

Hieff NGS™ EvoMax RNA Library Prep Kit (dNTP) (Compatible with Illumina & MGI)

12341ES

INSTRUCTIONS FOR USE

Ver. EN20260401

A large, abstract orange graphic at the bottom of the page, consisting of several overlapping, wavy, rounded shapes that create a sense of movement and depth.

CONTENT

PRODUCT DESCRIPTION	1
SPECIFICATIONS	1
COMPONENTS	1
STORAGE	2
NOTES	2
WORKFLOW	5
INSTRUCTIONS	5
APPENDICES	13

Product description

Hieff NGS™ EvoMax RNA Library Prep Kit (dNTP) is a Total RNA sequencing library preparation kit compatible with both Illumina and MGI platforms. This product is available in two formats: conventional tube format and pre-loaded plate format. The pre-loaded plate format does not require manual preparation or subpackaging, and can be directly adapted to automated library preparation, making it more convenient to use. The kit contains RNA fragmentation reagents, reverse transcription reagents, ds-cDNA synthesis reagents, and library amplification reagents. It can be seamlessly combined with mRNA purification kits or rRNA depletion kits for sequencing library construction. All reagents have undergone strict quality control and functional verification to ensure the stability and repeatability of library preparation to the greatest extent.

Specifications

Cat.No.	12341ES24 / 12341ES96 / 12341ES97 / 12341ES98
Size	24 T / 96 T / 96 T (automated) / 96 T (plate Format)

Components

Components No.	Name	12341ES08	12341ES24	12341ES96	12341ES97(Tube Automated, 96 T)	12341ES98(Plate Automated 96 T)
12341-A	Frag/Prime Buffer	150 µL	450 µL	2× 900 µL	2× 1064 µL	8× 266 µL
12341-B	1st Reaction Module 2.0	64 µL	192 µL	768 µL	960 µL	8×120 µL
12341-C	2nd Reaction Module(dNTP)	280 µL	840 µL	3× 1120 µL	3× 1280 µL	8× 480 µL
12341-D	Ligation Reaction Module	280 µL	840 µL	3× 1120 µL	3× 1280 µL	8×480 µL
12341-E	2×Super Canace™ II High-Fidelity Mix	200 µL	600 µL	2× 1200 µL	2× 1360 µL	8× 340 µL
*	Primer Mix*	/	/	/	/	/

[Note]:* Components marked with * are not included in this kit. When preparing libraries with uncomplete adapters, these components are not required. For libraries using full-length adapters, the components must be added separately.

This kit is compatible with both Illumina and MGI platforms; however, platform-specific primer mixes must be supplied separately (Cat# 13334 Primer Mix for MGI and Cat# 13335 Primer Mix for Illumina).

For the layout of plate-based automated reagent components, please refer to the figure below.

Unit: μL

	Frag/Primer Buffer	1st Reaction Module	2nd Reaction Module(dNTP)	Ligation Reaction Module	2 × Super Canace™ II High-Fidelity Mix							
	1	2	3	4	5	6	7	8	9	10	11	12
A	266	120	480	480	340							
B	266	120	480	480	340							
C	266	120	480	480	340							
D	266	120	480	480	340							
E	266	120	480	480	340							
F	266	120	480	480	340							
G	266	120	480	480	340							
H	266	120	480	480	340							

The left image shows the layout of the plate-based library preparation kit, where each component occupies one column. Each well contains enough reagent for 12 library preparation reactions.

When using the plate-based library preparation kit with automated instruments, the unused positions on the plate (columns 6–12) can be preloaded with beads, adapters, or other reagents according to experimental needs before starting the experiment.

Yeasten provides adapted scripts and tutorials for automation liquid handling workstations for use in automated library preparation.

Storage

This product should be stored at -25°C ~ -15°C for 1 year.

Notes

I. About Operation

1. For your safety and health, please wear a lab coat and disposable gloves during handling.
2. Before use, allow all kit components to thaw at room temperature. After thawing, invert the tubes several times to ensure thorough mixing. Briefly centrifuge and place on ice until ready for use.
3. It is recommended to perform all reaction steps in a PCR cycler with a heated lid. Preheat the PCR cycler to near the reaction temperature before use.
4. Use RNase-free consumables and regularly clean the working area. We recommend using ThermoFisher Scientific's RNAZap™ Wipes for RNase decontamination.
5. PCR products are highly prone to aerosol contamination, which can affect the accuracy of experimental results. It is recommended to enforce physical separation between the PCR setup area and the PCR product purification/detection area. Use dedicated pipettes and equipment for library preparation, and routinely clean each area (we recommend using ThermoFisher Scientific's DNAZap™ Wipes) to maintain a clean experimental environment.
6. If using a liquid handling workstation for automated library preparation, please consult Yeasen for the corresponding automation protocols.
7. Each well of the plate-based reagent kit is recommended for single-use only after opening.
8. This product is intended for research use only.

II. About Adapter Ligation

1. We offer both Illumina or MGI complete (barcoded) adapter kits and uncomplete adapter kits. Users may choose based on their experimental needs.
2. We recommend using high-quality commercial adapters. If using custom-synthesized adapters, please source them from a company experienced in NGS primer synthesis and ensure strict contamination control during

synthesis. Adapter annealing should be performed in a biosafety cabinet. Only one adapter should be handled at a time to prevent cross-contamination.

3. Prior to use, remove adapters and place them on ice or at 4°C for thawing. When working at room temperature, keep the lab temperature below 25°C to prevent adapter strand separation.

4. Adapter concentration that is too high or too low may reduce library preparation success rate. In this protocol, the volume of adapter added per reaction is fixed at 5 µL. Please refer to Table 1 below and dilute the adapter accordingly based on the initial RNA input amount. Use 0.1× TE buffer for adapter dilution. Diluted adapters can be stored at 4°C for up to 48 hours.

Table 1-1. Recommended Illumina Adapter Concentration Based on Input Total RNA Amount

Input Total RNA	Illumina Adapter stock concentration
10 ng	1 µM
100 ng	1.5 µM
500 ng	3 µM
≥1 µg	5 µM

Table 1-2. Recommended MGI Adapter Concentration Based on Input Total RNA Amount*

Input Total RNA	MGI Adapter stock concentration
10~99 ng	1 µM
100~499 ng	2 µM
500~4000 ng	5 µM

[Note]:* Adapter usage can be adjusted based on sample type and input amount.

III. About Library Amplification

1. The library amplification module in this kit contains our second-generation high-fidelity DNA polymerase, which significantly improves amplification uniformity compared to the first generation. Even low-copy genes can be amplified without bias.

2. The number of amplification cycles must be strictly controlled. Too few cycles may result in low library yield; too many cycles may lead to increased bias, duplication rate, chimera formation, and mutation accumulation.

3. Table 2 below lists the recommended number of amplification cycles based on input total RNA amount. The cycle numbers in Table 2 are suitable for most applications. For samples of poor quality (e.g., degraded FFPE samples), the number of cycles may be increased accordingly. For mRNA library preparation, note that mRNA content varies significantly across species and tissues. Adjust the number of cycles based on the starting amount, species type, and sample treatment conditions.

Table 2. Recommended Number of Amplification Cycles Based on Input Total RNA Amount

Input Total RNA	Number of cycles
10 ng	16
100 ng	14
500 ng	11
1 µg	10

[Note]: Library yield depends not only on input amount and amplification cycles but also on sample quality, fragmentation conditions, and size selection. Please consider all these factors when determining the optimal library preparation conditions.

IV. DNA Bead Purification and Size Selection

1. Several steps in the library preparation process require DNA purification using magnetic beads. We recommend using Hieff NGS™ DNA Selection Beads (Yeasten Cat#12601) or AMPure XP Beads (Beckman Cat#A63880) for DNA purification and size selection.
2. Allow the beads to equilibrate to room temperature before use; using cold beads may reduce yield and impair size selection performance.
3. Always vortex or pipette the beads thoroughly before each use to ensure homogeneity.
4. When transferring the supernatant, avoid aspirating the beads. Even trace amounts of bead carryover can negatively affect downstream library quality.
5. Freshly prepare 80% ethanol for bead washing; aged ethanol may reduce recovery efficiency.
6. Allow the beads to air-dry at room temperature before elution. Incomplete drying may leave residual ethanol that affects downstream reactions; excessive drying may cause bead cracking and reduce yield. Typically, 3–5 minutes of air drying is sufficient.
7. For long-term storage of purified or size-selected DNA, elute using 0.1× TE buffer. Eluted products can be stored at 4°C for 2 days or at –20°C for up to 1 month.

V. Library Quality Assessment

1. Library quality is typically assessed by measuring fragment size distribution and concentration.
2. Library concentration can be measured using:
dsDNA fluorescent dyes such as Qubit™ or PicoGreen™
qPCR-based absolute quantification methods
3. qPCR-based quantification is recommended: Fluorescent dye-based methods (e.g., Qubit™) cannot distinguish between fragments with one adapter, no adapters, or incomplete structures. qPCR quantifies only fully ligated libraries (i.e., those with adapters on both ends), excluding non-sequencable fragments.
4. Do not use spectrophotometry-based methods (e.g., NanoDrop™) for library quantification.
5. For fragment size distribution analysis, we recommend using instruments based on capillary electrophoresis or microfluidic technology, such as the Agilent Bioanalyzer 2100.

VI. Required Materials

1. mRNA enrichment kit: Hieff NGS™ mRNA Isolation Master Kit V2 (Yeasten Cat#12629)
2. rRNA depletion kit: Hieff NGS™ MaxUp Human rRNA Depletion Kit (rRNA & ITS/ETS) (Yeasten Cat#12257) or other rRNA depletion kits
3. RNA purification beads: Hieff NGS™ RNA Cleaner (Yeasten Cat#12602) or equivalent
4. DNA purification beads: Hieff NGS™ DNA Selection Beads (Yeasten Cat#12601) or AMPure XP Beads (Cat#A63880) or equivalent
5. RNA quality control: Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico Chip or equivalent
6. Adapters: Full-length adapters: Illumina (Cat#13519–13520 or equivalent) or MGI (Cat#13361–13362 or equivalent);
Truncated (short) adapters: Illumina (Cat#12414–12415, tube format; Cat#13327–13330, plate format, or equivalent) or MGI (Cat#13350–13353 or equivalent).

VII. Library Quality Control

Agilent 2100 Bioanalyzer DNA 1000 Chip or High Sensitivity Chip or equivalent; library quantification reagents.

VIII. Additional Materials

Absolute ethanol, nuclease-free water, low-binding pipette tips, PCR tubes, magnetic stand, PCR cyclor, etc.

Workflow

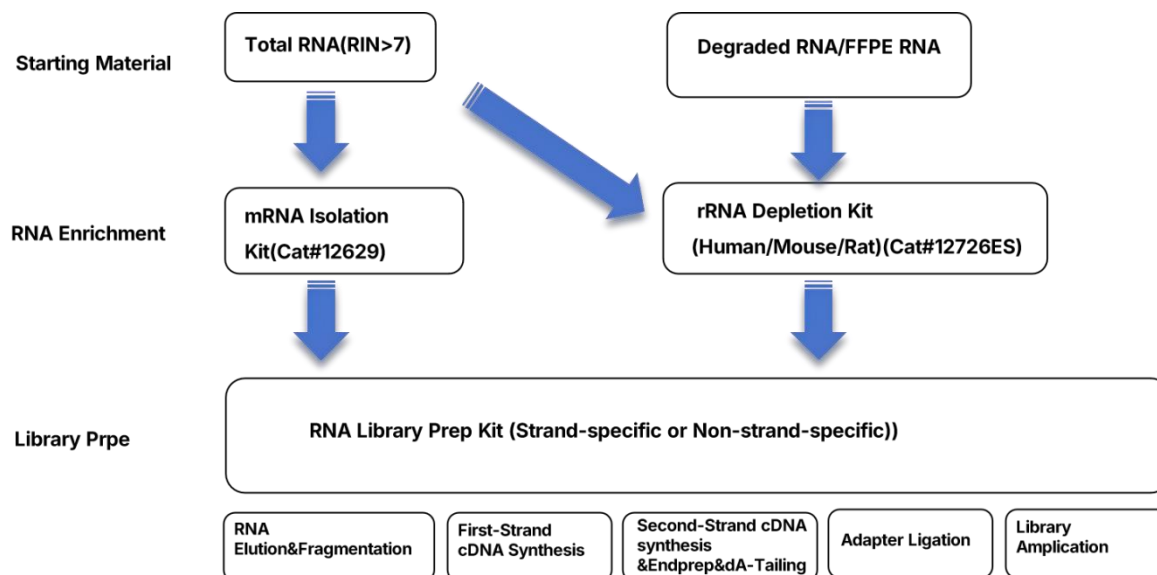


Figure 1. RNA Library Preparation Workflow

Instructions

Part I: Target RNA Enrichment and Fragmentation

This step prepares the target RNA before library preparation. Depending on the requirements, you can choose either the Poly(A) mRNA Isolation protocol (Protocol A) or the rRNA Depletion protocol (Protocol B).

[Note]: Yeasen Cat#12341 library preparation module does not include the reagents needed for this step; please prepare the necessary reagents based on your library preparation needs.

Protocol A: mRNA Purification and Fragmentation

1. Sample Requirements

This protocol uses the Hieff NGS™ mRNA Isolation Master Kit V2 (Yeasten Cat#12629) for mRNA enrichment. It is suitable for high-quality total RNA from eukaryotic organisms such as animals, plants, and fungi, with an input range of 10 ng to 4 µg (volume ≤ 50 µL). If the initial RNA concentration is low and the volume exceeds 50 µL, use the Hieff NGS™ RNA Cleaner (Yeasten Cat#12602) magnetic beads for concentration. The RNA should be tested using the Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico chip, with a required RIN value greater than 7 to ensure intact poly(A) tails. This kit uses oligo(dT) magnetic beads, which only capture poly(A)-tailed mRNA; non-coding RNAs and other RNAs without poly(A) tails are not applicable. Additionally, FFPE samples typically have degraded mRNA without complete poly(A) tails and thus cannot be used with this kit.

2. Procedure

- 1) Remove the mRNA Capture Beads 2.0 from 2-8°C and allow them to equilibrate to room temperature for about 30 minutes.
- 2) Prepare a nuclease-free centrifuge tube, add 10 ng to 4 µg of total RNA, and adjust the volume to 50 µL with

Nuclease-Free Water. Keep it on ice until use.

- 3) Invert or vortex the magnetic beads to mix thoroughly. Add 50 μ L of the bead suspension to the 50 μ L RNA sample and mix by pipetting up and down six times.
- 4) Place the mixture in a thermal cycler and run the following program: 65°C for 5 min, 25°C for 5 min, hold at 25°C to allow RNA to bind to the capture beads.
- 5) Place the sample on a magnetic rack and incubate at room temperature for 5 minutes. Once the solution clears, carefully remove the supernatant.
- 6) Remove the sample from the magnetic rack and resuspend the beads in 200 μ L Beads Wash Buffer 2.0. Mix thoroughly by pipetting up and down six times. Place back on the magnetic rack and incubate at room temperature for 5 minutes, then carefully remove the supernatant.
- 7) Repeat step 6 for a total of two washes.
- 8) Remove the sample from the magnetic rack and resuspend the beads in 50 μ L Tris Buffer 2.0. Mix thoroughly by pipetting up and down six times.
- 9) Place the sample in a thermal cycler and run the following program: 80°C for 2 minutes, hold at 25°C to elute the mRNA.
- 10) Remove the sample from the thermal cycler and add 50 μ L Beads Binding Buffer 2.0. Mix thoroughly by pipetting up and down six times.
- 11) Incubate at room temperature for 5 minutes to allow the mRNA to bind to the beads.
- 12) Place the sample on a magnetic rack and incubate at room temperature for 5 minutes. Carefully remove the supernatant.
- 13) Remove the sample from the magnetic rack and resuspend the beads in 200 μ L Beads Wash Buffer 2.0. Mix thoroughly by pipetting up and down six times. Place back on the magnetic rack and incubate at room temperature for 5 minutes, then remove all the supernatant.
- 14) Remove the sample from the magnetic rack and resuspend the beads in 18.5 μ L Frag/Prime Buffer. Mix thoroughly by pipetting up and down six times. Place the sample in a thermal cycler (preset to 94°C) and select the fragmentation program according to Table 3. Different species may require different fragmentation times; for example, try 94°C for 5 minutes. Analyze the purified mRNA size using the Agilent 2100 Bioanalyzer.

Table 3. mRNA Fragmentation Recommendations

Insert Size (bp)	Fragmentation Program
200-300	94°C, 10 min, 4°C, hold;
300-400	94°C, 7 min, 4°C, hold;
400-500	94°C, 5 min, 4°C, hold;

- 15) After the fragmentation program is complete, to prevent poly(A) tailed RNA from binding to the magnetic beads, immediately place the sample on a magnetic rack. Once the solution clears, transfer 17 μ L of the supernatant to a new nuclease-free microcentrifuge tube, and proceed immediately to the first-strand cDNA synthesis reaction (Part II, Step 1).

Protocol B: rRNA Depletion and RNA Fragmentation

1. Sample Requirements

This protocol uses the Hieff NGS™ MaxUp Human rRNA Depletion Kit (rRNA & ITS/ETS) (Yeasten Cat#12257) to remove rRNA from total RNA. It is suitable for 100 ng to 1 µg of total RNA (volume ≤ 10 µL) from human, mouse, or rat sources. It is also applicable to intact or partially degraded RNA samples (e.g., FFPE RNA).

2. Procedure

1) Probe Hybridization

- Remove the probe and hybridization buffer from -20°C, mix by inversion after thawing, and place on ice for later use.
- Prepare the RNA sample: Based on the input amount and concentration, calculate the required RNA volume and dilute to 10 µL with Nuclease-Free H₂O.
- Prepare the rRNA depletion reaction mixture in a 200 µL PCR tube according to Table 4.

Table 4. Probe Hybridization Reaction

Reagent	Volume (µL)
Hybridization Buffer	3
Human Probe Mix (rRNA & ITS/ETS)	2
Total RNA	10 (100 ng~1 µg)
Total	15

- Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution at the bottom of the tube.
- Place the PCR tube into a PCR thermocycler (with gradient cooling function) and perform the probe hybridization reaction according to the program specified in Table 5.

Table 5. Probe Hybridization Program

Temperature	Time
Heat Lid 105°C	On
95°C	2 min
95°C~22°C	0.1°C/s
42°C	5 min
4°C	hold

2) RNase H Digestion

- Remove the RNase H digestion reagents from -20°C, mix by inverting after thawing, and place on ice for later use. Prepare the RNase H digestion reaction mixture according to Table 6.

Table 6. RNase H Digestion Reaction

Reagent	Volume (µL)
RNase H Buffer	3
RNase H	2
Previous Product	15
Total	20

- Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution at the bottom of the

tube.

c. Place the PCR tube into a thermal cycler and set the program: Lid 50°C; 37°C for 30 min; 4°C hold, for RNase H digestion.

3) DNase I Digestion

a. Remove the DNase I digestion reagents from -20°C, mix by inverting after thawing, and keep on ice until use.

Prepare the DNase I digestion reaction mixture according to Table 7.

Table 7. DNase I Digestion Reaction

Reagent	Volume (μL)
DNase I Buffer	27.5
DNase I	2.5
Previous Product	20
Total	50

b. Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution at the bottom of the tube.

c. Place the PCR tube into a thermal cycler and set the program: Lid 50°C; 37°C for 30 min; 4°C hold, for DNase I digestion.

4) RNA Purification

a. Preparation: Remove the Hieff NGS™ RNA Cleaner (Yeasten Cat#12602) magnetic beads from the refrigerator and allow them to equilibrate to room temperature for at least 30 minutes. Prepare 80% ethanol using Nuclease-Free H₂O.

b. Vortex or invert the magnetic beads thoroughly to ensure uniform suspension.

c. Add 110 μL of Hieff NGS™ RNA Cleaner (2.2×, Beads:DNA = 2.2:1) to the previous product, mix thoroughly by pipetting, and incubate at room temperature for 5 minutes.

d. Place the PCR tube on a magnetic rack to separate the beads from the liquid. Once the solution is clear (~5 minutes), carefully remove the supernatant.

e. Keep the PCR tube on the magnetic rack, add 200 μL of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.

f. Repeat step e for a total of two washes. Use a 10 μL pipette to remove any remaining liquid.

g. Keep the PCR tube on the magnetic rack, open the lid, and air dry the beads at room temperature (5-10 minutes).

h. RNA Elution: Remove the PCR tube from the magnetic rack, add 18.5 μL of Frag/Prime buffer, and mix thoroughly by gentle pipetting. Incubate at room temperature for 5 minutes.

i. Briefly centrifuge the tube and place it back on the magnetic rack. Once the solution is clear (~3 minutes), carefully transfer 17 μL of the supernatant to a new nuclease-free PCR tube for RNA fragmentation.

Table 8. FFPE RNA Fragmentation Recommendations

DV200*	Fragmentation Program
>70%	94°C, 7 min, 4°C, hold;
50%~70%	94°C, 5 min, 4°C, hold;
20%~50%	85°C, 8 min, 4°C, hold;
<20% (high risk)	65°C, 8 min, 4°C, hold;

[Note]:*DV200 is used to assess RNA degradation. See Appendix III for details.

Part II: RNA Library preparation for Illumina™ & MGI™ Platforms

1. First Strand cDNA Synthesis

This step involves synthesizing the first strand of cDNA from enriched/fragmented target RNA. The enrichment of target RNA can be achieved using either a Poly(A) mRNA isolation protocol or an rRNA depletion protocol, depending on experimental requirements and sample conditions (see Part I for details).

Remove the first-strand synthesis reagents from -20°C, mix by pipetting, and add the components to the reaction mixture according to Table 9.

Table 9. 1st Strand cDNA Synthesis Reaction

Reagent	Volume (μL)
Frag/Prime Buffer with Fragmented RNA	17
1st Reaction Module 2.0	8
Total	25

2) Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution to the bottom of the tube.

3) Place the PCR tube into a thermal cycler and run the first-strand cDNA synthesis according to the program in Table 10. Proceed immediately to second-strand cDNA synthesis upon completion of the first-strand reaction.

Table 10. 1st Strand cDNA Synthesis Program

Temperature	Time
Heat Lid 105°C	On
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

2. Second Strand cDNA Synthesis / End Repair / dA-Tailing

1) Remove the second strand synthesis reagents from -20°C, mix by pipetting, and add the components to the reaction mixture according to Table 11.

Table 11. 2nd Strand cDNA Synthesis Reaction

Reagent	Volume (μL)
1st Strand cDNA	25
2nd Reaction Module (dNTP)*	35
Total	60

[Note]:*This kit is designed for constructing standard mRNA libraries. For strand-specific mRNA library preparation, please use Yeasen Cat#12340.

2) Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution to the bottom of the tube.

3) Place the PCR tube into a thermal cycler and perform the second-strand cDNA synthesis according to the reaction program specified in Table 12.

Table 12. 2nd Strand cDNA Synthesis Program

Temperature	Time
Heat Lid 105°C	on
16°C	30 min
72°C	15 min
4°C	Hold

3. Adapter Ligation

This step enables the ligation of specific Illumina or MGI adapters to the ends of the products generated from the end repair and dA-tailing reactions.

- 1) Refer to Table 1 in Appendix II, and dilute the adapter to an appropriate concentration based on the Input RNA amount.
- 2) Remove the adapter ligation reagents from -20°C, mix thoroughly by pipetting or vortexing, and then add the components to the reaction system according to Table 13.

Table 13. Adapter Ligation Reaction

Reagent	Volume (μL)
dA-tailed DNA	60
Ligation Reaction Module	35*
DNA Adapter***	5**
Total	100

[Note]: The Ligation Reaction Module is viscous. Please mix thoroughly by pipetting or vortexing, and perform a brief centrifugation before use.

** During the experiment, if using a short adapter, the DNA Adapter is a PE adapter.

*** The original concentration of Illumina adapters from our company is 15 μM, and that of MGI adapters is 10 μM. Please refer to Table 1 in Appendix II, and dilute the adapters according to the input amount, so that the final adapter addition volume is fixed at 5 μL.

- 3) Mix the reaction thoroughly by gentle pipetting, and briefly centrifuge to collect the solution to the bottom of the tube.
- 4) Place the PCR tube into a PCR thermocycler, and run the adapter ligation reaction using the program specified in Table 14.

Table 14. Adapter Ligation Program

Temperature	Time
Heat Lid	Off
20°C	15 min
4°C	Hold

4. Post Ligation Clean-Up

This protocol is suitable for purifying ligation products when the fragments are less than 200 bp by performing two rounds of purification to remove adapter residues. For insert sizes ≥ 200 bp, refer to the size selection protocol in Appendix II to obtain libraries of the desired length.

For Libraries with Insert Sizes <200 bp (Requires Two Rounds of Purification)

[Note]: Ensure that your sample RNA fragments are less than 200 nt during experimentation.

- 1) Preparation: Remove the Hieff NGS™ DNA Selection Beads from the refrigerator and allow them to equilibrate to room temperature for at least 30 minutes. Prepare freshly 80% ethanol.
- 2) Mixing: Vortex or invert the magnetic beads thoroughly to ensure a uniform suspension.
- 3) Add 60 µL of Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA = 0.6:1) to the Adapter Ligation product. Mix thoroughly by vortexing or pipetting, and incubate at room temperature for 5 minutes.
- 4) Briefly centrifuge the PCR tube and place it on a magnetic rack. Allow the solution to clear (~5 minutes), then carefully remove the supernatant.
- 5) While the PCR tube remains on the magnetic rack, add 200 µL of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 6) Repeat step 5 for a total of two washes.
- 7) Keep the PCR tube on the magnetic rack, remove the cap, and air-dry the beads until small cracks appear on the surface (not exceeding 5 minutes).
- 8) Remove the PCR tube from the magnetic rack and add 52 µL of ddH₂O. Mix thoroughly by vortexing or pipetting gently, and incubate at room temperature for 5 minutes. Briefly centrifuge the tube and place it back on the magnetic rack. Allow the solution to clear (~3 minutes), then carefully transfer 50 µL of the supernatant to a new PCR tube for a second round of purification.
- 9) Add 40 µL of Hieff NGS™ DNA Selection Beads (0.8×, Beads:DNA = 0.8:1) to the previous supernatant. Mix thoroughly by vortexing or pipetting, and incubate at room temperature for 5 minutes.
- 10) Briefly centrifuge the PCR tube and place it on a magnetic rack. Allow the solution to clear (~3 minutes), then carefully remove the supernatant.
- 11) While the PCR tube remains on the magnetic rack, add 200 µL of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 12) Repeat step 11 for a total of two washes.
- 13) Keep the PCR tube on the magnetic rack, remove the cap, and air-dry the beads until small cracks appear on the surface (not exceeding 5 minutes).
- 14) Remove the PCR tube from the magnetic rack and add 21 µL of ddH₂O. Mix thoroughly by vortexing or pipetting gently, and incubate at room temperature for 5 minutes. Briefly centrifuge the tube and place it back on the magnetic rack. Allow the solution to clear (~3 minutes), then carefully transfer 20 µL of the supernatant to a new PCR tube for library amplification.

5. Library Amplification

This step involves PCR amplification to enrich the purified or size-selected adapter-ligated products.

- 1) Prepare Amplification Reagents: Remove the amplification reagents from -20°C storage, mix well by pipetting, and prepare the amplification reaction according to Table 15.

Table 15-A. PCR System for Illumina Short-Adapter Libraries

Reagent	Volume (μL)
2×Super Canace™ II High-Fidelity Mix	25
Universal Primer/ i5 Primer*	2.5
Index Primer/ i7 Primer*	2.5
Adapter Ligated DNA	20
Total	50

Table 15-B. PCR System for Illumina Full-Length Adapter Libraries

Reagent	Volume (μL)
2×Super Canace™ II High-Fidelity Mix	25
Primer Mix**	5
Adapter Ligated DNA	20
Total	50

[Note]:*If you are using adapters without indexes, commonly referred to as short adapters (small Y adapters), please use the Index primers provided in the short adapter kit (Cat#12414-12415 or equivalent) for amplification.

**If you are using Indexed Adapters (Cat#13519~Cat#13520), commonly known as full-length adapters (large Y adapters), you can use the Hieff NGS™ Primer Mix for Illumina (Cat#13335) for amplification.

Table 15-C. PCR Reaction System for MGI Full-length Adapter Ligation Products

Reagent	Volume (μL)
2×Super Canace™ II High-Fidelity Mix	25
Primer Mix for MGI*	5
Adapter Ligated DNA	20

[Note]:*The primer mix for MGI is not included in this kit. For amplification, you may use the RNA Library Prep Primer Mix for MGI, Compatible with Complete Adaptor (Cat#13334).

2) Mix the reaction thoroughly by gentle pipetting or vortexing, and briefly centrifuge to collect the solution to the bottom of the tube.

3) Place the PCR tube into a PCR thermocycler, and perform PCR amplification using the reaction program outlined in Table 16.

Table 16. PCR Amplification Program

Temperature	Time	Cycles
98°C	1 min	1
98°C	10 sec	10~16 cycles*
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1
4°C	Hold	-

[Note]: The number of PCR cycles should be adjusted based on sample quality, input amount, and other library preparation conditions. For details, see Precautions section 3.

6. Post-Amplification Clean Up

1) Preparation: Remove the Hieff NGS™ DNA Selection Beads from the refrigerator and allow them to equilibrate to room temperature for at least 30 minutes. Prepare freshly 80% ethanol.

2) Mixing: Vortex or invert the magnetic beads thoroughly to ensure a uniform suspension.

3) Bead Binding: Add 45 μL of Hieff NGS™ DNA Selection Beads (0.9×, Beads:DNA = 0.9:1) to the amplification

product. Mix thoroughly by vortexing or pipetting, and incubate at room temperature for 5 minutes.

4) Bead Separation: Briefly centrifuge the PCR tube and place it on a magnetic rack. Allow the solution to clear (~5 minutes), then carefully remove the supernatant without disturbing the bead pellet.

5) First Wash: While the PCR tube remains on the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to wash the beads. Incubate at room temperature for 30 seconds, then carefully remove the supernatant.

6) Second Wash: Repeat step 5 for a total of two washes.

7) Drying: Keep the PCR tube on the magnetic rack, remove the cap, and air-dry the beads until small cracks appear on the surface (not exceeding 5 minutes).

8) Elution and Recovery: Remove the PCR tube from the magnetic rack and add 21 μ L of ddH₂O. Mix thoroughly by vortexing or pipetting gently, and incubate at room temperature for 5 minutes. Briefly centrifuge the tube and place it back on the magnetic rack. Allow the solution to clear (~3 minutes), then carefully transfer 20 μ L of the supernatant to a new PCR tube for library quantification and quality control.

7. Library Quality Control

Evaluate library quality by measuring concentration and fragment size distribution. See Section 5 for details.

Appendices

Appendix I: mRNA Fragmentation Results

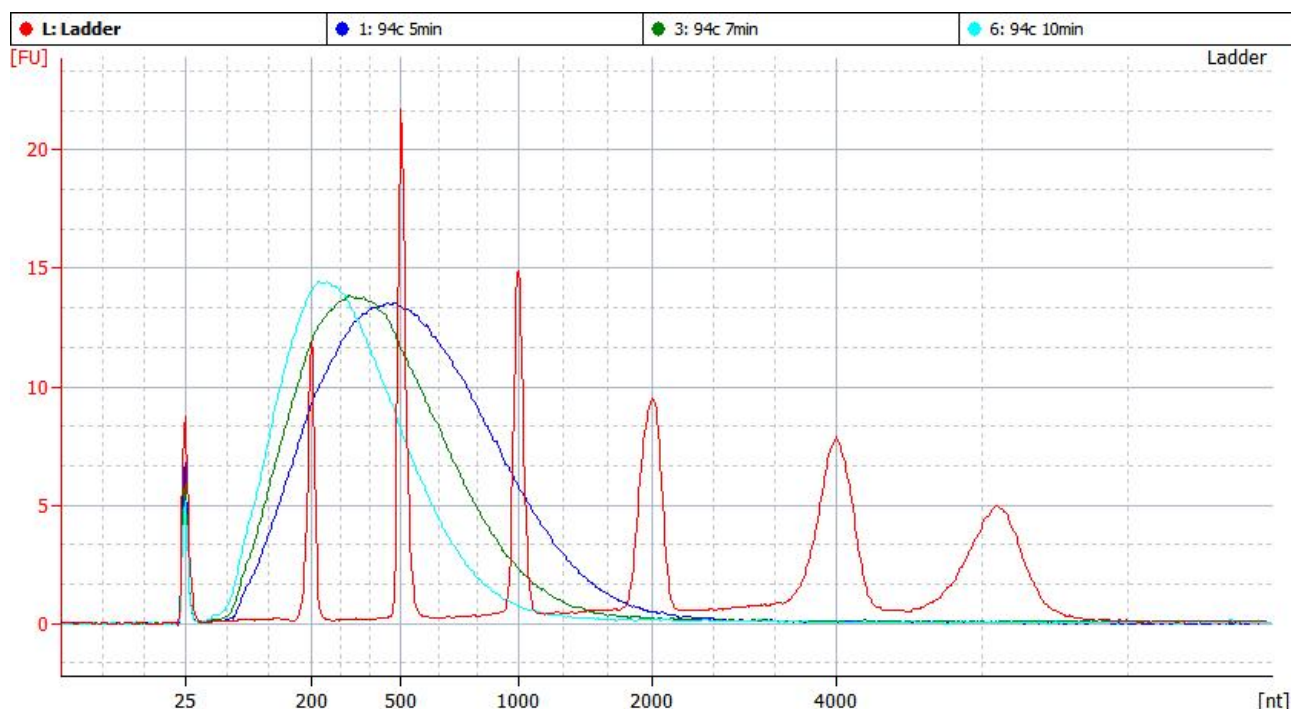


Figure 2. Different mRNA Fragmentation Times

Fragmentation of mRNA at 94°C for 10 min, 7 min, and 5 min. Fragment sizes were analyzed using Agilent 2100 Bioanalyzer.

[Note]: The RNA used in this result is Agilent's Universal Human Reference RNA. If using RNA from other sources, it is recommended to optimize the fragmentation time.

Appendix II: Size Selection for Illumina Platform

The selection scheme is suitable for RNA library preparation with fragmentations at 94°C for 10 min, 7 min, and 5

min, which can achieve insert sizes greater than 200 bp:

Scheme 1: Size selection After Adapter Ligation Purification

1. 0.6X Hieff NGS™ DNA Selection Beads for Adapter Ligation Product Purification

- 1) Preparation: Remove Hieff NGS™ DNA Selection Beads from the fridge and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol.
- 2) Vortex or invert the beads thoroughly to ensure a uniform suspension.
- 3) Add 60 μ L of Hieff NGS™ DNA Selection Beads (0.6 $\times$, Beads:DNA=0.6:1) to the adapter ligation product, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes.
- 4) Briefly centrifuge and place on a magnetic rack until the solution is clear (~5 minutes), then carefully remove the supernatant.
- 5) Keep the PCR tube on the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to wash the beads, incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 6) Repeat step 5 twice.
- 7) Keep the PCR tube on the magnetic rack, air dry the beads until they just begin to crack (no more than 5 minutes).
- 8) Remove the tube from the magnetic rack, add 102 μ L ddH₂O, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes. Briefly centrifuge and place on the magnetic rack until the solution is clear (~5 minutes), then carefully transfer 100 μ L of the supernatant to a new PCR tube for double-sided selection.

Dual-round bead size selection: For example, if the target library size is 410–510 bp after fragmentation at 94°C for 7 minutes, use the recommended bead ratios in Table 17 for short adapters and Table 18 for full-length adapters.

2. 0.65X /0.15X Hieff NGS™ DNA Selection Beads for Adapter Ligation Product Selection

- 1) Vortex or invert the beads thoroughly to ensure a uniform suspension.
- 2) Based on the required DNA fragment length, refer to Table 17. To the above 100 μ L of DNA, add 65 μ L (0.65 $\times$) of first-round selection beads, vortex or pipette mix 10 times.
- 3) Incubate at room temperature for 5 minutes.
- 4) Briefly centrifuge and place on the magnetic rack until the solution is clear (~5 minutes), then carefully transfer the supernatant to a clean centrifuge tube, leaving 1–2 μ L in the bottom.
- 5) Refer to Table 17 and add 15 μ L (0.15 $\times$) of second-round selection beads to the supernatant.
- 6) Vortex or pipette mix 10 times, and incubate at room temperature for 5 minutes.
- 7) Briefly centrifuge and place on the magnetic rack until the solution is clear (~3 minutes), then carefully remove the supernatant.
- 8) Keep the PCR tube on the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to wash the beads, incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 9) Repeat step 8.
- 10) Keep the PCR tube on the magnetic rack, air dry the beads until they just begin to crack (about 3 minutes).
- 11) Remove the tube from the magnetic rack, add 21 μ L ddH₂O, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes.
- 12) Briefly centrifuge and place on the magnetic rack until the solution is clear (~3 minutes), then carefully

transfer 20 μL of the supernatant to a clean tube.

Table 17. Recommended Bead Ratios for Short Adapter Libraries

Insert Size (bp)	200~300	250~350	350~450	450~550
Library Size (bp)	260~360	310~410	410~510	510~610
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Round 1 Beads (μL)	80 (0.8 $\times$)	75 (0.75 $\times$)	65 (0.65 $\times$)	60 (0.6 $\times$)
Round 2 Beads (μL)	15 (0.15 $\times$)	15 (0.15 $\times$)	15 (0.15 $\times$)	10 (0.1 $\times$)

Table 18. Recommended Bead Ratios for Full-length Adapter Libraries

Insert Size (bp)	200~300	250~350	350~450	450~550
Library Size (bp)	320~420	370~470	470~570	570~670
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
First Round Beads Volume (μL)	75 (0.75 $\times$)	70 (0.7 $\times$)	65 (0.65 $\times$)	60 (0.6 $\times$)
Second Round Beads Volume (μL)	15 (0.15 $\times$)	15 (0.15 $\times$)	15 (0.15 $\times$)	10 (0.1 $\times$)

[Note]: The recommended double-sided selection ratios in Tables 17 and 18 apply to Hieff NGS™ DNA Selection Beads; “ $\times$ ” represents the sample DNA volume. For example, if the desired insert size peak is 300 bp and the sample DNA volume is 100 μL , then for short adapter connection post-selection, the first round selection bead volume would be $0.65 \times 100 \mu\text{L} = 65 \mu\text{L}$, and the second round selection bead volume would be $0.15 \times 100 \mu\text{L} = 15 \mu\text{L}$.

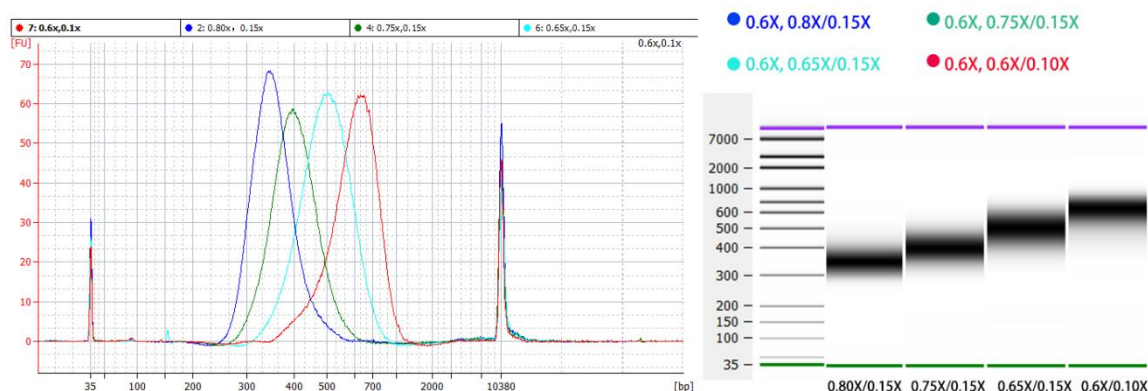


Figure 3. Different Fragmentation Conditions Library Peak Profiles

1 μg of 293 total RNA was fragmented at 94°C for 10 min, 7 min, and 5 min. The library sizes were obtained according to the recommended bead ratios in Table 17.

Appendix III: Size Selection for MGI Platform

The selection scheme is suitable for RNA library preparation with fragmentations at 94°C for 10 min, 7 min, and 5 min, achieving insert sizes greater than 200 bp:

Scheme 1: Size Selection After Adapter Ligation Purification

1. 0.6X Hieff NGS™ DNA Selection Beads for Adapter Ligation Product Purification

- 1) Preparation: Remove Hieff NGS™ DNA Selection Beads from the fridge and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol.
- 2) Vortex or invert the beads thoroughly to ensure a uniform suspension.

- 3) Add 60 μL of Hieff NGS™ DNA Selection Beads (0.6 $\times$, Beads:DNA=0.6:1) to the adapter ligation product, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes.
- 4) Briefly centrifuge and place on a magnetic rack until the solution is clear (~5 minutes), then carefully remove the supernatant.
- 5) Keep the PCR tube on the magnetic rack, add 200 μL of freshly prepared 80% ethanol to wash the beads, incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 6) Repeat step 5 twice. A total of two washes were performed.
- 7) Keep the PCR tube on the magnetic rack, air dry the beads until they just begin to crack (no more than 5 minutes).
- 8) Remove the tube from the magnetic rack, add 102 μL ddH₂O, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes. Briefly centrifuge and place on the magnetic rack until the solution is clear (~5 minutes), then carefully transfer 100 μL of the supernatant to a new PCR tube for double-sided selection.

[Note]: The Ligation Enhancer contains a high concentration of polyethylene glycol (PEG), which can affect the efficiency of magnetic bead binding during double-size selection. To avoid interference, a preliminary purification step is required prior to performing the two-round size selection.

2. Dual-round bead size selection: Selection for Library Size of 380 bp to 480 bp with Fragmentation at 94°C for 7 Minutes.

- 1) Vortex or invert the beads thoroughly to ensure a uniform suspension.
- 2) Based on the required DNA fragment length, refer to Table 21. To the above 100 μL of DNA, add 65 μL (0.65 $\times$) of first-round selection beads, vortex or pipette mix 10 times.

[Note]: The recommended double-sided selection ratios in this table apply to Hieff NGS™ DNA Selection Beads; “ $\times$ ” represents the sample DNA volume. For example, if the desired insert size peak is 300 bp and the sample DNA volume is 100 μL , then for short adapter connection post-selection, the first round selection bead volume would be $0.65 \times 100 \mu\text{L} = 65 \mu\text{L}$, and the second round selection bead volume would be $0.15 \times 100 \mu\text{L} = 15 \mu\text{L}$.

- 3) Incubate at room temperature for 5 minutes.
- 4) Briefly centrifuge and place on the magnetic rack until the solution is clear (~5 minutes), then carefully transfer the supernatant to a clean centrifuge tube, leaving 1-2 μL in the bottom.
- 5) Refer to Table 19 and add 15 μL (0.15 $\times$) of second-round selection beads to the supernatant.
- 6) Vortex or pipette mix 10 times, and incubate at room temperature for 5 minutes.
- 7) Briefly centrifuge and place on the magnetic rack until the solution is clear (~3 minutes), then carefully remove the supernatant.
- 8) Keep the PCR tube on the magnetic rack, add 200 μL of freshly prepared 80% ethanol to wash the beads, incubate at room temperature for 30 seconds, then carefully remove the supernatant.
- 9) Repeat step 8.
- 10) Keep the PCR tube on the magnetic rack, air dry the beads until they just begin to crack (about 3 minutes).
- 11) Remove the tube from the magnetic rack, add 21 μL ddH₂O, vortex or pipette mix thoroughly, and incubate at room temperature for 5 minutes.
- 12) Briefly centrifuge and place on the magnetic rack until the solution is clear (~3 minutes), then carefully transfer 20 μL of the supernatant to a clean tube.

Table 19. Recommended Bead Ratios for Short Adapter Libraries

Insert Size (bp)	200~300	300~400	400~500	500~600
Library Size (bp)	280~380	380~480	480~580	580~680
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
First Round Beads Volume (μL)	70(0.7×)	65(0.65×)	58(0.58×)	50(0.5×)
Second Round Beads Volume (μL)	20(0.2×)	15(0.15×)	15(0.15×)	15(0.15×)

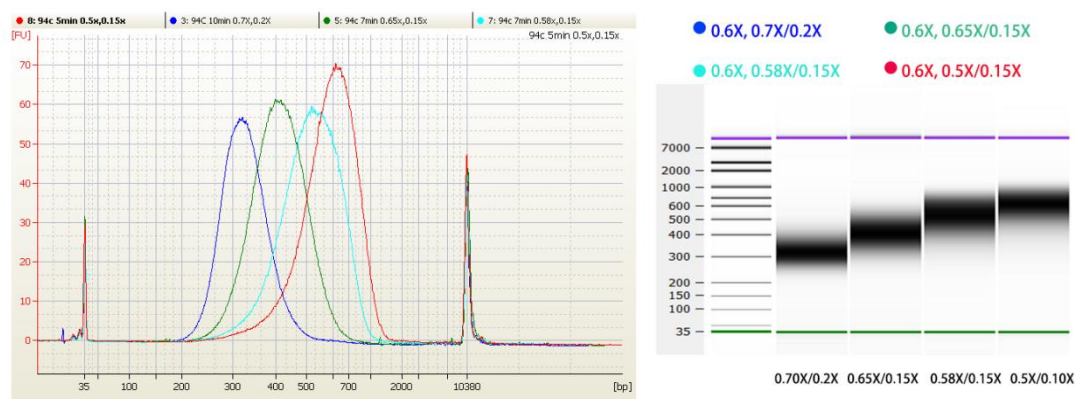


Figure 4. Different Selection Ratio Library Peak Profiles

1 μg of 293 total RNA was fragmented at 94°C for 10 min, 7 min, and 5 min. The library sizes were obtained according to the recommended bead ratios in Table 21.

Appendix IV: FFPE Sample Library preparation Instructions

1. FFPE RNA Quality Evaluation

rRNA removal library preparation schemes can be used for low-quality total RNA samples such as FFPE. However, due to significant variations in FFPE sample quality, library preparation conditions need to be adjusted based on the sample. Commonly used parameters for evaluating RNA sample quality are RIN values, but for degraded FFPE samples, DV200 provides a better indication of sample quality. DV200 indicates the proportion of RNA fragments longer than 200 nt in the sample. For severely degraded FFPE samples, DV200 values provide a better reflection of sample quality.

Calculation Method for DV200:

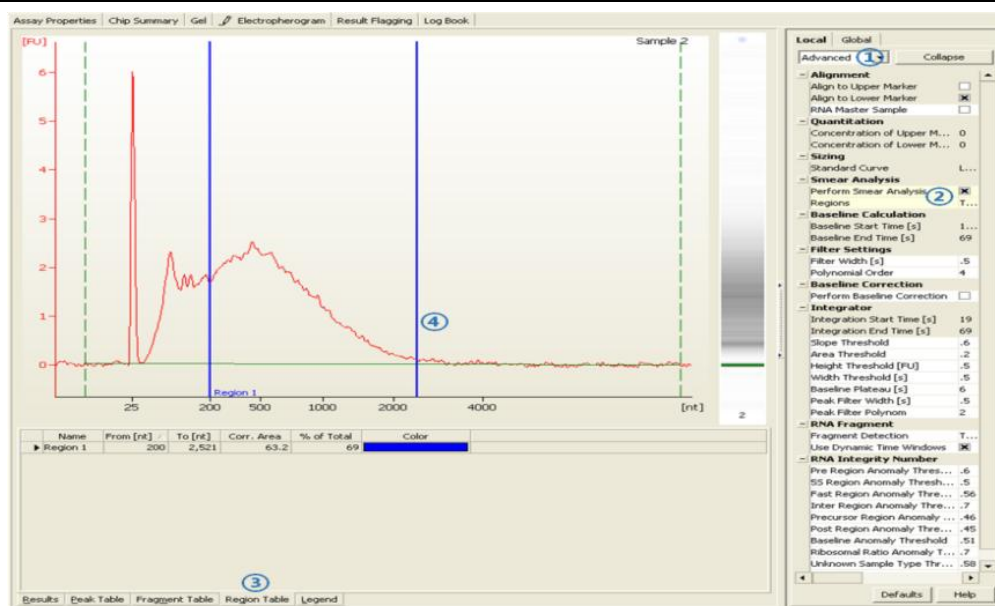


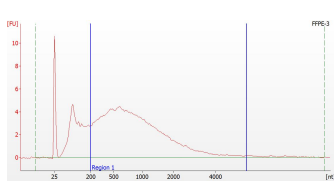
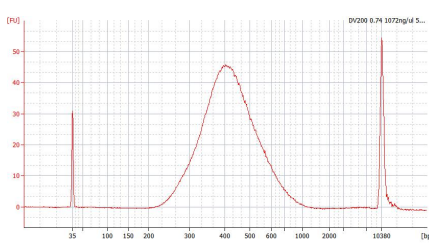
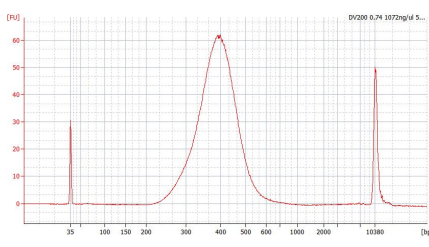
Figure 5: DV200 Proportion

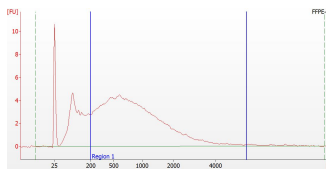
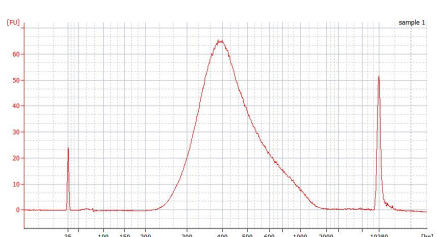
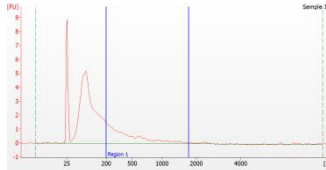
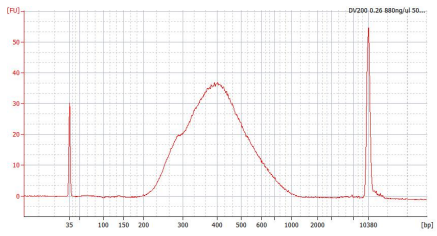
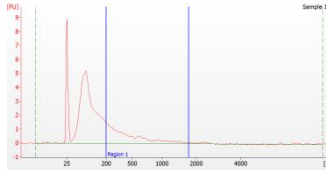
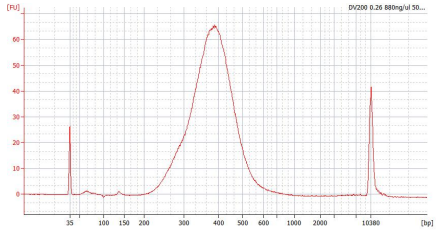
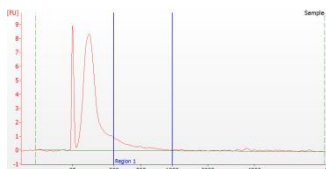
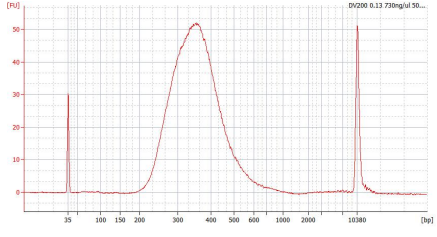
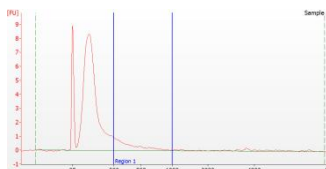
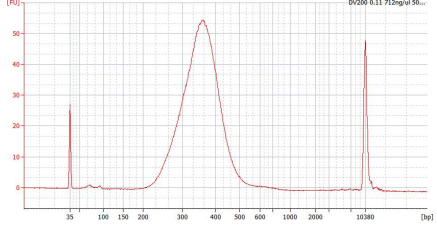
In a completed Agilent 2100 Bioanalyzer result, under Local select Advanced, check Perform Smear Analysis under Smear Analysis options, choose Region Table page, right-click, select Add Region to adjust the indicator line range to obtain the selected fragment range percentage % of Total.

2. FFPE RNA Library preparation Example

Table 20 shows different quality FFPE sample library distributions under various library preparation conditions for reference. For severely degraded FFPE RNA (DV200<50%) and low input amounts, we recommend two rounds of purification after adapter ligation to reduce library loss.

Table 20. Different Quality FFPE Library Peak Profiles

RNA Sample QC	Library Preparation Conditions	Library Distribution QC
 RIN=2.2; DV200=74%	Input RNA: 500 ng Fragmentation condition: 94°C, 7 min Double purification after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 717.2 ng	
	Input RNA: 500 ng Fragmentation condition: 94°C, 7 min Purification and size selection after adapter ligation: 0.6×; 0.7× / 0.15× Strand-specific library amplification: 13 cycles Library yield: 437.8 ng	

 RIN=2.2; DV200=74%	Input RNA: 100 ng Fragmentation condition: 94°C, 7 min Double purification after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 15 cycles Library yield: 206.8 ng	
 RIN=2.5; DV200=26%	Input RNA: 500 ng Fragmentation condition: 85°C, 8 min Double purification after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 207 ng	
 RIN=2.5; DV200=26%	Input RNA: 500 ng Fragmentation condition: 85°C, 8 min Purification and size selection after adapter ligation: 0.6×; 0.70× / 0.15× Strand-specific library amplification: 13 cycles Library yield: 98.56 ng	
 RIN=2.5; DV200=11%	Input RNA: 500 ng Fragmentation condition: 65°C, 8 min Double purification after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 354.2 ng	
 RIN=2.5; DV200=11%	Input amount: 500 ng Fragmentation condition: 65°C, 8 min Double purification after adapter ligation: 0.6×; 0.70× / 0.15× Strand-specific library amplification: 13 cycles Library yield: 172.48 ng	

Note:

Note:



To enable success of our customers
Together to make a healthier and brighter world