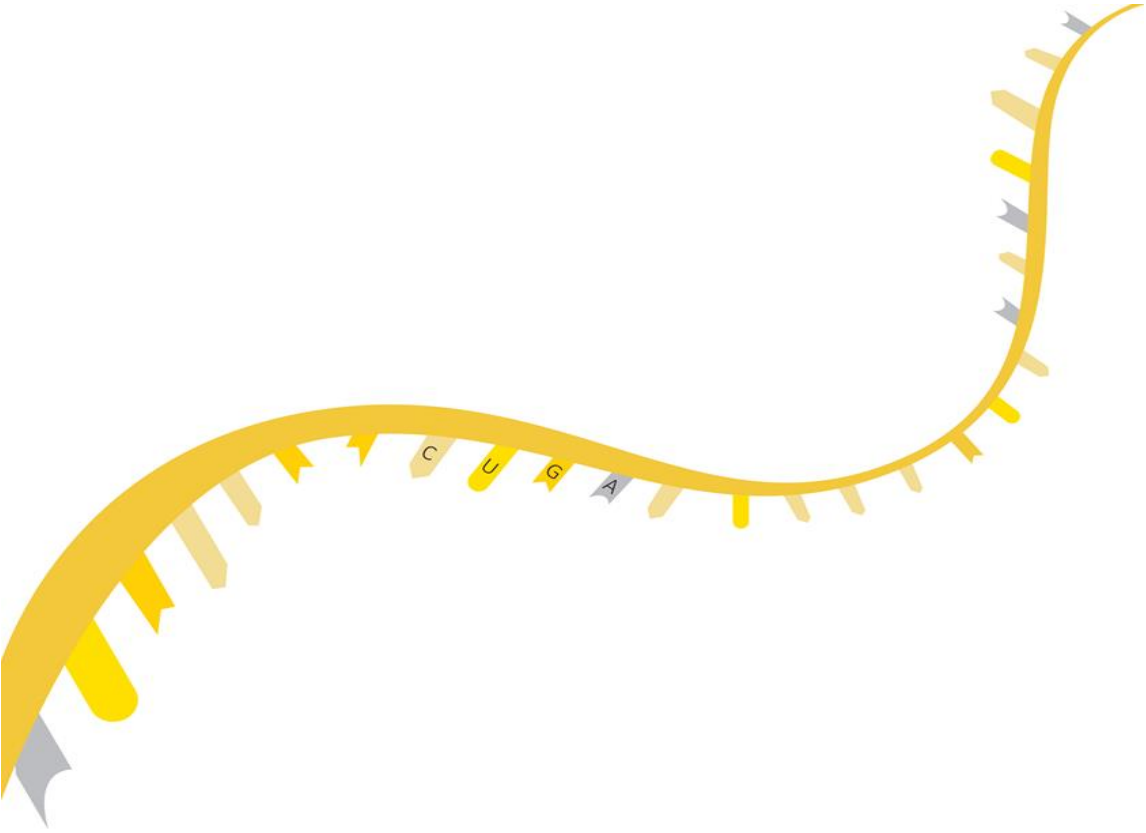
mRNA-seq Lib Prep Kit for Illumina®

RK20302



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Version: N17H17v4.0

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1. Product Overview

- The kit is compatible with the Illumina sequencing platform.
- The kit is applicable to animal, plant, and eukaryote (e.g., fungi) RNA samples. The total initial RNA input is 10 ng-1 μg, and the recommended RNA integrity number (RIN) is ≥ 7.
- The kit contains the RNA Truncated Adapter and needs to be combined with the PCR Index to add a unique index to each sample. The truncated adapter exhibits higher ligation efficiency and reduces adapter dimers compared with the full-length adapter.
- The mRNA-seq Lib Prep Kit for Illumina® contains the Poly(A) mRNA Capture Module, mRNA-seq Lib Prep Module for Illumina, and Unique Dual Index for Illumina. It contains all buffers and enzymes required for mRNA library preparation.
- All reagents provided in the kit have undergone rigorous quality control. Each lot of the kits has been verified through library preparation and sequencing to ensure stable performance.

2. Kit Components

Kit Components		Tube Name and Color	24 rxns	96 rxns
Poly(A) mRNA Capture Module (RK20340)	○	2X Oligo d(T)$_{25}$ Capture Beads	1.2 mL	4.8 mL
	○	mRNA Binding Buffer	1.2 mL	4.8 mL
	○	Washing Buffer	9.6 mL	38.4 mL
	○	Tris Buffer	1.2 mL	4.8 mL
mRNA-seq Lib Prep Kit for Illumina (RK20350)	●	2X Frag/Elute Buffer	144 µL	576 µL
	●	RT Reagent	192 µL	768 µL
	●	First Strand Synthesis Enzyme Mix	48 µL	192 µL
	●	Second Strand Synthesis Reaction Buffer	192 µL	768 µL
	●	Second Strand Synthesis Enzyme Mix	96 µL	384 µL
	○	Nuclease-free Water	1 mL × 2	8 mL
	●	End-prep Buffer	240 µL	960 µL
	●	End-prep Enzyme Mix	72 µL	288 µL
	●	Ligation Buffer	396 µL	1,584 µL
	●	Ligase Mix	72 µL	288 µL
	●	2X PCR Mix	600 µL	1,200 µL × 2
	○	Low EDTA TE	1 mL × 3	10 mL

○ denotes the color of the tube cap.

The kit contains the Truncated Adapter, so PCR is a must to ensure an intact library structure and a unique index must be added to each sample. The kit can be combined with the following adapter kits for Illumina as needed:

Kit Name	Cat. No.
Unique Dual Index for Illumina MidiSet (24 indices)	RK21623
Unique Dual Index for Illumina Set_A (48 indices)	RK21624
Unique Dual Index for Illumina Set_B (48 indices)	RK21625
Unique Dual Index for Illumina Set_C (48 indices)	RK21626
Unique Dual Index for Illumina Set_D (48 indices)	RK21627

3. Storage

mRNA-seq Lib Prep Kit for Illumina® contains three packages:

Kit Name	storage
Poly(A) mRNA Capture Module	2-8°C
mRNA-seq Lib Prep Module for Illumina	-20°C
Unique Dual Index for Illumina	-20°C

The Oligo $d(T)_{25}$ Capture Beads should not be stored at -20°C, otherwise the mRNA capture efficiency will be compromised.

4. Additional Materials Required

Magnetic beads for purification: AFTMag NGS DNA Clean Beads (ABclonal, Cat.NO. RK20257).

RNA quality control: Agilent RNA 6000 Pico Chip.

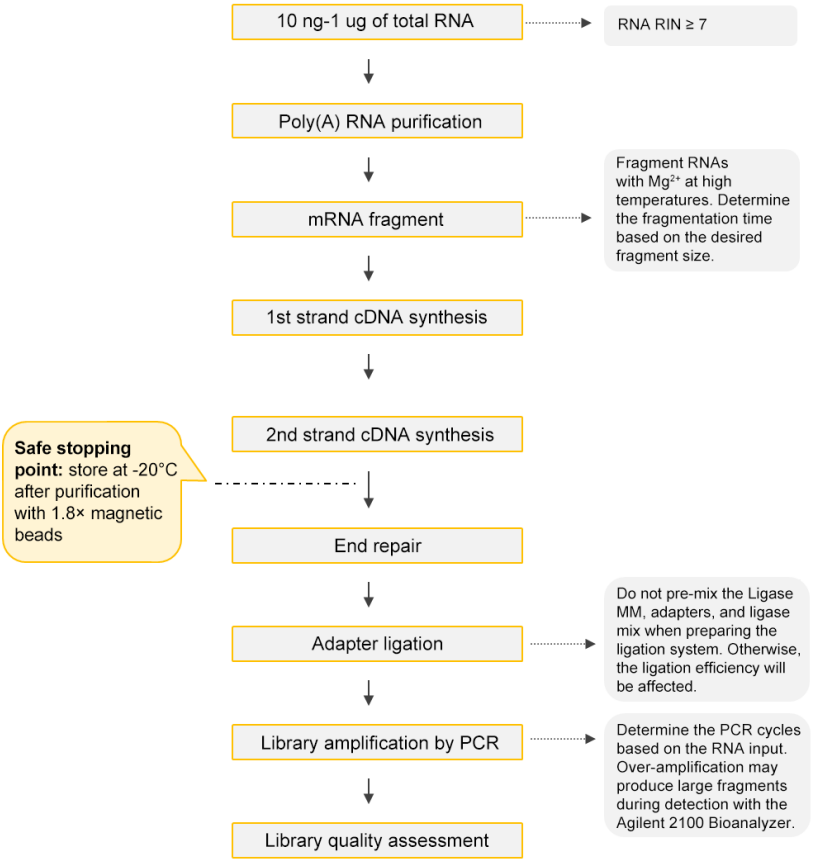
Library quality control: Agilent High Sensitivity DNA Chips and Agilent DNA 1000 Chip.

Other reagents: freshly prepared 80% ethanol.

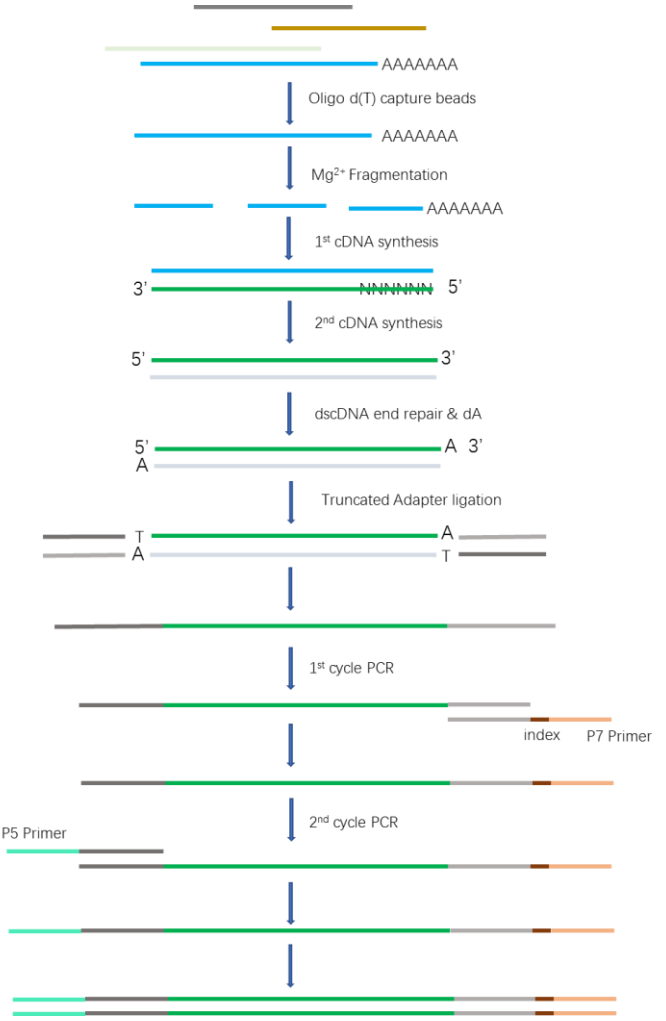
Other instruments: magnetic rack, PCR system, etc.

5. Workflow Diagram

Flow diagram:



Schematic diagram:



6. Precautions

◆ The total RNA input should be ≥ 10 ng. Less input may affect library preparation.

◆ The OD260/OD280 ratio of RNAs should be 1.8-2.1. A higher or lower ratio may represents genome or protein contamination. The agarose gel electrophoresis should show three clear bands.

◆ Masks and gloves should be worn during mRNA library preparation. The mRNAs should be purified at room temperature. The diluted RNA sample should be placed on ice and used for the next operation as soon as possible to avoid RNA degradation.

◆ The conditions for mRNA fragmentation and subsequent size selection should be determined according to the recommended parameter ranges in the Instructions for Use. Otherwise, the library size and yield will be compromised.

◆ The magnetic beads should be taken out half an hour in advance and brought to room temperature. During purification with magnetic beads, the Low EDTA TE must be added for elution after complete alcohol volatilization, namely when the bead color changes from bright brown to frosted brown. Incomplete alcohol volatilization or excessive drying (leading to surface cracks) of beads may reduce the library yield.

◆ During purification with magnetic beads, the supernatant should be pipetted cautiously to avoid disturbing the magnetic beads. Otherwise, the library fragment size and library yield will be compromised.

◆ During size selection of the ligation products, the tubes should stand at room temperature for 5 minutes after each round of adding beads and then on the magnetic rack for another 5 minutes. The supernatant should not be discarded.

◆ The PCR Index should be used cautiously to avoid cross contamination between the reagent and the sample.

7. Protocol

1 mRNA Capture and Fragmentation

1.1. Thaw RNA on ice, dissolve 10-1,000 ng of total RNA in 50 μL of nuclease-free water, and place the solution on ice for later use.

1.2. Vortex-mix 2X Oligo (dT)25 Capture Beads after returning to room temperature. Add 50 μL of the beads to the RNA solution, and mix by pipetting.

1.3. Incubate the mixture in the PCR system (heating lid temperature ≥ 75° C).

Temperature	Time
65°C	5 min
25°C	5 min

1.4. After incubation, let the centrifuge tube stand on the magnetic rack for about 2 min until the solution becomes clear, and remove and discard the supernatant.

1.5. Add 200 μL of Washing Buffer, and mix the solution by pipetting. Let the centrifuge tube stand on the magnetic rack until the solution becomes clear, and remove and discard the supernatant.

1.6. Remove the PCR tube from the magnetic rack, add 50 μL of Tris Buffer, mix the solution well by pipetting, and incubate the mixture in the PCR system (heating lid temperature 105°C).

Temperature	Time
80°C	2 min

1.7. After the solution cools to room temperature, add 50 μL of mRNA Binding Buffer, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

1.8. Let the centrifuge tube stand on the magnetic rack for about 2 min until the solution becomes clear, and remove and discard the supernatant.

1.9. Add 200 µL of Washing Buffer, and mix the solution by pipetting. Let the centrifuge tube stand on the magnetic rack until the solution becomes clear, and remove and discard the supernatant.

1.10. Centrifuge instantaneously after capping, place the tube on the magnetic rack, and remove all the residual liquid with a 10 µL pipette..

1.11 Prepare the Frag/Elute Buffer according to the table below:

Reagent	Volume
2X Frag/Elute Buffer*	6 µL
Nuclease-free Water*	6 µL
Total volume	12 µL

* : The pre-mix can be prepared in advance based on the number of samples. No additional amount is needed.

1.12 Add 11 µL of Frag/Elute Buffer, mix the solution well by pipetting, and elute and fragment RNAs as per the table below (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-300 nt	94°C 15 min, 4°C hold
300-450 nt	94°C 10 min, 4°C hold
400-700 nt	94°C 5 min, 4°C hold

1.13 After cooling to 4°C, take out the centrifuge tube, centrifuge it instantaneously, and let the tube stand on the magnetic rack until the solution becomes clear. Transfer 10 µL of the supernatant into another PCR tube, and immediately use it for the first strand cDNA synthesis.

2 First Strand cDNA Synthesis

2.1 Thaw RT Reagent at room temperature, and prepare the following system on ice:

Reagent	Volume
Fragmented mRNAs	10 μL
RT Reagent*	8 μL
First Strand Synthesis Enzyme Mix*	2 μL
Total volume	20 μL

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

2.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (heating lid temperature 105°C).

Temperature	Time
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

3 Second Strand cDNA Synthesis

3.1 Take Second Strand Synthesis Reaction Buffer out of the refrigerator, thaw it on ice, and successively add the reagents listed in the table below.

Reagent	Volume
First strand cDNA (Step 2.2)	20 μL
Second Strand Synthesis Reaction Buffer*	8 μL
Second Strand Synthesis Enzyme Mix*	4 μL
Nuclease-free Water*	48 μL
Total volume	80 μL

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

3.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled)

Temperature	Time
16°C	1 h

3.3 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

3.4 Add 144 μL of AFTMag NGS DNA Clean Beads (1.8X) into each incubated sample, and mix them well by pipetting.

3.5 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

3.6 Place the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

3.7 Repeat Step 3.6, wash the magnetic beads once with 80% ethanol, and remove all of the residual supernatant with a 10 μL pipette.

3.8 Dry the magnetic beads for 2-3 minutes, add 40 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

3.9 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 37 μL of the supernatant into another PCR tube.

- The double-stranded cDNA elute can be stored at -20°C for no more than 24 hours.

4 End Repair

4.1 Take the End-prep Buffer out of the refrigerator, thaw it on ice, and prepare the systems listed in the table below.

Reagent	Volume
Double-stranded cDNA (Step 3.9)	37 μL
End-prep Buffer*	10 μL
End-prep Enzyme Mix*	3 μL
Total volume	50 μL

* : The pre-mix can be prepared in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

4.2 Mix the prepared system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system according to the table below (heating lid temperature 75°C).

Temperature	Time
20°C	30 min
65°C	30 min
4°C	Hold

5 Adapter Ligation

5.1 Thaw the Ligation Buffer and RNA Truncated Adapter on ice and prepare the adapter ligation system on ice.

Reagent	Volume
End-prep DNA (Step 4.2)	50 μL
Ligation Buffer*	16.5 μL
Truncated Adapter**	2.5 μL
Ligase Mix	3 μL
Total volume	About 70 μL

* : Ligation Buffer contains PEG and is viscous, so it needs to be pipetted slowly to avoid volume errors, which may affect the subsequent size selection.

** : The adapter is a truncated adapter and is not applicable to PCR-free library preparation, so the ligation products must be amplified.

Note: Do not pre-mix the Ligase Mix and RNA Truncated Adapter when preparing the ligation system. Otherwise, adapter dimers will be produced, thus affecting the ligation efficiency.

5.2 Mix the ligation system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled).

Temperature	Time
22°C	15 min

6 Size Selection

After the end of ligation, the ligation products can be purified with two options: direct purification or size selection:

When the total RNA input < 100 ng, direct purification is preferred.

When the total RNA input ≥ 100 ng, size selection is preferred according to the table below (Option 2).

Option 2. Bead Proportions for Size Selection and Library Size Distribution

Fragmentation Condition	**94°C 15 min**	**94°C 10 min**	**94°C 5 min**
RNA fragment size	200-300 nt	300-450 nt	400-600 nt
Library fragment size	320-420 bp	420-570 bp	520-720 bp
Proportion of beads (1st round)	0.35X (35 μL)	0.3X (30 μL)	0.25X (25 μL)
Proportion of beads (2nd round)	0.2X (20 μL)	0.2X (20 μL)	0.15X (15 μL)

Refer to the Appendix for more size selection options (Options 1, 2, and 3).

Both Options 1 and 2 are cautiously performed in Ligation Buffer. The fragments obtained with Option 2 are slightly larger and have a narrower size distribution range compared with those in Option 1. Option 3 is implemented in the aqueous phase. In this option, the ligation products are purified first before size selection (refer to the Appendix for specific procedures).

Direct Purification of Ligation Products

6.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

6.2 After the end of ligation, add 56 μL of AFTMag NGS DNA Clean Beads (0.8X) into the ligation products, and mix them well by pipetting.

6.3 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

6.4 Place the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.5 Repeat Step 6.4, wash the magnetic beads once with 80% ethanol, and remove all the residual supernatant with a 10 μL pipette.

6.6 Air dry the magnetic beads for 2-3 minutes, add 23 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

6.7 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 20 μL of the supernatant into a new PCR tube for later use.

Size Selection of Ligation Products (fragmentation conditions: 94°C 10 min)

6.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

6.2 **Add 30 μL of nuclease-free water into the ligation system to get a 100 μL volume.**

6.3 Add 30 μL of AFTMag NGS DNA Clean Beads (0.30X), and mix the solution well by pipetting.

6.4 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear (**Do not discard the supernatant**).

6.5 Transfer the supernatant into another centrifuge tube, add 20 μL of AFTMag NGS DNA Clean Beads (0.2X), and mix them well by pipetting.

6.6 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

6.7 Place the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.8 Repeat Step 6.7, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

6.9 Air dry the magnetic beads for 2-3 minutes, add 23 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

6.10 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 20 μL of the supernatant into a new PCR tube for later use.

7 Library Amplification by PCR

7.1 After purification of ligation products, enrich the library with the Adapter Kit.

Reagent	Volume
Purification products	20 μL
2X PCR Mix	25 μL
UDI Primer**	5 μL
Total volume	50 μL

**: UDI primer is premixed P5 and P7 index labeled primer. Caution is required when using UDI Primer. Both need to be pipetted very cautiously. Each pipette tip is for single use to avoid cross contamination between samples and reagents.

7.2 Mix the reaction system by pipetting, microcentrifuge it, and incubate it in the PCR system according to the table below **(heating lid temperature 105°C)**.

Temperature	Time	Cycles
98°C	45s	1
98°C	10s	
60°C	15s	8-16*
72°C	30s	
72°C	1 min	1
4°C	hold	

***Recommended PCR cycles:**

Total RNA Input	Direct Purification PCR Cycles	Size Selection PCR Cycles
10 ng	15-16	-
100 ng	12-13	14-15
1 μg	8-9	10-11

7.3 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

7.4 After the end of the reaction, add 40 μL of AFTMag NGS DNA Clean Beads (0.8X) into each PCR tube and mix them well by pipetting.

7.5 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and remove and discard the supernatant.

7.6 Hold the PCR tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

7.7 Repeat Step 7.6, wash the magnetic beads once with 80% ethanol, and remove all the residual supernatant with a 10 μL pipette.

7.8 Air dry the magnetic beads for 2-3 minutes, add 31 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

7.9 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 30 μL of the library into a new centrifuge tube for later use.

8. Appendix

1. Size Distribution of RNA Fragments

Fragment mRNAs purified from 1 μg of mouse tissue total RNA using 1X Frag/Elute Buffer at 94°C for 5, 10, and 15 minutes, respectively, place them on the magnetic rack, pipette the supernatant, and use Agencourt RNAClean XP Beads (2.2X volume) for purification. Use the Agilent RNA 6000 Pico Chip to analyze the fragment size distribution.

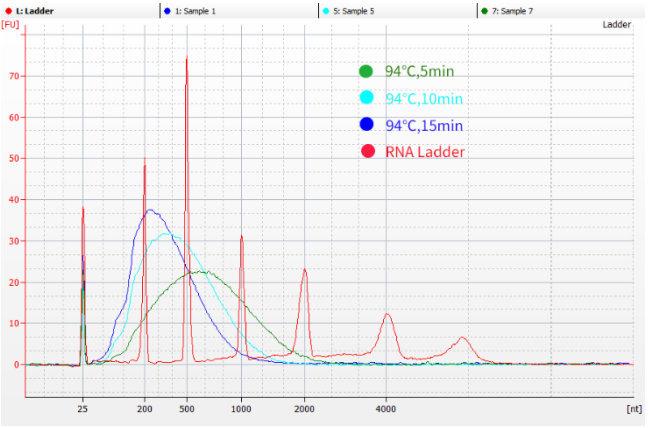


Figure 1. Size Distribution of mRNA Fragments (Agilent 2100 Bioanalyzer)

2. Library Size of Different RNA Fragmentation Time and Size Selection Programs

Different size selection options can help obtain fragments with varying main-peak patterns and those with varying size distribution ranges. ABclonal provides multiple size selection options to meet different needs. Options 1 and 2 are carried out in Ligation Buffer. The fragments obtained with Option 2 have smaller size distribution ranges and narrower library peak patterns compared with Option 1.

Option 3 is implemented in the aqueous phase, and library fragments selected with this option show stable size distribution ranges. Refer to Appendix 2.3 for specific procedures.

2.1 Size Selection of Adapter Ligation Products (Option 1)

Option 1. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 μL)	0.15X (15 μL)	520-720 bp
94°C 10 min	0.30X (30 μL)	0.2X (20 μL)	420-570 bp
94°C 15 min	0.35X (35 μL)	0.2X (20 μL)	320-420 bp

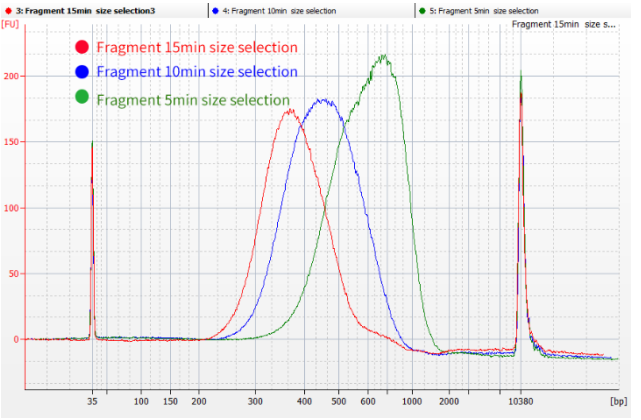


Figure 2. Library Fragment Size Distribution of Option 1 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and

analyze them using Agilent High Sensitivity DNA Chips and the Agilent 2100 Bioanalyzer.

2.2 Size Selection of Adapter Ligation Products (Option 2)

Option 2. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 µL)	0.1X (10 µL)	500-700
94°C 10 min	0.3X (30 µL)	0.1X (10 µL)	450-550
94°C 15 min	0.35X (35 µL)	0.1X (10 µL)	350-450

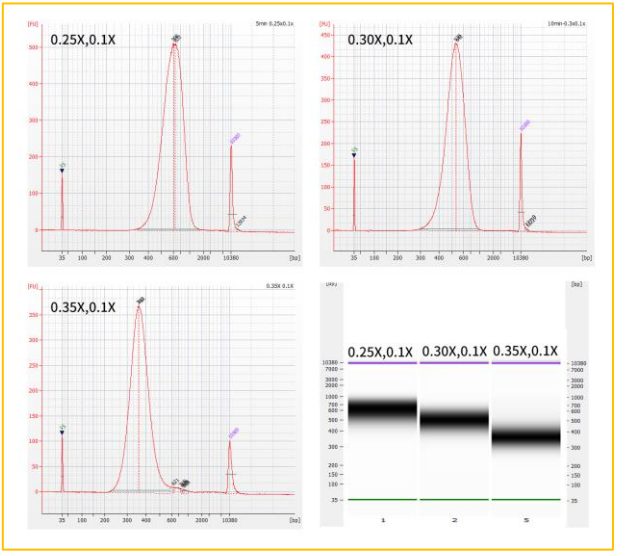


Figure 3. Library Fragment Size Distribution of Option 2 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions in Option 2, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and analyze their peak patterns using the Agilent 2100 Bioanalyzer.

2.3 Size Selection of Adapter Ligation Products (Option 3)

During adapter ligation, PEG contained in Ligation Buffer enables very sensitive size selection. Thus, a bead volume error tends to cause fragment size deviations. If high-quality fragments are required, it is recommended to conduct size selection in the aqueous phase. Specifically, purify the ligation products with 1.0X magnetic beads after ligation, elute them with 103 μL of water, and take 100 μL of the elution product for size selection as per the table below.

Option 3. Bead Proportions for Size Selection and Library Size Distribution

Fragmentation Condition	Purification of PCR Products	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	Purify with 1.0X magnetic beads	0.55X (55 μL)	0.1X (10 μL)	600-720
94°C 10 min		0.6X (60 μL)	0.1X (10 μL)	500-600
		0.65X (65 μL)	0.1X (10 μL)	420-500
94°C15 min		0.75X (75 μL)	0.1X (10 μL)	360-420
		0.8X (80 μL)	0.1X (10 μL)	320-360

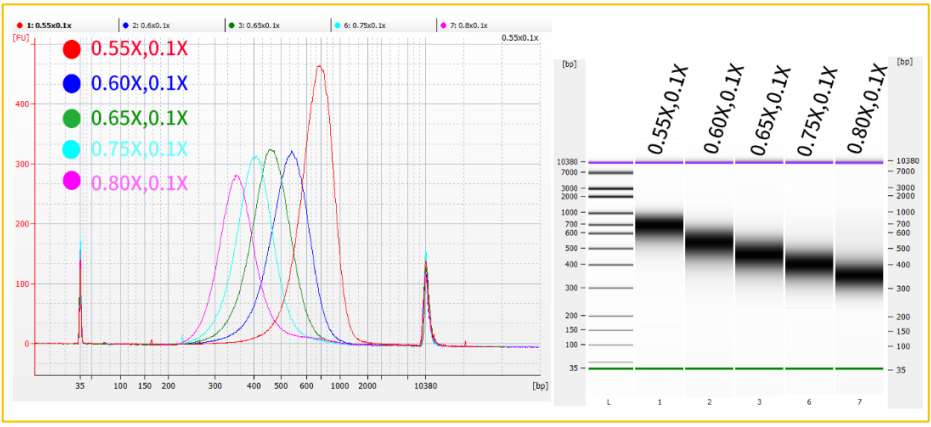


Figure 4. Library Fragment Size Distribution of Option 3 (Agilent 2100 Bioanalyzer)

Option 3: Operating Procedures for Size Selection in Aqueous Phase

2.3.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and vortex-mix or shake it well prior to use.

2.3.2 After the end of ligation (Step 5.2), add 70 μL of AFTMag NGS DNA Clean Beads (1.0X) into the ligation products, and mix them well by pipetting.

2.3.3 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and remove and discard the supernatant.

2.3.4 Place the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.5 Repeat Step 2.3.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

2.3.6 Air dry the magnetic beads for 2-3 minutes, add 102.5 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.7 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 100 μL of the supernatant into a new PCR tube for size selection.

2.3.8 Add 65 μL of AFTMag NGS DNA Clean Beads (0.65X 100 μL) into the purified ligation products, and mix them well by pipetting.

2.3.9 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear (Do not discard the supernatant).

2.3.10 Transfer 160 μL of the supernatant into another centrifuge tube, add 10 μL of AFTMag NGS DNA Clean Beads (0.1X 100 μL) and mix them well by pipetting.

2.3.11 Let the mixture stand at room temperature for 5 minutes and then on the magnetic rack for another 5 minutes until the solution becomes clear, and cautiously remove and discard the supernatant.

2.3.12 Place the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.13 Repeat Step 2.3.12, wash the magnetic beads once with 80% ethanol, and remove all the residual supernatant with a 10 μL pipette.

2.3.14 Dry the magnetic beads for 2-3 minutes, add 22 μL of Low EDTA TE after complete alcohol volatilization (namely when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.15 Let the mixture stand at room temperature for 2 minutes and then on the magnetic rack for another 1 minute until the solution becomes clear, and cautiously pipette 20 μL of the supernatant into a new PCR tube for later use.

2.4 The above results are internal test data. The ligation system is sensitive and closely associated with the size selection system, so the operating habits of operators or pipette use errors may cause fragment size deviations.

- When larger library fragments are obtained, it is recommended to increase the volume of beads in the first round.
- When smaller library fragments are obtained, it is recommended to reduce the volume of beads in the first round.
- The bead proportion can be adjusted by 0.01X-0.05X based on the size deviations.

3. FQAs

◆ What if Box 1 is mistakenly stored at -20°C?

The Oligo $d(T)_{25}$ Capture Beads may be damaged at -20°C, so Box 1 cannot be used any longer. It is recommended to purchase the ABclonal Poly(A) mRNA Purification Module (RK20341).

◆ Can the RNA degraded to RIN < 7 be used for RNA-seq library preparation?

For severely degraded human/mouse/rat RNA samples (e.g., FFPE samples), it is recommended to prepare libraries using the ABclonal Whole RNA-seq Lib Prep Kit for Illumina (RK20303).

For plant or other eukaryote cell RNA samples, if RNAs are degraded but their electrophoresis with the agarose gel shows the separate presence of 28S and 18S bands, it is recommended to increase the total RNA input and appropriately increase PCR cycles, which can also obtain desired libraries.

◆ How should the quality of prepared libraries be determined?

Before sequencing, the prepared library is detected using the Agilent 2100 Bioanalyzer. The library is deemed conforming if there is no burr-like peak, no detectable peak at 130 bp (adapter dimer), or no fragment peak with a large peak area at the right side of the library peak.

The library concentration is determined with the Qubit fluorometer. The library molarity determined with the Agilent Bioanalyzer can be calibrated by the qPCR, while the library molarity obtained with the qPCR can be calibrated by the Agilent Bioanalyzer.

◆ What if library preparation with conforming total RNA (RIN > 7) fails?

The RIN of total RNA is a metric for total RNA quality assessment and cannot definitely indicate the abundance and integrity of poly(A) RNAs. For some samples, the total RNA is intact, but many mRNAs are degraded. The degradation causes a large loss during poly(A) RNA purification. Thus, library preparation fails.

In this case, the abundance and integrity of purified poly(A) RNAs can be assessed as follows: After Step 1.0, add 6 μL of Tris Buffer, heat the solution at 80°C for 2 minutes, let the tube stand on the magnetic rack until the solution becomes clear, and pipette 1 μL of the supernatant (namely intact poly(A) RNAs) for analysis with the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Chip.

Determine solutions based on analysis results:

- If the mRNA abundance is low, increase the total RNA input.
- If the mRNA integrity is poor, adjust the mRNA fragmentation time.

For more information, please consult our technical support team.

4. Sequence Information of Adapter and PCR Index Primer

Please refer to the Unique Dual Index for Illumina Kit manual.

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