Manual

Version 2.0

Product name: RNA-Seq Library Construction Kit

Cat #: NGRR-100, NGRR-200, NGRR-300

Description:

As more than 60% of the genome is now believed to be transcribed, understanding the changes in the coding genes (< 2% of the genome) as well as the noncoding regulatory transcriptome is critical for a clear elucidation of genome. Deep sequencing of transcriptomes (RNA-Seq) provides a comprehensive amount of information including highly sensitive and accurate gene-expression levels, alternative splice forms, novel fusion transcripts, and mutational changes that have a direct impact on protein functions, and so on. Disease related RNA-Seq has revealed unexpected complexity including the presence of proteins that are unique to the specific conditions.
Sufficient reagents are supplied in the MCLAB RNA-Seq Library Construction Kit (Illumina®-compatible) to prepare cDNA libraries from 8 samples for high-throughput sequencing through the Illumina platform. Overview of the process is shown below (Figure 1).

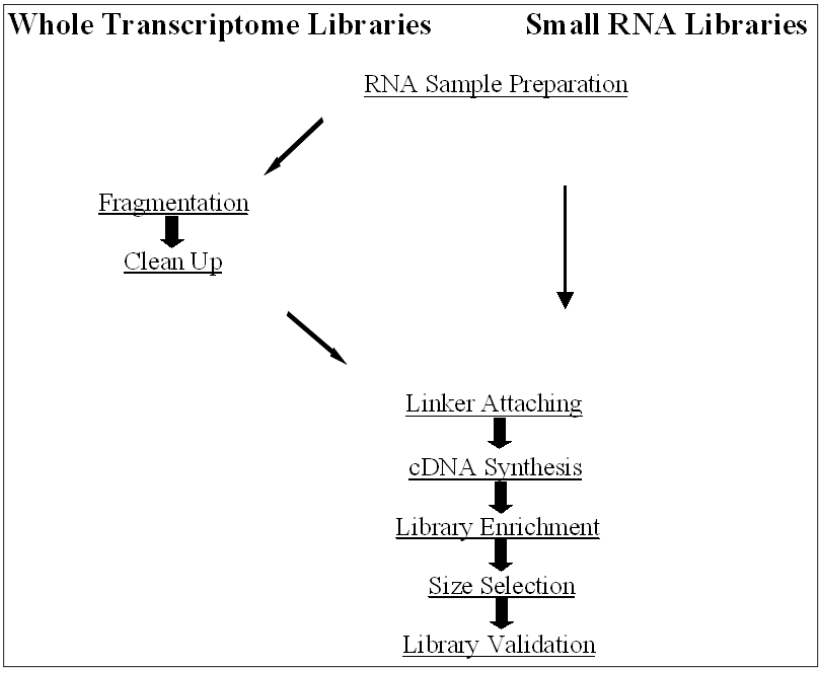


Figure 1 The MCLAB RNA-Seq Library Construction Procedure

• For whole transcriptome libraries, follow the procedure on page 4.
• For small RNA libraries, follow the procedure on page 9.

List of Components

Sufficient reagents are supplied in the MCLAB RNA-Seq Library Construction Kit (Illumina®-compatible) to prepare cDNA libraries from 8 samples for high-throughput sequencing through the Illumina platform. Upon receipt of the kit, immediately store the components at –20 °C in a freezer without a defrost cycle.

Box 1:

Fragmentation Solution	27 µl
Fragmentation Enzyme Mix	36 µl
Linker-A	13.5 µl
Linker Enzyme Mix-A	18 µl
Linker Addition Buffer	18 µl
Reaction Enhancer	13.5 µl
Linker-B	13.5 µl
Linker Enzyme Mix-B	13.5 µl

Box 2:

RT Primer	22.5 µl
RT Mix	22.5 µl
PCR Mix	225 µl
Primer-1	18 µl
Library Elution Buffer	1.5 mL

Box 3:

Index Primer 1	18 µl
Index Primer 2	18 µl
Index Primer 3	18 µl
Index Primer 4	18 µl
Index Primer 5	18 µl
Index Primer 6	18 µl
Index Primer 7	18 µl
Index Primer 8	18 µl

Additional Materials Required

The following equipments, consumables and materials are required but not included for both libraries:
Thermal cycler with heated lid
NanoDrop Spectrophotometer (Thermo Scientific)
Microcentrifuge
Vortexer
Benchtop Cooler (optional)
Pipettors, positive displacement or air-displacement
PCR Tubes & Caps, RNase-free, 0.2-mL
Non-Stick RNase-free Microfuge Tubes (0.5 mL, 1.5 mL)
Pipette tips, RNase-free
Nuclease-free Water
Agilent 2100 Bioanalyzer (Agilent)
Agilent DNA 1000 Kit (Agilent)
Agilent High Sensitivity DNA Kit (optional)
RNA 6000 Pico Chip Kit (optional)

The following items are required but not supplied for whole transcriptome libraries:
Poly(A) + RNA Selection Kit (optional)
Ribo-Zero™ rRNA Removal Kit (Epicentre, optional)
Zymo RNA Clean & Concentrate Kit (Zymo)
100mM EDTA
Ethanol, 100%, ACS reagent grade or equivalent
Agencourt AMPure® XP System (Beckman Coulter)
Magnetic stand

The following items are required but not supplied for small RNA libraries:
miRNA Isolation Kit
Gel Electrophoresis device
Novex® Hi-Density TBE Sample Buffer (Invitrogen)
10 bp DNA Ladder (Invitrogen)
Novex 6% TBE Gels 1.0 mm, 10 well (Invitrogen)
Novex TBE Running Buffer (Invitrogen)
Razor Blade (optional)
x-tracta Gel Extraction Tool (Sigma, optional)
GenElute™ Gel Extraction Kit (Sigma)
Spin Columns and Elution Tubes (Invitrogen, optional)
Ultra Pure Ethidium Bromide (optional)
SYBR® Gold Gel Stain (Invitrogen, optional)
Glycogen (optional)
Isopropanol (optional)
Dark Reader or UV Transilluminator(optional)
Tube Shaker or Rotator

Construct Whole Transcriptome Libraries

Guidelines for RNA sample to construct library

- The MCLAB RNA-Seq Library Construction Kit can be used with poly(A)+ RNA or with rRNA-depleted RNA. If only interest in mRNA, we recommend using the poly(A)+ RNA selection kit to purify the intact total RNA. Check the profile of the poly(A)+ RNA on an Agilent® 2100 Bioanalyzer™ Instrument to confirm the absence of 18S and 28S rRNA. To maximize the removal of rRNA, we recommend using one of the Epicentre's Ribo-Zero™ rRNA Removal Kits. Duplex-Specific thermostable nuclease (DSN) enzyme normalization kit from Illumina involves the degradation of abundant DNA molecules derived from rRNA, tRNA, and housekeeping genes while preserving DNA molecules derived from less abundant transcripts.
- Use high-quality RNA as your starting material.
- The RNA sample from human, animal, plant, viral or bacterial species should be dissolved in RNase-free water and free of contaminating salts, metal ions, ethanol, and phenol.
- RNA extracted from FFPE cell/tissue has been tested successfully but the quality of FFPE RNA can be highly variable due to the fixation procedure, age of the sample, storage conditions, etc.

RNA Sample Preparation
Prepare 10–500 ng poly(A)+ RNA or rRNA-depleted total RNA in maximum 25 µL RNase-free water to construct whole transcriptome libraries.

Kit Procedure
MCLAB recommends that store the enzyme mix in a benchtop cooler (–20˚C) to avoid repeated freeze-thaws. Remove all the components to thaw and store on ice.
Centrifuge the tubes briefly before opening.

A. Fragment the RNA

1. Run and hold the thermal cycler at 92°C.
2. In a 0.2-ml PCR tube on ice, assemble the following reaction mixture:

	Component	Volume
	Fragmentation Solution	3 µL
	Poly(A)+ RNA or rRNA-depleted total RNA	X µL *
	Nuclease-free Water	Add till to final 28 µL
Total Volume	28 µL	

Total Volume 28 µL
* Maximum volume at 25 µL.

3. Flick the tube or pipette up and down a few times to mix, then spin briefly.
4. Incubate the reaction in the pre-heated thermal cycler at 92°C for 2 minutes.
5. Place the tube on ice and immediately add 3 µL of 100 mM EDTA.
6. Flick the tube or pipette up and down a few times to mix.
7. Add 4 µL Fragmentation Enzyme Mix.
8. Incubate the reaction in the pre-heated thermal cycler at 37°C for 45 minutes.
9. Place the tube on ice and go to the next step.

B. Clean up the Fragmented RNA
MCLAB recommends use RNA Clean & Concentrator-5 kit from Zymo to purify fragmented RNA.

1. Transfer fragmented RNA sample to a new 1.5 mL tube, add RNA Binding Buffer to a total volume of 75 µL and mix thoroughly.
2. Add 75 µL 100% ethanol, invert the tube to mix well.
3. Transfer the mixture to the Zymo-Spin column and centrifuge at 12,000 rpm for 1 minute.
4. Add 400 µL RNA Prep Buffer to the column and centrifuge at 12,000 rpm for 1 minute. Discard the flow through.
5. Add 800 µL RNA Wash Buffer to the column and centrifuge at 12,000 rpm for 0.5 minute. Discard the flow through.
6. Add 400 µL RNA Wash Buffer to the column and centrifuge at 12,000 rpm for 2 minutes.
7. Transfer the column to a new 1.5 mL tube.
8. Add 12 µL Nuclease-free water to the center of the column membrane, wait for 1 minute.
9. Centrifuge at 10,000 rpm for 0.5 minute to recover approximately 10 µL of fragmented RNA from the column.

The RNA can be fragmented and purified by other methods upon availability. If so, the fragmented RNA must be dissolved in about 10 µl of Nuclease-Free Water. To use the RiboMinus™ Concentration Module (Invitrogen), please refer to Appendix 2.

C. Performance Specifications of the Fragmented RNA (optional)
Use the RNA 6000 Pico Chip Kit with the Agilent 2100 Bioanalyzer instrument to assess the yield and size distribution of the fragmented RNA.

1. Quantitate the yield of the fragmented RNA using the Quant-iT RNA Assay Kit on the Qubit Fluorometer. Refer to the Invitrogen Quant-iT™ RNA Assay Kit Protocol or the Qubit®Fluorometer Instruction Manual for instructions.
2. Assess the size distribution of the fragmented RNA using the RNA 6000 Pico Chip Kit with the Agilent 2100 Bioanalyzer instrument:

a. Dilute 1 µL of the sample 1:10 with Nuclease-free Water.
b. Follow the manufacturer's instructions for performing the assay, run the diluted sample on an Agilent 2100 Bioanalyzer Instrument with the RNA 6000 Pico Chip Kit.
c. Using the 2100 expert software, review the size distribution.

The fragmentation procedure should produce a distribution of RNA fragments with sizes from near 100 nt to several hundred nt, depending on your sample type. The average sizes should between 100 and 200 nt. If the fragmented RNA profile does not meet the specifications, fragmentation optimization is recommended.

D. Attach Linkers to the RNA Targets
The RNA samples are attached with the linkers, which is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for the Illumina sequencing platform at the other end. The linkers constrain the orientation of the RNA in this reaction to facilitate generating strand specific sequencing data.

Pre-heat a thermal cycler to 68°C.

1. Set up reaction in a sterile, nuclease-free 200 µL PCR tube on ice as below:

	Component	Volume
	Purified RNA Fragments	6 µL
	Linker-A	1.5 µL
Total Volume	7.5 µL	

2. Flick the tube or slowly pipette up and down a few times to mix well, then spin briefly.
3. Incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
4. Set up mixture in a new sterile, nuclease-free 200 µL PCR tube on ice as below:

	Component	Volume
	Linker Addition Buffer	2 µL
	Linker Enzyme Mix-A	2 µL
Total Volume	4 µL	

5. Mix well, then spin briefly.
6. Transfer these 4 µL mixture to the reaction tube from Step 3. Mix well and then spin briefly.
7. Incubate the tube at 25°C for 1 hour.
8. Aliquot 1.5 µL Reaction Enhancer to a new PCR tube, incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
9. Directly add 1.5 µL Reaction Enhancer into the Linker Addition reaction tube on the thermal cycler, mix well, continue incubate the tube at 25°C for another 15 minutes, and then store the tube on ice.
10. Transfer 1.5 µL Linker-B into a new PCR tube.
11. Incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
12. Add 1.5 µL Linker Enzyme Mix-B to the tube from last step and mix well.
13. Transfer these 3 µL mixture to the reaction tube from Step 8. Mix well and then spin briefly.
14. Incubate the tube at 30°C for 50 minutes, and then place on ice.

E: Synthesize cDNA

1. Aliquot 2.5 µL RT Primer to a new PCR tube, incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
2. Transfer 8.5 µL linkers attached RNA into this PCR tube on ice. Store the left RNA at -80°C.
3. Add 2 µL RT Mix to this tube, mix well and then spin briefly.
4. Incubate the tube at 42°C for 50 minutes, and then place on ice.

F: Enrich cDNA Library

1. Set up PCR reaction in a new PCR tube on ice as below:

	Component	Volume
	PCR Mixture	25 µL
	Primer-1	2 µL
	Index Primer *	2 µL
	Nuclease-free water	8.5 µL
Total Volume	37.5 µL	

* Total 8 options included for multiplex library construction (Appendix 5).

Master mix should be made for multiple reactions with 5% extra reagents.

2. Transfer 12.5 µL cDNA to a PCR reaction tube. Store the left cDNA at -20°C. Gently mix thoroughly and then centrifuge briefly.
3. Place the tube in the thermal cycler using the following PCR cycling conditions:
   - One circle at 98°C for 30 seconds;
   - Followed by 12* cycles of: 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 15 seconds;
   - 72°C for 10 minutes;
   - 4°C hold.

*: Limiting the cycle number prevents PCR bias introducing into the RNA profile of the sample and minimizes duplicate sequencing reads. PCR reaction cycle is generally limited which depends on starting material's resource and quality. If necessary, adjust the number of cycles according to the amount of input fragmented RNA.

G: Purify the Amplified DNA Library

MCLAB recommends using the AMPure XP system (Beckman Coulter) to purify the libraries. The AMPure XP System has shown better performance on removing PCR primer-dimers.

1. Remove the AMPure XP beads to room temperature for at least 30 minutes.
2. Prepare 400 µL of fresh 80% ethanol at room temperature for each sample.
3. Vortex the AMPure XP beads until they are a homogeneous suspension.
4. Transfer 40 to 50 µL (cut off around 180 or 150nt) of the beads to a microfuge tube (or each well independently if using 96-well plate format).
5. Add 45 µL amplified library into the bead tube.
6. Mix thoroughly by pipetting.
7. Incubate the tube at room temperature for 15 minutes.
8. Place the tube in a magnetic stand at room temperature for at least 5 minutes.
9. Remove and discard the supernatant from tube using a pipette without disturbing the beads.
10. Remain the tube on the magnetic stand, add 200 µL of 80% ethanol to the tube without disturbing the beads.
11. Incubate the tube at room temperature for at least 30 seconds, then remove and discard all of the supernatant without disturbing the beads.
12. Repeat steps 10 and 11 for a second round of 80% ethanol wash.
13. Briefly spin the tube, put back to the magnetic stand for 1 minute
14. Remove any remaining liquid at the bottom of the tube. Allow the tube to air-dry on the magnetic stands for 5 minutes at room temperature or until see small cracks in the dried bead pellete surface.
15. Remove from the magnetic stand, add 12 µL of Library Elution Buffer to the tube, thoroughly resuspend the beads by pipetting.
16. Incubate the tube at room temperature for 2 minutes.
17. Place the tube on the magnetic stand at room temperature for at least 5 minutes.
18. Transfer about 10 µL of the clear supernatant to an appropriate collection tube for library validation.

Please refer to Appendix 3 if using the MinElute PCR Purification system (Qiagen).

H: Validate the Library

Before proceeding with cluster generation for sequencing on the Genome Analyzer, HiSeq or loading to the MiSeq cartridge, MCLAB recommends performing the following analysis to assess library quantity and quality.

1. Assess the yield and size distribution of the amplified DNA use the Agilent 2100 Bioanalyzer Instrument

with the DNA 1000 Kit (Figure 2), or High Sensitivity DNA Kit if necessary. Determine the median peak size (bp) and molar concentration (nM) of the library.

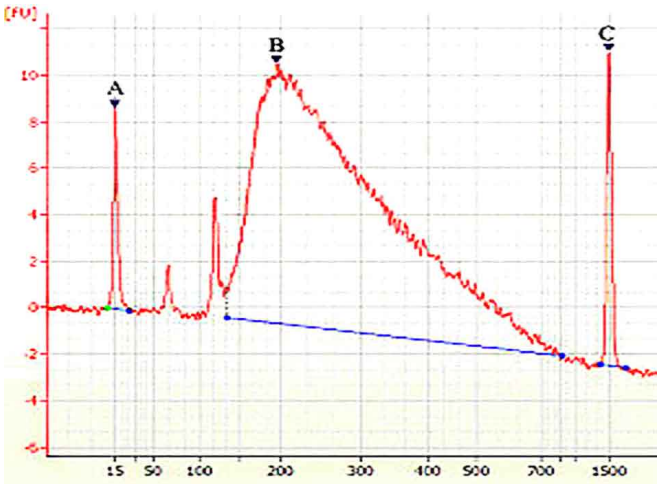


Figure 2. Purified Final RNA-Seq Library Bioanalyzer Profile on a DNA-1000 Chip.
Peak A: Lower Marker; Peak B: RNA-Seq Library; Peak C: Upper Marker

2. Measure the concentration of the purified DNA with the use of the PicoGreen reagent (Life Technologies, Carlsbad, CA). A qPCR quantitation method using a KAPA Biosystems (Woburn, MA) Library Quant Kit or other SYBR Green qPCR kits determine the levels of adapter-bound fragments. The recovery of your experimental RNA depends on its source and quality.

Construct Small RNA Libraries

Guidelines for Small RNA Library Construction

- For this protocol, starting RNA sample must contains the small RNA fraction (microRNA or miRNA, 10–40 nt).
- The amount of small RNA in samples varies widely according to the tissue source and the RNA isolation method. A survey by Agilent provides a guide for the relative proportion of miRNA of 40 different tissues (Tissot 2008, Appendix 4).
- Low amounts of microRNA in starting material can result in poor yield. MCLAB recommends using the following products:
- Recommended RNA source: Use Ambion® FirstChoice® Total RNA, which is certified to contain miRNA and other small RNAs.
- Recommended RNA isolation kits: MCLAB recommends using precipitation-based methods over column-based methods for total RNA purification for maximal recovery of microRNA.
- Degrade RNA sample may affect the quantitation of small RNA in the sample and interfere with small RNA ligation resulting low yield.
- For optimal results, use RNA that has been size selected for miRNA. When total RNA is used in the MCLAB RNA-Seq Library Construction Kit procedure, the resulting reaction products comprise a larger size range than those produced from small RNA-enriched samples.

Kit Procedure

MCLAB recommends that store enzyme mix in a benchtop cooler (–20˚C) to avoid repeated freeze-thaws. Remove all components to thaw and store on ice.
Centrifuge tubes briefly before opening.

A: Attach Linkers to the RNA Targets
The RNA samples are attached with the linkers, which is a set of oligonucleotides with a single-stranded degenerate sequence at one end and a defined sequence required for Illumina sequencing platform at the other end. The linkers constrain the orientation of the RNA in this reaction to facilitate generating strand specific sequencing data.

Pre-heat a thermal cycler to 68°C.
1. Set up reaction in a sterile, nuclease-free 200 µL PCR tube on ice as below:

	Component	Volume
	RNA sample	6 µL
	Linker-A	1.5 µL
Total Volume	7.5 µL	

2. Flick the tube or slowly pipette up and down a few times to mix well, then spin briefly.
3. Incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
4. Set up the mixture in a new sterile, nuclease-free 200 µL PCR tube on ice as below:

	Component	Volume
	Linker Addition Buffer	2 µL
	Linker Enzyme Mix-A	2 µL
Total Volume	4 µL	

5. Mix well, then spin briefly.
6. Transfer these 4 μL mixture to the reaction tube from Step 3. Mix well and then spin briefly.
7. Incubate the tube at 25°C for 1 hour.
8. Aliquot 1.5 μL Reaction Enhancer to a new PCR tube, incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
9. Directly add this 1.5 μLReaction Enhancer into the Linker Addition reaction tube on the thermal cycler from Step 7, mix well, continue incubate the tube at 25°C for another 15 minutes, and then store the tube on ice.
10. Transfer 1.5 μL Linker-B into a new PCR tube.
11. Incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
12. Add 1.5 μL Linker Enzyme Mix-B to the tube from last step and mix well.
13. Transfer these 3 μL mixture to the reaction tube from Step 9. Mix well and then spin briefly.
14. Incubate the tube at 30°C for 50 minutes, and then place on ice.

B: Synthesize cDNA

1. Aliquot 2.5 μL RT Primer to a new PCR tube, incubate the tube at 68°C for 2 minutes and then immediately place the tube on ice.
2. Transfer 8.5 μL linkers attached RNA into this PCR tube on ice. Store the left RNA at -80°C.
3. Add 2 μL RT Mix to this tube, mix well and then spin briefly.
4. Incubate the tube at 42°C for 50 minutes, and then place on ice.

C: Enrich cDNA Library

1. Set up PCR reaction in a new PCR tube on ice as below:

	Component	Volume
	PCR Mixture	25 μL
	Primer-1	2 μL
	Index Primer *	2 μL
	Nuclease-free water	8.5 μL
Total Volume	37.5 μL	

* Total 8 options included for multiplex library construction (Appendix 5).

Master mix should be made for multiple reactions with 5% extra reagents.

2. Transfer 12.5 μL cDNA to a PCR reaction tube. Store the remaining cDNA at -20°C. Gently mix thoroughly and then centrifuge briefly
3. Place the tube in the thermal cycler using the following PCR cycling conditions:
   - One circle at 98°C for 30 seconds;
   - Followed by 12* cycles of: 98°C for 10 seconds, 60°C for 30 seconds, 72°C for 15 seconds;
   - 72°C for 10 minutes;
   - 4°C hold.

*: Limiting the cycle number prevents PCR bias introducing into the RNA profile of the sample and minimizes duplicate sequencing reads. PCR reaction cycle is generally limited which depends on starting material's resource and quality. You may test several PCRs with different cycles to see the minimum number of cycles needed to get a visible product on the gel (Figure 3). The fewer number of PCR cycles used to amplify your libraries, the less biased your libraries will be for the products that are more efficiently amplified.

D. Size-select the amplified cDNA

1. Prepare samples with 5X Novex Hi-Density TBE Sample Buffer or equivalent. Please load less than 30 μL

cDNA library each well (Novex® 6% TBE Gel 1.0 mm, 10 well).

2. Load 50-100ng/lane of 10bp DNA ladder (Invitrogen) in the outer side of the sample wells.
3. Run the gel for 60 minutes at 145 V or until the blue front dye exits the gel.
4. Stain the gel with Ethidium Bromide and then view the gel on a Dark Reader transilluminator or a UV transilluminator. Work quickly to limit its exposure to UV radiation or use SYBR® Gold nucleic acid gel stain (Invitrogen).
   You may see two close bands on your gel – the higher band (~140 bp) corresponds to the majority of the microRNA library, while the lower band (~120 bp) corresponds to a linker–linker ligation product (Figure 3 and 4). To get better separation of your target library from the linkers, MCLAB recommends running your library on a long gel at low voltage (and prefer in a cold room).

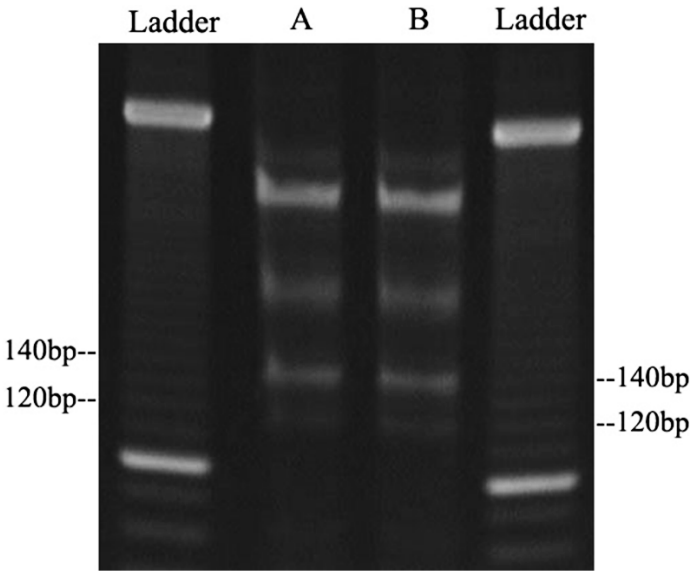


Figure 3 Duplicate Small RNA Library Samples (A and B) on 6% TBE Gel

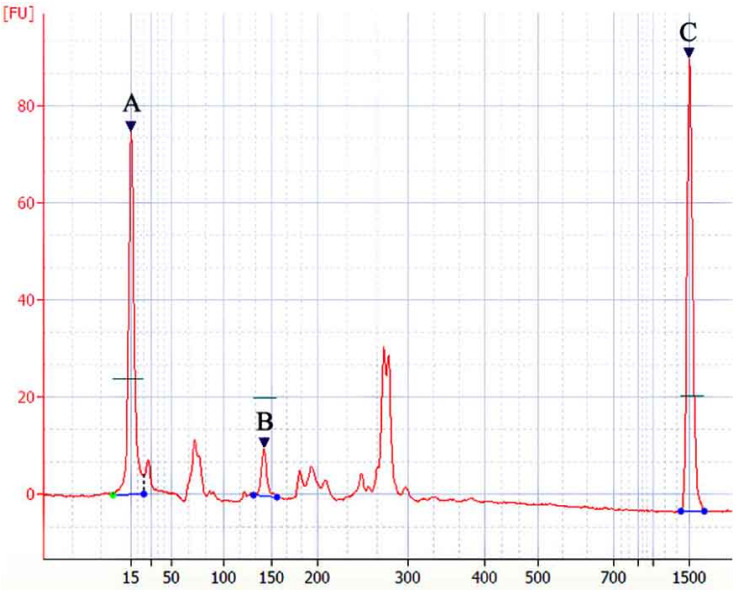


Figure 4. Small RNA Library Bioanalyzer Profile on a DNA-1000 Chip before size selection
Peak A: Lower Marker; Peak B: miRNA Library; Peak C: Upper Marker

5. Using the ladder bands and the ruler for reference, cut the band from the gel with a clean razor blade. Prepare a razor blade by treating it with DEPC H2O. Make sure to wash the razor blade with DEPC H2O between cutting out different libraries to avoid non-multiplex library cross-contamination or choose x-tracta Gel Extraction Tool from Sigma. Make sure to avoid cutting this lower band out of the gel.
6. Shred the gel piece:
   a. Use a 21-gauge needle to puncture through the bottom-center of a 0.5-mL microcentrifuge tube.
   b. Place the gel piece in the punctured 0.5-mL tube, then place the 0.5-mL tube into a larger, 1.5-mL, nuclease-free microcentrifuge tube.
   c. Spin for 3 minutes at top speed to shred the gel.
7. Purify the library:
   a. Add 180 µL of Library Elution Buffer to the shredded gel pieces.
   b. Incubate the mixture over 3 hours or overnight at room temperature, with gentle agitation.
   c. Cut a pipette tip to make a larger opening and use it to transfer the combined Library Elution Buffer, which contains eluted DNA and gel slurry, from each sample to a Spin Column (Invitrogen).
   d. Spin the column at top speed for 5 minutes to remove gel pieces.

The DNA library is now in the flow-through.

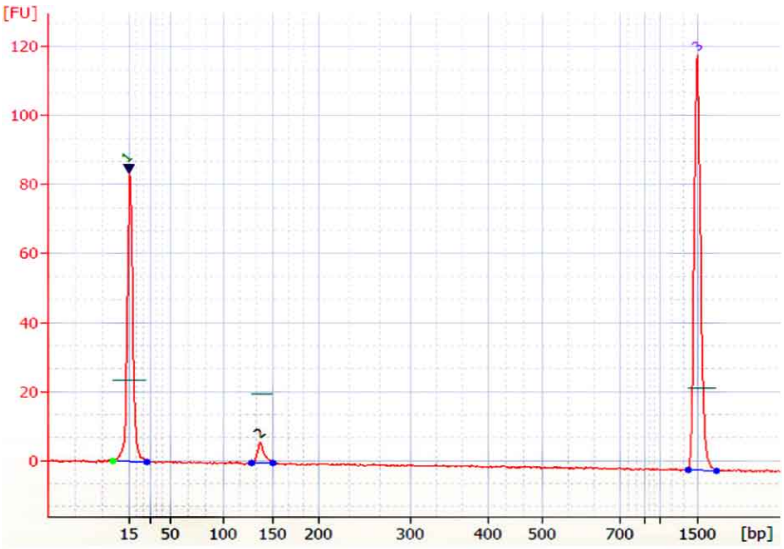
E: Concentrate library (Optional)

1. Add 1/100 volume of glycogen and 0.7 volume of isopropanol to each sample.
2. Mix thoroughly, then incubate the sample at room temperature for 5 minutes.
3. Spin the sample at the top speed for 20 minutes at room temperature.
4. Carefully remove and discard the supernatant, then air dry the pellet.
5. Resuspend the DNA pellet in 20 µL of Library Elution Buffer.

F: Library Validation

Before proceeding with cluster generation for sequencing on the Genome Analyzer, HiSeq or loading to MiSeq cartridge, MCLAB recommends performing the following analysis to assess library quantity and quality.

1. Assess the yield and size distribution of the size selected DNA use the Agilent 2100 Bioanalyzer Instrument with the DNA 1000 Kit (Figure 5), or High Sensitivity DNA Kit if necessary. Determine the peak size (bp) and molar concentration (nM) of the library.



Peak	Size [bp]	Conc. [ng/ul]	Molarity [nmol/l]	Observations
1	15	4.2	424.2	Lower Marker
2	144	0.22	2.3	miRNA Library
3	1500	2.1	2.1	Upper Marker

Figure 5 MicroRNA Library Bioanalyzer Profile on a DNA-1000 Chip (1:2 diluted)

2. Measure the concentration of the purified DNA with the use of the PicoGreen reagent (Life Technologies, Carlsbad, CA). A qPCR quantitation method using a KAPA Biosystems (Woburn, MA) Library Quant Kit or other SYBR Green qPCR kits to determine the levels of adapter-bound fragments. The recovery of your experimental RNA depends on its source and quality.

3. For further validation, small-scale Sanger sequencing of cloned RNAs is recommended to verify the correct size distribution of libraries as well as enrichment for tissue or cell-specific miRNAs before proceeding to high-throughput sequencing.

Troubleshooting

A general strategy is to save remaining samples from each key step for performing appropriate analysis, such as fragmentation clean up, linker addition, cDNA synthesis, library enrichment and size selection.

The other key strategy is to set parallel reaction with control RNA (Human Brain Total RNA from Ambion was used for this kit optimization) during the MCLAB RNA-Seq Library Construction Kit procedure, especially for first-time users.

Reference:

Levin JZ, et al. Nat Methods 2010; 7: 709–715

Appendix 1: Cautions and Recommendations

- Follow good laboratory practice guidelines for nucleic acid experiment.
- Set up reactions on ice to minimize the risk of RNA degradation.
- Include 5–10% excess volume in the master mix to compensate for pipetting error.
- Clean the lab bench and pipettors with an RNase decontamination solution.
- Wear clean gloves and a clean lab coat.
- Use a positive-displacement pipette.
- Use RNase-free pipette tips containing hydrophobic filters to minimize the risk of cross contamination.
- Centrifuge tubes before opening and keep reactions and components capped as much as possible.

Appendix 2: Fragmented RNA Clean up

RiboMinus™ Concentration Module (Invitrogen)
Prepare the 1.5 mL Wash Buffer (W5) with 6 mL 100% ethanol, then store at room temperature.

1. Transfer fragmented RNA sample to a new 1.5 mL tube, add Nuclease-free water to a total volume at 100 uL and mix thoroughly.
2. Add 100 µL binding buffer and 250 µL 100% ethanol to the fragmented RNA, then mix well.
3. Transfer the RNA sample containing Binding Buffer (L3) and ethanol to the Spin Column:

a. Place the Spin Column in a clean 1.5-mL Wash Tube.
b. Load 450 µL of the RNA sample containing Binding Buffer (L3) and ethanol onto the Spin Column.
c. Spin the column at 12,000 × g for 1 minute.
d. Discard the flow-through.

4. Wash the RNA:

a. Return the Spin Column to the Wash Tube.
b. Add 500 µL of Wash Buffer (W5) with ethanol to the Spin Column.
c. Spin the column at 12,000 × g for 1 minute.
d. Discard the flow-through.
e. Return the Spin Column in the Wash Tube.
f. Spin the column at maximum speed for 2 minutes.

5. Elute the RNA in a clean Recovery Tube:

a. Place the Spin Column in a clean Recovery Tube.
b. Add 12 µL of RNase-Free Water to the center of the Spin Column.
c. Wait 1 minute, then spin the column at maximum speed for 1 minute.

Appendix 3: Purify the Amplified DNA Library

It is often to see libraries purified using the column with primer-dimers' contamination. Recommend using MinElute PCR Purification Kit (Qiagen) for purifying libraries made from very low quality RNA sample (such as FFPE RNA) with an average size less than 180 nt.

1. Remove excess PCR primers by adding 1 µl of Exonuclease I to each reaction and incubate the reactions at 37°C for 15 minutes.
2. Purify the library using the MinElute Kit procedure described by the manufacturer.
3. Add 12 µl Library Elution Buffer to elute DNA.

Appendix 4:

The relative proportion of miRNA of 40 different tissues (Tissot 2008)

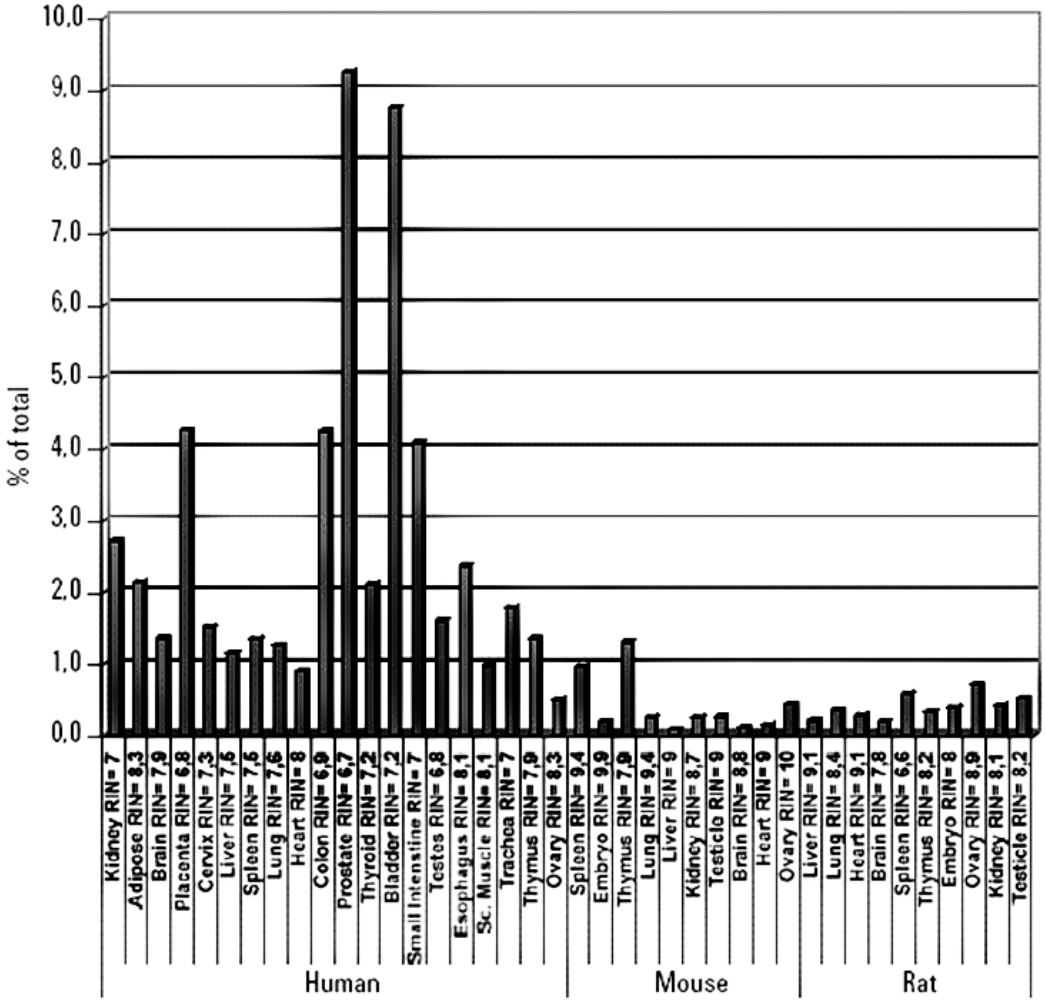


Figure 6. miRNA ratio in total RNA of 40 different tissues

Appendix 5:

Index Primer Codes for entry on sample sheet

Index Primer 1: ATGTCA
Index Primer 2: CCGTCC
Index Primer 3: GTCCGC
Index Primer 4: GTGAAA
Index Primer 5: GTGGCC
Index Primer 6: GTTTCG
Index Primer 7: CGTACG
Index Primer 8: GAGTGG