Fast RNA-seq Lib Prep Kit V2

RK20306



Instruction Manual

Version: N19H22V4.3

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1. Overview

Fast RNA-seq Lib Prep Kit V2 (ABclonal, Cat. RK20306) is an efficient, fast, and highly-compatible RNA library preparation kit. The kit is applicable to total RNA samples or purified mRNA samples from eukaryotes, including animals, plants, and fungi. The kit takes 10-1000 ng of RNA sample to prepare sequencing libraries for MGI and Illumina high-throughput sequencing platforms. The kit is compatible with Full Adapters and Truncated Adapters of MGI and Illumina sequencing platforms.

The kit integrates second strand cDNA synthesis, dsDNA end repair, and dA-tailing into one step, significantly shortening the library preparation time from 7 hours to 3 hours. To meet clients' needs, the kit offers two types of Second Strand & dA Buffers: Second Strand & dA Buffer for non-strand-specific library preparation and Second Strand & dA Buffer with dUTP for strand-specific 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 Compents

Tube Name and Color	24 rxns	96 rxns
Frag/Elute Buffer	168 µL	672 µL
RT Strand Specificity Reagent	96 µL	384 µL
First Strand Synthesis Enzyme Mix	48 µL	192 µL
Second Strand & dA Buffer	240 µL	960 µL
Second Strand & dA Buffer with dUTP	240 µL	960 µL
Second Strand & dA Enzymes	120 µL	480 µL
Nuclease-free Water	2 mL	8 mL
Ligation Buffer	396 µL	1,584 µL
Ligase Mix T5	72 µL	288 µL
2X PCR Master Mix	600 µL	1.2mL X 2
10X ILM PCR Primers	120 µL	480 µL
MGI PCR Primer Mix	120 µL	480 µL
Low EDTA TE	1 mL X 2	10 mL

Note: 1. ○ denotes the color of the tube cap.

2. The kit provides both the Second Strand & dA Buffer and the Second Strand & dA Buffer with dUTP. Use the Second Strand & dA Buffer for non-strand-specific library preparation or the Second Strand & dA Buffer with dUTP for strand-specific library preparation.

3. The kit contains the MGI PCR Primer Mix (for MGI) and 10X ILM PCR Primers (for Illumina).

3. Storage

All components should be strored at-20℃.

4. Additional Materials

4.1 RNA enrichment kit

Poly(A) mRNA Capture Module (ABclonal, Cat. RK20340).

rRNA Depletion Module (H/M/R) (ABclonal, Cat. RK20348).

4.2 Adapters

For Illumina （Cat. RK21622~RK21627, RK21701~RK21703);

For MGI (Cat. Cat. RK21686~RK21689).

For details, refer to the summary table of compatible adapters in the Appendix Table.

4.3 Magnetic beads for purification

AFTMag NGS DNA Clean Beads (ABclonal, Cat. RK20257) or Agencourt AMPure XP beads (Beckman Coulter, Inc., #A63881), Agencourt RNAClean XP Beads (Beckman Coulter, Inc., #A63987), or other equivalent magnetic beads for nucleic acid purification.

4.4 RNA sample concentration and quality evaluation

Qubit fluorometer.

Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. Q32855).

Nanodrop.

Agilent RNA 6000 Pico Chips (Agilent #5067-1513).

4.5 Library quality control

Qubit fluorometer.

ABQubit dsDNA Quantitation Kit (ABclonal, Cat. RK30140).

Agilent High Sensitivity DNA Chips (Agilent #5067-4626).

Agilent DNA 1000 Chips (Agilent #5067-1504).

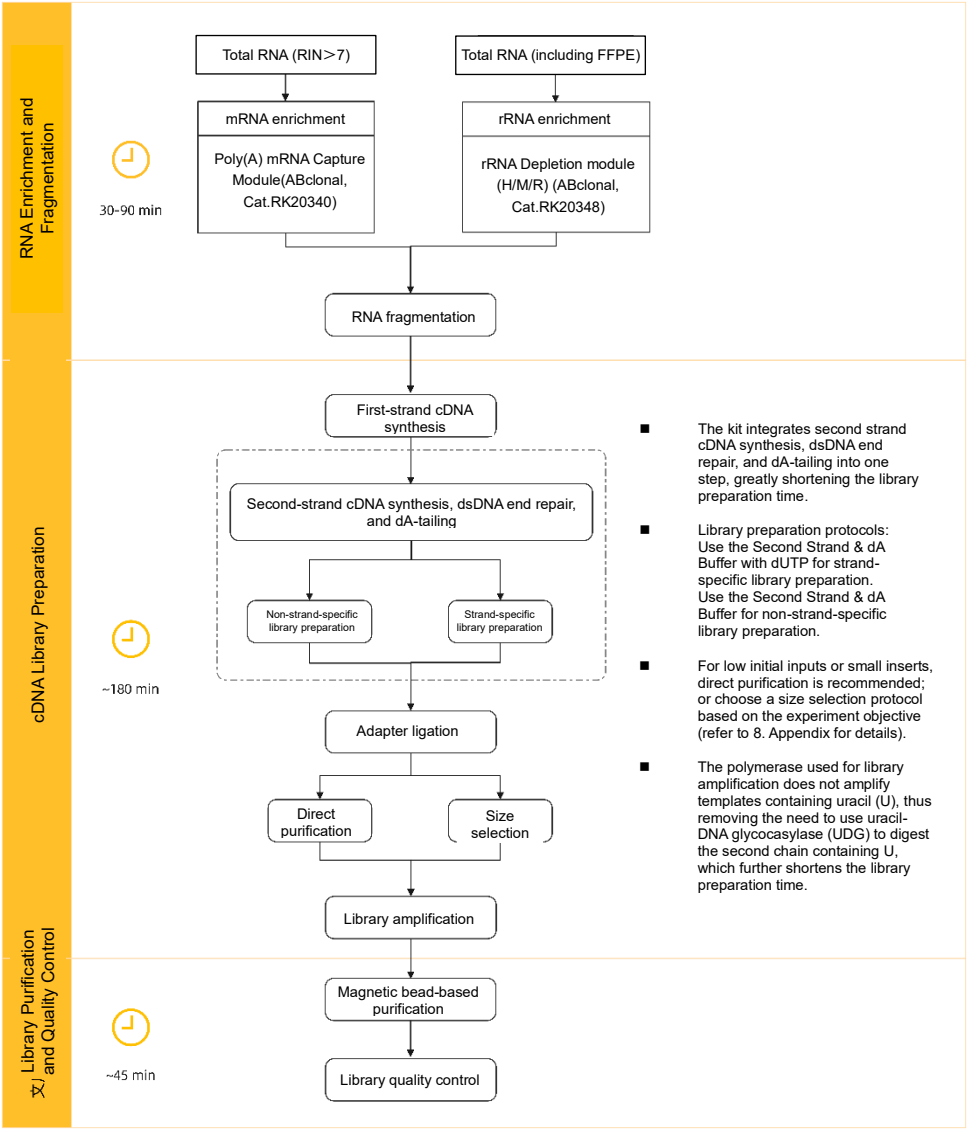
4.6 Other reagents and consumables

Freshly prepared 80% ethanol.

Magnetic rack.

PCR system.

5. Workflow Diagram



6. Precautions

6.1 Quality Control of RNA Samples

6.1.1 The kit is suitable for an initial total RNA input of 10-1,000 ng. It is recommended to quantify the total RNA using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. Q32855). A lower RNA input may affect library preparation.

6.1.2 RNA enrichment using Poly (A) mRNA Capture Module must be performed with a High-quality total RNA samples (RIN > 7), otherwise the 3' bias may be introduced during library preparation. Therefore, for RNA samples with RIN < 7, it is recommended to use the rRNA Depletion Module for RNA enrichment.

6.1.3 For plant or other eukaryotic cell RNA samples, if RNAs are degraded but 28S and 18S bands are visible in agarose gel electrophoresis, it is recommended to increase the total RNA input and appropriately increase PCR cycles to obtain sufficient libraries.

6.2 Use of Magnetic Beads

6.2.1 The magnetic beads should be taken out half an hour in advance and brought to room temperature.

6.2.2 The 2X Oligo (dT)25 Capture Beads and AFTMag NGS DNA Clean Beads should not be stored at -20°C. Once frozen, the beads will agglomerate and cannot be separated. Therefore, frozen magnetic beads cannot be used any longer and new ones need to be purchased.

6.2.3 During purification with AFTMag NGS DNA Clean Beads, the Low EDTA TE must be added for elution after ethanol has fully evaporated, namely 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.

6.3 Library Quality Control

6.3.1 The library quality is deemed 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.

6.3.2 The possible reason why library preparation with good-quality total RNA (RIN > 7) fails and the solutions are as follows:

The RIN of total RNA is a metric for total RNA quality assessment and cannot fully reflect 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 1.10 (Page 11), add 6 µL of Tris Buffer, heat the solution at 80°C for 2 min, let the tube stand on the magnetic rack until the solution becomes clear, and take 1 µL of the supernatant (the intact poly(A) RNAs) for analysis with the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Chip.

If analysis results show that the mRNA abundance is low, increase the total RNA input. If the mRNA integrity is

poor, adjust the mRNA fragmentation time.

6.4 Adapters for Library Preparation

6.4.1 ABclonal provides both full and truncated adapters (as detailed in the Appendix Table). Clients can select appropriate adapters as needed.

6.4.2 If a PCR-free library is prepared, use full adapters with full indices in the adapter ligation step and remove excess adapters from the ligation product before sequencing.

6.4.3 The kit is compatible with Full Adapters and Truncated Adapters of MGI and Illumina sequencing platforms.

6.4.4 The adapter quality and concentration directly affect the ligation efficiency and library yield. Excessive adapters may produce many adapter dimers, while insufficient adapters may compromise the ligation efficiency and library yield. Table 1 lists the recommended adapter concentrations for different total RNA inputs.

Table 1. Recommended Adapter Concentrations

Total RNA	Recommended Illumina Adapter Concentrations	Recommended MGI Adapter Concentrations
1 μg	15 μM	15 μM
100 ng	3 μM	7.5 μM
10 ng	1.5 μM	3 μM

6.5 Operating Procedures

6.5.1 Masks and gloves should be worn during mRNA library preparation.

6.5.2 After magnetic beads are added, all poly(A) mRNA enrichment steps should be conducted at room temperature.

6.5.3 The RNA sample should be placed on ice and used for the next step as soon as possible to avoid RNA degradation.

6.5.4 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.

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

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

6.5.7 The reagent for each step can be pre-mixed in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

7. Protocols (RNA Library Preparation for Illumina)

7. 1 RNA Enrichment and Fragmentation

Select one of the following RNA enrichment and fragmentation methods based on the experiment objective: Protocol 1: Poly(A) mRNA capture and fragmentation; Protocol 2: rRNA depletion and fragmentation.

Protocol 1: Poly(A) mRNA Capture and Fragmentation

Use the Poly(A) mRNA Capture Module (ABclonal, Cat. RK20340) for mRNA capture. This kit works with eukaryotic total RNA samples (with poly(A) tail) with RNA RIN ≥ 7. Prepare RNA samples on ice and perform all the other steps at room temperature.

7.1.1 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.

7.1.2 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.

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

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

7.1.4 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.

7.1.5 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.

7.1.6 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

7.1.7 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.

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

7.1.9 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.

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

7.1.11 Prepare the 1X Frag/Elute Buffer according to the following table:

	Reagents	Volume
●	Frag/Elute Buffer	7 µL
○	Nuclease-free Water	14 µL
	Total Volume	21 µL

7.1.12 Add 21 µL of 1X Frag/Elute Buffer, mix the solution well by pipetting, and elute and fragment RNAs as per the following table (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-350 nt	94°C 15 min, 4°C hold
300-400 nt	94°C 12 min, 4°C hold
350-500 nt	94°C 8 min, 4°C hold
450-600 nt	94°C 6 min, 4°C hold

7.1.13 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 19 µL of the supernatant into a new PCR tube, and immediately use it for Step 2. First Strand cDNA Synthesis.

Protocol 2: rRNA Depletion and Fragmentation

Use the ABclonal rRNA Depletion Module (H/M/R) (Cat. RK20348) for rRNA depletion. The method effectively depletes cytoplasmic rRNA (including 5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA) and mitochondrial rRNA (12S rRNA and 16S rRNA) from total RNA.

Probe/rRNA Hybridization

7.1.1 Take 10-1,000 ng of total RNA, dilute to 12 µL with nuclease-free water, and place on ice for later use.

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

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

7.1.3 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.

7.1.4 Load the system 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

7.1.5 After hybridization, transfer the sample from the PCR system onto ice, and proceed immediately with RNase H digestion. During hybridization, thaw the 10X RNase H Buffer on ice in advance.

RNase H Digestion

7.1.6 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

7.1.7 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.

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

Temperature	Time
37°C	30 min

7.1.9 After RNase H digestion, transfer the sample from the PCR system onto ice, and proceed immediately with DNase I digestion. During RNase H digestion, thaw the 10X DNase I Buffer on ice in advance.

DNase I Digestion

7.1.10 Prepare the DNase I digestion pre-mix according to the following:

	Reagents	Volume
●	10X DNase I Buffer	5 μL
●	DNase I	2.5 μL
○	Nuclease-free Water	22.5 μL
	Total Volume	30 μL

7.1.11 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.

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

Temperature	Time
37°C	30 min

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

Purification of rRNA-depleted RNA

7.1.14 Prepare the 1X Frag/Elute Buffer according to the following table:

	Reagents	Volume
	Frag/Elute Buffer	7 μL
	Nuclease-free Water	14 μL
	Total Volume	21 μL

7.1.15 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 prior to use.

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

7.1.17 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.

7.1.18 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.

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

7.1. 20 Dry the magnetic beads for 2-3 min, add 21 μL of 1X Frag/Elute Buffer after the ethanol has fully evaporated, and mix them well by pipetting.

7.1. 21 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 μL of the supernatant into a new centrifuge tube.

7.1. 22 Place the supernatant in the PCR system for RNA fragmentation (heating lid temperature 105°C).

Target Fragment Size	Fragmentation Condition
200-350 nt	94°C 15 min, 4°C hold
300-400 nt	94°C 12 min, 4°C hold
350-500 nt	94°C 8 min, 4°C hold
450-600 nt	94°C 6 min, 4°C hold

7.1. 23 After cooling to 4°C, centrifuge the tube instantaneously and then place it on ice. Proceed immediately with Step 2. First Strand cDNA Synthesis.

7.2 First Strand cDNA Synthesis

7.2.1 Thaw RT Strand Specificity Reagent at room temperature, mix it well, and prepare the following system on ice:

	Reagents	Volume
	Fragmented mRNA**	19 μL
●	RT Strand Specificity Reagent	4 μL
●	First Strand Synthesis Enzyme Mix	2 μL
	Total Volume	25 μL

** : The 2X Frag/Elute Buffer contains the Random Primer required for the First strand cDNA synthesis. Ensure that the 2X Frag/Elute Buffer is added when performing this step.

7.2.2 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

During the reaction, thaw the Second Strand Buffer on ice.

7.3 Second Strand cDNA Synthesis

7.3.1 Add the reagents listed in the table below.

	Reagents	Volume
	First Strand cDNA	25 μL
●	Second Strand & dA Buffer /Second Strand & dA Buffer with dUTP *	10 μL
●	Second Strand & dA Enzymes	5 μL
●	Nuclease-free Water	10 μL
	Total Volume	50 μL

Note: Use the Second Strand & dA Buffer with dUTP for strand-specific library preparation; use the Second Strand & dA Buffer for non-strand-specific library preparation.

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

Temperature	Time
20°C	45 min
72°C	15 min
4°C	Hold

During the reaction, thaw the Ligation Buffer and adapter on ice.

7.4 Adapter Ligation

7.4.1 Thaw the Ligation Buffer and adapter on ice and prepare the adapter ligation system on ice.

	Reagents	Volume
	ds cDNA (generated in Step 3.2)	50 μL
●	Ligation Buffer	16.5 μL
●	Adapter*	5 μL
●	Ligase Mix T5	3 μL
	Total Volume	74.5 μL

Note: 1. Do not pre-mix the Ligase Mix T5 and adapter. Otherwise, adapter dimers will be produced, thus affecting the ligation efficiency.

2. The stock concentration of the adapter provided by ABclonal is 15 μM. Dilute the adapter according to recommended adapter concentrations in Table 1 and add nuclease-free water to make a ligation system of 74.5 μL.

3. Full adapters contain different indices. Distinguish indices when adding adapters and change tips in time to avoid contamination of samples and indices.

7.4.2 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

7.5 Purification of Ligation Products

This is a direct purification protocol suitable for library preparation scenarios without fragment size requirements. To select library fragments of specific sizes or purify products from low initial input, refer to 8. Appendix: Size Selection of Ligation Products and Two-Round Purification Protocols.

7.5.1 Add 60 μL of AFTMag NGS DNA Clean Beads (0.8X) to each 74.5 μL ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

7.5.2 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

7.5.3 Hold the centrifuge tube on the magnetic rack, add 200 µL of 80% ethanol to rinse the beads, incubate for 30s, and remove and discard the supernatant.

7.5.4 Repeat Step 5.3.

7.5.5 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 µL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

7.5.6 Remove the PCR tube from the magnetic rack and add 22 µL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

7.5.7 Let the PCR tube stand on the magnetic rack for 2 min and transfer 20 µL of the supernatant into a new PCR tube.

7.5.8 Safe Stopping Point: Purified adapter ligation products can be stored at 4°C for 1-2 weeks, or at -20°C for a longer time.

7.6 Library Amplification

7.6.1 Prepare the PCR system based on the adapter kit selected.

Table 2. PCR System for Illumina - Truncated Adapter (Dual Index with UDI/UMI)

	Component	Volume
	Purified adapter ligation products	20 µL
●	2X PCR Master Mix	25 µL
○	UDI Primer	5 µL
	Total Volume	50 µL

Table 3. PCR System for Illumina - Full Adapter

	Component	Volume
	Purified adapter ligation products	20 µL
●	2X PCR Master Mix	25 µL
●	10X ILM PCR Primers	5 µL
	Total Volume	50 µL

Note: For adapters and indices compatible with the kit, refer to 9. Appendix Table.

7.6.2 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	10~17* (Refer to Table 4)
60°C	15s	
72°C	30s	
72°C	1 min	1
4°C	Hold	

Table 4. Recommended PCR Cycles for Illumina - Adapter

Total RNA	Ligation Products Cycles without Size Selection	Ligation Products Cycles with Size Selection
10 ng	15-17	/
100 ng	12-14	13-15
1 μg	10-12	11-13

Note: Since the content of extracted mRNA varies across different species and tissues, the actual number of amplification cycles needs to be adjusted according to the quality of extracted total RNA, species and tissue type, and how samples are treated.

7.7 PCR Product Purification

7.7.1 Add 40 μL of AFTMag NGS DNA Clean Beads (0.8X) to each PCR product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

7.7.2 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

7.7.3 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol to rinse the beads, incubate for 30s, and remove and discard the supernatant.

7.7.4 Repeat Step 7.3.

7.7.5 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

7.7.6 Remove the PCR tube from the magnetic rack and add 22 μL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

7.7.7 Let the PCR tube stand on the magnetic rack for 2 min and transfer 20 μL of the supernatant into a new PCR tube for later use.

7. Protocols (RNA Library Preparation for MGI)

7.1 RNA Enrichment and Fragmentation

Select one of the following RNA enrichment and fragmentation methods based on the experiment objective: Protocol 1: Poly(A) mRNA capture and fragmentation; Protocol 2: rRNA depletion and fragmentation.

Protocol 1: Poly(A) mRNA Capture and Fragmentation

Use the Poly(A) mRNA Capture Module (ABclonal, Cat. RK20340) for mRNA capture. This kit works with eukaryotic total RNA samples (with poly(A) tail) with RNA RIN ≥ 7. Prepare RNA samples on ice and perform all the other steps at room temperature.

7.1.1 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.

7.1.2 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.

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

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

7.1.4 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.

7.1.5 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.

7.1.6 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

7.1.7 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.

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

7.1.9 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.

7.1.10 Centrifuge instantaneously after capping, place the tube on the magnetic rack, and remove all the

residual liquid with a 10 μL pipette.

7. 1. 11 Prepare the 1X Frag/Elute Buffer according to the following table:

	Reagents	Volume
●	Frag/Elute Buffer	7 μL
○	Nuclease-free Water	14 μL
	Total Volume	21 μL

7.1.12 Add 21 μL of 1X Frag/Elute Buffer, mix the solution well by pipetting, and elute and fragment RNAs as per the following table (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-350 nt	94°C 15 min, 4°C hold
300-400 nt	94°C 12 min, 4°C hold
350-500 nt	94°C 8 min, 4°C hold
450-600 nt	94°C 6 min, 4°C hold

7.1.13 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 19 μL of the supernatant into a new PCR tube, and immediately use it for Step 2. First Strand cDNA Synthesis.

Protocol 2: rRNA Depletion and Fragmentation

Use the ABclonal rRNA Depletion Module (H/M/R) (Cat. RK20348) for rRNA depletion. The method effectively depletes cytoplasmic rRNA (including 5S rRNA, 5.8S rRNA, 18S rRNA, and 28S rRNA) and mitochondrial rRNA (12S rRNA and 16S rRNA) from total RNA.

Probe/rRNA Hybridization

7.1.1 Take 10-1,000 ng of total RNA, dilute to 12 μL with nuclease-free water, and place on ice for later use.

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

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

7.1.3 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.

7.1.4 Load the system 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

7.1.5 After hybridization, transfer the sample from the PCR system onto ice, and proceed immediately with RNase H digestion. During hybridization, thaw the 10X RNase H Buffer on ice in advance.

RNase H Digestion

7.1.6 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

7.1.7 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.

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

Temperature	Time
37°C	30 min

7.1.9 After RNase H digestion, transfer the sample from the PCR system onto ice, and proceed immediately with DNase I digestion. During RNase H digestion, thaw the 10X DNase I Buffer on ice in advance.

DNase I Digestion

7.1.10 Prepare the DNase I digestion pre-mix according to the following:

	Reagents	Volume
●	10X DNase I Buffer	5 μL
●	DNase I	2.5 μL
○	Nuclease-free Water	22.5 μL
	Total Volume	30 μL

7.1.11 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.

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

Temperature	Time
37°C	30 min

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

Purification of rRNA-depleted RNA

7.1.14 Prepare the 1X Frag/Elute Buffer according to the following table:

	Reagents	Volume
	Frag/Elute Buffer	7 μL
	Nuclease-free Water	14 μL
	Total Volume	21 μL

7.1.15 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 prior to use.

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

7.1.17 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.

7.1.18 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.

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

7.1. 20 Dry the magnetic beads for 2-3 min, add 21 μL of 1X Frag/Elute Buffer after the ethanol has fully evaporated, and mix them well by pipetting.

7.1. 21 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 μL of the supernatant into a new centrifuge tube.

7.1. 22 Place the supernatant in the PCR system for RNA fragmentation **(heating lid temperature 105°C)**.

Target Fragment Size	Fragmentation Condition
200-350 nt	94°C 15 min, 4°C hold
300-400 nt	94°C 12 min, 4°C hold
350-500 nt	94°C 8 min, 4°C hold
450-600 nt	94°C 6 min, 4°C hold

7.1. 23 After cooling to 4°C, centrifuge the tube instantaneously and then place it on ice. Proceed immediately with Step 2. First Strand cDNA Synthesis.

7.2 First Strand cDNA Synthesis

7.2.1 Thaw RT Strand Specificity Reagent at room temperature, mix it well, and prepare the following system on ice:

	Reagents	Volume
	Fragmented mRNA**	19 μL
●	RT Strand Specificity Reagent	4 μL
●	First Strand Synthesis Enzyme Mix	2 μL
	Total Volume	25 μL

** : The 2X Frag/Elute Buffer contains the Random Primer required for the First strand cDNA synthesis. Ensure that the 2X Frag/Elute Buffer is added when performing this step.

7.2.2 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

During the reaction, thaw the Second Strand Buffer on ice.

7.3 Second Strand cDNA Synthesis

7.3.1 Add the reagents listed in the table below.

	Reagents	Volume
	First Strand cDNA	25 μL
●	Second Strand & dA Buffer /Second Strand & dA Buffer with dUTP *	10 μL
●	Second Strand & dA Enzymes	5 μL
●	Nuclease-free Water	10 μL
	Total Volume	50 μL

Note: Use the Second Strand & dA Buffer with dUTP for strand-specific library preparation; use the Second Strand & dA Buffer for non-strand-specific library preparation.

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

Temperature	Time
20°C	45 min
72°C	15 min
4°C	Hold

During the reaction, thaw the Ligation Buffer and adapter on ice.

7.4 Adapter Ligation

7.4.1 Thaw the Ligation Buffer and adapter on ice and prepare the adapter ligation system on ice.

	Reagents	Volume
	ds cDNA (generated in Step 3.2)	50 μL
●	Ligation Buffer	16.5 μL
●	Adapter*	5 μL
●	Ligase Mix T5	3 μL
	Total Volume	74.5 μL

Note: 1. Do not pre-mix the Ligase Mix T5 and adapter. Otherwise, adapter dimers will be produced, thus affecting the ligation efficiency.

2. The stock concentration of the adapter provided by ABclonal is 15 μM. Dilute the adapter according to recommended adapter concentrations in Table 1 and add nuclease-free water to make a ligation system of 74.5 μL.

3. Full adapters contain different indices. Distinguish indices when adding adapters and change tips in time to avoid contamination of samples and indices.

7.4.2 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

7.5 Purification of Ligation Products

This is a direct purification protocol suitable for library preparation scenarios without fragment size requirements. To select library fragments of specific sizes or purify products from low initial input, refer to 8. Appendix: Size Selection of Ligation Products and Two-Round Purification Protocols.

7.5.1 Add 60 μL of AFTMag NGS DNA Clean Beads (0.8X) to each 74.5 μL ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

7.5.2 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

7.5.3 Hold the centrifuge tube on the magnetic rack, add 200 µL of 80% ethanol to rinse the beads, incubate for 30s, and remove and discard the supernatant.

7.5.4 Repeat Step 5.3.

7.5.5 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 µL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

7.5.6 Remove the PCR tube from the magnetic rack and add 22 µL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

7.5.7 Let the PCR tube stand on the magnetic rack for 2 min and transfer 20 µL of the supernatant into a new PCR tube.

7.5.8 Safe Stopping Point: Purified adapter ligation products can be stored at 4°C for 1-2 weeks, or at -20°C for a longer time.

7.6 Library Amplification

7.6.1 Prepare the PCR system based on the adapter kit selected.

Table 5. PCR System for MGI - Truncated Adapter (Dual Index with UDI/UMI)

	Component	Volume
	Purified adapter ligation products	20 µL
●	2X PCR Master Mix	25 µL
○	MGI UDI Primer	5 µL
	Total Volume	50 µL

Table 6. PCR System for MGI- Full Adapter

	Component	Volume
	Purified adapter ligation products	20 µL
●	2X PCR Master Mix	25 µL
●	MGI PCR Primer Mix	5 µL
	Total Volume	50 µL

Note: For adapters and indices compatible with the kit, refer to 9. Appendix Table.

7.6.2 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	11~18* (Refer to Table 7)
60°C	15s	
72°C	30s	
72°C	1 min	1
4°C	Hold	

Table 7. Recommended PCR Cycles for MGI - Adapter

Total RNA	Ligation Products Cycles without Size Selection	Ligation Products Cycles with Size Selection
10 ng	16-18	/
100 ng	13-15	14-16
1 μg	11-13	12-14

Note: Since the content of extracted mRNA varies across different species and tissues, the actual number of amplification cycles needs to be adjusted according to the quality of extracted total RNA, species and tissue type, and how samples are treated.

7.7 PCR Product Purification

7.7.1 Add 40 μL of AFTMag NGS DNA Clean Beads (0.8X) to each PCR product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

7.7.2 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

7.7.3 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol to rinse the beads, incubate for 30s, and remove and discard the supernatant.

7.7.4 Repeat Step 7.3.

7.7.5 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

7.7.6 Remove the PCR tube from the magnetic rack and add 22 μL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

7.7.7 Let the PCR tube stand on the magnetic rack for 2 min and transfer 20 μL of the supernatant into a new PCR tube for later use.

8. Appendix

8.1 Size Selection of Ligation Products and Two-Round Purification Protocols

Since magnetic bead-based size selection will lead to sample loss, it is recommended to add 2 more PCR cycles.

Protocol 1: Size Selection

This protocol is suitable for libraries with inserts larger than 200 bp. Purify adapter ligation products using AFTMag NGS DNA Clean Beads (1.0X) before proceeding with two-round size selection using magnetic beads. The steps are as follows (fragmentation at 94℃ for 12 min, full adapter ligation, 420-520 bp size selection):

8.1.1 After ligation, add 74.5 μL of AFTMag NGS DNA Clean Beads (1.0X) to the ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

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

8.1.3 Repeat Step 3.

8.1.4 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

8.1.5 Remove the PCR tube from the magnetic rack and add 102.5 μL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

8.1.6 Let the PCR tube stand on the magnetic rack for 2 min until the solution becomes clear, and cautiously transfer 100 μL of the supernatant into a new PCR tube.

8.1.7 Add 60 μL of AFTMag NGS DNA Clean Beads (0.60X) to the 100 μL of purified ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

8.1.8 Transfer the PCR tube to the magnetic rack and let it stand for 2 min until the solution becomes clear (Do not discard the supernatant).

8.1.9 Transfer 155 μL of the supernatant into a new centrifuge tube, add 10 μL of AFTMag NGS DNA Clean Beads (0.1X), and mix well by pipetting. Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 2 min until the solution becomes clear, and cautiously remove and discard the supernatant.

Note: Change the proportion of the two rounds of beads (highlighted in grey) according to the adapter type and library fragment size (refer to Table 11 or Table 12).

8.1.10 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.

8.1.11 Repeat Step 11.

8.1.12 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

8.1.13 Remove the PCR tube from the magnetic rack, add 22 μL of Low EDTA TE, and mix well by pipetting. 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 library amplification.

Table 8. Magnetic Bead Proportions in Size Selection (Full Adapter Ligation System)

Fragmentation Condition	**94°C 15 min**	**94°C 12 min**	**94°C 8 min**	**94°C 6 min**
RNA Fragment Size	200-350 nt	300-400 nt	350-500 nt	450-600 nt
Library Fragment Size	320-470 bp	420-520 bp	470-620 bp	570-720 bp
Proportion of Beads (1st Round)	0.65X (65 μL)	0.60X (60 μL)	0.55X (55 μL)	0.5X (50 μL)
Proportion of Beads (2nd Round)	0.1X (10 μL)	0.1X (10 μL)	0.1X (10 μL)	0.1X (10 μL)

Table 9. Magnetic Bead Proportions in Size Selection(Truncated Adapter Ligation System)

Fragmentation Condition	94°C 15 min	94°C 12 min	94°C 8 min	94°C 6 min
RNA Fragment Size	200-350 nt	300-400 nt	350-500 nt	450-600 nt
Library Fragment Size	320-470 bp	420-520 bp	470-620 bp	570-720 bp
Proportion of Beads (1st Round)	0.70X (70 μL)	0.65X (65 μL)	0.60X (60 μL)	0.55X (55 μL)
Proportion of Beads (2nd Round)	0.1X (10 μL)	0.1X (10 μL)	0.1X (10 μL)	0.1X (10 μL)

Note: As the same size selection proportion may yield different fragment sizes for full and truncated adapters, the proportion of magnetic beads may be adjusted as needed. To obtain larger fragments, reduce the proportion of beads in the first round; to obtain smaller fragments, increase the proportion of beads in the first round.

Protocol 2: Two-round Purification

This protocol is suitable for low input RNA (≤ 10 ng) or low quality samples (such as FFPE samples with DV200 < 30%), for which two rounds of purification are recommended after adapter ligation. The steps are as follows:

8.1.1 After ligation, add 60 μL of AFTMag NGS DNA Clean Beads (0.8X) to the ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

8.1.2 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

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

8.1.4 Repeat Step 3.

8.1.5 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

8.1.6 Remove the PCR tube from the magnetic rack and add 102.5 μL of Low EDTA TE to resuspend the magnetic beads. Let the mixture stand at room temperature for 1 min to fully release the DNA from the beads.

8.1.7 Let the PCR tube stand on the magnetic rack for 2 min until the solution becomes clear, and cautiously transfer 100 μL of the supernatant into a new PCR tube.

8.1.8 Add 100 μL of AFTMag NGS DNA Clean Beads (1.0X) to the 100 μL of purified ligation product, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 min.

8.1.9 Transfer the PCR tube to the magnetic rack and let it stand for 2 min. After the solution becomes clear, carefully remove the supernatant without disturbing the beads.

8.1.10 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.

8.1.11 Repeat Step 10.

8.1.12 Keep the PCR tube on the magnetic rack, remove the residual ethanol with a 10 μL pipette, and uncap to air dry until there is no residual ethanol *(bead color changes from bright brown to frosted brown)*.

8.1.13 Remove the PCR tube from the magnetic rack, add 22 μL of Low EDTA TE, and mix well by pipetting. 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 library amplification.

8.2 RNA Library Preparation for FFPE Samples or Other Degraded Samples

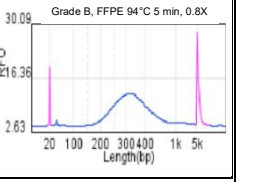
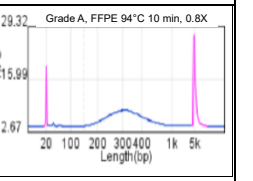
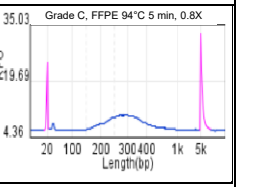
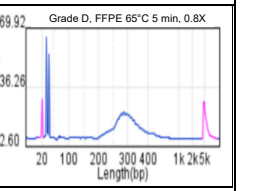
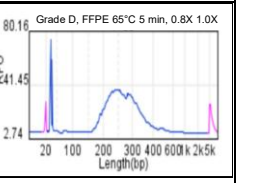
8.2.1 FFPE samples or other degraded samples have poor RNA integrity. Therefore, it is recommended to use the rRNA Depletion Module for RNA enrichment.

8.2.2 Due to FFPE sample damage or degradation, library preparation conditions need to be optimized and modified, and the number of amplification cycles needs to be increased appropriately.

8.2.3 Formalin fixation leads to the fragmentation of RNA in FFPE samples, rendering RIN unsuitable for RNA quality control. Therefore, DV200 is often used to evaluate the quality of RNA extracted from FFPE samples. DV200 refers to the percentage of RNA fragments > 200 nt in total RNA. DV200 is directly related to the

successful sequencing of FFPE-derived RNA. A DV200 over 70% indicates high RNA quality, while a value between 50%-70% indicates moderate RNA quality, in which case a higher initial input is required for transcriptome analysis. A DV200 below 20% indicates highly degraded RNA, which is not recommended for further testing. Refer to the library preparation results of FFPE samples of different grades in Table 13 to modify the library preparation protocol.

Table 10. Library Preparation Results of FFPE Samples of Different Grades

Initial Input (ng)	DV200	Fragmentation Condition	Purification of Ligation Products	PCR Amplification Cycles	Library Yield (ng/μL) (31 μl Elution)	Library Peak Patterns
200 ng	> 70%	94°C 10 min	0.8X	14	30.8	
200 ng	50%-70%	94°C 5 min	0.8X	15	30.2	
500 ng	30%-50%	94°C 3 min	0.8X	16	5.22	
500 ng	< 30%	65°C 5 min	0.8X	19	3.22	
500 ng	< 30%	65°C 5 min	0.8X; 1.0X	19	1.07	

9. Appendix Table

Table 11. Summary of Compatible Adapters and Indices

Type	Product Name	Catalog
UDI (8-base)	Unique Dual Index for Illumina MiniSet (8 indices)	RK21622
	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
UDI (8-base)-UMI	Unique Dual Index (with UMI) for Illumina MidiSet (24 indices)	RK21701
	Unique Dual Index (with UMI) for Illumina Set_A (48 indices)	RK21702
	Unique Dual Index (with UMI) for Illumina Set_B (48 indices)	RK21703
UDI (10-base)	Truncated DNA Adapter (UDI) Kit for MGI MiniSet	RK21686
	Truncated DNA Adapter (UDI) Kit for MGI MidiSet	RK21687
	Truncated DNA Adapter (UDI) Kit for MGI Set A	RK21688
	Truncated DNA Adapter (UDI) Kit for MGI Set B	RK21689

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