Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit

Cat#12308

INSTRUCTION FOR USE

Yeasen Biotechnology (Shanghai) Co., Ltd.

Table of Contents

Product Information

Product Name	Cat#	Specification
Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit	12308ES24	24 T
	12308ES96	96 T

Product Description

Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit is a total RNA sequencing library construction kit for the Illumina® and MGI® sequencing platform, including RNA fragmentation reagents, reverse transcription reagents, conventional and strand-specific ds-cDNA synthesis reagents, and library amplification reagents. The sequencing library can be constructed followed by the mRNA purification kit or rRNA removal kit. The two-strand synthesis module is equipped with two buffers to meet the need for conventional library or strand-specific library. Among them, dTTP is replaced with dUTP in the strand-specific two-strand synthesis Buffer, so dUTP can be added to the second strand of cDNA. The high-fidelity DNA polymerase used in this kit cannot amplify the DNA template containing uracil, achieving strand specificity. All reagents provided have undergone strict quality control and functional verification, ensuring the stability and reproducibility of library construction to the greatest extent.

Product Components

Components			12308ES24	12308ES96
12308-A	🔴	2× Frag/Prime Buffer	250 μL	930 μL
12308-B	🔴	1st Strand Enzyme Mix	48 μL	192 μL
12308-C	🟤	Strand Specificity Reagent	150 μL	580 μL
12308-D	🟢	2nd Strand Buffer (dNTP)	720 μL	2×1440 μL
12308-E	🟣	2nd Strand Buffer (dUTP)	720 μL	2×1440 μL
12308-F	🟢	2nd Strand Enzyme Master Mix	120 μL	480 μL
12308-G	🔵	Ligation Enhancer	720 μL	2×1440 μL
12308-H	🔵	Novel T4 DNA Ligase	120 μL	480 μL
12308-I	◌	2×Super Canace® II High-Fidelity Mix	600 μL	2×1200 μL
12308-K	○	Nuclease Free H_2O	300 μL	1000 μL

Note: This kit is compatible with both Illumina and MGI platforms, but additional Illumina® or MGI® Primer Mix (Cat# 13335 Primer Mix for Illumina®and Cat# 13334 Primer Mix for MGI®) are required.

Shipping and Storage

The Hieff NGS™ Ultima Dual-mode mRNA Library Prep Kit components in Box I are shipped with ice packs and can be stored at 2-8°C for one year.

The Hieff NGS™ Ultima Dual-mode mRNA Library Prep Kit components in Box II are shipped with dry ice and can be stored at -20°C for one year.

Cautions

1 Operation

1.1 For your safety and health, please wear personal protective equipment (PPE), such as laboratory coats and disposable gloves, when operating with this product. This product is for research use ONLY!

1.2 Thaw components at room temperature. Mix thoroughly by inverting up and down several times, spin down briefly and place on ice for use.

1.3 It is recommended to perform each step reaction in a thermal cycler with a heated lid. The thermal cycler should be preheated to the set temperature prior to use.

1.4 Supplies free of RNase contamination and cleaning the experimental area regularly are necessary. ThermoFisher's RNAZap™

high-efficiency nucleic acid removal spray was recommended to remove RNase contamination.

1.5 Improper operations may very likely cause aerosol contaminations, impacting the accuracy of result. Mandatory physical isolation of PCR reaction mixing regions and PCR product purification assay regions is recommended. Equipped with equipment such as specialized pipettes for library construction.

2 Adapter Ligation

2.1 Illumina® or MGI® Long Adapter (Barcoded Adapter) kits and short Adapter kits are available for customers to choose according to their experimental requirements.

2.2 Selecting high-quality, commercial adapters was recommended. If self-made adapters are selected, please entrust a company with experience in NGS primer synthesis and remark the need for strict contamination control. In addition, it is recommended to prepare DNA annealing solution in a clean bench and only operate one type of adapter each time to prevent cross-contamination.

2.3 Please thaw the adapters on the ice or at 4°C; when operating at room temperature, the laboratory temperature should not exceed 25°C to prevent the adapters from denaturing.

2.4 The concentration of the adapter directly affects the ligation efficiency and library yield. The adapter volume added to the kit is fixed to 5 μl. The adapters are recommended to be diluted with 0.1×TE buffer and the diluted adapters can be stored at 4°C for 48 hours. Table 1 lists the recommended adapter amount for different amounts of input RNA.

Table 1-1 The recommended Illumina® adapter amount for different input RNA

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 The recommended MGI® adapter amount for different input RNA

Input Total RNA	MGI® Adapter stock concentration
100-499 ng	2 μM
500-4000 ng	5 μM

*The Adapter usage can be adjusted according to different types of Total RNA samples and input amount.

3 Library Amplification

3.1 On the basis of the first-generation DNA polymerase, the high-fidelity DNA polymerase in the kit has greatly improved its amplification uniformity and exhibits no amplification bias.

3.2 If Indexed Adapter (also known as long adapter or large Y adapter) is ligated to the target DNA, primer mix provided in this kit can be used for amplification; if "short adapter" or "small Y adapter" is used for DNA ligation, index primers are needed for amplification.

3.3 Amplification cycle numbers should be strictly controlled. Insufficient amplification may lead to low library yield; Over-amplification may introduce increased bias, errors, duplicated read, chimeric products and accumulation of expansion mutations. Table 2 lists the recommended cycle numbers for PCR amplification.

Table 2 The recommended number of cycles to generate RNA library*

Input Total RNA	Number of cycles	
	Non-stranded	Stranded
10 ng	15	15
100 ng	14	14
500 ng	12	13
1 μg	11	12

Note：*The yield of the library is not only related to the input quantity and the number of amplification cycles, but also affected by the quality of samples, fragmentation conditions and sorting conditions. In the process of library construction, choose the most appropriate conditions according to the actual situation.

4 Bead-based DNA Cleanup and Size Selection

4.1 There are multiple steps in the library construction process that require DNA purification magnetic beads. We recommend Hieff NGS™ DNA Selection Beads (Yeasen Cat#12601) or AMPure® XP magnetic beads (Beckman Cat#A63880) for DNA purification and size-selection.

4.2 The magnetic beads should be equilibrated at room temperature prior to use, otherwise the yield will decrease and the size selecting effect will be affected.

4.3 The magnetic beads should be mixed well by vortex or pipetting prior to use.

4.4 Do not aspirate the beads when transferring the supernatant, even trace amounts of the beads may impact the following reactions.

4.5 The 80% ethanol should be freshly prepared, otherwise it will affect the recovery efficiency.

4.6 The magnetic beads should be dried at room temperature before eluting the product. Insufficient dryness will easily cause ethanol residual to affect subsequent reactions; excessive dryness will cause the magnetic beads to crack and reduce the purification yield. Normally, drying at room temperature for 3-5 minutes is enough to allow the beads to fully dry.

4.7 If needed, the purified or size-selected DNA samples eluted in TE buffer can be stored at 4°C for 1-2 weeks or at -20°C for a month.

5 Library Quality Analysis

5.1 Normally, the quality of the constructed library can be evaluated by length distribution and concentration detection.

5.2 Library concentration detection: methods based on double-stranded DNA fluorescent dyes, such as Qubit®, PicoGreen®, etc.; absolute quantification based on qPCR.

5.3 Methods based on spectral detection, such as NanoDrop®, etc., is not applicable to library concentration detection.

5.4 qPCR is recommended for library concentration detection: Through Qubit®, PicoGreen® and other methods based on double-stranded DNA fluorescent dyes, it cannot effectively distinguish between products ligated to adapters at one end, products not ligated to adapters at both ends, and other incomplete double-strand products. Absolute quantification of qPCR is based on the principle of PCR amplification, which only quantifies the complete library of the adapter at both ends of the sample (the library that can be sequenced), excluding the interference of non-sequencing libraries that are not ligated to the adapter at either single-ended or double-ended ends.

5.5 Library length distribution detection can be performed by Agilent Bioanalyzer 2100 and other equipment based on the principle of capillary electrophoresis or microfluidics.

6 Other Materials

6.1 mRNA enrichment Kit：Hieff NGS™ mRNA Isolation Master Kit (Yeasen Cat#12603).

6.2 rRNA depletion Kit：Hieff NGS™ MaxUp rRNA Depletion Kit (Human/Mouse/Rat) (Yeasen Cat#12253).

6.3 RNA Cleaner： Hieff NGS™ RNA Cleaner (Yeasen Cat#12602) or other equivalent products.

6.4 DNA Cleaner：Hieff NGS™ DNA Selection Beads (Yeasen Cat#12601) or AMPure® XP Beads (A63880) or other equivalent products.

6.5 RNA quality control：Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico Chip or other equivalent products.

6.6 Adapters：Complete Adapter for Illumina® (Yeasen Cat#13519-13520 or other equivalent products) or Complete Adapter for MGI® (Yeasen Cat#13360-13362 or other equivalent products).

6.7 Library quality control：Agilent 2100 Bioanalyzer DNA 1000 Chip/ High Sensitivity Chip or other equivalent products.

6.8 Other materials: ethanol, Sterilized ddH_2O, PCR tube, magnetic stand, thermal cycler etc.

Instructions

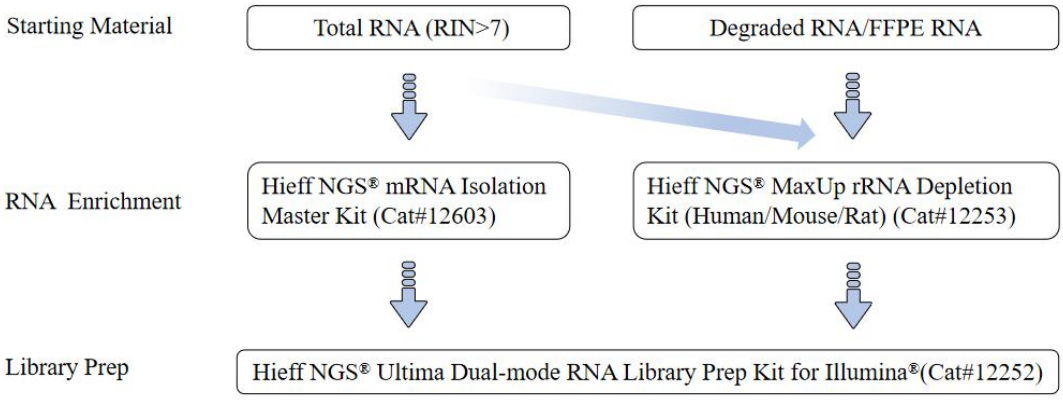


Figure 1 The workflow of RNA library construction kit

Instructions

Part 1：Enrichment and fragmentation of target RNA

Preparation of target RNA before library construction. According to the requirements of library construction, Poly(A) mRNA Isolation protocol (Scheme A) or rRNA Depletion protocol (Scheme B) can be chose. Yeasen kit (Cat#12308) does not include the reagents used in this step, please prepare the corresponding reagents in line with the requirement of library construction.

Scheme A：mRNA Purification and Fragmentation

Sample requirements

The Hieff NGS™ mRNA Isolation Master Kit (Yeasen Cat#12603) is applicable for mRNA enrichment. This kit is suitable for high-quality total RNA from eukaryotes such as animals, plants, and fungi with a starting input of 10 ng - 4 μg (volume≤50 μL). If the initial RNA concentration is low and the volume exceeds 50 μL, it is recommended to condensed the RNA with Hieff NGS™ RNA Cleaner magnetic beads. To ensure that the mRNA has a complete poly(A) tail structure, RNA needs to be detected by the Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico chip and the RIN value must be >7. Oligo (dT) magnetic beads was applied in the mRNA isolation module of this kit, so that only mRNA with poly(A) tail can be extracted; other RNAs without poly(A) tail, such as non-coding RNA, no poly(A) tail mRNA etc. were washed away. In addition, this kit is not compatible with FFPE samples since the mRNA in the FFPE sample is severely degraded and usually does not have a complete poly(A) tail structure.

Operations Steps

1. Take out the mRNA Capture Beads from 2-8°C, and equilibrate at room temperature for at least 30 min.
2. Dilute 10 ng-4 μg of total RNA with Nuclease-free Water to a final volume of 50 μL in a nuclease-free 0.2ml PCR tube and keep on ice.
3. Mix the magnetic beads by inverting upside down or vortexing. Add 50 μL of the magnetic beads into 50 μL total RNA sample and pipette 6 times to mix well. Spin down briefly to the bottom of the tube.
4. Incubate the mixture of magnetic beads and RNA in a thermal cycler and run the following program: 65°C, 5 min; 25°C, 5 min;

25°C, hold.

5. Place the tube on a magnetic stand for 5 minutes to separate mRNA from total RNA. Carefully remove the supernatant.

6. Remove the tube from the magnetic stand and resuspend the magnetic beads with 200 μL Beads Wash Buffer. Pipette the entire volume up and down 6 times to mix thoroughly. Place the tube on a magnetic stand for 5 min, and carefully remove the supernatant.

7. Repeat step 6.

8. Remove the tube from the magnetic stand. Add 50 μL Tris Buffer to resuspend the magnetic beads and pipette 6 times to mix thoroughly.

9. Put the sample in a thermal cycler and run the following program to elute the mRNA: 80°C, 2 min; 25°C, hold.

10. Remove the sample from the thermal cycler. Add 50μL Beads Binding Buffer and pipette repeatedly 6 times to mix thoroughly.

11. Incubate at room temperature for 5 minutes to allow mRNA to bind to the magnetic beads.

12. Place the tube on the magnetic stand for 5 minutes, and carefully remove the supernatant.

13. Remove the tube from the magnetic stand, resuspend the magnetic beads with 200 μL Beads Wash Buffer, pipette repeatedly 6 times to mix thoroughly. Place the tube on the magnetic stand at room temperature for 5 minutes. Remove and discard all of the supernatant.

[Note]: A 10 μL pipette is required to aspirate the remaining liquid. Prepare 1× Frag/Prime Buffer in advance (use Nuclease Free H_2O equal volume mixing configuration, such as configuring a reaction system: 9.5 μL 2×Frag/Prime Buffer + 9.5 μL Nuclease Free H_2O)

14. Remove the tube from the magnetic stand and resuspend the magnetic beads with 19 μL Frag/Prime Buffer. Pipette 6 times to mix thoroughly and place the tube in the thermal cycler (preheated at 94°C). Table 3 lists the recommended time for mRNA fragmentation. (There are differences in the effect of fragmentation of different species, and customers may first make a gradient of fragmentation time, such as 94 ° C for 5 min. mRNA isolation product size was analyzed by Agilent 2100.)

Table 3 The recommended time for mRNA Fragmentation

Inserted DNA size (bp)	Fragmentation time
200-300	94°C, 10 min
300-400	94°C, 7 min
400-500	94°C, 5 min

15. Immediately, place the tube on the magnetic stand to prevent the combination between poly(A) tail RNA and the magnetic beads. When the solution is clear, transfer 17 μL of the supernatant to a new nuclease-free PCR tube (Part II/III- Step 1).

Scheme B: rRNA Depletion and RNA Fragmentation

Sample requirements

Hieff NGS® MaxUp rRNA Depletion Kit (Human/Mouse/Rat) (Yeasen Cat#12253) is applicable to remove rRNA from total RNA. Suitable for 100 ng~1 μg (volume ≤ 11 μL) total RNA samples from human, mouse, and rat; suitable for complete RNA or partially degraded RNA (such as FFPE RNA) samples.

Operation Steps

Step 1 Probe Hybridization to RNA

1.1 Dilute 10 ng–1 μg of total RNA with Nuclease-free water to a final volume of 11 μL in a PCR tube. Keep the RNA **on ice.**

1.2 Prepare the following RNA/Probe hybridization reaction **on ice** according to Table 4.

Table 4 RNA/Probe hybridization reaction

Components	Volume (μL)
Hybridization Buffer	3
Probe Mix(H/M/R)	1
Total RNA	11 (100 ng~1 μg)
Total	15

1.3 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the

liquid from the side of the tube.

1.4 Place tube in a thermocycler and run the following program with the heated lid set to 105°C.

Table 5 Reaction program of RNA/Probe hybridization

Temperature	Time
Hot lid 105°C	On
95°C	2 min
95°C-22°C	0.1°C/s
22°C	5 min
4°C	hold

Step 2 RNase H Digestion

2.1 Prepare the following RNase H digestion reaction **on ice** according to Table 6.

Table 6 RNase H digestion reaction

Components	Volume (μL)
RNase H Buffer	3
RNase H	2
Hybridized RNA (Step 1.4)	15
Total	20

2.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.3 Place tube in a thermocycler and run the following program: hot lid 50°C; 37°C, 30 min; 4°C, hold.

Step 3 DNase I Digestion

3.1 Prepare the following DNase I digestion reaction on ice according to Table 7.

Table 7 DNase I digestion reaction

Components	Volume (μL)
DNase I Buffer	27.5
DNase I	2.5
RNase H treated RNA (Step 2.3)	20
Total	50

3.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

3.3 Place tube in a thermocycler and run the following program: hot lid 50°C; 37°C, 30 min; 4°C, hold.

Step 4 RNA Purification

4.1 Equilibrate the Hieff NGS™ RNA Cleaner (Cat#12602) to room temperature and resuspend the beads thoroughly by vortexing before use.

4.2 Add **110 μL (2.2×)** beads to the RNA solution from Step 3.3 and mix thoroughly by pipetting up and down at least 10 times.

4.3 Incubate at room temperature for 5 minutes to bind RNA to the beads.

4.4 Place the tube on a magnetic stand to separate the beads from the supernatant. When the solution is clear (about 3 mins), discard the supernatant. Be careful not to touch the beads with the pipette tips.

4.5 Keep the tube on the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to the tube. Incubate at room temperature for 30 seconds and then discard the supernatant. Be careful not to touch the beads with the pipette tips.

4.6 Repeat Step 4.5 once for a total of two washes.

4.7 Remove residual ethanol with 10 μL - pipette tips. Keep the tube on the magnetic stand and air dry-the beads for up to 5 minutes with the lid open.

4.8 Remove the tube from the magnetic stand. Elute the RNA from the beads by adding 19 μL of Frag/Prime buffer. Mix thoroughly by pipetting up and down at least 5 times and briefly spin the tube.

4.9 Incubate for 5 minutes at room temperature. Place the tube on the magnetic stand until the solution is clear (~ 3 minutes).

4.10 Transfer 17 μL of the supernatant to a nuclease-free tube for fragmentation according to Table 3. Table 8 recommends the fragmentation conditions of FFPE samples of different quality.

11. After fragmentation, please put it on ice immediately and perform the first-chain synthesis reaction (Part II/III-Step 1).

Table 8 Recommended FFPE RNA fragmentation conditions

DV_{200}*	Fragmentation time
>70%	94°C, 7 min
50%~70%	94°C, 5 min
20%~50%	85°C, 8 min
<20%(library construction is risky)	65°C, 8 min

[Note]* Sample quality of degraded RNA was determined using DV200 index, as described in Appendix III

Part 2：RNA library construction for Illumina® platform

Step 1 1st Strand Synthesis

This step perform 1st cDNA synthesizes for the enrichment/fragmentation target RNA by Poly(A) mRNA Isolation or rRNA Depletion scheme. See Part I for details

1.1 Prepare the following 1st Strand Synthesis reaction according to Table 9.

Table 9 1st Strand cDNA synthesis reaction

Components	**Volume (μL)**
Frag/Prime Buffer with Fragmented RNA	17
Strand Specificity Reagent	6
1st Strand Enzyme Mix	2
Total	25

1.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

1.3 Place tube in a thermocycler and run the following program according to Table 10.

Table 10 Reaction program of 1st Strand Synthesis

Temperature	**Time**
Hot lid 105°C	On
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

Step 2 2nd Strand Synthesis/end repair/dA-Tailing

2.1 Prepare the following 2nd Strand Synthesis/**end repair/** dA-Tailing reaction according to Table 11.

Table 11 2nd Strand Synthesis/ end repair/ dA-Tailing synthesis reaction

Components	**Volume (μL)**
1st Strand cDNA	25
2nd Strand Buffer (dNTP or dUTP)*	30
2nd Strand Enzyme Master Mix	5
Total	60

[Note] Buffer containing dNTP was designed for normal mRNA library construction; while buffer containing dUTP was applied for strand-specific mRNA

library construction.

2.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.3 Place tube in a thermocycler and run the following program according to Table 12.

Table 12 Reaction program of 2nd Strand Synthesis/end repair/dA-Tailing Synthesis

Temperature	Time
Hot lid 105°C	on
16°C	30 min
72°C	15 min
4°C	Hold

Step 3 Adapter Ligation

Specific Illumina® adapters can be ligated into the products of end repair and dA tailing.

3.1 Dilute the adapter to the appropriate concentration according to Table 1-1.

3.2 Thaw the reagents in Table 13. Mix thoroughly and place them on ice for later use.

3.3 Add the following reagents to the product of step 2.3.

Table 13 The reaction for Adapter Ligation

Components	Volume (μL)
dA-tailed DNA (Step 2.3 product)	60
Ligation Enhancer	30*
Novel T4 DNA Ligase	5
DNA Adapter	5**
Total	100

Note: * Ligation Enhancer should be mixed thoroughly followed by a quick spin prior to use.

**The original adapter concentration of Yeasen is 15 μM. Please dilute the adapter according to the input amount as shown in Table 1-1, so that the added volume of the adapter is fixed at 5 μL.

3.4 Mix thoroughly by vortexing at low speed or pipetting several times. Spin the reaction solution briefly to the bottom of the tube.

3.5. Place the PCR tube on a thermocycler and run the reaction programs in the Table 14.

Table 14 The reaction program for Adapter Ligation

Temperature	Time
Hot lid 105°C	Off
20°C	15 min
4°C	Hold

Step 4 Clean Up Post Ligation

This plan is suitable for fragments <200 bp, and the adapter residue is removed by two purifications; when the inserted fragments are ≥200 bp, library is obtained by purification and size selection refer to Appendix II.

Suitable for libraries with inserts <200 bp(two rounds of purification are required)

4.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature (~ 30 min). Prepare 80% ethanol.

4.2 Resuspend the beads thoroughly by vortexing or shaking.

4.3 Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to the adapter ligation product. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.4 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), carefully discard the supernatant.

4.5 Keep the tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to the tube, and incubate at room temperature for 30 sec. Carefully remove the supernatant.

4.6 Repeat step 4.5 once for a total of two washes.

4.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube on the magnetic stand, and air-dry beads for 5 minutes while

the tube is on the magnetic stand with the lid open.

4.8 Remove the tube from the magnetic stand, elute DNA target from the beads with 52 μl ddH2O. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min. Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 50 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

4.9 Add 40 μL Hieff NGS™ DNA Selection Beads (0.8×, Beads:DNA=0.8:1) to the step 4.8. Mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.10 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), carefully remove the supernatant.

4.11 Keep the tube on the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to the tube and incubate at room temperature for 30 sec. Carefully remove the supernatant.

4.12 Repeat step 4.11 once for a total of two washes.

4.13 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube on the magnetic stand, air dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

4.14 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.15 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Step 5 Library Amplification

In this step, the adapter-ligated DNA is amplified with PCR amplification after cleanup or size selection.

5.1 Thaw the reagents in Table 15. Mix thoroughly and place them on ice for later use.

5.2 Add the following reagents to the product of step 4:

Table 15-A PCR reaction for short adapter ligation

Components	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 reaction for short adapter ligation

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

Note: *If you use an adapter without index, commonly known as a short adapter (small Y adapter), the Index primer provided in the short adapter reagent (Cat#12414~ Cat#12415) is recommended for amplification. **If you use an adapter with index (Cat#12615~ Cat#12618), commonly known as long adapter (big Y adapter), Hieff NGS™ Primer Mix for Illumina® (Yeasen Cat#13335) is needed.

5.3 Mix thoroughly by vortexing or pipetting several times. Spin the reaction solution briefly.

5.4 Place the PCR tube to a thermocycler and run the reaction programs in Table 16.

Table 16 Reaction programs for PCR amplification

Temperature	Time	Cycles
98°C	1 min	1
98°C	10 sec	11~15cycles*
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1
4°C	Hold	-

[Note] * The number of library amplification cycles should be adjusted according to the sample quality, input amount and other library construction conditions. See Caution 3 for details.

Step 6 Cleanup and Size Selection of PCR product

6.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature for at least 30 min. Prepare 80% ethanol.

6.2 Resuspend the beads thoroughly by vortex or shaking.

6.3 Add 45 μL Hieff NGS™ DNA Selection Beads (0.9×, Beads:DNA=0.9:1) to the PCR product. Mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

6.4 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), carefully discard the supernatant.

6.5 Keep the tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to the tube, and incubate at room temperature for 30 sec. Carefully remove the supernatant.

6.6 Repeat step 6.5 once for a total of two washes.

6.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube on the magnetic stand, and air-dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

6.8 Remove the tube from the magnetic stand. Elute DNA target from the beads with 21 μL ddH_2O and mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

6.9 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Step 7 DNA Library Quality Control

The quality of the constructed DNA library can be evaluated by concentration detection and size distribution detection. Please refer to Caution 5 for details.

Part 3：RNA library construction for MGI® platform

Step 1 1st Strand Synthesis

This step perform 1st cDNA synthesizes for the enrichment/fragmentation target RNA by Poly(A) mRNA Isolation or rRNA Depletion scheme. See Part I for details.

1.1 Assemble the following 1st Strand Synthesis digestion reaction according to Table 17.

Table 17 1st Strand cDNA synthesis reaction

Components	Volume (μL)
Frag/Prime Buffer with Fragmented RNA	17
Strand Specificity Reagent	6
1st Strand Enzyme Mix	2
Total	25

1.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

1.3 Place tube in a thermocycler and run the following program according to Table 18.

Table 18 Reaction program of 1st Strand Synthesis

Temperature	Duration
Hot lid 105°C	On
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

Step 2 2nd Strand Synthesis/dA-Tailing

2.1 Assemble the following 2nd Strand Synthesis/dA-Tailing digestion reaction according to Table 19.

Table 19 2nd Strand Synthesis/dA-Tailing synthesis reaction

Components	Volume (μL)

1st Strand cDNA	25
2nd Strand Buffer (dNTP or dUTP)*	30
2nd Strand Enzyme Master Mix	5
Total	60

[Note] To construct a normal mRNA library, use a buffer containing dNTP; for constructing a strand-specific mRNA library, use a buffer containing dUTP.

2.2 Mix thoroughly by gently pipetting up and down at least 10 times. Briefly spin down the tube in a microcentrifuge to collect the liquid from the side of the tube.

2.3 Place tube in a thermocycler and run the following program according to Table 20.

Table 20 Reaction program of 2nd Strand Synthesis/dA-Tailing Synthesis

Temperature	Duration
Hot lid 105°C	on
16°C	30 min
72°C	15 min
4°C	Hold

Step 3 Adapter Ligation

This step is to ligate the MGI® adapter to the product of step 2.3.

3.1 Dilute the adapter to the appropriate concentration according to Table 1-2.

3.2 Thaw the reagents in Table 21. Mix thoroughly and place them on ice for later use.

3.3 Add the following reagents to the product of step 2.3.

Table 21 The reaction system for Adapter Ligation

Components	Volume (μL)
dA-tailed DNA (Step 2.3 product)	60
Ligation Enhancer	30*
Novel T4 DNA Ligase	5
DNA Adapter	5**
Total	100

Note: *Ligation Enhancer is relatively viscous. Please turn it upside down, mix well by vigorously vortexing and centrifuge briefly before use.

**The original concentration of the adapter of our company is 10 μM. Please dilute the adapter according to the instructions in Table 1-2, so that the added volume of the adapter is fixed at 5 μl.

3.4 Mix thoroughly by vortexing at low speed or pipetting several times. Spin the reaction solution briefly to the bottom of the tube.

3.5. Place the PCR tube on a thermocycler and run the reaction programs in the Table 22.

Table 22 The reaction programs for Adapter Ligation

Temperature	Duration
Hot lid 105°C	Off
20°C	15 min
4°C	Hold

Step 4 Post Ligation Clean Up

This scheme is suitable for fragments <200 bp, and the adapter residue in the system is removed by two purifications; when the inserted fragments are ≥200 bp, refer to the sorting scheme in Appendix III to obtain a library of the target length through purification and sorting.

Suitable for libraries with inserts <200 bp (Need to be purified twice)

4.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature (~ 30 min). Prepare 80% ethanol.

4.2 Resuspend the beads thoroughly by vortexing or shaking the bottle.

4.3 Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to the adapter ligation product. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.4 Spin the tube briefly and place it on the magnetic stand. When the solution is clear (about 5 min), aspirate the supernatant and discard.

4.5 Keep the tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

4.6 Repeat step 4.5 once for a total of two washes.

4.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube on the magnetic stand, open the lid and air-dry the beads until cracks just appear (about 5 min).

4.8 Remove the tube from the magnetic stand, add 52 μL ddH_2O directly. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min. Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 3 min), transfer 50 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

4.9 Add 40 μL Hieff NGS™ DNA Selection Beads (0.8×, Beads:DNA=0.8:1) to the step 4.8. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.10 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 3 min), aspirate the supernatant and discard.

4.11 Keep the tube on the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

4.12 Repeat step 4.11 once for a total of two washes.

4.13 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube in the magnetic stand, open the lid and air-dry the selection beads until cracks just appear (about 5 min).

4.14 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

4.15 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Step 5 Library Amplification

In this step, the adapter-ligated DNA is amplified with PCR amplification after cleanup or size selection.

5.1 Thaw the reagents in Table 23. Mix thoroughly and place them on ice for later use.

5.2 Add the following reagents to the product of step 4:

Table 23 PCR reaction system for adapter ligation

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

[Note] Primer mix for MGI is not included, and you need Yeasen Cat#13334(Hieff NGS™ Primer Mix for MGI®).

5.3 Mix thoroughly by vortexing or pipetting several times. Spin the reaction solution briefly to the bottom of the tube.

5.4 Place the PCR tube to a thermocycler and run the reaction programs in Table 24.

Table 24 Reaction programs for PCR amplification

Temperature	Duration	Cycles
98°C	1 min	1
98°C	10 sec	11~15cycles*
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1
4°C	Hold	-

[Note]: *The number of library amplification cycles needs to be adjusted according to the sample quality, input volume and other conditions for library construction. See Caution 3 for details.

Step 6 Cleanup and Size Selection of PCR product

6.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature for at least 30 min. Prepare 80% ethanol.

6.2 Resuspend the beads thoroughly by vortexing or shaking the bottle.

6.3 Add 45 μL Hieff NGS™ DNA Selection Beads (0.9×, Beads:DNA=0.9:1) to the PCR product. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

6.4 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 5 min), aspirate the supernatant and discard.

6.5 Keep the tube in the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

6.6 Repeat step 6.5 once for a total of two washes.

6.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube in the magnetic stand, open the lid and air-dry the beads until cracks just appear (about 5 min).

6.8 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

6.9 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Step 7 DNA Library Quality Control

The quality of the constructed DNA library can be evaluated by concentration detection and size distribution detection. For details, please refer to Caution 5.

Appendix I: Demonstration of mRNA Fragmentation Effects

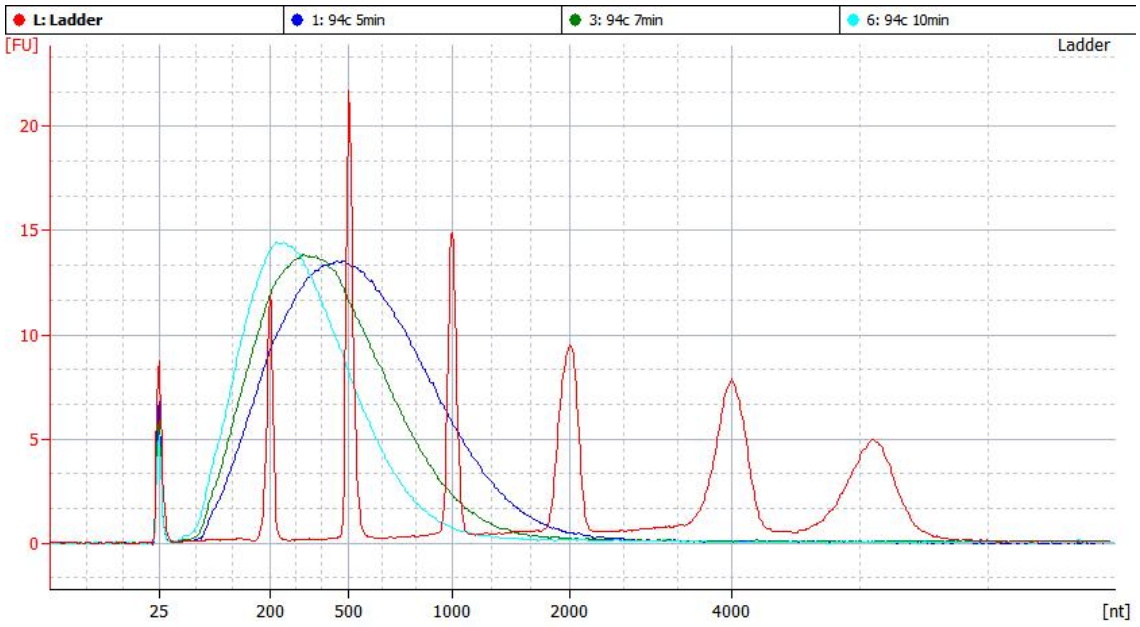


Figure 2 The range of RNA fragments corresponding to different fragmentation time of mRNA.

[Note] They were treated at 94°C for 10 min, 94°C for 7 min and 94°Cfor 5 min, respectively. After that, mRNA was purified by 2.2x magnetic beads and detected by Agilent 2100 Bioanalyzer.

Appendix II: Illustration for library size selection for Illumina®

The plan is suitable for the construction of a library with fragmented RNA at 94°C, 10 min, 94°C, 7 min and 94°C, 5 min, and a library with an insert larger than 200 bp can be obtained:

Plan A: Size Selection after Adapter-ligated DNA

1 Adapter-ligated DNA purification with 0.6×Hieff NGS™ DNA Selection Beads

1.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature (~ 30 min). Prepare 80% ethanol.

1.2 Resuspend the beads thoroughly by vortexing or shaking.

1.3 Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to the adapter ligation product. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

1.4 Spin the tube briefly and place it carefully discard the supernatant.

1.5 Keep the tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to the tube, and incubate at room temperature for 30 sec. Carefully remove the supernatant.

1.6 Repeat step 1.5 once for a total of two washes.

1.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube on the magnetic stand, and air-dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

1.8 Remove the tube from the magnetic stand and elute the DNA with 102 μL ddH_2O directly. Mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min. Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 100 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

2 Two-round size selection (The protocol below is for libraries with a 410–510 bp insert size with fragmentation at 94°C for 7 min. For libraries with different size fragment inserts, refer to the recommended ratio)

2.1 Resuspend the beads thoroughly by vortexing or shaking.

2.2 Refer to Table 17 or 18, add 65 μL (0.65×) magnetic beads to the above 100 μL DNA. Mix thoroughly by vortex or pipetting 10 times.

2.3 Incubate at room temperature for 5 min.

2.4 Spin the tube briefly and place it on the magnetic stand. When the solution is clear (about 5 min), transfer the supernatant to a new PCR tube.

2.5 Refer to Table 17, add 15 μL (0.15×) of magnetic beads to the supernatant.

2.6 Mix thoroughly by vortexing or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.

2.7 Spin the tube briefly and place it on a magnetic stand. When the solution is clear (about 5 min), carefully remove the supernatant.

2.8 Keep the tube on the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to the tube and incubate at room temperature for 30 sec. Carefully remove the supernatant.

2.9 Repeat step 2.8 once for a total of two washes.

2.10 Remove residual ethanol with a 10 μL - pipette tip. Air dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

2.11 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

2.12 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Table 17 Recommended conditions for beads-based size selection for short adapter

Inserted DNA size (bp)	**200~300**	**250~350**	**350~450**	**450~550**
Final DNA library size (bp)	**260~360**	**310~410**	**410~510**	**510~610**
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	80 (0.8×)	75 (0.75×)	65 (0.65×)	60 (0.6×)
Volume ratio in the 2nd round (Beads:DNA)	15 (0.15×)	15 (0.15×)	15 (0.15×)	10 (0.1×)

Table 18 Recommended conditions for beads-based size selection for long adapter

Inserted DNA size (bp)	**200~300**	**250~350**	**350~450**	**450~550**
Final DNA library size (bp)	**320~420**	**370~470**	**470~570**	**570~670**
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	75 (0.75×)	70 (0.7×)	65 (0.65×)	60 (0.6×)
Volume ratio in the 2nd round (Beads:DNA)	15 (0.15×)	15 (0.15×)	15 (0.15×)	10 (0.1×)

[Note]: "×" in the table indicates the volume of DNA sample. For example, if the insert DNA is 300 bp and the sample DNA volume is 100 μL, the volume of magnetic beads used in the first round of size selection is 0.65×100 μL=65 μL; the volume of magnetic beads used in the second round is 0.15× 100 μL=15 μL.

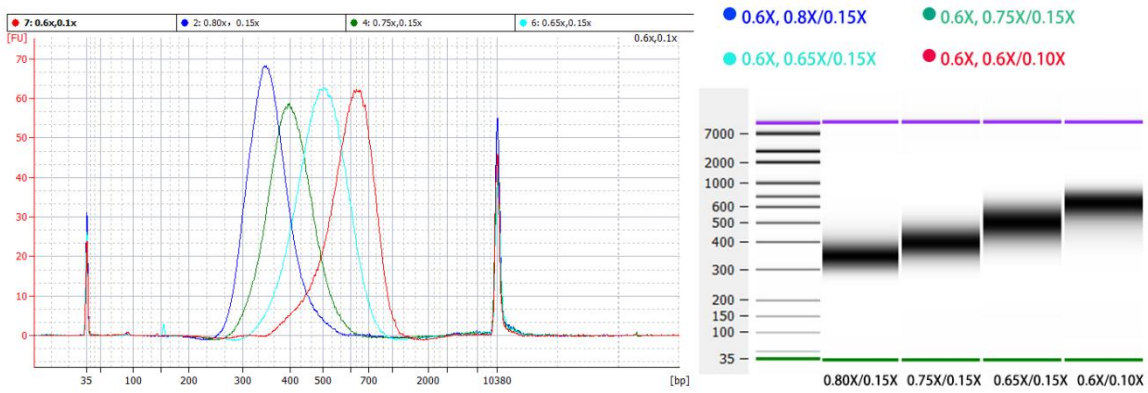


Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°C for 10 min, 94°C for 7 min and 94°C for 5 min, according to the recommended using different bead/DNA ratios of Table 17.

Plan B：Size Selection of Adapter-ligated DNA

Refer to Table 19 or Table 20 to perform size selection

2.1 Resuspend the beads thoroughly by vortexing or shaking.

2.2 Refer to Table 19, add 20 μL (0.2×) magnetic beads to the above 100 μL DNA. Mix thoroughly by vortex or pipetting 10 times.

2.3 Incubate at room temperature for 5 min.

2.4 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer the supernatant to a new PCR tube.

2.5 Refer to Table 19, add 10 μL (0.10×) magnetic beads to the supernatant.

2.6 Mix thoroughly by vortex or pipetting up and down at least 10 times. Incubate at room temperature for 5 min.

2.7 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), carefully remove the supernatant.

2.8 Keep the tube on the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads and incubate at room temperature for 30 sec. Carefully remove the supernatant.

2.9 Repeat step 2.8 once for a total of two washes.

2.10 Remove residual ethanol with a 10 μL - pipette tip. and air-dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

2.11 Remove the tube from the magnetic stand. Elute DNA from the beads with 21 μL ddH_2O and mix thoroughly by vortex or pipetting up and down 10 times. Incubate at room temperature for 5 min.

2.12 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Table 19 Recommended conditions for beads-based size selection for short adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Final DNA library size (bp)	260~360	310~410	410~510	510~610
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	25 (0.25×)	25 (0.25×)	20 (0.2×)	18 (0.18×)
Volume ratio in the 2nd round (Beads:DNA)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

Table 20 Recommended conditions for beads-based size selection for long adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Final DNA library size (bp)	320~420	370~470	470~570	570~670
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	25 (0.25×)	18 (0.18×)	20 (0.2×)	18 (0.18×)
Volume ratio in the 2nd round (Beads:DNA)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

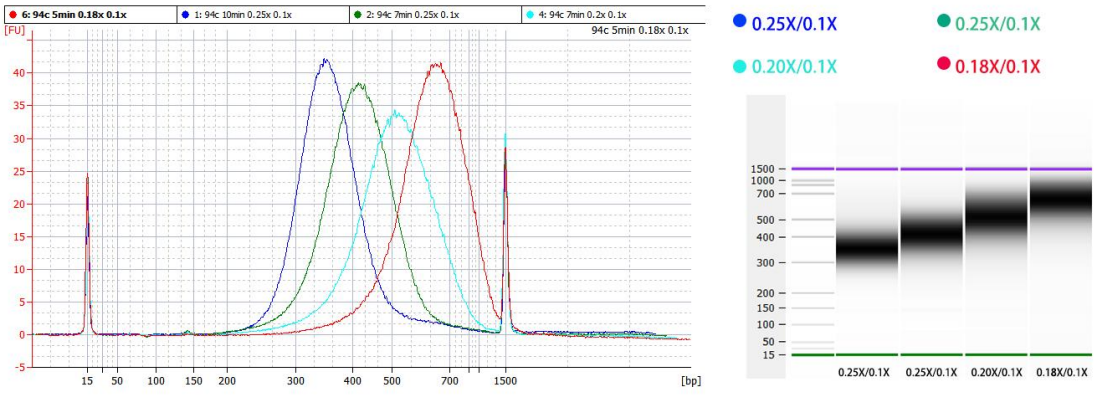


Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°C for 10 min, 94°C for 7 min and 94°Cfor 5 min, according to the recommended using different bead/DNA ratios of Table 19.

Appendix III: Illustration for library size selection for MGI®

The sorting scheme is suitable for constructing a library with fragmented RNA at 94°C, 10 min, 94°C, 7 min and 94°C, 5 min. The product is a library with an insert larger than 200 bp:

Plan A: Selection after purification of the linker ligation product

1 Purification adapter ligation product with 0.6×Hieff NGS™ DNA Selection Beads

1.1 Equilibrate the Hieff NGS™ DNA Selection Beads at room temperature (~ 30 min). Prepare 80% ethonal.

1.2 Resuspend the beads thoroughly by vortexing or shaking the bottle.

1.3 Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to the adapter ligation product. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

1.4 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 5 min), aspirate the supernatant and discard.

1.5 Keep the tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

1.6 Repeat step 1.5 once for a total of two washes.

1.7 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube in the magnetic stand, open the lid and air-dry the beads until cracks just appear (about 5 min).

1.8 Remove the tube from the magnetic stand and elute the DNA,add 102 μL ddH_2O directly. Mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min. Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 100 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

[Note]: The high concentration of PEG contained in Ligation Enhancer had an effect on the double round separation of magnetic beads, so the double round separation had to be carried out after a round of purification.

2 Two-round sorting. (Take the 380-480bp library which undergo the 94°C, 7min fragmentation l as an example. Other library sizes are sorted by magnetic beads according to the recommended ratio.)

2.1 Resuspend the beads thoroughly by vortexing or shaking the bottle.

2.2 Refer to Table 17, add the first round of magnetic beads 65 μL (0.65×) to the above 100 μL DNA elute. Mix thoroughly by vortexing or pipetting 10 times.

[Note]: "×" in the table indicates the volume of DNA sample. For example, if the insert length of the library is 300 bp and the sample DNA volume is 100 μL, the volume of magnetic beads used in the first round of sorting is 0.65×100 μL=65 μL; the volume of magnetic beads used in the second round of sorting is 0.15× 100 μL=15 μL.

2.3 Incubate at room temperature for 5 min.

2.4 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 5 min), transfer the supernatant to a new PCR tube.

2.5 Refer to Table 21, add 15 μL (0.15×) of magnetic beads for the second round of sorting to the supernatant.

2.6 Mix thoroughly by vortexing or pipetting up and down at least 10 times.Incubate at room temperature for 5 min.

2.7 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 3 min), aspirate the supernatant and discard.

2.8 Keep the tube in the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

2.9 Repeat step 2.8 once for a total of two washes.

2.10 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube in the magnetic stand, open the lid and air-dry the selection beads until cracks just appear (about 3 min).

2.11 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

2.12 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 3 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Table21 Recommended conditions for beads-based size selection for short adapter

Inserted DNA library size (bp)	200~300	300~400	400~500	500~600
Final DNA library size (bp)	**280~380**	**380~480**	**480~580**	**580~680**
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	70 (0.7×)	65 (0.65×)	58 (0.58×)	50 (0.5×)
Volume ratio in the 2nd round (Beads:DNA)	20 (0.2×)	15 (0.15×)	15 (0.15×)	15 (0.15×)

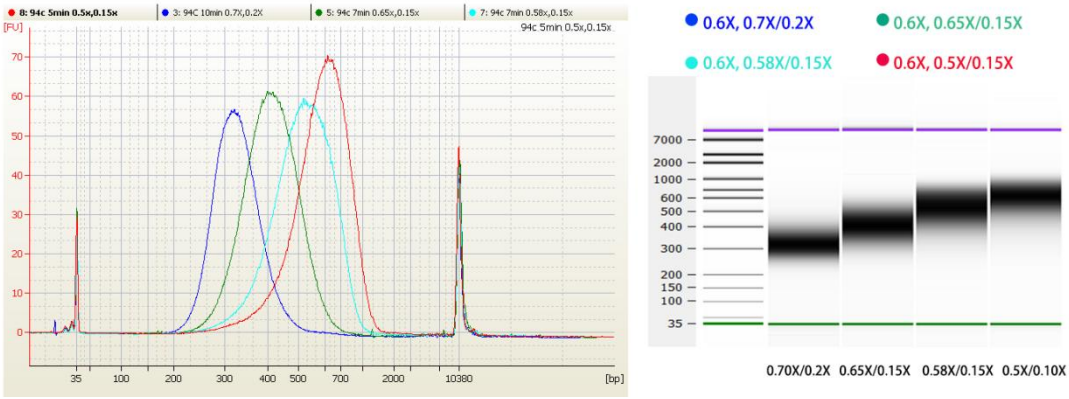


Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min, according to the recommended ratio of magnetic beads in Table 21

Plan B：Direct sorting of linker ligation products (take 94°C, 7 min fragmentation, sorting library size of 410 bp ~ 510 bp as an example, other library sizes are sorted by magnetic beads according to the recommended ratio)

The total RNA of 500 ng or more is used for mRNA capture and then the library is built. It is recommended to sort directly. The system is relatively viscous and needs to be added carefully. Samples with slightly poor RNA quality may have residual adapters.

2.1 Resuspend the beads thoroughly by vortexing or shaking the bottle.

2.2 Refer to Table 18, add the first round of magnetic beads 20 μL (0.2×) to the above 100 μL DNA elute. Mix thoroughly by vortexing or pipetting 10 times.

2.3 Incubate at room temperature for 5 min.

2.4 Spin the tube briefly and place it onto magnetic stand. When the solution is clear (about 5 min), transfer the supernatant to a new PCR tube.

2.5 Refer to Table 22, add 10 μL (0.10×) of magnetic beads for the second round of sorting to the supernatant.

2.6 Mix thoroughly by vortexing or pipetting up and down at least 10 times. Incubate at room temperature for 10 min.

2.7 Spin the tube briefly and place it on the magnetic stand. When the solution is clear (about 3 min), aspirate the supernatant and discard.

2.8 Keep the tube in the magnetic stand. Add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads and incubate at room temperature for 30 sec. Aspirate the ethanol and discard.

2.9 Repeat step 2.8 once for a total of two washes.

2.10 Remove residual ethanol with a 10 μL - pipette tip. Keep the tube in the magnetic stand, open the lid and air-dry the selection beads until cracks just appear (about 3 min).

2.11 Remove the tube from the magnetic stand. Add 21 μL ddH_2O and mix thoroughly by vortexing or pipetting up and down 10 times. Incubate at room temperature for 5 min.

2.12 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 3 min), transfer 20 μL of supernatant to a new PCR tube carefully without touching the magnetic beads.

Table 22 Recommended conditions for beads-based size selection for short adapter

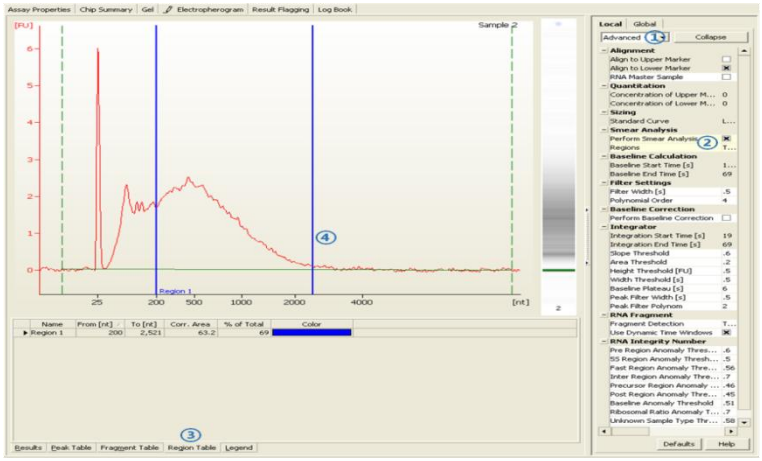
Inserted DNA library size (bp)	200~300	300~400	400~500	500~600
Final DNA library size (bp)	280~380	380~480	480~580	580~680
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
Volume ratio in the 1st round (Beads:DNA)	25 (0.25×)	20 (0.2×)	15 (0.15×)	15 (0.15×)
Volume ratio in the 2nd round (Beads:DNA)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

Appendix IV: FFPE sample library

1 FFPE RNA quality control

The rRNA depletion library construction can be used for low-quality Total RNA samples such as FFPE, but the library construction conditions required to be adjusted according to the FFPE sample quality The conventional parameter for evaluating the quality of RNA samples is the RIN value, however, the RIN value cannot be used to accurately evaluate the quality of the degraded sample such as FFPE. DV_{200} indicated the proportion of RNA fragments larger than 200 nt in the sample is required to evaluate the quality of sample. For severely degraded FFPE samples, the DV_{200} value can better reflect the quality of the sample.

DV_{200} calculation method：



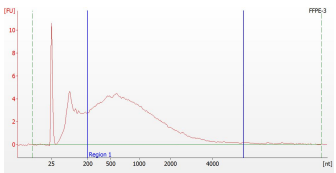
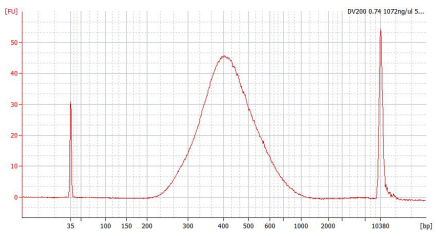
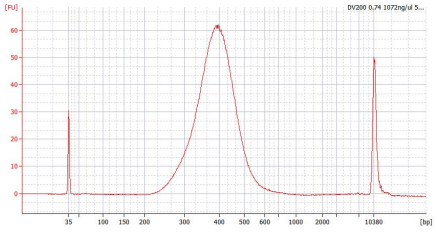
① Select “Advanced” under “Local”

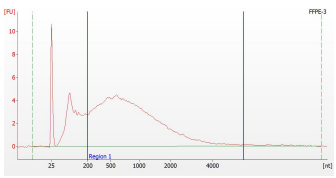
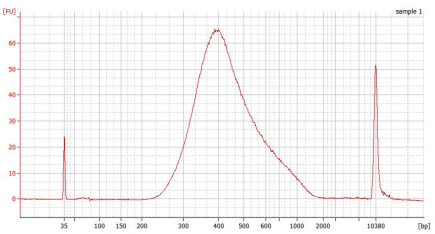
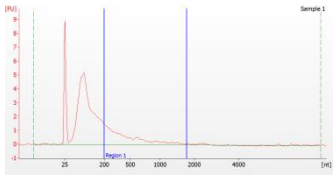
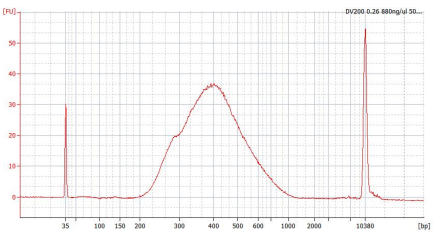
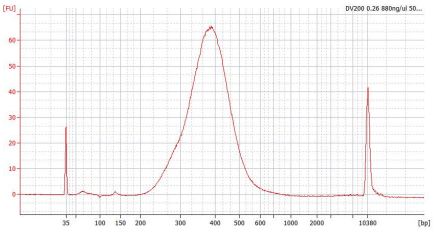
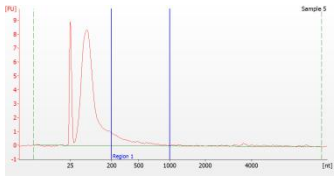
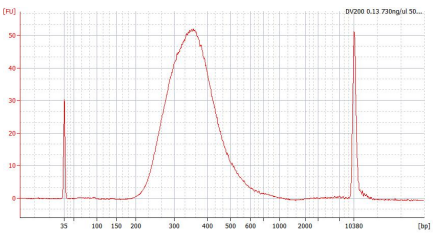
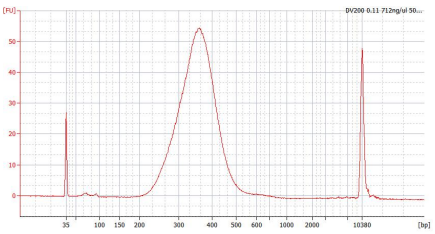
② Check the “Perform Smear Analysis” option under “Smear Analysis”

③ Select the “Region Table” page, right-click with the mouse, and select “Add Region”

④ Adjust the range of the indicator line to get the percentage of the selected segment range “% of Total”

2 FFPE RNA library example

RNA sample quality control	conditions	Library distribution quality control
 RIN=2.2; DV_{200}=74%	Input RNA：500 ng Fragmentation conditions：94℃，7 min Two purifications after ligation of the adapter：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：717.2 ng	
	Input RNA：500 ng Fragmentation conditions：94℃，7min Purification/size selection after adapter ligation：0.6×；07×/0.15× Chain-specific library amplification：13cycles Library yield：437.8 ng	

 RIN=2.2; DV_{200}=74%	Input RNA：100 ng Fragmentation conditions：94℃，7 min Purification/size selection after adapter ligation：0.6×；0.8× Chain-specific library amplification：15cycles Library yield： 206.8ng	
 RIN=2.5; DV_{200}=26%	Input RNA：500 ng Fragmentation conditions：85℃，8 min Purification/size selection after adapter ligation：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：207 ng	
	Input RNA：500 ng Fragmentation conditions：85℃，8 min Purification/ size selection after adapter ligation：0.6×；0.70×/0.15× Chain-specific library amplification：13cycles Library yield：98.56 ng	
 RIN=2.5; DV_{200}=11%	Input RNA：500 ng Fragmentation conditions：65℃，8 min Purification/ size selection after adapter ligation：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：354.2 ng	
	Input RNA：500 ng Fragmentation conditions：65℃，8 min Purification/ size selection after adapter ligation：0.6×；0.70×/0.15× Chain-specific library amplification：13cycles Library yield：172.48 ng	

BRING VALUES TO CUSTOMERS
BUILD A HEALTHIER AND HAPPIER WORLD

Yeasen Biotechnology (Shanghai) Co., Ltd.

Tel: 400-6111-883
Sales: marketing@yeasen.com
Support: marketing@yeasen.com
Service: marketing@yeasen.com
Web: www.yeabio.com