Hyaseq Strand-Specific RNA Library Prep Kit (dUTP)

Cat.No.: HYD212

Product Description

Hyaseq Strand-Specific RNA Library Prep Kit (dUTP) is a strand-specific RNA library preparation kit compatible with Illumina and MGI platforms. It comprises reagents for RNA fragmentation, reverse transcription, strand-specific ds-cDNA synthesis and library amplification. This kit can be coupled with mRNA purification or rRNA depletion kits for library construction. Its optimized reverse transcription system delivers high strand specificity without actinomycin D, enhancing operational safety. All reagents are strictly quality tested to guarantee stable and reproducible library preparation.

Components

Components	24 T	96 T
Frag/Prime Buffer	450 µL	2×900 µL
1st Reaction Module V2	192 µL	768 µL
2nd Reaction Module (dUTP)	840 µL	3×1120 µL
Ligation Reaction Module	840 µL	3×1120 µL
2×HiFi Mix for Library Amplification	600 µL	2×1200 µL

Note: When the recommended short adapters are used, Primer Mix is not required. If long adapters are selected, Primer Mix becomes an essential reagent for the library preparation workflow. However, Primer Mix is not included in this kit and must be prepared or obtained separately.

Storage

Store at -25 ~ -15 °C, valid for 1 year.

Notes

1. About operation

1) For your personal safety and health, always wear a laboratory coat and disposable gloves during all experimental procedures.
2) Thaw all kit components at room temperature prior to use. Upon complete thawing, invert the tubes repeatedly to achieve homogeneous mixing. Briefly centrifuge each component and place it on ice until ready for setup.
3) All reaction steps are recommended to be performed using a thermal cycler equipped with a heated lid. Preheat the thermal cycler close to the targeted reaction temperature before starting the experiment.
4) Only use RNase-free consumables throughout the entire workflow. Routinely sanitize the working area to avoid nuclease contamination. ThermoFisher RNAZap™ Decontamination Solution is highly recommended for efficient RNase elimination.
5) Improper manipulation of PCR products may lead to aerosol contamination and adversely affect experimental reliability. Strict physical segregation must be implemented between the PCR setup zone and post-PCR purification/detection zone. Use dedicated pipettes and accessories exclusively for library preparation. Regular decontamination of workstations with ThermoFisher DNAZap™ Decontamination Solution is required to maintain a nuclease-free working environment.
6) For Research Use Only.

2. About Adapter Ligation

1) Long and short adapter kits for Illumina® and MGI® are available for selection based on experimental needs.
2) High-grade commercial adapters are recommended. For custom adapters, engage vendors proficient in NGS primer synthesis and require strict contamination control. Perform adapter annealing in a biosafety cabinet, and handle only one adapter type at a time to prevent cross-contamination.
3) Thaw adapters at 4 °C or on ice prior to use. Maintain ambient temperature below 25 °C during operation to avoid adapter denaturation.

4) Suboptimal adapter concentration will compromise library yield. For this plate-based protocol, add a fixed volume of 5 μL adapters. Dilute adapters with 0.1× TE buffer per Table 1 according to initial RNA input. 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

*Adjust the adapter volume accordingly based on sample type and input amount.

3. About Library Amplification

1) The library amplification components contain our 2nd-generation high-fidelity DNA polymerase. Optimized from the 1st version, it delivers greatly improved amplification uniformity and enables unbiased amplification even for low-copy genes.
2) Amplification cycles must be strictly controlled. Insufficient cycles result in low library yield, while excessive cycles cause amplification bias, elevated duplication rates, increased chimeric products and accumulated mutations. Table 2 lists the recommended amplification cycles corresponding to different input total RNA amounts.
3) The recommended cycles in Table 2 apply to most samples. For poor-quality samples such as severely degraded FFPE tissues, increase cycles appropriately as needed. For mRNA library construction, adjust cycles based on input quantity, species and sample conditions, as mRNA content varies greatly across species and tissues.

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	12
1 μg	11

*Library yield is affected not only by RNA input and amplification cycles, but also by sample quality, fragmentation and size selection conditions. Please optimize experimental parameters comprehensively to achieve optimal results.

4. About Bead-based DNA Cleanup and Size Selection

1) DNA purification beads are required in multiple steps. We recommend Hyasen NGS DNA Selection Beads (Cat.No: HYD511) or AMPure® XP Beads (Cat.No: A63880) for DNA purification and size selection.
2) Equilibrate beads to room temperature before use; otherwise, low yield and poor size selection may occur.
3) Fully resuspend beads by vigorous vortexing or pipetting up and down prior to each use.
4) Do not carry over beads during supernatant transfer. Even trace amounts will compromise final library quality.
5) Prepare 80% ethanol freshly for bead washing, as stale solution reduces recovery efficiency.
6) Air-dry beads at room temperature before elution. Incomplete drying leaves residual ethanol and impairs downstream reactions; over-drying causes bead cracking and lower yield. Typically, 3–5 minutes of room-temperature drying is sufficient.
7) For storage, elute purified or size-selected DNA with 0.1× TE Buffer. Products can be stored for 2 days at 4 °C or 1 month at −20 °C.

5. About Library Quality Analysis

1) Completed libraries are routinely assessed by size distribution and concentration analysis.
2) Library concentration can be measured via double-stranded DNA fluorescent dye assays (e.g., Qubit®, PicoGreen®) or absolute qPCR quantification.

3) qPCR is the preferred method for library quantification. Fluorescent dye-based methods such as Qubit® fail to distinguish products with single-adapter ligation, no adapters or other incomplete dsDNA structures. By contrast, absolute qPCR specifically quantifies fully adapter-ligated, sequence-ready libraries and eliminates interference from non-sequencable fragments.
4) Spectrophotometric methods such as NanoDrop® are not recommended for library quantification.
5) Library size distribution can be analyzed using capillary electrophoresis or microfluidic devices, e.g., Agilent 2100 Bioanalyzer.

6. Other Material

1) mRNA Enrichment Kit: Hyaseq mRNA Capture Kit V2 (Cat.No: HYD217)
2) rRNA Depletion Kit: Hyaseq rRNA & ITS/ETS Depletion Kit (Human/Mouse/Rat) (Cat.No: HYD218) or equivalent rRNA depletion kits.
3) RNA Purification Beads: NGS RNA Clean Beads (Cat.No: HYD513) or equivalent products.
4) DNA Purification Beads: NGS DNA Selection Beads (Cat.No: HYD511) , AMPure® XP Beads (A63880) or equivalent products.
5) RNA Quality Control: Agilent 2100 Bioanalyzer RNA 6000 Nano / Pico Chip or equivalent products.
6) Adapters: Complete Adapter for Illumina® (Cat.No: HYD311-312) or Complete Adapter for MGI® (Cat.No: HYD315-316), or equivalent products
7) Absolute ethanol, nuclease-free ultrapure water, low-retention pipette tips, PCR tubes, magnetic stand, thermal cycler, etc.

Instructions

1. Target RNA Enrichment and Fragmentation

This step covers target RNA preparation prior to library construction. Two protocols are available: Protocol A for poly(A) mRNA Capture and Protocol B for rRNA depletion. Reagents for this step are not included in the kit; please prepare them separately as required.

Protocol A: mRNA purification and fragmentation

1. Sample requirements

This protocol uses Hyaseq mRNA Capture Kit V2 (Cat.No: HYD217) for mRNA enrichment. It is suitable for high-quality total RNA from eukaryotes including animals, plants and fungi, with input ranging from 10 ng to 4 μg and a maximum volume of 50 μL.

If the RNA volume exceeds 50 μL due to low concentration, concentrate the sample using NGS RNA Clean Beads (Cat.No: HYD513). RNA integrity must be verified with an Agilent 2100 Bioanalyzer using RNA 6000 Nano/Pico Chip, and a RIN value > 7 is required to ensure intact poly(A) tails. This kit adopts oligo(dT) magnetic beads for mRNA isolation, which only captures RNA with intact poly(A) tails. It is not applicable to non-polyadenylated RNA such as non-coding RNA and tailless mRNA. In addition, mRNA from FFPE samples is severely degraded and generally lacks complete poly(A) tails, so this kit is not recommended for library construction with FFPE samples.

2. Operation steps

1) Take Hyaseq mRNA Capture Kit V2 from 2–8 °C and equilibrate to room temperature for approximately 30 min.
2) Transfer 10 ng–4 μg total RNA into a nuclease-free centrifuge tube, and adjust the total volume to 50 μL with nuclease-free water. Place the tube on ice until use.
3) Resuspend beads thoroughly by inverting or vortexing. Add 50 μL bead suspension to the 50 μL RNA sample, then pipette up and down 6 times for complete mixing.
4) Incubate the mixture in a thermal cycler: 65 °C for 5 min → 25 °C for 5 min → hold at 25 °C to allow RNA binding to beads.
5) Place the tube on a magnetic stand at room temperature for 5 min to separate mRNA. Carefully remove the supernatant.

6) Remove the tube from the stand. Resuspend beads in 200 µL Beads Wash Buffer V2 and pipette up and down 6 times thoroughly. Place the tube back on the magnetic stand for 5 min at room temperature, then discard the supernatant gently.
7) Repeat Step 6 for a second wash.
8) Take the tube off the stand, add 50 µL Tris Buffer 2.0 and pipette up and down 6 times to fully resuspend beads.
9) Incubate in a thermal cycler: 80 °C for 2 min → hold at 25 °C to elute mRNA.
10) Remove the tube from the cycler, add 50 µL Beads Binding Buffer V2, and mix well by pipetting up and down 6 times.
11) Incubate at room temperature for 5 min to re-bind mRNA to beads.
12) Place the tube on a magnetic stand for 5 min at room temperature, then carefully remove the supernatant.
13) Take the tube off the stand, resuspend beads in 200 µL Beads Wash Buffer V2 and pipette thoroughly 6 times. Return the tube to the magnetic stand, incubate for 5 min at room temperature, and remove all supernatant.
14) Remove the tube from the stand. Resuspend beads in 18.5 µL Frag/Prime Buffer and mix by pipetting 6 times. Place the tube in a preheated thermal cycler (94 °C). Refer to Table 3 for recommended fragmentation programs. Fragmentation efficiency varies among species; it is recommended to set a time gradient (e.g., 94 °C for 5 min) and verify fragment size using an Agilent 2100 Bioanalyzer.

Table 3. mRNA Fragmentation Recommendations

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

15) Once fragmentation is complete, immediately place the tube on a magnetic stand to prevent re-binding of poly(A)-tailed RNA to beads. After the solution clears, transfer 17 µL supernatant to a new nuclease-free centrifuge tube and proceed directly to first-strand synthesis.

Scheme B: rRNA Depletion and RNA Fragmentation

1. Sample requirements

This protocol uses Hyaseq rRNA & ITS/ETS Depletion Kit (Human/Mouse/Rat) (Cat.No.: HYD218) to deplete rRNA from total RNA. It is applicable to 100 ng–1 μg total RNA (volume ≤ 10 μL) derived from human, mouse and rat, including intact and partially degraded RNA such as FFPE RNA.

2. Operation steps

1) Probe Hybridization

a. Remove probes and Hybridization Buffer from −20 °C, thaw, mix by inverting, and keep on ice until use.

b. Prepare RNA sample: Calculate the required volume based on input amount and concentration, then dilute to 10 μL with nuclease-free water.

c. Prepare the rRNA depletion reaction mix in a 200 μL PCR tube as specified in Table 4.

Table 4. Probe Hybridization Reaction

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

d. Mix gently by pipetting, then spin down briefly to collect liquid at the tube bottom.

e. Place the tube in a thermal cycler (gradient cooling supported) and run the probe hybridization program listed 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
22°C	5 min
4°C	hold

2) RNase H digestion

a. Take RNase H digestion reagents from −20 °C, thaw, mix by inverting and keep on ice. Prepare the RNase H digestion reaction mix following Table 6.

Table 6. RNase H Digestion Reaction

Components	Volume
RNase H Buffer	3 μL
RNase H	2 μL
Last step product	15 μL

b. Mix gently by pipetting, then spin down briefly to collect liquid at the bottom.

c. Place the tube in a thermal cycler with the heated lid set to 75 °C. Run the program: 65 °C for 30 min, then hold at 4 °C for RNase H digestion.

3) DNase I digestion

a. Thaw DNase I digestion reagents removed from −20 °C, mix by inverting and keep on ice. Prepare the DNase I digestion reaction mix per Table 7.

Table 7. DNase I Digestion Reaction

Components	Volume
DNase I Buffer	27.5 μL
DNase I	2.5 μL
Last step product	20 μL

b. Mix gently by pipetting, then spin down briefly to collect liquid at the tube bottom.

c. Place the tube in a thermal cycler with the heated lid set to 50 °C. Run the program: 37 °C for 30 min, then hold at 4 °C for DNase I digestion.

4) RNA Purification

a. Preparations: Take NGS RNA Clean Beads (Cat.No.: HYD513) from storage and equilibrate to room temperature for at least 30 min. Prepare fresh 80% ethanol with nuclease-free water.

b. Thoroughly resuspend beads by vortexing or repeated inversion.

c. Add 110 μL NGS RNA Clean Beads (2.2× ratio, beads:sample = 2.2:1) to the product from the previous step. Mix well by pipetting and incubate at room temperature for 5 min.

d. Place the tube on a magnetic stand for separation. After approximately 5 min when the solution clears, carefully remove the supernatant.

e. Keep the tube on the stand. Add 200 µL freshly prepared 80% ethanol for washing. Incubate for 30 sec at room temperature, then discard the supernatant gently.

f. Repeat Step e for a second wash. Remove residual liquid completely using a 10 µL pipette.

g. Keep the tube on the stand and leave the lid open to air-dry beads at room temperature for 5–10 min.

h. RNA elution: Remove the tube from the stand. Add 18.5 µL Frag/Prime Buffer, mix gently by pipetting, and incubate at room temperature for 5 min.

i. Briefly spin down the tube and place it on the magnetic stand. After about 3 min when the solution clears, transfer 17 µL supernatant to a new nuclease-free PCR tube for RNA fragmentation.

j. Fragmentation conditions should be adjusted according to sample quality. For high-quality RNA, follow the mRNA fragmentation protocol in Table 3. Table 8 lists recommended conditions for FFPE samples of varying quality. Since fragmentation performance varies across samples, optimize conditions empirically to obtain ideal results.

k. Place samples on ice immediately after fragmentation, then proceed to first-strand synthesis.

Table 8. FFPE RNA Fragmentation Recommendations

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

*DV200 is used to assess RNA degradation. See Appendix III for details.

2. RNA Library preparation for Illumina & MGI Platforms

1. 1st Strand cDNA Synthesis

This step generates first-strand cDNA from enriched and fragmented target RNA. Select either the poly(A) mRNA Capture protocol or rRNA Depletion protocol for RNA enrichment based on experimental requirements and sample conditions (see Section 1: Target RNA Enrichment and Fragmentation).

1) Thaw 1st strand synthesis reagents from −20 °C, mix by pipetting, and assemble the reaction system as listed in Table 9.

Table 9. 1st Strand cDNA Synthesis Reaction

Components	Volume
Frag/Prime Buffer with Fragmented RNA	17 µL
1st Reaction Module V2	8 µL

2) Mix gently by pipetting, then spin down briefly to collect all liquid at the tube bottom.
3) Place the tube in a thermal cycler and run the program specified in Table 10 for 1st strand cDNA synthesis. Proceed directly to 2nd strand cDNA synthesis upon completion.

Table 10. 1st Strand cDNA Synthesis Program

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

2. 2nd Strand Synthesis / End Repair / dA-Tailing

1) Thaw reagents for second-strand synthesis from −20 °C and mix by pipetting. Prepare the reaction mixture as specified in Table 11.

Table 11. 2nd Strand cDNA Synthesis Reaction

Components	Volume
1st Strand cDNA	25 µL
2nd Reaction Module (dUTP)*	35 µL

2) Mix gently by pipetting, then spin down briefly to collect liquid at the tube bottom.
3) Place the tube in a thermal cycler and run the program listed in Table 12 for reaction.

Table 12. 2nd Strand cDNA Synthesis Program

Temperature	Time

Heat Lid 105℃	On
16℃	30 min
72℃	15 min
4℃	Hold

3. Adapter Ligation

This step ligates Illumina® or MGI® adapters to the ends of end-repaired and dA-tailed products.

1) Dilute adapters to the appropriate concentration according to input RNA amount (see Table 1 in Notes Section 2).
2) Thaw ligation reagents from −20 °C and mix by pipetting. Prepare the ligation reaction mix as shown in Table 13.

Table 13. Adapter Ligation Reaction

Components	Volume
dA-tailed DNA	60 µL
Ligation Reaction Module	35 µL*
DNA Adapter***	5 µL**

*The ligation reaction mixture is viscous. Mix thoroughly and spin down briefly before use.

**For short adapters, use PE adapters.

***Stock concentration: 15 µM for Illumina® adapters and 10 µM for MGI® adapters. Dilute adapters according to input quantity as instructed in Table 1 of Note 2, and keep the added volume fixed at 5 µL.

3) Mix gently by pipetting, then spin down briefly to collect liquid at the tube bottom.
4) Place the tube in a thermal cycler and run the program listed in Table 14 for adapter ligation.

Table 14. Adapter Ligation Program

Temperature	Time
Heat Lid	Off
20℃	15 min
4℃	Hold

4. Post Ligation Clean Up

This protocol uses two rounds of purification to remove residual adapters for inserts shorter than 200 bp. For inserts ≥ 200 bp, follow the size selection protocol in Appendix II to obtain libraries of target size via purification and selection.

Libraries with Inserts < 200 bp (Two Rounds of Purification Required)

Note: Ensure RNA fragments are shorter than 200 nt before use.

1) Preparations: Take NGS DNA Selection Beads from storage and equilibrate to room temperature for at least 30 min. Prepare 80% ethanol.
2) Resuspend beads completely by vortexing or repeated inversion.
3) Add 60 μL NGS DNA Selection Beads (0.6×, beads:DNA = 0.6:1) to the adapter ligation product. Mix by vortexing or pipetting, then incubate at room temperature for 5 min.
4) Briefly spin down the tube and place it on a magnetic stand. After approximately 5 min when the solution clears, carefully remove the supernatant.
5) Keep the tube on the stand. Add 200 μL freshly prepared 80% ethanol to wash the beads. Incubate for 30 sec at room temperature, then discard the supernatant gently.
6) Repeat Step 5 for a second wash.
7) Keep the tube on the stand with the lid open. Air-dry beads until slight cracks appear (do not exceed 5 min).
8) Remove the tube from the stand, add 52 μL ddH_2O, and mix thoroughly by vortexing or gentle pipetting. Incubate at room temperature for 5 min. Briefly spin down, place on the magnetic stand and wait about 3 min until the solution clears. Transfer 50 μL supernatant to a new PCR tube for a second round of purification.
9) Add 40 μL NGS DNA Selection Beads (0.8×, beads:DNA = 0.8:1) to the sample above. Mix by vortexing or pipetting, then incubate at room temperature for 5 min.
10) Briefly spin down the tube and place on a magnetic stand. After around 3 min when the solution clears, carefully remove the supernatant.
11) Keep the tube on the stand. Add 200 μL freshly prepared 80% ethanol for washing. Incubate for 30 sec at room temperature, then remove the supernatant gently.
12) Repeat Step 11 for a second wash.
13) Keep the tube on the stand with the lid open. Air-dry beads until slight cracks appear (do not exceed 5 min).

14) Remove the tube from the stand, add 21 μL ddH_2O and mix well by vortexing or gentle pipetting. Incubate at room temperature for 5 min. Briefly spin down and place on the magnetic stand. After about 3 min when the solution clears, transfer 20 μL supernatant to a new PCR tube for amplification.

5. Library Amplification

This step amplifies and enriches purified or size-selected adapter-ligated products.

1) Thaw amplification reagents from −20 °C and mix by pipetting. Prepare the reaction mixture as specified in Table 15.

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

Components	Volume
2×HiFi Mix for Library Amplification	25 μL
Universal Primer/ i5 Primer*	2.5 μL
Index Primer/i7 Primer*	2.5 μL
Adapter Ligated DNA	20 μL

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

Components	Volume
2×HiFi Mix for Library Amplification	25 μL
Primer Mix**	5 μL
Adapter Ligated DNA	20 μL

*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.No.: HYD333-334 or equivalent) for amplification.

**If you are using Indexed Adapters (Cat.No.: HYD311~312), commonly known as full-length adapters (large Y adapters), you can use the NGS RNA Library Prep Primer Mix for Illumina (Cat.No.: HYD314) for amplification.

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

Components	Volume
2×HiFi Mix for Library Amplification	25 μL
Primer Mix for MGI*	5 μL
Adapter Ligated DNA	20 μL

* This primer mix for MGI is not contained in this kit and can be needed with the NGS Primer Mix for MGI, Compatible with Complete Adaptor (Cat.No.: HYD318).

2) Mix gently by pipetting or vortexing, then spin down briefly to collect liquid at the tube bottom.

3) Place the tube in a thermal cycler and run the program listed in Table 16 for PCR amplification.

Table 16. PCR Amplification Program

Temperature	Time	Cycle
98℃	1 min	1
98℃	10 s	11~16 cycles*
60℃	30 s	
72℃	30 s	
72℃	5 min	1
4℃	Hold	-

*Adjust the PCR cycle number according to sample quality, input amount and other library preparation conditions. See Note 3 for details.

6. Post Amplification Clean Up

1) Preparations: Take NGS DNA Selection Beads out of storage and equilibrate to room temperature for no less than 30 min. Prepare 80% ethanol.

2) Fully resuspend beads by vortexing or repeated inversion.

3) Add 45 μL NGS DNA Selection Beads (0.9×, beads:DNA = 0.9:1) to the amplified product. Mix by vortexing or pipetting, then incubate at room temperature for 5 min.

4) Briefly spin down the tube and place it on a magnetic stand. After approximately 5 min when the solution clears, carefully remove the supernatant.

5) Keep the tube on the stand. Add 200 μL freshly prepared 80% ethanol for washing. Incubate for 30 sec at room temperature, then gently remove the supernatant.

6) Repeat Step 5 for a second wash.
7) Leave the tube on the stand with the lid open. Air-dry beads until slight cracks appear (do not exceed 5 min).
8) Remove the tube from the stand, add 21 μL ddH_2O, and mix thoroughly by vortexing or gentle pipetting. Incubate at room temperature for 5 min. Briefly spin down and place on the magnetic stand. After around 3 min when the solution clears, transfer 20 μL supernatant to a new PCR tube for library quantification and quality control.

7. Library Quality Control

Generally, library quality is assessed via concentration detection and fragment size analysis. Refer to Note 5 for details.

Appendix

Appendix I: mRNA Fragmentation Results

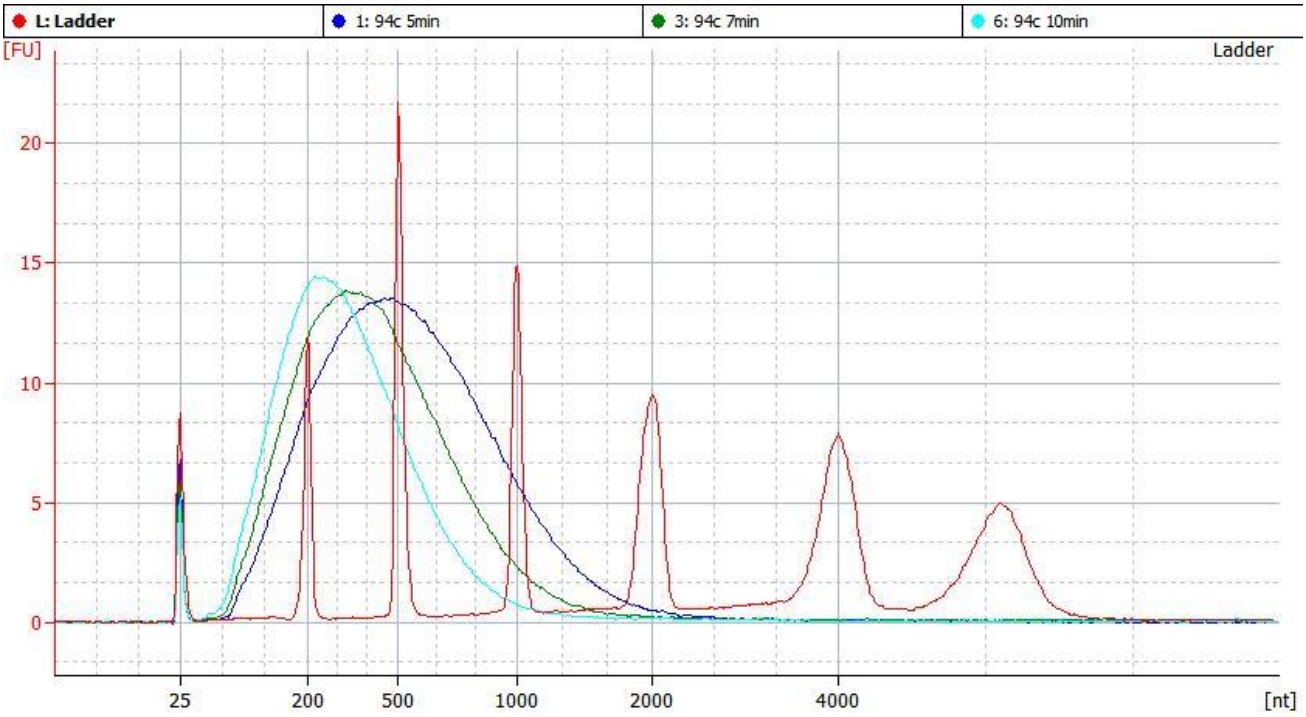


Figure 1. mRNA Fragmentation with Different Shearing Times

mRNA fragment size distribution under different shearing conditions: 94 °C for 10 min, 94 °C for 7 min, and 94 °C for 5 min. Sheared mRNA was purified with 2.2× magnetic beads and analyzed using an Agilent 2100 Bioanalyzer.

Note: These results were obtained using Agilent Universal Human Reference RNA. If using RNA from other sources, it is recommended to optimize the fragmentation time.

Appendix II: Size Selection Conditions for the Illumina® Platform

This selection protocol is applicable to libraries prepared from RNA fragmented at 94 °C for 10 min, 7 min, or 5 min, yielding libraries with insert sizes > 200 bp.

1. Purification of Adapter-Ligated Products with 0.6× NGS DNA Selection Beads

1) Preparations: Remove NGS DNA Selection Beads from storage and equilibrate to room temperature for at least 30 min. Prepare fresh 80% ethanol.
2) Thoroughly resuspend the beads by vortexing or repeated inversion.
3) Add 60 µL NGS DNA Selection Beads (0.6× ratio, beads:DNA = 0.6:1) to the adapter ligation product. Mix by vortexing or pipetting, then incubate at room temperature for 5 min.

4) Briefly spin down the tube and place it on a magnetic stand. After approximately 5 min when the solution clears, carefully remove the supernatant.
5) Keep the tube on the magnetic stand. Add 200 µL freshly prepared 80% ethanol to wash the beads. Incubate for 30 sec at room temperature, then carefully remove the supernatant.
6) Repeat Step 5 for a second wash.
7) Keep the tube on the magnetic stand. Air-dry the beads with the lid open until slight cracks appear (do not exceed 5 min).
8) Remove the tube from the magnetic stand. Add 102 µL ddH_2O, mix thoroughly by vortexing or gentle pipetting, and incubate at room temperature for 5 min. Briefly spin down the tube, place it on the magnetic stand, and wait about 5 min until the solution clears. Carefully transfer 100 µL of the supernatant to a new PCR tube for double-sided size selection.

Double-Sided Size Selection (Example: 94 °C, 7 min fragmentation, target insert size 410–510 bp)

Use the ratios in Table 17 when using short adapters (small Y-adapters) with the paired-end 384-index primers: NGS RNA 384 CDI Primer for Illumina, Set 1–Set 2 (Cat. No: HYD333–334).

Use the ratios in Table 18 when using long adapters (large Y-adapters) with the indexed adapters: NGS Complete Adapter Kit for Illumina, Set 1–Set 2 (Cat. No: HYD311–312).

2. Size Selection of Adapter-Ligated Products with 0.65× / 0.15× NGS DNA Selection Beads

1) Thoroughly resuspend the beads by vortexing or repeated inversion.
2) Based on the desired DNA fragment size, refer to Table 17 and add 65 µL (0.65×) of the first-round selection beads to the 100 µL DNA sample. Mix by vortexing or pipetting 10 times.
3) Incubate at room temperature for 5 min.
4) Briefly spin down the tube and place it on a magnetic stand. After approximately 5 min when the solution clears, carefully transfer the supernatant to a clean centrifuge tube, leaving 1–2 µL of liquid at the bottom.
5) Refer to Table 17 and add 15 µL (0.15×) of the second-round selection beads to the supernatant.
6) Mix by vortexing or pipetting 10 times, then incubate at room temperature for 5 min.
7) Briefly spin down the tube and place it on a magnetic stand. After approximately 3 min when the solution clears, carefully remove the supernatant.
8) Keep the tube on the magnetic stand. Add 200 µL freshly prepared 80% ethanol to wash the beads. Incubate for 30 sec at room temperature, then carefully remove the supernatant.
9) Repeat Step 8.

10) Keep the tube on the magnetic stand. Air-dry the beads with the lid open until slight cracks appear (about 3 min).
11) Remove the tube from the magnetic stand. Add 21 μL ddH_2O, mix thoroughly by vortexing or gentle pipetting, and incubate at room temperature for 5 min.
12) Briefly spin down the tube and place it on a magnetic stand to separate beads from liquid. After approximately 3 min when the solution clears, carefully transfer 20 μL of the supernatant to a clean tube.

Table 17. Recommended Bead Ratios for Short Adapter Libraries

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

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

Inserted Size (bp)	**200~300**	**250~350**	**350~450**	**450~550**
Library Size (bp)	**320~420**	**370~470**	**470~570**	**570~670**
Fragmentation	94℃ 10 min	94℃ 7 min	94℃ 7 min	94℃ 5 min
Volume Beads in the 1st round (μL)	75 (0.75×)	70 (0.70×)	65 (0.65×)	60 (0.6×)
Volume Beads in the 2nd round (μL)	15 (0.15×)	15 (0.15×)	15 (0.15×)	10 (0.1×)

Note: The recommended double-sided selection ratios in Tables 17 and 18 apply to NGS DNA Selection Beads; "×" 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 0.65×100 μL = 65 μL, and the second round selection bead volume would be 0.15×100 μL = 15 μL.

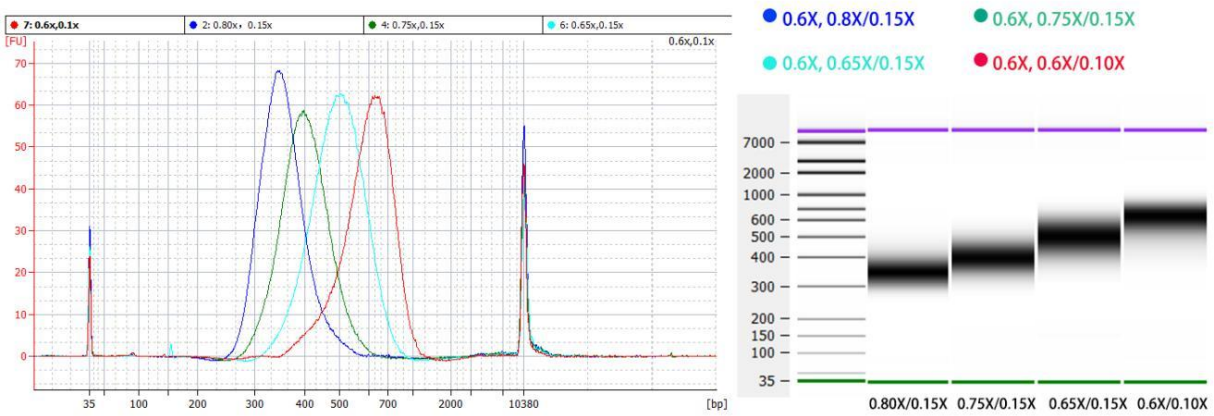


Figure 3. Different Fragmentation Conditions Library Peak Profiles

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 Conditions for the MGI® Platform

This protocol is designed for libraries constructed from RNA fragmented at 94 °C for 10 min, 7 min or 5 min, to obtain libraries with insert sizes greater than 200 bp.

1. Purification of Adapter-Ligated Products with 0.6× NGS DNA Selection Beads

1) Preparations: Take NGS DNA Selection Beads from storage and equilibrate to room temperature for at least 30 min. Prepare fresh 80% ethanol.
2) Fully resuspend beads by vortexing or repeated inversion.
3) Add 60 μL NGS DNA Selection Beads (0.6×, beads:DNA = 0.6:1) to the adapter ligation product. Mix by vortexing or pipetting, then incubate at room temperature for 5 min.
4) Briefly spin down the tube and place it on a magnetic stand. After approximately 5 min when the solution clarifies, carefully remove the supernatant.
5) Keep the tube on the stand. Add 200 μL freshly prepared 80% ethanol for washing. Incubate for 30 sec at room temperature, then gently remove the supernatant.
6) Repeat Step 5 for a second wash.
7) Keep the tube on the stand. Air-dry beads with the lid open until slight cracks appear (do not exceed 5 min).
8) Remove the tube from the stand, add 102 μL ddH_2O, and mix thoroughly by vortexing or gentle pipetting. Incubate at room temperature for 5 min. Briefly spin down the tube and place it on the

magnetic stand. After about 5 min when the solution clarifies, transfer 100 µL supernatant to a new PCR tube for double-sided size selection.

Note: High-concentration PEG contained in Ligation Enhancer may interfere with double-sided bead selection. Perform one round of purification prior to size selection.

2. Double-Sided Size Selection

The following takes RNA fragmented at 94 °C for 7 min for target insert size of 380 bp–480 bp as an example. Adjust bead ratios accordingly for other target sizes.

1) Fully resuspend beads by vortexing or repeated inversion.
2) Refer to Table 19 based on target fragment size. Add 65 µL (0.65×) first-round selection beads to the 100 µL sample. Mix by vortexing or pipetting 10 times.

Note: The ratios listed in this table apply exclusively to NGS DNA Selection Beads. The symbol "×" refers to the volume of sample DNA. For example, for a library with a main insert peak of 300 bp after short adapter ligation with 100 µL sample, add 65 µL (0.65×) beads for the first round and 15 µL (0.15×) beads for the second round.

3) Incubate at room temperature for 5 min.
4) Briefly spin down the tube and place it on a magnetic stand. After around 5 min when the solution clarifies, transfer the supernatant to a clean centrifuge tube, leaving 1–2 µL liquid at the bottom.
5) Refer to Table 19 and add 15 µL (0.15×) second-round selection beads to the supernatant.
6) Mix by vortexing or pipetting 10 times, then incubate at room temperature for 5 min.
7) Briefly spin down the tube and place it on a magnetic stand. After approximately 3 min when the solution clarifies, carefully remove the supernatant.
8) Keep the tube on the stand. Add 200 µL freshly prepared 80% ethanol for washing. Incubate for 30 sec at room temperature, then gently remove the supernatant.
9) Repeat Step 8.
10) Keep the tube on the stand. Air-dry beads with the lid open until slight cracks appear (about 3 min).
11) Remove the tube from the stand, add 21 µL ddH_2O, and mix thoroughly by vortexing or gentle pipetting. Incubate at room temperature for 5 min.
12) Briefly spin down the tube and place it on the magnetic stand. After about 3 min when the solution clarifies, transfer 20 µL supernatant to a clean tube.

Table 19. Recommended Bead Ratios for Short Adapter Libraries

Inserted Size (bp)	**200~300**	**300~400**	**400~500**	**500~600**
Library Size (bp)	**280~380**	**380~480**	**480~580**	**580~680**
Fragmentation	94℃ 10 min	94℃ 7 min	94℃ 7 min	94℃ 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×)

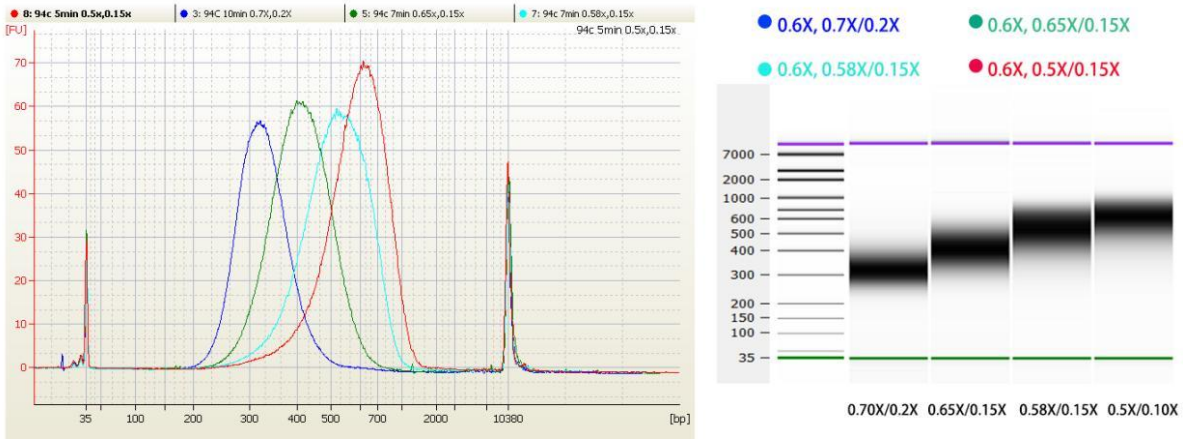


Figure 4. Different Selection Ratio Library Peak Profiles

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 19.

Appendix IV: Library Preparation for FFPE Samples

1. Quality Assessment of FFPE-Derived RNA

The rRNA depletion library preparation workflow is compatible with low-quality total RNA such as FFPE RNA. Given the large variation in quality among different FFPE samples, relevant experimental conditions shall be adjusted accordingly.

RIN value is a conventional indicator for RNA quality evaluation. However, it cannot fully reflect the actual quality of degraded FFPE RNA. The DV200 metric is therefore adopted as a supplementary parameter. DV200 represents the percentage of RNA fragments longer than 200 nt, which enables more accurate quality assessment for severely degraded FFPE samples.

Calculation of DV200:

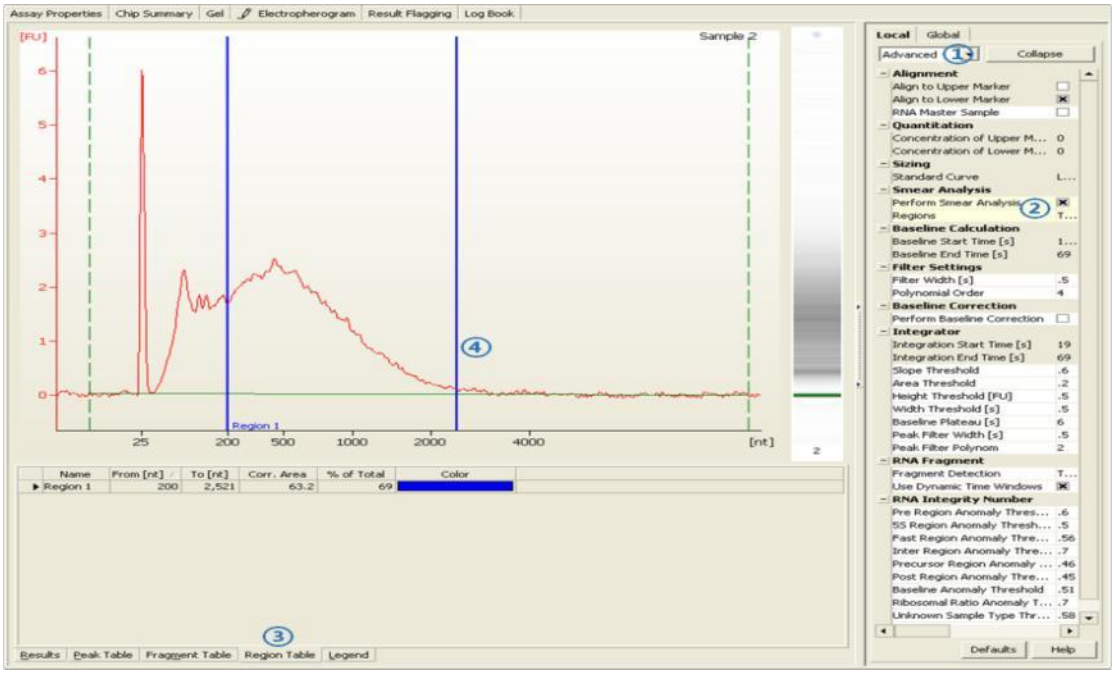


Figure 5. DV200 Proportion

On the result interface of Agilent 2100 Bioanalyzer: Go to Local > Advanced, and check Perform Smear Analysis under Smear Analysis. Navigate to the Region Table tab, right-click and select Add Region. Adjust the marker lines to define the target fragment range, and the corresponding percentage (% of Total) will be displayed.