TM-310-UG
Ref# U19D25001

TailorMix miRNA Sample Preparation 12-reaction Kit (Version 3)

Catalog Numbers: TM-310-A | TM-310-B | TM-310-C | TM-310-D

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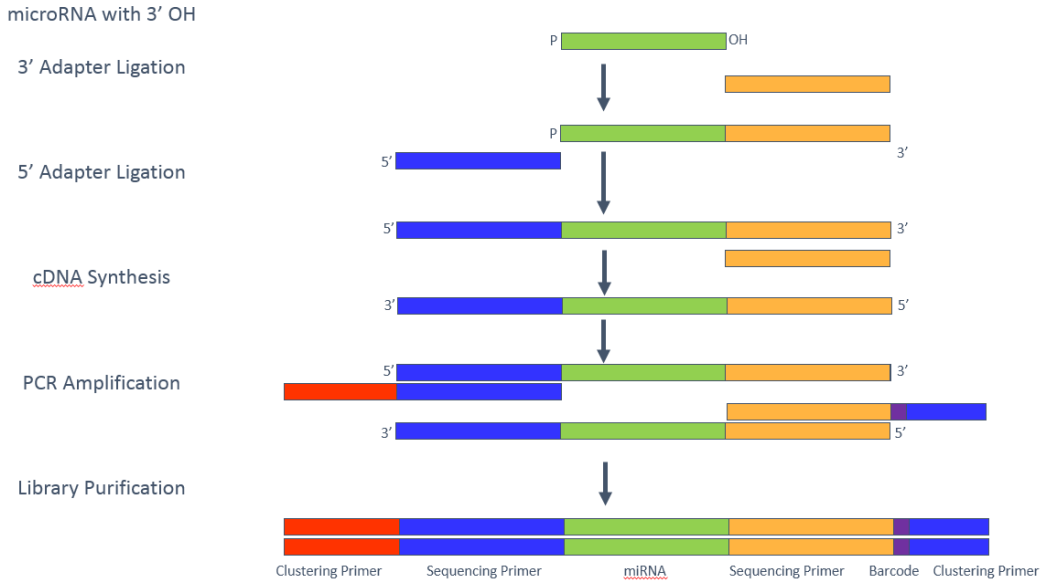
44846 Osgood Road
Fremont, CA 94539
U.S.A.
Tel: 510.870.0965
www.seqmatic.com
info@seqmatic.com

Introduction

The TailorMix miRNA Sample Preparation Kit (Version 3) from SeqMatic offers a high sensitivity solution for generating miRNA libraries from low concentration RNA samples. Our kits enable 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.

Figure 1 TailorMix miRNA Sample Preparation V3 Overview



Features

- Wide dynamic range
  - Requires as little as 1ng of 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.

Kit Components

Each TailorMix miRNA Sample Preparation Kit (Version 3) contains two sets of reagents (12 samples), one set of 12 unique index primers, and one set of gel purification kit.

Set 1: TM310 Reagent Set 1 of 2 (store at -20 °C)

1. Mix A310
2. Mix B310
3. Mix C310
4. Mix D310
5. Mix E310
6. Mix F310
7. Mix G310
8. Mix H310

Set 2: TM310 Reagent Set 2 of 2 (store at 4 °C)

1. TailorMag Purification Beads (TPB)

Set 3: Index Primer Set (store at -20 °C)

Set A		
1.	PCR Primer	
2.	Custom Ladder	
	Index Primers	**Index Sequence**
3.	Index 1	ATCACG
4.	Index 2	CGATGT
5.	Index 3	TTAGGC
6.	Index 4	TGACCA
7.	Index 5	ACAGTG
8.	Index 6	GCCAAT
9.	Index 7	CAGATC
10.	Index 8	ACTTGA
11.	Index 9	GATCAG
12.	Index 10	TAGCTT
13.	Index 11	GGCTAC
14.	Index 12	CTTGTA

Set B		
1.	PCR Primer	
2.	Custom Ladder	
	Index Primers	**Index Sequence**
3.	Index 13	AGTCAA
4.	Index 14	AGTTCC
5.	Index 15	ATGTCA
6.	Index 16	CCGTCC
7.	Index 17	GTAGAG
8.	Index 18	GTCCGC
9.	Index 19	GTGAAA
10.	Index 20	GTGGCC
11.	Index 21	GTTTCG
12.	Index 22	CGTACG
13.	Index 23	GAGTGG
14.	Index 24	GGTAGC

Set C		
Tube#	**Index Primers**	**Index Sequence**
1	Index 73	GGCAGA
2	Index 74	CACTCG
3	Index 75	ACATAC
4	Index 76	AATGAG
5	Index 77	GTCACC
6	Index 78	AGGCTT
7	Index 79	TCTGTC
8	Index 80	GAAGTG
9	Index 81	ACAAGA
10	Index 82	TTCGGA
11	Index 83	TACAAC
12	Index 84	CGGTGA
13	PCR Primer	n/a

Set D		
Tube#	**Index Primers**	**Index Sequence**
1	Index 85	CTTGCC
2	Index 86	AGACCA
3	Index 87	GACTTA
4	Index 88	AAGTTC
5	Index 99	GGTGTT
6	Index 90	TACTGT
7	Index 91	GTTAGG
8	Index 92	TCATCG
9	Index 93	ATCGAC
10	Index 94	CTATGG
11	Index 95	TAGGAT
12	Index 96	ATGCGC
13	PCR Primer	n/a

Set 4: Gel Purification Kit (store at room temperature)

1. Gel Cutter Tools
2. Gel Breaker Tubes

Consumables Preparation

The kit contains all necessary reagents to perform the experiment with the exception of common consumables and instruments. Please make sure all equipment is available before starting this experiment (Table 1).

Table 1 List of Consumables and Equipment

Consumables and Equipment	Supplier
1.5 ml nuclease-free microcentrifuge tubes	General lab supplier
200 µL, clean, nuclease-free PCR tubes	General lab supplier
Nuclease-free water	General lab supplier
TE buffer	General lab supplier
Tween-20	General lab supplier
Cooler block (optional)	SeqMatic, 6388-001
Thermal cycler	General lab supplier
Ethanol	General lab supplier
TailorMag 12 Tube PCR Magnetic Stand	SeqMatic, TM-700
5X TBE buffer	General lab supplier
8% TBE Gels 1.0 mm, 10 Well	LIFE Technologies, EC6215BOX
Hi-Density TBE Sample Buffer (5X)	LIFE Technologies, LC6678
100bp DNA Ladder (Optional)	SeqMatic, R-100L
Ethidium Bromide 10mg/ml	BioRad, 161-0433
UV transilluminator	General lab supplier
SYBR® Gold Nucleic Acid Gel Stain (Optional)	LIFE Technologies, S-11494
Dark reader transilluminator (Optional)	General lab supplier
XCell SureLock® Mini-Cell	LIFE Technologies, EI0001
Electrophoresis power supply	General lab supplier
Bench top microcentrifuge	General lab supplier
Tube shaker or thermal mixer	General lab supplier
2100 Bioanalyzer	Agilent Technologies
Agilent High Sensitivity DNA Kit	Agilent Technologies, 5067-4626

Related Products

TailorMag 12 Tube PCR Magnetic Stand (version 2)	Cat. TM-710
TailorMag Purification Beads	Cat. TM-601
Gel Cutter Tips (pack of 25)	Cat. TC-201
Gel Breaker Tubes (pack of 100)	Cat. TC-200

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 1 ng to 10 ng of purified high quality human total RNA as input. Micro RNA populations vary among different tissue types and species, therefore 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.

Micro RNA Library Sample Preparation Protocol

3' Adapter Ligation

1. Thaw Mix C310 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 A310	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 B310	2
Mix C310	6.5
Total	**16.5**

Note: Mix C310 is a highly viscous reagent. Handle with care and pipette slowly to ensure the correct amount of Mix C310 is dispensed for each reaction.

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

Note: Reaction mix is viscous. Handle with care to ensure the reagents are thoroughly mixed.

Ligation Product Clean Up

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

8. Prepare 80% ethanol for wash steps.

9. Mix 30 µL of TPB with each 3'-adapter ligated sample from Step 6. Vortex mix thoroughly and incubate at room temperature for 5 minutes. Pulse spin if necessary.

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.

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

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

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

12. 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.

13. Air dry sample tube at room temperature.

Note: TailorMag Purification Beads are dried within 5 to 10 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.

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

5’ Adapter Ligation

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

Reagent	Volume (µL)
3’ Adapter Ligated RNA from Step 14 (contains TPB)	7
Mix D310	3
Mix E310	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 D310 and Mix E310 to the sample in consecutive steps.

16. Vortex mix thoroughly and incubate at 25°C for 1 hour and then place the tube on ice.

cDNA Synthesis

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

18. 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 F310	2
Mix G310	1
Total	**15**

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

19. 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 with TPB could be stored at -20°C for up to seven days.

PCR Amplification

Note: This protocol has been optimized using 1 - 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.

20. 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 H310	8
PCR Primer	1
Index Primer*	1
Total	**25**

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

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

1) 95°C for 30 seconds
2) PCR cycles (See Table 2 for cycling numbers):
    i. 95°C for 5 seconds
    ii. 60°C for 15 seconds
    iii. 72°C for 1 minute
3) 72°C for 5 minutes
4) Hold at 4°C

Table 2 PCR cycle number reference

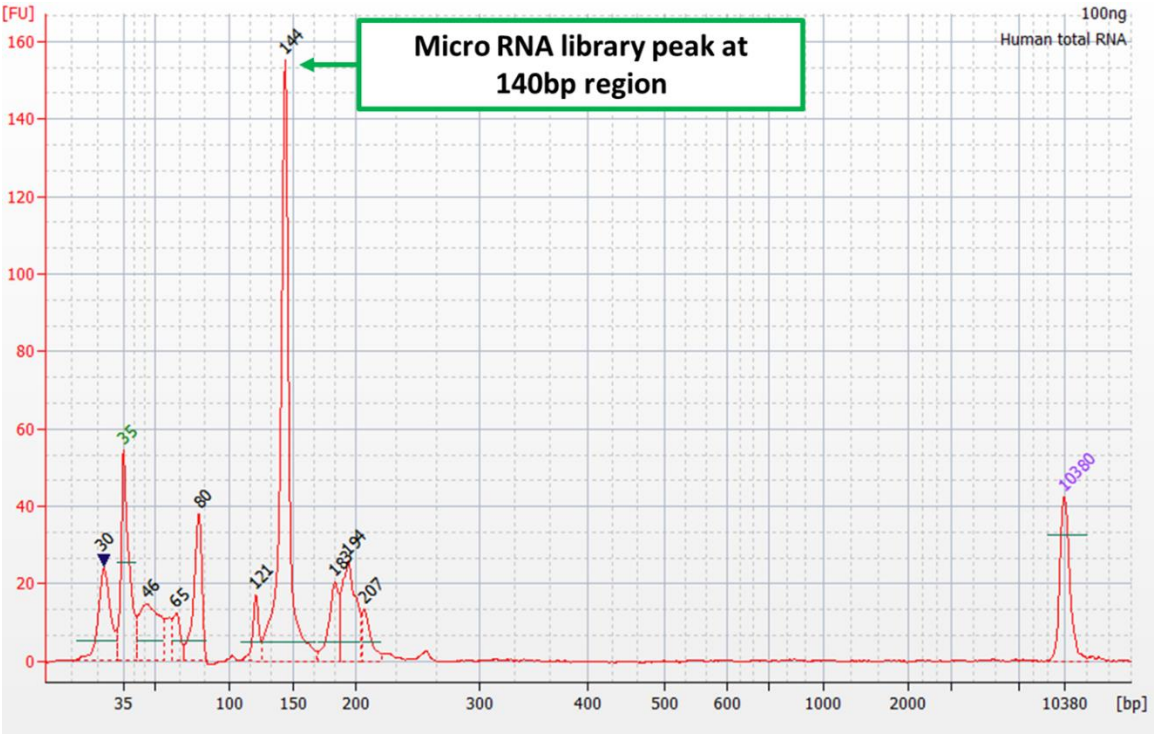
RNA input	Recommended PCR cycle number
1,000 ng	15
100 ng	18
10 ng	18
1 ng or lower	21

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

22. 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 Tissue Total RNA Sample



Library Size-Selection

Note: Library size-selection could be performed on individual PCR products or on pool of PCR products. See Appendix A for details.

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

24. Assemble the gel electrophoresis apparatus.

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

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

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

28. 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.

29. 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.

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

31. Run the gel at 145V for 65 to 75 minutes 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.

Recover Purified Library

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

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

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

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

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

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

37. 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

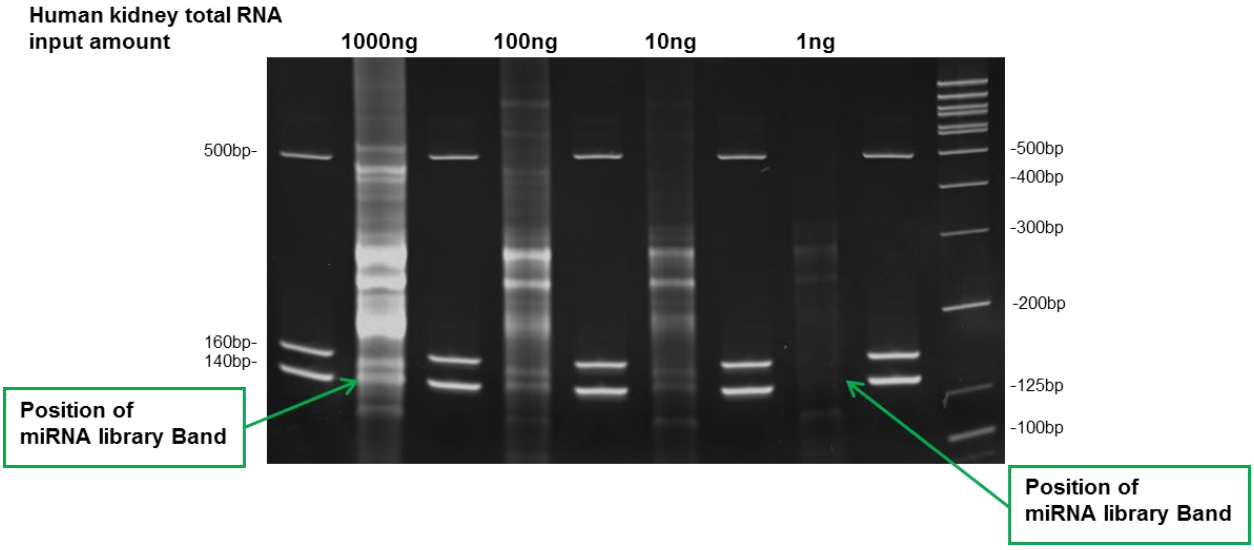


Figure 4 Close up of gel cutting position for miRNA libraries

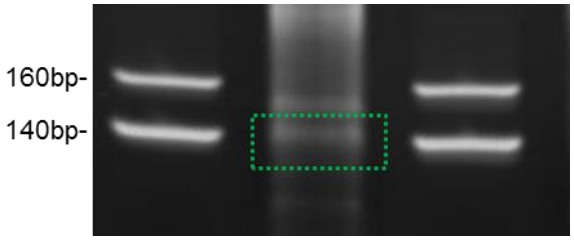
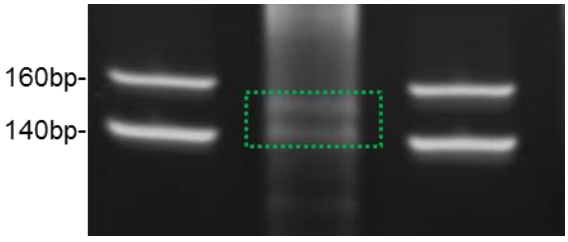


Figure 5 Close up of gel cutting position for isolating miRNA and other small RNA species



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

39. 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.

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

41. 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.

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

    Note: Do NOT heat up gel buffer mix.

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

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

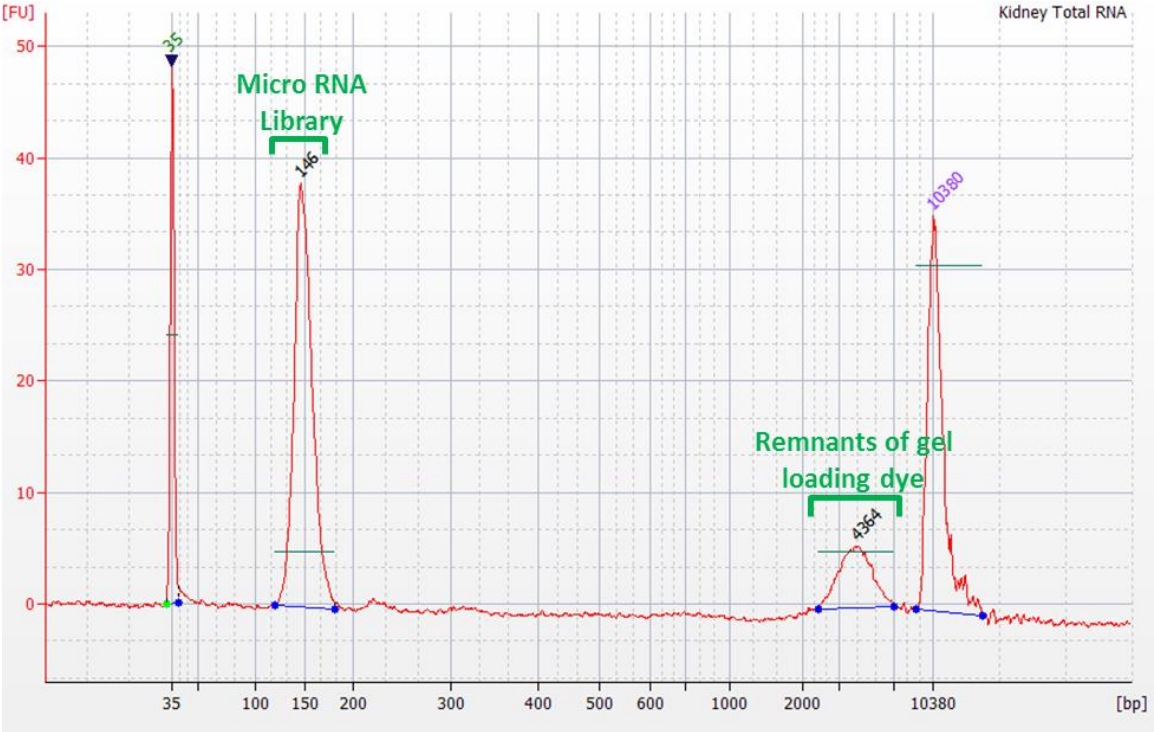
Library Validation

45. 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



Appendix A: Library Pooling prior to size-selection

Normalize Pooling of PCR Products

1) QC the PCR products with Agilent BioAnalyzer High Sensitivity DNA assay (step 22).

2) Under the "Region Table" tab of the assay file, record units of "Corr. Area" of the 140bp peak.

   Note: Adjust region range according to target library size.

3) Normalize and pool PCR products based on the "Corr. Area" of the 140bp peaks.

Library Size-Selection, Purification and Validation

4) Follow step 23 to step 44 of standard protocol.

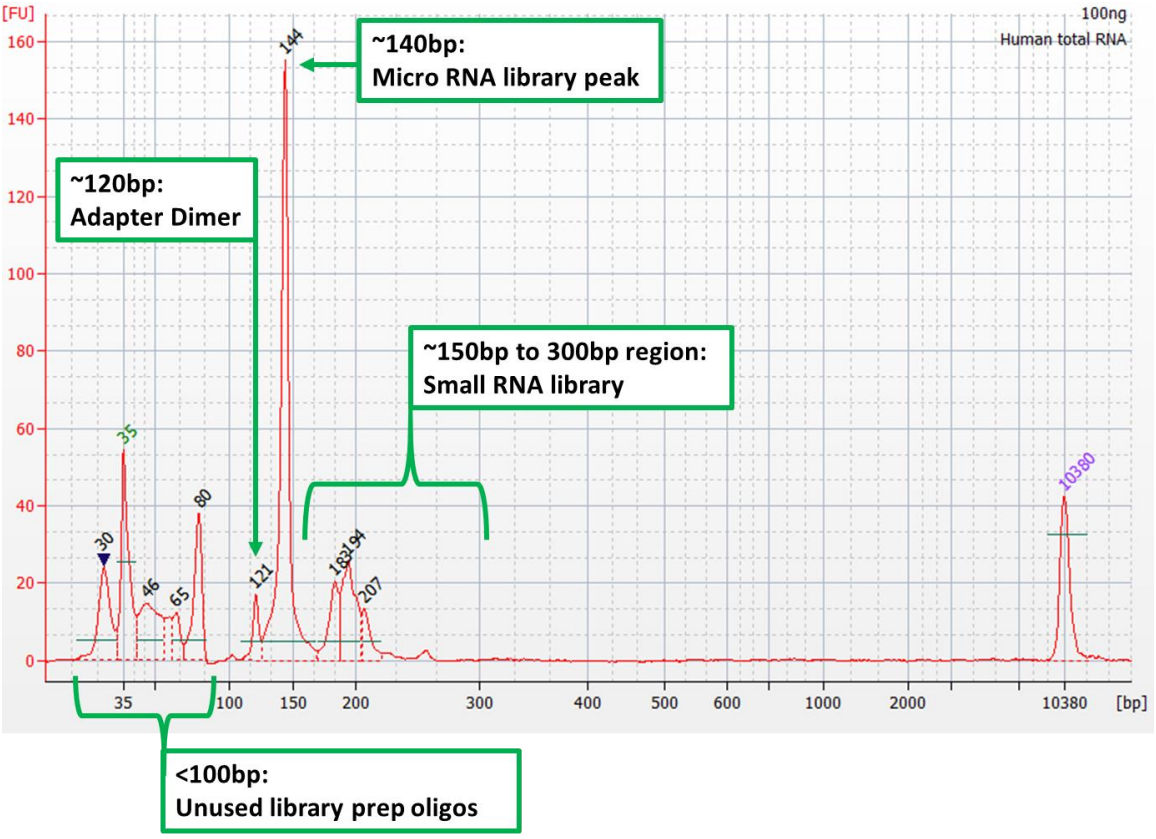
   Note: Add 2.5 µL of Hi-Density TBE Sample Buffer per 25 µL of PCR product and pipet mix thoroughly.

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

Appendix B: Frequently Asked Questions

Q1: What is the typical profile of the TailorMix Micro RNA kit PCR products on a Bioanalyzer DNA High Sensitivity assay?

A1: Each sample has its unique PCR product profile. However, four regions could be identified in most of the Bioanalyzer DNA High Sensitivity assay electropherograms:



	Position	Content
Region 1	<100bp, multiple peaks	Unused library prep oligos, such as adapters and primers
Region 2	A peak at approximately the 120 bp position	Adapter Dimers
Region 3	A peak at approximately the 140 bp position	Micro RNA libraries
Region 4	From about 150bp to 300bp, may contain multiple peaks	Small RNA libraries

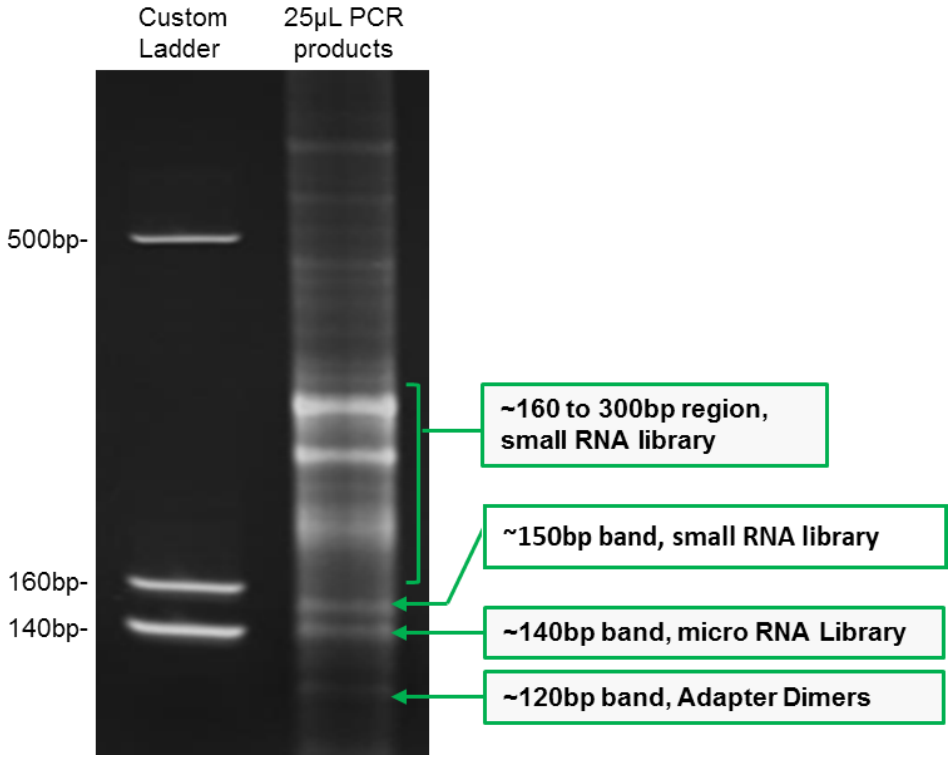
Appendix B: Frequently Asked Questions (Continued)

Q2: What is the typical profile of the TailorMix Micro RNA kit PCR products on an 8% PAGE gel?

A2: Each sample has its unique PCR product profile. In most of the gel images, the adapter dimer band is visible as a band located below the 140bp marker band.

For low yield libraries, the targeted micro RNA library band might not be visible on the gel image. To maximize library yield for such libraries, a gel cut that includes both the 140bp and 150bp region is recommended (see Figure 5).

As an example, below is the profile of the PCR product from 100ng total human kidney total RNA.



PAGE gel used:	8% TBE Gels 1.0 mm, 10 Well (LIFE Technologies, EC6215BOX)
Voltage:	145V
Run Time:	65 minutes
Gel staining conditions:	1µg/ml ethidium bromide solution for 1 minute

Appendix B: Frequently Asked Questions (Continued)

Q3: Can I prepare a small RNA library with the TailorMix miRNA Sample Preparation kit?

A3: Yes. Simply extend the gel cutting region to include the desired small RNA library bands. The same gel cutter can be used to make the serial cuts and the gel sections will be stacked within the gel cutter. After the gel fragmentation step (Step 41), add in additional TE buffer with 0.1% Tween-20 to ensure all gel fragments is submerged in the buffer.

Q4: What if I cannot see a library band within the 140bp to 160bp region on the PAGE gel?

A4: If the library band is not visible on the PAGE gel, excise the region between the two custom ladder markers (Figure 5). The gel purification step would help concentrating the libraries. Check final library yield with the Bioanalyzer DNA High Sensitivity assay.

Avoid cutting into the 120bp adapter dimer band (below the 140bp ladder band), which may appear stronger in the low yield libraries.

Q5: What if I cannot see a library peak at the 140bp region on the Bioanalyzer DNA High Sensitivity assay of the PCR products?

A5: Sometimes the strong background of the PCR products may mask the presence of the library peak. The gel purification step could help concentrating the library products for sequencing. On the PAGE gel, excise the region between the two custom ladder markers (Figure 5). Check final library yield with the Bioanalyzer DNA High Sensitivity assay.

Avoid cutting into the 120bp adapter dimer band as it may appear stronger than the micro RNA library band in low yield libraries.

Q6: What if my final library contains part of the 120bp band?

A6: The 120bp PCR products contain adapter dimers, therefore avoid collecting this product during size-selection as much as possible. Small percentage of the 120bp product does not affect quality of the sequencing data.

Appendix B: Frequently Asked Questions (Continued)

Q7: What if the 120bp band and 140bp band are very close to each other on the PAGE gel?

A7: The separation of PCR product bands may vary due to variations of the electrophoresis equipment. Optimize run time and voltage of the electrophoresis equipment to maximize separation of the 140bp micro RNA library band from the 120bp adapter dimer band.

Q8: How could I avoid cutting into the 120bp band?

A8: Avoid cutting into the 120bp band by adjusting the cutting position upward (Figure 5). It is safe to include part of the 150bp band in the final micro RNA library.

Q9: Do I have to incubate my gel cut in TE buffer with 0.1% Tween-20 overnight?

A9: Longer incubation time increases library recovery rate and this is especially important for low yield libraries (for example, libraries that do not show obvious library peak in the Bioanalzyer DNA High Sensitivity Assay or libraries that do not have a visible library band in the PAGE gel). Optimization is required to shorten the incubation time.

To check the recovery rate, before transferring the eluate to a new collection tube, take 1 µL of the eluate and check library concentration with the Bioanalyzer DNA High Sensitivity Assay. Extend the incubation time to an overnight incubation if recovery is low.

Q10: What if I see a strong 150bp band and a weak 140bp band in my PCR products?

A10: The micro RNA library is most abundant in the 140bp region. If the intensity of the 150bp band is significantly higher than the 140bp band, avoid the 150bp products as much as possible during gel cut.