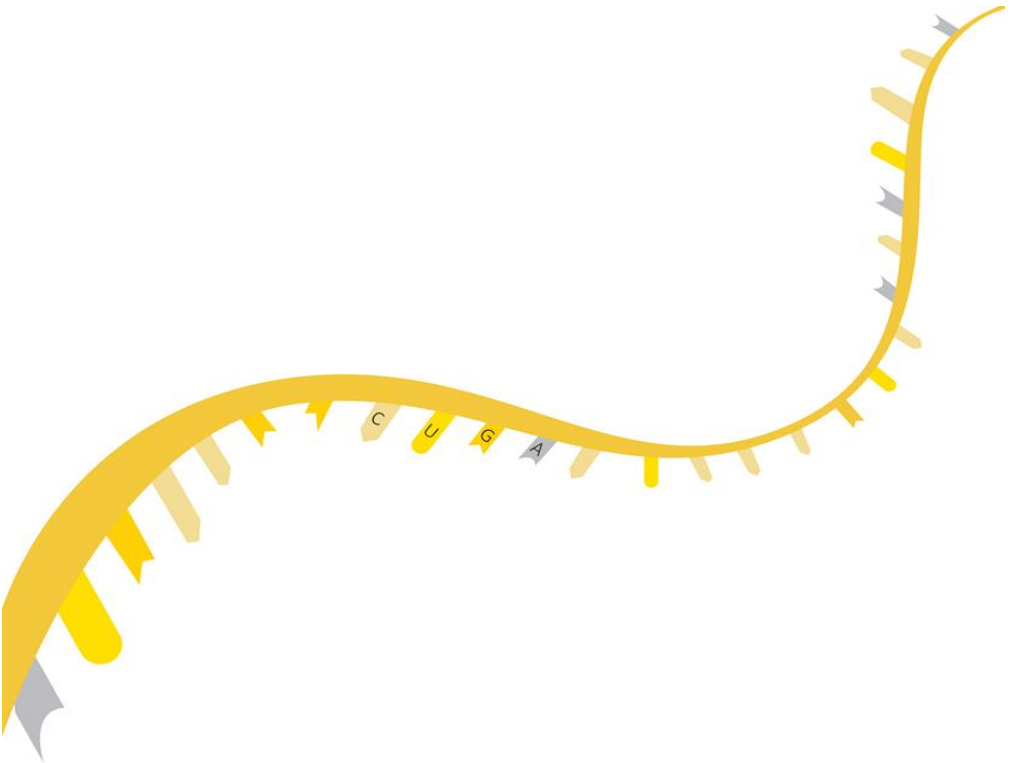
Whole RNA-seq Lib Prep Kit for Illumina®

RK20303



www.abclonal.com

Version: N17H18v3.5

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1. Product Overview

- The kit is compatible with Illumina sequencing platforms.
- The rRNA Depletion Module (H/M/R) effectively depletes cytoplasmic rRNA (including 5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA) and mitochondrial rRNA (12S rRNA and 16S rRNA) from human, mouse, and rat total RNA.
- This product is applicable for both intact total RNA and degraded RNA samples, such as FFPE RNA. The initial total RNA input is 10 ng-1 μg.
- Strand-specific library preparation is recommended for this kit. Whole RNA-seq data can be used to analyze all non-ribosomal mRNAs and some LncRNAs. Strand-specific library preparation enables more accurate gene expression quantification and RNA analysis, facilitating a comprehensive understanding of genetic structure.
- The kit contains the RNA Truncated Adapter and uses 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 Whole RNA-seq Lib Prep Kit for Illumina® contains the rRNA Depletion Module (H/M/R), Stranded RNA-seq Lib Prep Kit for Illumina and ABclonal RNA Adapter Module. It contains all buffers and enzymes required for Whole RNA-seq 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 (RK20303M)	96 rxns (RK20303L)
rRNA Depletion Module (H/M/R) (RK20348)	Probe Hybridization Buffer	48 µL	192 µL
	rRNA Probe Mix (H/M/R)	24 µL	96 µL
	RNase H	48 µL	192 µL
	10X RNase H Buffer	48 µL	192 µL
	DNase I	60 µL	240 µL
	10X DNase I Buffer	120 µL	480 µL
Stranded RNA-seq Lib Prep Kit for Illumina (RK20349)	2X Frag/Elute Buffer	144 µL	576 µL
	RT Strand Specificity Reagent	192 µL	768 µL
	First Strand Synthesis Enzyme Mix	48 µL	192 µL
	Second Strand Synthesis Reaction Buffer with dUTP	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 X 2
	UDG Enzyme	12 µL	48 µL
	Low EDTA TE	1 mL X 3	10 mL

denotes the color of the tube cap.

The kit contains the Truncated Adapter, so PCR is required to ensure an intact library structure and add a unique index 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

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

Kit Name	storage
rRNA Depletion Module (H/M/R)	-20°C
Stranded mRNA-seq Lib Prep Module for Illumina	-20°C
Unique Dual Index for Illumina	-20°C

4. Additional Materials Require

Agencourt RNAClean XP Beads (Beckman Coulter Inc., Cat. No. A63987)

AFTMag NGS DNA Clean Beads (ABclonal，Cat.NO. RK20257)

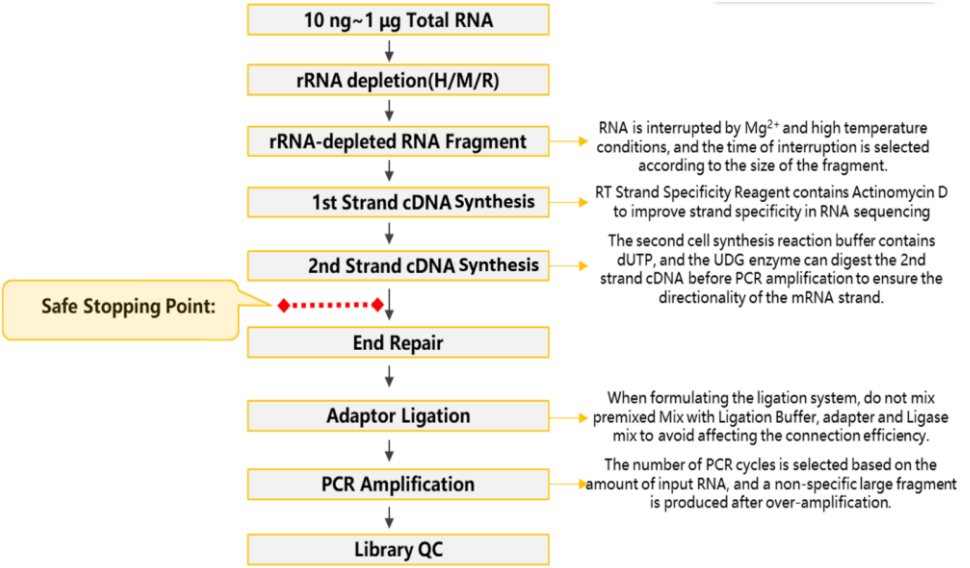
Freshly prepared 80% ethanol

Magnetic rack

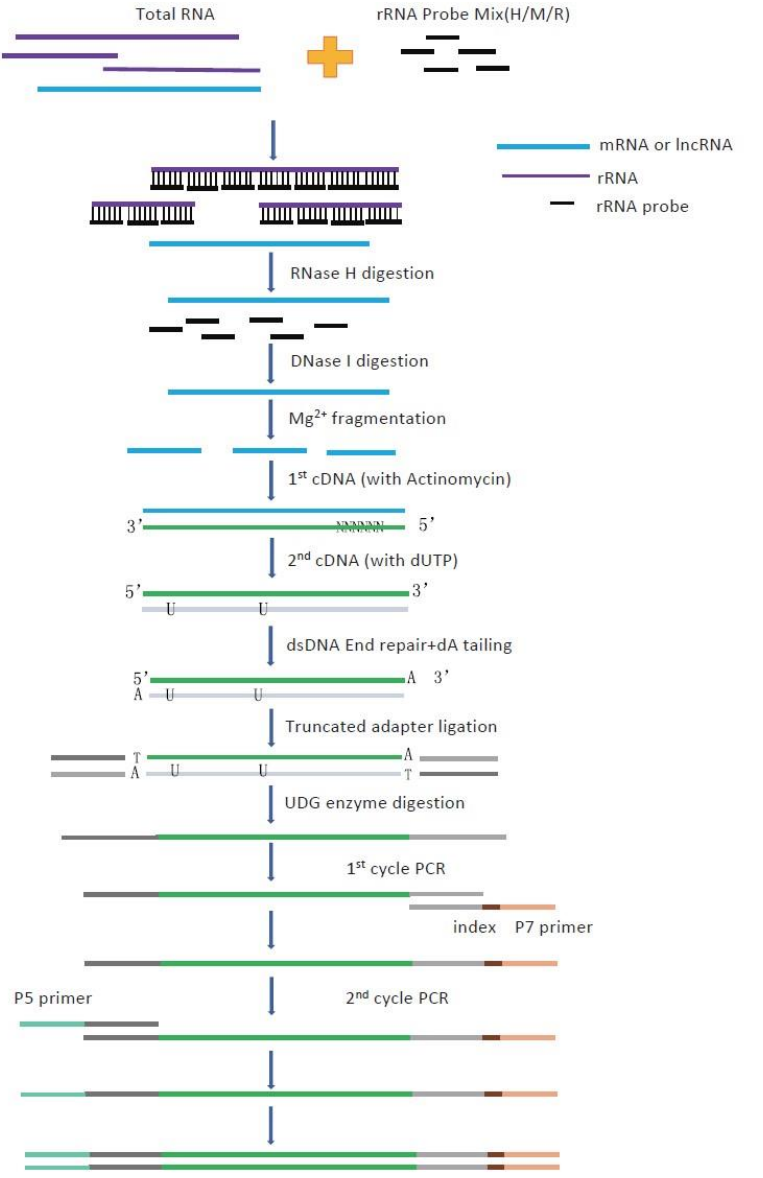
PCR system

5. Workflow Diagram

Flow diagram:



Schematic diagram:



6. Precautions

◆ The conditions for RNA 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.

◆ For FFPE RNA or highly degraded RNA samples, RNA fragmentation conditions and size selection methods should be determined on a case-by-case basis due to poor mRNA integrity, or the library yield may be compromised. For specific procedures, refer to Appendix 2.

◆ 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 the ethanol has fully evaporated, e.g. when the bead color changes from bright brown to frosted brown. Residual ethanol or over-drying (leading to cracks) of beads may reduce the library yield.

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

7. Protocol

1 rRNA Depletion

1.1 Probe/rRNA Hybridization

1.1.1 Take 10 ng-1 μg of total RNA, dilute to 12 μL with nuclease-free water, and place on ice for later use.

1.1.2 Thaw the Probe Hybridization Buffer on ice and prepare the probe hybridization pre-mix according to the following:

Reagent	Volume
Probe Hybridization Buffer	2 μL
rRNA Probe Mix (H/M/R)	1 μL
Total Volume	**3 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.1.3 Add 3 μL of the probe hybridization pre-mix into 12 μL of prepared RNA solution, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.1.4 Load the sample into the PCR system (**heating lid temperature 105°C**) to hybridize the probes to rRNA:

Temperature	Time
95°C	2 min
95°C-22°C	Ramp down to 22°C at 0.1°C/sec
22°C	5 min

1.1.5 After hybridization, transfer the sample from the PCR system onto ice, and proceed immediately with RNase H digestion.

1.2 RNase H Digestion

1.2.1 Thaw the 10X RNase H Buffer on ice and prepare the RNase H digestion pre-mix according to the following:

	Reagents	Volume
●	10X RNase H Buffer	2 μL
●	RNase H	2 μL
○	Nuclease-free Water	1 μL
	Total Volume	**5 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.2.2 Add 5 μL of the RNase H digestion pre-mix into the solution prepared in Step 1.1.5 to make a reaction system of 20 μL, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.2.3 Load the reaction system into the PCR system (heating lid temperature ≥ 45°C) for RNase H digestion:

Temperature	Time
37°C	30 min

1.2.4 After RNase H digestion, transfer the sample from the PCR system onto ice, and proceed immediately with DNase I digestion.

1.3 DNase I Digestion

1.3.1 Thaw the 10X DNase I Buffer on ice and prepare the DNase I digestion pre-mix according to the following:

Reagent	Volume
10X DNase I Buffer*	5 μL
DNase I*	2.5 μL
Nuclease-free Water*	22.5 μL
Total Volume	**30 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.3.2 Add 30 μL of the DNase I digestion pre-mix into the solution prepared in Step 1.2.4 to make a reaction system of 50 μL, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.3.3 Load the reaction system into the PCR system (**heating lid temperature ≥ 45°C**) for DNase I digestion:

Temperature	Time
37°C	30 min

1.3.4 After DNase I digestion, transfer the sample from the PCR system onto ice, and proceed immediately with RNA purification.

1.4 Purification of rRNA-depleted RNA

1.4.1 Take Agencourt RNAClean XP Beads out of the 2-8°C environment in advance, let it stand for 30 min until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

1.4.2 After DNase I digestion, add 110 μL of Agencourt RNAClean XP Beads (2.2X) into each reaction tube, and mix them well by pipetting.

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

1.4.4 Hold 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.

1.4.5 Repeat Step 1.4.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

1.4.6 Dry the magnetic beads for 2-3 min, add 7 μL of nuclease-free water after the ethanol has fully evaporated, and mix them well by pipetting.

1.4.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 5 μL of the supernatant into a new centrifuge tube.

1.4.8 Add 5 μL of 2X Frag/Elute Buffer, mix the solution well by pipetting, and fragment the RNA as per the following table (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

Notes: For FFPE RNA samples or highly degraded samples (RIN < 6), fragmentation time can be shortened. Refer to Appendix 2 for specific durations.

1.4.9 After cooling to 4°C, centrifuge the tube instantaneously and then place it on ice. Proceed immediately with the first strand cDNA synthesis.

2 First Strand cDNA Synthesis

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

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

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

2.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system.

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 with dUTP out of the refrigerator, thaw it on ice, and add the reagents listed in the table below.

Reagents	Volume
First strand cDNA	20 μL
Second Strand Synthesis Reaction Buffer with dUTP*	8 μL
Second Strand Synthesis Enzyme Mix*	4 μL
Nuclease-free Water*	48 μL
Total Volume	**80 μL**

* : The mix of Second Strand Buffer, Second Strand Enzyme Mix, and nuclease-free water can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

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	60 min

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 mix well by vortexing or oscillation 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 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

3.6 Hold 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 the residual liquid with a 10 μL pipette.

3.8 Dry the magnetic beads for 2-3 min, add 39 μL of Low EDTA TE after the ethanol has fully evaporated, and mix them well by pipetting.

3.9 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 37 μL of the supernatant into a new centrifuge 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 mix of End-prep Buffer and End-prep Enzyme Mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

4.2 Mix the prepared system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system according to the table below.

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 is viscous as it contains PEG, so it needs to be pipetted slowly to avoid volume errors that may affect the subsequent size selection.

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

Note: Do not pre-mix the Ligase Mix and 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 following table.

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)

Notes: 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 (Step 5.2) 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 mix well by vortexing or oscillation prior to use.

6.2 After 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 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.4 Hold 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 liquid with a 10 μL pipette.

6.6 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (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 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 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 mix well by vortexing or oscillation 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 min and then on the magnetic rack for another 5 min until the solution becomes clear (**Do not discard the supernatant)**.

6.5 Transfer the supernatant into a new 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 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.7 Hold 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 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (***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 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 μL of the supernatant into a new PCR tube for later use.

7 PCR Amplification

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

Reagent	Volume
Purification products	19.5 μL
2X PCR Mix	25 μL
UDG Enzyme	0.5 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 the tube, and incubate it in the PCR system according to the table below (heating lid temperature 105°C).

Temperature	Time	Cycles
37°C	10 min	1
98°C	1 min	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 mix well by vortexing or oscillation prior to use.

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

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

7.6 Hold 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.

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

7.8 Dry the magnetic beads for 2-3 min, add 31 μL of Low EDTA TE after the ethanol has fully evaporated (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 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 30 μL of the library into a new centrifuge tube for later use.

8. Appendix

1 Fragment Size Distribution of rRNA-depleted Library

Different size selection options can help obtain fragments with varying main-peak patterns and 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.

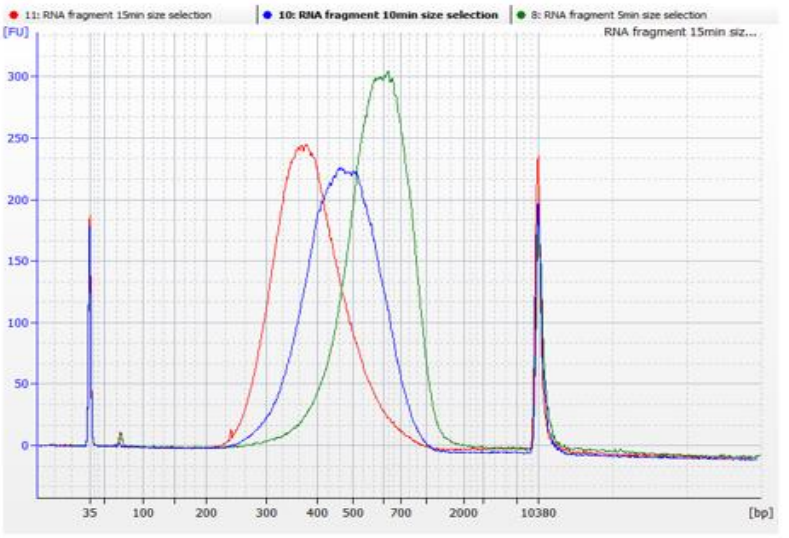
Option 3 is implemented in the aqueous phase, and library fragments selected with this option show stable size distribution ranges. Refer to Appendix 1.3 for specific procedures.

1.1 Size Selection of Adapter Ligation Products (Option 1)

Use 1 μg of intact total RNA (RIN > 7) as input. Fragment RNA using the conditions recommended in the Instructions for Use (Step 1.4.8) and perform size selection according to the program recommended in Step 5.2. Figure 1 illustrates the size distribution of library fragments obtained from PCR amplification.

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
94°C 10 min	0.30X (30 μL)	0.2X (20 μL)	420-570
94°C 15 min	0.35X (35 μL)	0.2X (20 μL)	320-420



Red peak: RNA fragment 15 min size selection library
Blue peak: RNA fragment 10 min size selection library
Green peak: RNA fragment 5 min size selection library

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

Use 1 μg of 293T 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.

1.2 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

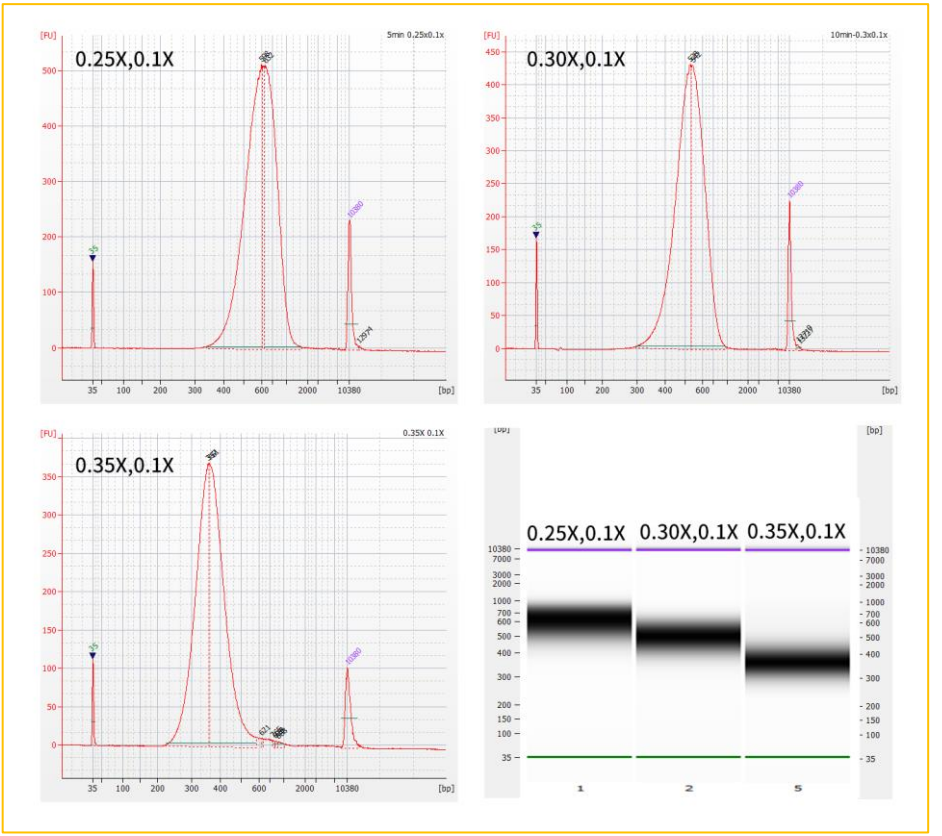


Figure 2. Library Fragment Size Distribution of Option 2

(Agilent 2100 Bioanalyzer)

1.3 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 following table.

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

Fragmentation Condition	Purification of Ligation Products	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	1.0× magnetic bead purification	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°C 15 min		0.75X (75 μL)	0.1X (10 μL)	360-420
		0.8X (80 μL)	0.1X (10 μL)	320-360

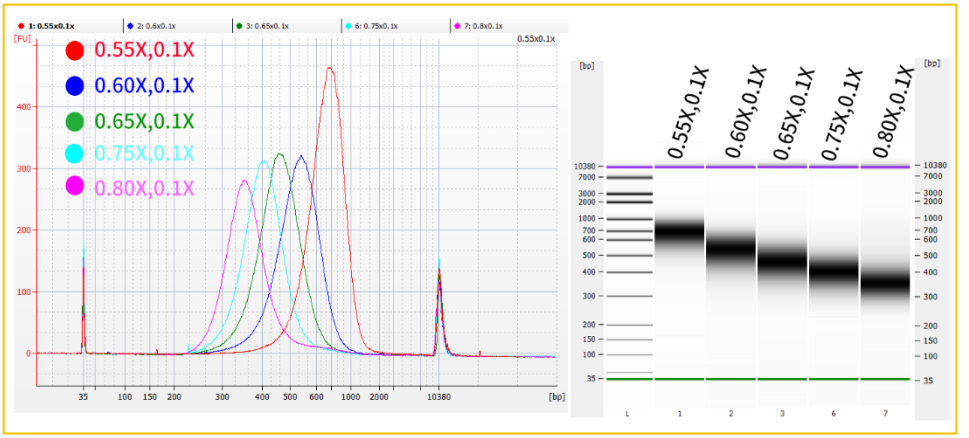


Figure 3. Library Fragment Size Distribution of Option 3

(Agilent 2100 Bioanalyzer)

Option 3: Operating Procedures for Size Selection in Aqueous Phase

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

1.3.2 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.

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

1.3.4 Hold 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.

1.3.5 Repeat Step 1.3.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

1.3.6 Dry the magnetic beads for 2-3 min, add 102.5 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

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

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

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

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

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

1.3.12 Hold 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.

1.3.13 Repeat Step 1.3.12, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

1.3.14 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

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

1.4 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 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.

2 Treatment of FFPE or Degraded RNA Samples

2.1 Due to the poor integrity of degraded mRNA samples, the fragmentation conditions for intact RNA in Step 1.4.8 are not applicable. It is recommended to choose direct purification after adapter ligation. If size selection is done after adapter ligation, the library yield will be compromised.

In this case, 2-3 more PCR cycles can be added to improve the library yield.

Table 1. Fragmentation Conditions for RNA Samples of Varying Quality

RNA RIN	Fragmentation Condition
> 7	94°C 15 min
2-6	94°C 7 min
< 2	65°C 5 min

2.2 Examples of Library Preparation for FFPE Samples

2.2.1 Library preparation example (RIN = 3.8)

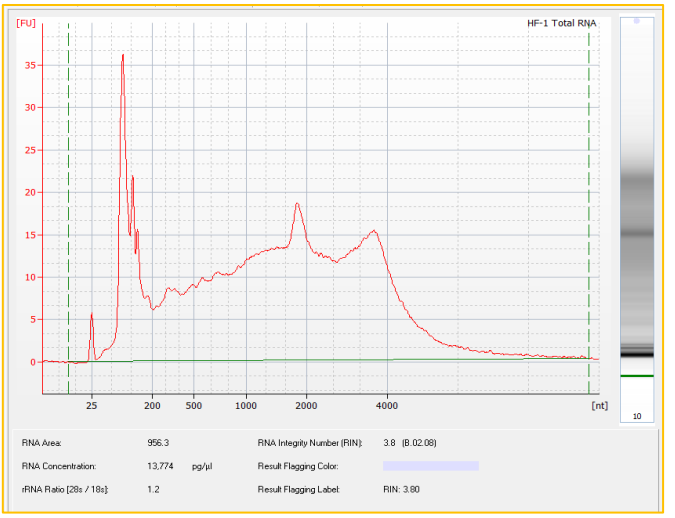


Figure 4. FFPE RNA Sample (Agilent 2100 Bioanalyzer)

FFPE human rectal cancer sample, stored at -80°C for 1 year. Isolate total RNA using RNeasy FFPE Kit (QIAGEN, Cat. No. 73504) and analyze the sample with Agilent Bioanalyzer RNA Pico Chips.

✧ Library prepared without size selection

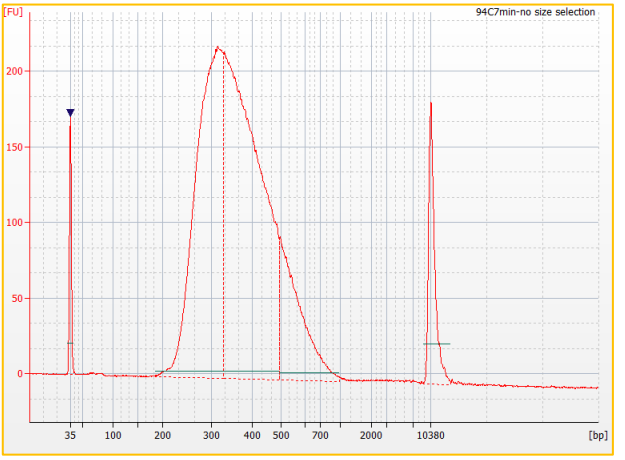


Figure 5. FFPE RNA Library after rRNA Depletion (Agilent 2100 Bioanalyzer)

Use 100 ng of RNA from FFPE human rectal cancer sample (RIN = 3.8) as input. After rRNA depletion, fragment the RNA at 94°C for 7 min. Skip size selection. Run 12 PCR cycles to obtain the library (18.9 ng/μL X 30 μL), dilute to 2 ng/μL, and analyze with Agilent Bioanalyzer High Sensitivity DNA Chips.

✧ Library prepared with size selection

Table 2. FFPE RNA Library after rRNA Depletion and Size Selection

RNA Input	Fragmentation Condition	Size Selection Program	Peak (bp)	PCR Cycles	Library Concentration (ng/μL)	Volume (μL)
100 ng	94°C 7 min	0.35X, 0.2X	390	14	12.2	30
100 ng	94°C 5 min	0.30X, 0.2X	420	14	11.5	30
100 ng	94°C 5 min	0.25X, 0.15X	540	14	6.4	30

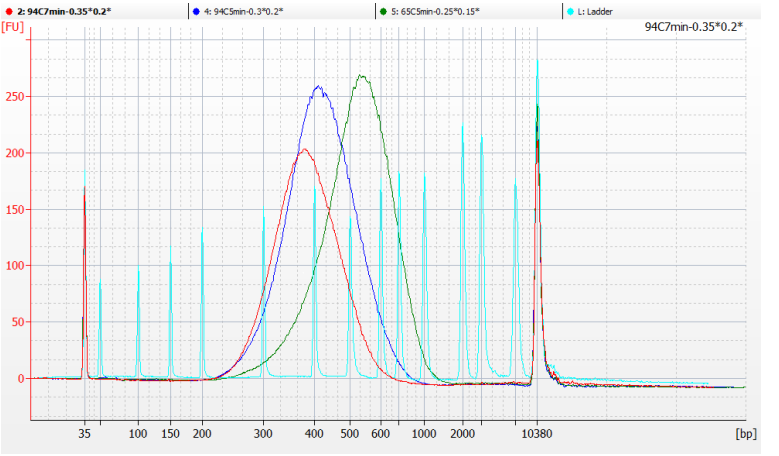


Figure 6. FFPE RNA Library after rRNA Depletion and Size Selection (Agilent 2100 Bioanalyzer)

Use 100 ng of RNA from FFPE human rectal cancer sample (RIN = 3.8) as input. After rRNA depletion, fragment the RNA using different durations and perform size

selection. Run 14 PCR cycles to obtain the libraries, dilute to 2 ng/μL, and analyze with Agilent Bioanalyzer High Sensitivity DNA Chips.

2.2.2 Library preparation example (RIN = 1.9)

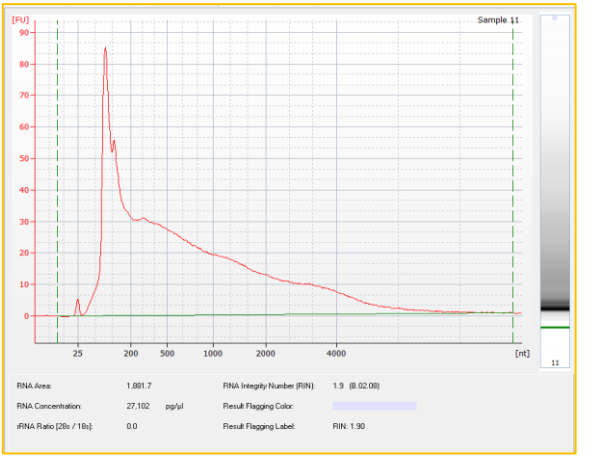


Figure 7. FFPE RNA Sample (Agilent 2100 Bioanalyzer)

FFPE mouse tissue sample, stored at room temperature for 1 year. Isolate total RNA using RNeasy FFPE Kit (QIAGEN, Cat. No. 73504) and analyze the sample with Agilent Bioanalyzer RNA Pico Chips.

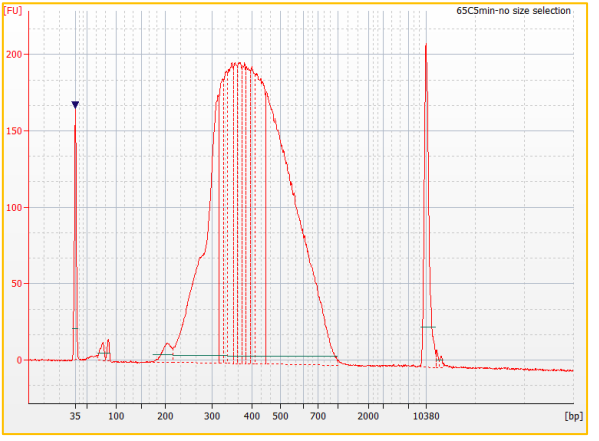


Figure 8. FFPE RNA Library after rRNA Depletion (Agilent 2100 Bioanalyzer)

Use 100 ng of RNA from FFPE mouse tissue sample (RIN = 1.9) as input. After rRNA depletion, treat the RNA at 65°C for 5 min (no 94°C fragmentation). Skip size selection. Run 12 PCR cycles to obtain the library (4.34 ng/μL X 30 μL), dilute to 2 ng/μL and analyze with Agilent Bioanalyzer High Sensitivity DNA Chips.

3 FAQs

◆ How should the quality of prepared libraries be determined?

Before sequencing, the prepared library is assayed using the Agilent 2100 Bioanalyzer. The library quality is acceptable if there is no abnormal jagged 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.

4 Sequence Information of Adapter and PCR Index Primer

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

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