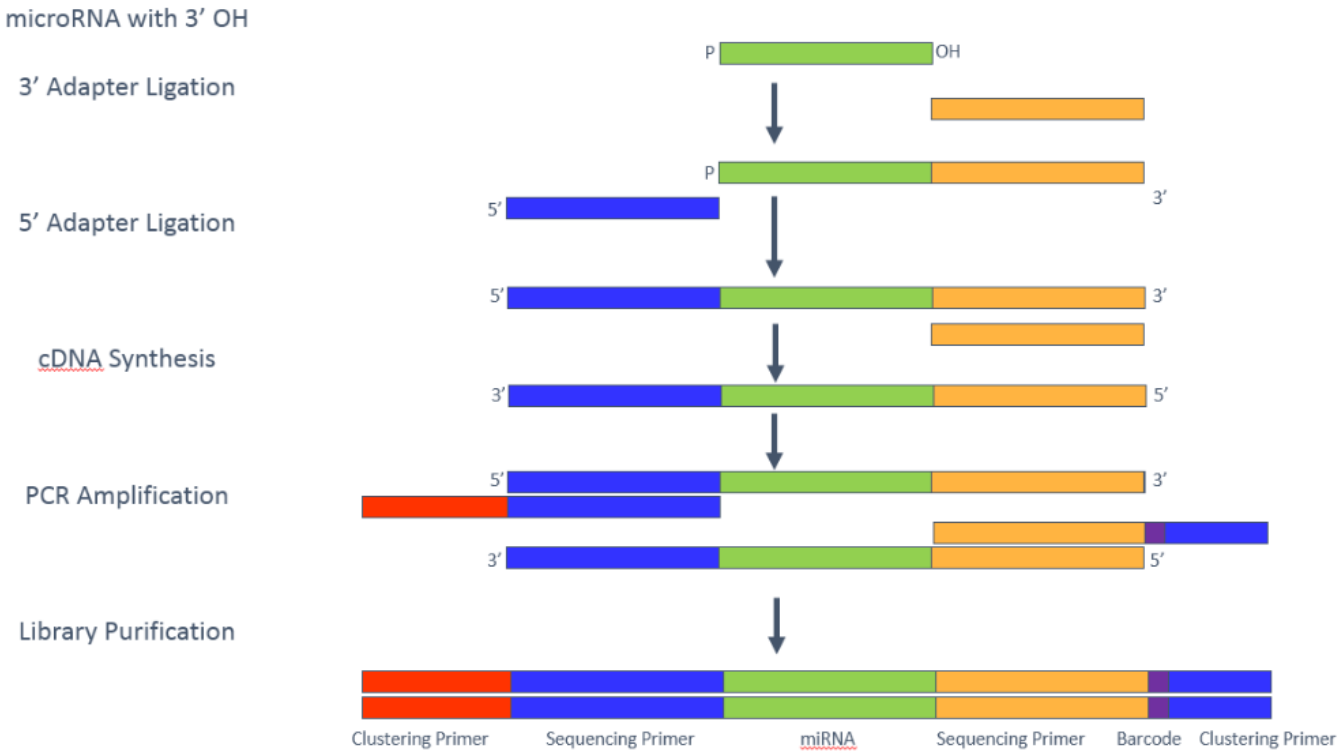
Guidelines

Figure 1 TailorMix miRNA Sample Preparation 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

The TailorMix Gel-Free miRNA Sample Preparation protocol has been optimized using 900 ng of purified high quality human kidney total RNA as input and confirmed with as low as 150ng RNA input. Because miRNA populations vary among different tissue types and species, the use of total RNA from other tissue or species may require optimization. Low yield libraries may require gel-base size selection for optimal results (See the Appendix, Table 1 for suggestions on Library Size Selection Methods).

Sample Pooling Guidelines

The TailorMix Gel-free mi RNA 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. Contact SeqMatic for more information about the 96-reaction sample preparation kit (sales@seqmatic.com).

Protocol

Step 1.

3’ Adapter Ligation

1. Thaw Mix C400 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 A400	2
Total	**8**

4. Vortex 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 B400	2
Mix C400	6.5
Total	**16.5**

Note: Mix C400 is a highly viscous reagent. Handle with care and pipette slowly to ensure the correct amount of Mix C400 is dispensed for each reaction.

6. Vortex mix thoroughly and pulse spin. Incubate at 25°C for 1 hour.

Step 2.

Ligation Product Clean Up

1. Vortex the TailorMag Purification Beads (TPB) until they are evenly suspended.

2. Prepare 80% ethanol for rinse step.

3. Add 30 µL of TPB with each 3’-adapter ligated sample from Step 6. Votex mix thoroughly and

incubate at room temperature for 15 minutes.

Reagent	Volume (µL)
3’-adapter ligated sample from Step 6	16.5
TailorMag Purification Beads (TPB)	30
Total	**46.5**

Note: Do NOT perform strong centrifugation because it will separate TPB from the sample.

4. Place the sample tube on the magnetic stand at room temperature for 5 minutes, or until solution clears up.

5. Carefully remove and discard 40 µL of the supernatant.

Note: Sample recovery may be affected if the TPB pellet is disrupted.

6. Keep sample tube on the magnetic stand. Gently rinse the TPB pellet with 150 µL of 80% ethanol without disrupting the TPB pellet. Discard the rinse solution.

Tip: Point pipette tip towards opposite direction as the TPB pellet. Gently pipette the 80% ethanol up and down once, then discard the rinse solution.

7. Air dry sample tube at room temperature.

Note: TailorMag Purification Beads are dried within 5 to 15 minutes at room temperature. Proceed to Step 14 when the appearance of the TPB pellet turns form glossy/shiny (wet) to matte (dry). Sample recovery may be affected if beads are over-dried and appear powdery.

8. Remove sample tube from the magnetic stand. Add 7 µL of nuclease free water to the dried TPB pellet. Vortex to resuspend and pulse spin. Incubate sample resuspension at room temperature for 2 minutes.

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 14 (contains TPB)	7
Mix D400	3
Mix E400	2
Total	**12**

Note: Presence of TPB does not interfere with the enzymatic reaction.

Note: To minimize the presence of the artifact products, add Mix D400 and Mix E400 to the sample in consecutive steps.

2. Vortex mix thoroughly and pulse spin. 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 16 (contains TPB)	12
Mix F400	2
Mix G400	1
Total	**15**

Note: Presence of TPB does not interfere with the enzymatic reaction.

3. Vortex mix thoroughly and pulse spin. Incubate at 50°C for 1 hour and then place the tube on ice.

Safe Stopping Point: First strand cDNA could be stored at -20°C for up to seven days.

Step 5.

PCR Amplification

Note: This protocol has been optimized using 900 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 additional optimization.

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

Reagent	Volume (µL)
First strand cDNA from Step 19 (contains TPB)	15
Mix H400	13
PCR Primer	1
Index Primer*	1
Total	**30**

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

Note: Presence of TPB does not interfere with the enzymatic reaction.

2. Vortex mix thoroughly and pulse spin. Amplify the samples in the thermal cycler using the following PCR cycling conditions:

- 95°C for 10 minutes
- 15 cycles of:
    1. 95°C for 5 seconds
    2. 60°C for 15 seconds
- 72°C for 1 minute
- 72°C for 5 minutes
- Hold at 4°C

Safe Stopping Point: PCR products could be stored at -20°C for up to seven days.

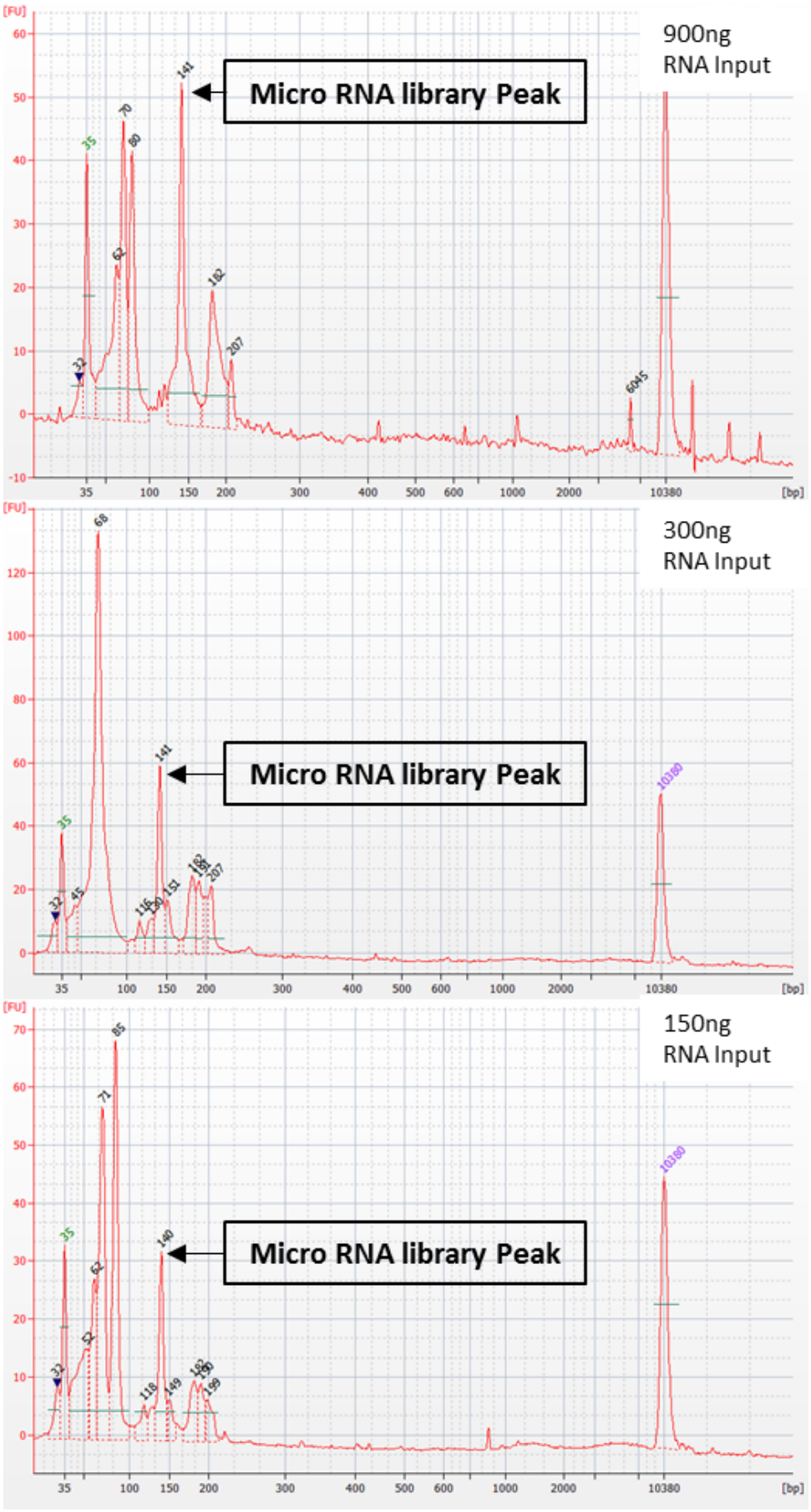
3. PCR yield can be monitored by running an Agilent BioAnalyzer High Sensitivity DNA assay using a dilution of 1 µL of PCR product and 9 µL of nuclease-free water. A typical result shows a distinct peak at approximately 140bp (Figure 2).

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

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

Figure 2 BioAnalyzer High Sensitivity DNA assay of PCR Product (10x diluted)

from Human Kidney Tissue Total RNA



Step 6.

The TailorMix Gel-Free miRNA Sample Preparation protocol enables the generation of micro RNA libraries form as low as 150ng Human kidney total RNA (Figure 3 and Figure 4). However miRNA populations vary among different samples, the use of total RNA from other tissue or species may cause variations in PCR profiles.

Gel-free size selection is suitable for libraries which has a strong 140bp library product peak in compare to the 120bp artifact product peak. It is recommended to use the PAGE-size selection approach for low yield libraries (weak 140bp library product) and libraries that have a strong 120bp artifact product peak. See Table 2 in the Appendix for examples.

Step 7.

Gel-Free Library Purification

Note: Sample volume may change after PCR. To ensure purification efficiency, bring sample volume back to 30 µL before starting Gel-Free Purification steps if necessary.

1. Vortex the TailorMag Purification Beads (TPB) until they are evenly resuspended.

2. Prepare 80% ethanol for rinse step.

3. Add TPB to each sample in the PCR tube according to the following table. Votex mix thoroughly and pulse spin. Incubate at room temperature for 5 minutes.

Reagent	Volume (µL)
PCR from Step 21 (contains TPB)	30
TailorMag Purification Beads (TPB)	30
Total	**60**

Note: Do NOT perform strong centrifugation because it will separate TPB from the sample.

4. Place the sample tube on the TailorMag PCR-tube magnetic stand at room temperature for 5 minutes, or until solution clears up. DO NOT DISCARD SUPERNATANT.

5. Keep sample tube on the magnetic stand. Transfer 55 µL of the clear supernatant to fresh sample tubes.

Note: Do not disrupt the TPB pellet. Contamination of TPB pellet to the next step may affect final library quality.

6. Add TPB to clear supernatant from Step 27. Vortex mix thoroughly and pulse spin. Incubate at room temperature for 5 minutes.

Reagent	Volume (µL)
Clear Supernatant from Step 27	55
TailorMag Purification Beads (TPB)	11
Total	**66**

Note: Do NOT perform strong centrifugation because it will separate TPB from the sample.

7. Place the sample tube on the magnetic stand at room temperature for 5 minutes, or until solution clears up.

8. Remove and discard 60 µL of the supernatant.

Note: Sample recovery may be affected if the TPB pellet is disrupted.

9. Keep sample tube on the magnetic stand. Gently rinse the TPB pellet with 150 µL of 80% ethanol without disrupting the TPB pellet. Discard the rinse solution.

Tip: Point pipette tip towards opposite direction as the TPB pellet. Gently pipette the 80% ethanol up and down once, then discard the rinse solution.

10. Air dry sample tube at room temperature.

Note: TailorMag Purification Beads are dried within 5 to 15 minutes at room temperature. Proceed to Step 33 when the appearance of the TPB pellet turns form glossy/shiny (wet) to matte (dry). Sample recovery may be affected if beads are over-dried and appear powdery.

11. Remove sample tube from the magnetic stand. Add 27 µL of TE buffer to the dried TPB pellet. Vortex to resuspend and pulse spin. Incubate resuspension at room temperature for 2 minutes.

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 library from Step 33 on a High Sensitivity DNA chip to check the size, purity and concentration of the sample.

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

Note: If high percentage of 120bp peak remains, use PAGE size selection gel to extract the 140bp micro RNA library. See Table 2 in the Appendix for references.

Figure 3 TailorMix Gel-Free miRNA libraries from Human Kidney Tissue Total RNA

BioAnalyzer High Sensitivity DNA assay profiles