

Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit(Stranded & Non-stranded)

12308ES

INSTRUCTIONS FOR USE

Ver. EN20260724

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CONTENT

PRODUCT DESCRIPTION.....	1
SPECIFICATIONS.....	1
COMPONENTS.....	1
STORAGE.....	2
INSTRUCTIONS.....	2
NOTES.....	12
APPENDIX.....	13

Product description

Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit is a total RNA library preparation 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 routine 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 preparation to the greatest extent.

Specifications

Cat.No.	12308ES08 / 12308ES24 / 12308ES96/12308ES98
Size	8 T / 24 T / 96 T/1000 T

Components

Components No.	Name	12308ES08	12308ES24	12308ES96	12308ES98
12308-A	 2× Frag/Prime Buffer	80 µL	250 µL	930 µL	9.7 mL
12308-B	 1st Strand Enzyme Mix	16 µL	48 µL	192 µL	2 mL
12308-C	 Strand Specificity Reagent	50 µL	150 µL	580 µL	6.042 mL
12308-D	 2nd Strand Buffer (dNTP)	240 µL	720 µL	2×1440 µL	30 mL
12308-E	 2nd Strand Buffer (dUTP)	240 µL	720 µL	2×1440 µL	30 mL
12308-F	 2nd Strand Enzyme Master Mix	40 µL	120 µL	480 µL	5 mL
12308-G	 Ligation Enhancer	240 µL	720 µL	2×1440 µL	30 mL
12308-H	 Novel T4 DNA Ligase	40 µL	120 µL	480 µL	5 mL
12308-I	 2×Super Canace™ II High-Fidelity Mix	200 µL	600 µL	2×1200 µL	25 mL
12308-K	 Nuclease Free H ₂ O	100 µL	300 µL	1000 µL	1000 mL
12308-*	* Primer mix	NA	NA	NA	NA

[Note]*: Primer mix is a necessary reagent for the library with completed adapter(short adapter not required), but it is not included in this kit and needs to be configured separately. The components of this kit are compatible with both Illumina and MGI platforms, but additional primer mixes specific to Illumina or MGI(Cat# 13335 Primer Mix for Illumina and Cat# 13334 Primer Mix for MGI) are required.

Storage

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

Instructions

Please read carefully before experimenting:

I. About Adapter Ligation

1. Our company can provide Illumina or MGI complete adapter (Barcoded Adapter) kits and short adapter kits, and customers can choose according to experimental requirements.
2. We recommend the use of high-quality commercial adapters. If customers use homemade adapters, please entrust a company with experience in NGS primer synthesis and note that strict anti-contamination control is required. In addition, when performing adapter annealing operations, please complete it in a clean bench. Only operate one adapter at a time to prevent cross contamination.
3. When using adapters, please take out the adapters in advance and thaw them at 4°C or on an ice box; when operating at room temperature, the laboratory temperature should not exceed 25°C to prevent the adapters from melting.
4. During the library preparation process, too high or too low adapter concentrations will lead to a low library preparation success rate. In the operation plan of this kit, the adapter volume added is fixed at 5 µL. Please refer to Table 1 to dilute the adapter according to the initial RNA input. Please choose 0.1×TE buffer as the adapter diluent. The diluted adapter can be stored at 4°C for 48 h.

Table 1-1 Recommended Input Total RNA Amount and Illumina Adapter Concentration

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 Input Total RNA Amount and MGI Adapter Concentration

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

[Note]: * The amount of Adapter used can be adjusted according to different types of total RNA samples and input amounts.

II. About Library Amplification

1. The library amplification component in this kit is composed of our company's second-generation high-fidelity DNA polymerase. Based on the first generation, it greatly enhances the uniformity of amplification, and even low-copy genes can be amplified without preference.
2. The library amplification step requires strict control of the number of amplification cycles. Insufficient cycles will lead to low library yield; excessive cycles will lead to increased library preference, increased repetitiveness, increased chimeric products, accumulation of amplified mutations and other adverse consequences. Table 2 lists

the recommendations for the input total RNA amount and the corresponding amplification cycle number for library amplification using this kit.

3. The recommended cycle number in Table 2 can meet most library preparation needs. If your sample quality is poor (such as severely degraded FFPE samples), the cycle number can be appropriately increased according to the actual situation. Please pay special attention when constructing mRNA libraries. Since the content of mRNA in Total RNA extracted from different species and tissues varies greatly, the number of amplification cycles needs to be appropriately adjusted according to the starting amount of library preparation, species type and sample processing conditions in the experiment.

Table 2 Recommended Input Total RNA Amount and Amplification Cycle Number*

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] : * Since the library yield is not only related to the input amount and the number of amplification cycles, but also sample quality, fragmentation conditions, sorting conditions, etc. will affect the yield. During the library preparation process, please consider the actual situation and choose the most appropriate library preparation conditions.

III. Bead-based Cleanup and Size Selection

1. There are multiple steps in the library preparation process that require the use of DNA purification magnetic beads. We recommend using Hieff NGS™ DNA Selection Beads (Yeasten Cat#12601) or AMPure XP magnetic beads (Beckman Cat#A63880) for DNA purification and sorting.
2. The magnetic beads should be equilibrated to room temperature before use, otherwise the yield will decrease and the sorting effect will be poor.
3. The magnetic beads should be fully vortexed or pipetted up and down to mix thoroughly before each use.
4. When transferring the supernatant, do not aspirate the magnetic beads, even a trace amount of residue will affect the quality of the subsequent library.
5. The 80% ethanol used for washing the magnetic beads should be prepared fresh, otherwise it will affect the recovery efficiency.
6. The magnetic beads should be dried at room temperature before eluting the product. Insufficient drying can easily cause anhydrous ethanol residues to affect subsequent reactions; excessive drying can cause the magnetic beads to crack and reduce the purification yield. Normally, drying at room temperature for 3-5 min is sufficient to allow the beads to dry fully.
7. If the DNA purification or length sorting products need to be stored, they can be eluted with 0.1×TE Buffer. The products can be stored at 4°C for 2 days and at -20°C for 1 month.

IV. Library Quality Analysis

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

2. Library concentration detection can be performed using: methods based on double-stranded DNA fluorescent dyes, such as Qubit®, PicoGreen®, etc.; methods based on qPCR absolute quantification.
3. It is recommended to use the qPCR method for library concentration detection: When using concentration determination methods based on double-stranded DNA fluorescent dyes such as Qubit®, it is impossible to effectively distinguish products with single-end connected to Adapter, products without Adapter connected to both ends, and other incomplete double-stranded structure products; qPCR absolute quantification is based on the principle of PCR amplification, and only quantifies libraries with complete Adapters at both ends in the sample (i.e., libraries that can be sequenced), which can exclude the interference of non-sequenceable libraries that are not connected to Adapters at either one end or both ends.
4. Library concentration detection cannot be performed using spectral detection methods, such as NanoDrop®.
5. Library length distribution detection can be performed using devices based on capillary electrophoresis or microfluidics, such as Agilent Bioanalyzer 2100.

V. Other Materials

1. **mRNA enrichment Kit:** Hieff NGS™ mRNA Isolation Master Kit V2(Yeasen Cat#12629).
2. **rRNA depletion Kit:** Hieff NGS™ MaxUp rRNA Depletion Kit ((rRNA & ITS/ETS)) (Yeasten Cat#12257).
3. **RNA Cleaner:** Hieff NGS™ RNA Cleaner (Yeasten Cat#12602) or other equivalent products.
4. **DNA Cleaner:** Hieff NGS™ DNA Selection Beads (Yeasten Cat#12601) or AMPure® XP Beads (A63880) or other equivalent products.
5. **RNA quality control:** Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico Chip or other equivalent products.
6. **Adapters:** Complete Adapter for Illumina™ (Yeasten Cat#13519-13520 or other equivalent products) or Complete Adapter for MGI™ (Yeasten Cat#13360-13362 or other equivalent products).
7. **Library quality control:** Agilent 2100 Bioanalyzer DNA 1000 Chip/ High Sensitivity Chip or other equivalent products.
8. **Other materials:** ethanol, Sterilized ddH₂O, PCR tube, magnetic stand, thermal cycler etc.

The workflow of RNA library preparation kit

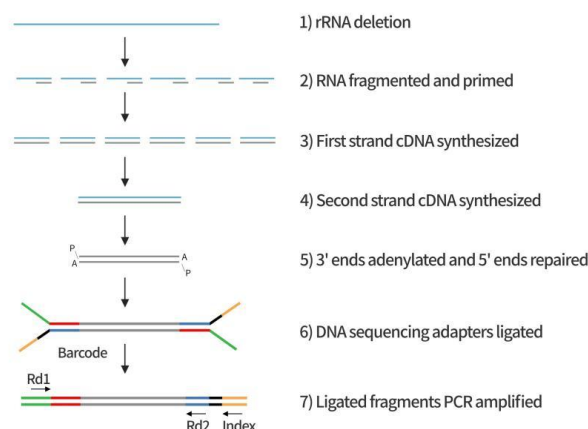


Figure 1. The workflow of RNA library preparation kit

Protocol

Part I. Enrichment and fragmentation of target RNA

Preparation of target RNA before library preparation. According to the requirements of library preparation, 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 preparation.

Scheme A: mRNA Purification and Fragmentation

1. Sample requirements

The Hieff NGS™ mRNA Isolation Master Kit V2(Yeasen Cat#12629) 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.

2. Operations Steps

- 2.1. Take out the mRNA Capture Beads from 2-8°C, and equilibrate at room temperature for at least 30 min.
- 2.2. 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.
- 2.3. 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.
- 2.4. 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.
- 2.5. Place the tube on a magnetic stand for 5 minutes to separate mRNA from total RNA. Carefully remove the supernatant.
- 2.6. 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.
- 2.7. Repeat step 6.
- 2.8. Remove the tube from the magnetic stand. Add 50 µL Tris Buffer to resuspend the magnetic beads and pipette 6 times to mix thoroughly.
- 2.9. Put the sample in a thermal cycler and run the following program to elute the mRNA: 80°C, 2 min; 25°C, hold.
- 2.10. Remove the sample from the thermal cycler. Add 50 µL Beads Binding Buffer and pipette repeatedly 6 times to mix thoroughly.
- 2.11. Incubate at room temperature for 5 minutes to allow mRNA to bind to the magnetic beads.
- 2.12. Place the tube on the magnetic stand for 5 minutes, and carefully remove the supernatant.

2.13. Remove the tube from the magnetic stand, resuspend the magnetic beads with 200 μ L 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 μ L pipette is required to aspirate the remaining liquid. Prepare 1 $\times$ Frag/Prime Buffer in advance (use Nuclease Free H₂O equal volume mixing configuration, such as configuring a reaction system: 9.5 μ L 2 $\times$ Frag/Prime Buffer + 9.5 μ L Nuclease Free H₂O)

2.14. Remove the tube from the magnetic stand and resuspend the magnetic beads with 19 μ L 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 library size (bp)	Fragmentation time
200-300	94°C, 10 min, 4°C hold
300-400	94°C, 7 min, 4°C hold
400-500	94°C, 5 min, 4°C hold

2.15. 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 μ L of the supernatant to a new nuclease-free PCR tube (Part II/III- Step 1).

Scheme B: rRNA Depletion and RNA Fragmentation

1. Sample requirements

Hieff NGS™ MaxUp Human rRNA Depletion Kit (rRNA & ITS/ETS) (Yeast Cat#12257) is applicable to remove rRNA from total RNA. Suitable for 100 ng~1 μ g (volume $\leq$ 11 μ L) total RNA samples from human, mouse, and rat; suitable for complete RNA or partially degraded RNA (such as FFPE RNA) samples.

2. Operation Steps

Step1 Probe Hybridization to RNA

1. Take out the probe and hybridization buffer from -20°C, thaw and mix by inversion, and place on ice for later use.
2. Prepare RNA samples: Calculate the RNA sampling volume based on the input amount and sample concentration, and dilute to 11 μ L with Nuclease Free H₂O.
3. Prepare the rRNA removal reaction system in a 200 μ L PCR tube according to Table 4.

Table 4 RNA/Probe hybridization reaction

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

4. Use a pipette to gently pipette and mix, and centrifuge the reaction solution to the bottom of the tube.
5. Place the above PCR tube in a PCR instrument (which can be set to a gradient cooling mode) and perform the probe hybridization reaction according to the reaction program shown in Table 5.

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

Step2 RNase H Digestion

1. Take out the RNase H digestion reagent from -20 °C, thaw it, mix it by inversion, and place it on ice for later use. Prepare the RNase H digestion reaction system as shown in 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

[Note] : RNase H Buffer and RNase H need to be added separately. If the mix is required due to a large amount of sample, please prepare it before use, otherwise it will affect the removal effect.

2. Use a pipette to gently pipette and mix, and centrifuge the reaction solution to the bottom of the tube.
3. Place the above PCR tube in a PCR thermocycler and set the reaction program: hot cover 50°C; 37°C, 30 min; 4°C, hold, and perform RNase H digestion reaction.

Step 3. DNase I Digestion

1. Take out the DNase I digestion reagent from -20 °C, thaw it, mix it by inversion, and place it on ice for later use. Prepare the DNase I digestion reaction system as shown in 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

2. Use a pipette to gently blow and mix, and centrifuge the reaction solution to the bottom of the tube.
3. Place the above PCR tube in the PCR instrument and set the reaction program: hot cover 50°C; 37°C, 30min; 4°C, hold, and perform DNase I digestion reaction.

Step 4 RNA purification

1. Preparation: Take the Hieff NGS™ RNA Cleaner (Yeast Cat#12602) magnetic beads out of the refrigerator and equilibrate at room temperature for at least 30 min. Prepare 80% ethanol with Nuclease Free H₂O.
2. Vortex or invert the magnetic beads to ensure thorough mixing.
3. Pipette 110 μL Hieff NGS™ RNA Cleaner (2.2×, Beads:DNA=2.2:1) into the product of the previous step, pipette thoroughly to mix, and incubate at room temperature for 5 min.

4. Place the PCR tube in a magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 5 min), carefully remove the supernatant.
5. Keep the PCR tube in the magnetic rack, add 200 μ L Nuclease free H₂O freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
6. Repeat step 5 for a total of two rinses. Use a 10 μ L pipette to remove any remaining liquid.
7. Keep the PCR tube in the magnetic rack and dry the magnetic beads at room temperature with the lid open (5~10 min).

[Note]: Prepare 1 $\times$ Frag/Prime Buffer in advance (mix with Nuclease Free H₂O in equal volumes, such as a reaction system: 9.5 μ L 2 $\times$ Frag/Prime Buffer + 9.5 μ L Nuclease Free H₂O)

8. RNA elution: Remove the PCR tube from the magnetic rack, add 19 μ L Frag/Prime buffer, use a pipette to gently pipette until fully mixed, and let stand at room temperature for 5 min.
9. Centrifuge the PCR tube briefly and place it in the magnetic rack. After the solution is clear (about 3 min), carefully transfer 17 μ L of supernatant to a new Nuclease Free PCR tube for RNA fragmentation.
10. RNA fragmentation conditions need to be adjusted according to sample quality. For high-quality RNA samples, the fragmentation conditions can refer to the mRNA fragmentation conditions (Table 3). Table 8 recommends the fragmentation conditions for FFPE samples of different quality. However, there will be certain differences in the fragmentation effects of different samples. Customers can compare different fragmentation conditions according to their own sample conditions and choose appropriate fragmentation conditions.
11. After the fragmentation is completed, please place it on ice immediately and enter the first-chain synthesis reaction (Part II/Part III-Step 1).

Table 8 Recommended FFPE RNA fragmentation conditions

DV200*	Fragmentation time
>70%	94°C, 7 min, 4°C hold
50%~70%	94°C, 5 min, 4°C hold
20%~50%	85°C, 8 min, 4°C hold

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

Part II: Illumina platform RNA library preparation

Step 1 Synthesis of the first strand cDNA (1st Strand cDNA Synthesis)

This step synthesizes the enriched/fragmented target RNA into a single-strand cDNA. The enrichment of the target RNA can be done by selecting the Poly(A) mRNA isolation scheme or the rRNA Depletion scheme according to the experimental requirements and sample conditions, see Part I for details.

1. Take out the first-strand synthesis reagent from -20°C, invert and mix, and then centrifuge. Prepare the reaction solution for the first-strand cDNA synthesis as shown in Table 9.

Table 9 First-strand cDNA synthesis reaction system

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

2. Use a pipette to gently blow and mix, and centrifuge the reaction solution to the bottom of the tube.
3. Place the above PCR tube in a PCR instrument, set the reaction program as shown in Table 10, and synthesize the first-strand cDNA. After the reaction is completed, immediately synthesize the second-strand cDNA.

Table 10 First-strand cDNA synthesis reaction program

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&Endprep&dA-Tailing

1. Take out the second strand synthesis reagent from -20 °C, thaw and mix by inversion; prepare the second strand cDNA synthesis/dA-Tailing reaction solution as shown in Table 11.

Table 11 Second Strand cDNA Synthesis Reaction System

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

[Note]: *If you are constructing a general mRNA library, please use a buffer containing dNTP; if you are constructing a chain-specific mRNA library, please use a buffer containing dUTP.

2. Use a pipette to gently blow and mix, and centrifuge the reaction solution to the bottom of the tube.
3. Place the above PCR tube in a PCR instrument, set the reaction program as shown in Table 12, and synthesize the second-strand cDNA.

Table 12 Second-strand cDNA synthesis reaction program

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

Step 3 Adapter Ligation

This step connects specific Illumina adapters to the ends of the products after end repair and dA tail addition.

1. Refer to Table 1-1 and dilute the adapter to the appropriate concentration according to the amount of Input RNA.
2. Thaw each reagent in Table 13, invert and mix, and place on ice for later use.
3. Continue to prepare the reaction system shown in Table 13 in the PCR tube after step 2.

Table 13 Adapter Ligation System

Components	Volume (μL)
dA-tailed DNA	60
Ligation Enhancer	30*

Novel T4 DNA Ligase	5
DNA Adapter	5**
Total	100

[Note]: * Before using Ligation Enhancer, please turn it upside down, shake it, mix it thoroughly and centrifuge it for a short time before use.

** The original concentration of our adapter is 15 μ M. Please dilute the adapter according to the input amount according to the instructions in Table 1-1 of Note 2, so that the adapter addition volume is fixed at 5 μ L.

4. Use a pipette to gently blow and mix, and centrifuge it briefly to collect the reaction solution to the bottom of the tube.

5. Place the PCR tube in the PCR instrument, set the reaction program shown in Table 14, and perform the adapter ligation reaction:

Table 14 Adapter Ligation Reaction Program

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

Step 4 Post Ligation Clean Up

This protocol is applicable to fragments <200 bp. Residual adapters in the system are removed by two purifications. When the insert fragment is $\geq$ 200 bp, refer to the sorting protocol in Appendix 2 to obtain a library of target length by purification and sorting.

Applicable to libraries with insert fragments <200 bp (two rounds of purification are required)

1. Preparation: Take the Hieff NGS™ DNA Selection Beads out of the refrigerator and equilibrate at room temperature for at least 30 min. Prepare 80% ethanol.
2. Vortex or invert the beads to ensure thorough mixing.
3. Pipette 60 μ L Hieff NGS™ DNA Selection Beads (0.6 $\times$, Beads:DNA=0.6:1) into the Adapter Ligation product, vortex or pipette to mix, and incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it on a magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 5 min), carefully remove the supernatant.
5. Keep the PCR tube in the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
6. Repeat step 5 for a total of two rinses.
7. Keep the PCR tube in the magnetic rack, open the lid and air dry the magnetic beads until they just crack (no more than 5 min).
8. Remove the PCR tube from the magnetic rack, add 52 μ L ddH₂O, vortex or use a pipette to gently blow until fully mixed, and let it stand at room temperature for 5 min. Centrifuge the PCR tube briefly and place it on a magnetic rack to stand. After the solution is clear (about 3 min), carefully transfer 50 μ L of supernatant to a new PCR tube and perform another round of purification.
9. Pipette 40 μ L Hieff NGS™ DNA Selection Beads (0.8 $\times$, Beads:DNA=0.8:1) into the product of the previous step, vortex or pipette to mix, and incubate at room temperature for 5 min.

10. Centrifuge the PCR tube briefly and place it on a magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 3 min), carefully remove the supernatant.
11. Keep the PCR tube in the magnetic rack, add 200 μ L freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
12. Repeat step 11 for a total of two rinses.
13. Keep the PCR tube in the magnetic rack, open the lid and air dry the magnetic beads until they just crack (no more than 5 min).
14. Remove the PCR tube from the magnetic rack, add 21 μ L ddH₂O, vortex or use a pipette to gently pipette to mix thoroughly, and let it stand at room temperature for 5 min. Centrifuge the PCR tube briefly and place it on a magnetic stand. After the solution becomes clear (about 3 min), carefully transfer 20 μ L of the supernatant to a new PCR tube for PCR amplification.

Step 5 Library Amplification

This step will perform PCR amplification and enrichment on the purified or length-sorted adapter ligation products.

1. Thaw the reagents in Table 15, invert and mix, and place on ice for later use.
2. Prepare the reaction system shown in Table 15 in a sterile PCR tube.

Table 15-A PCR reaction system for short adapter-ligated products

Components	Volume (μ L)
2 $\times$ 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 system for long adapter-ligated products

Components	Volume (μ L)
2 $\times$ Super Canace™ II High-Fidelity Mix	25
Primer Mix**	5
Adapter Ligated DNA	20
Total	50

[Note]: *If you are using an adapter without an index, commonly known as a short adapter (small Y adapter), please use the index primer provided in the short adapter reagent (Cat#12414-12415) for amplification.

**If you are using an Indexed Adapter (Cat#13519~ Cat#13520), commonly known as a long adapter (large Y adapter), you can use the Hieff NGS™ Primer Mix for Illumina in Cat#13335 for amplification.

3. Use a pipette to gently blow or oscillate to mix, and briefly centrifuge to collect the reaction solution to the bottom of the tube.
4. Place the PCR tube in the PCR instrument, set the reaction program shown in Table 16, and perform PCR amplification.

Table 16 PCR amplification reaction program

Temperature	Time	Cycles
Hot lid 105°C	on	
98°C	1 min	1
98°C	10 sec	11~15 cycles*
60°C	30 sec	
72°C	30 sec	
72°C	5 min	1

4°C	Hold	-
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[Note]: *The number of library amplification cycles needs to be adjusted according to the sample quality, input amount and other library preparation conditions, see Note 3 for details.

Step 6 Post Amplification Clean Up

1. Preparation: Take the Hieff NGS™ DNA Selection Beads out of the refrigerator and equilibrate at room temperature for at least 30 min. Prepare 80% ethanol.
2. Vortex or invert the beads to ensure thorough mixing.
3. Pipette 45 µL Hieff NGS™ DNA Selection Beads (0.9×, Beads:DNA=0.9:1) into the Adapter Ligation product, vortex or pipette to mix, and incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the beads and liquid. After the solution is clear (about 5 min), carefully remove the supernatant.
5. Keep the PCR tube in the magnetic rack, add 200 µL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
6. Repeat step 5 for a total of two rinses.
7. Keep the PCR tube in the magnetic rack, open the lid and air dry the magnetic beads until they just crack (no more than 5 min).
8. Remove the PCR tube from the magnetic rack, add 21 µL ddH₂O, vortex or use a pipette to gently blow until fully mixed, and let it stand at room temperature for 5 min. Centrifuge the PCR tube briefly and place it in the magnetic rack to stand. After the solution is clarified (about 3 min), carefully transfer 20 µL of supernatant to a new PCR tube for library quantification and quality inspection.

Step 7 Library quality control

Usually, the quality of the constructed library can be evaluated by concentration detection and length distribution detection. For details, please refer to Note 5.

Part III: MGI platform RNA library preparation

The operation steps are the same as Part II, but it is important to use the MGI platform adapter. If the full-length adapter(complete adapter) is used, the library amplification step can be amplified with the Hieff NGS™ Primer Mix for MGI™ in Cat#13334 (*This primer mix for MGI is not included in this kit). If the UDI adapter is used, the library amplification step uses the barcode primer of the UDI adapter kit.

Notes

1. This product is for research use only.
2. Please thaw the components of the kit at room temperature before use. After thawing, turn it upside down several times to mix thoroughly, centrifuge it briefly, and place it on ice for later use.
3. It is recommended to perform each step of the reaction in a PCR instrument with a hot cover. The PCR instrument should be preheated to near the reaction temperature before use.
4. Please use consumables without RNase contamination and clean the experimental area regularly. It is recommended to use ThermoFisher's RNAZap™ high-efficiency nucleic acid removal spray to remove RNase contamination.

5. PCR products are very likely to produce aerosol contamination due to improper operation, which will affect the accuracy of experimental results. It is recommended to physically isolate the PCR reaction system preparation area and the PCR product purification and detection area; equip with equipment such as special pipettes for library preparation; and clean each experimental area regularly (it is recommended to use ThermoFisher's DNAZap™ high-efficiency nucleic acid removal spray) to ensure the cleanliness of the experimental environment.

6. Please operate with lab coats and disposable gloves, for your safety.

Appendix

Appendix 1: Demonstration of mRNA fragmentation effect

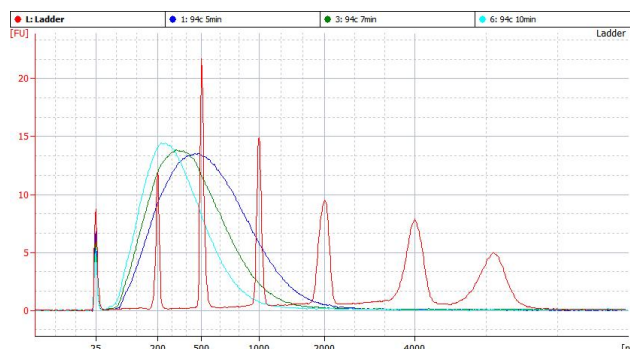


Figure 2 The range of RNA fragments corresponding to different mRNA shearing times.

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

Appendix II: DNA size selection for the Illumina platform

The sorting scheme is suitable for RNA fragmentation at 94°C, 10 min, 94°C, 7 min, and 94°C, 5 min. It can obtain libraries with insert fragments larger than 200 bp:

Scheme 1: Purification of adapter ligation products and size selection

Purification of 0.6X Hieff NGS™ DNA Selection Beads adapter ligation products

1. Preparation: Take the Hieff NGS™ DNA Selection Beads magnetic beads out of the refrigerator and equilibrate at room temperature for at least 30 min. Prepare 80% ethanol.
2. Vortex or invert the magnetic beads to ensure sufficient mixing.
3. Pipette 60 μ L Hieff NGS™ DNA Selection Beads (0.6 $\times$, Beads:DNA=0.6:1) into the Adapter Ligation product, vortex or pipette to mix, and incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it in a magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 5 min), carefully remove the supernatant.
5. Keep the PCR tube in the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
6. Repeat step 5 for a total of two rinses.
7. Keep the PCR tube in the magnetic rack, open the lid and air dry the magnetic beads until they just crack (no more than 5 min).
8. Remove the PCR tube from the magnetic rack, add 102 μ L ddH₂O, vortex or use a pipette to gently blow until fully mixed, and let it stand at room temperature for 5 min. Centrifuge the PCR tube briefly and place it in a magnetic

rack to stand. After the solution is clear (about 5 min), carefully transfer 100 μ L of supernatant to a new PCR tube and prepare for double-round size selection.

Double round DNA size selection (using 94°C, 7min RNA fragmentation, and selection library size of 410 bp~510 bp as an example. Other library sizes are sorted by magnetic beads according to the recommended ratio)

When short adapters (small Y adapters) are selected for post-ligation purification, and double-ended 384 Index Primers: Hieff NGS™ RNA 384 CDI Primer Kit for Illumina, Set 1~Set 2 (Cat#12414~Cat#12415) or Hieff NGS™ Stubby UDI Primer Kit for Illumina, PE adapter plus, set1~set 4(Cat#12327-12330) are used for RNA library preparation, the sorting ratio is based on Table 17. When long adapters (large Y adapters) are selected, Indexed Adapters: Hieff NGS™ Complete Adapter Kit for Illumina, Set 1~Set 4 (Cat#13519~Cat#13520) or Hieff NGS™ Full UDI Adapter Kit for Illumina, set1~set4 (Cat#12333~12336) are used for post-ligation purification, and the sorting ratio is based on Table 18.

1. Please vortex or invert the magnetic beads to ensure mixing.
2. According to the DNA fragment length requirements, refer to Table 17, add 65 μ L (0.65 $\times$) of the first round of sorting magnetic beads to the above 100 μ L DNA, and vortex or pipette 10 times to mix.
3. Incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it on a magnetic rack. After the solution is clear (about 5 min), carefully transfer the supernatant to a clean centrifuge tube, leaving 1-2 μ L of solution at the bottom of the tube.
5. Refer to Table 17 and add 15 μ L (0.15 $\times$) of the second round of sorting magnetic beads to the supernatant.
6. Vortex or pipette 10 times to mix, and let it stand at room temperature for 5 min.
7. Centrifuge the PCR tube briefly and place it on a magnetic rack. After the solution is clear (about 3 min), carefully remove the supernatant.
8. Keep the PCR tube in the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
9. Repeat step 8.
10. Keep the PCR tube in the magnetic rack, open the lid and dry the magnetic beads until they just crack (about 3 min).
11. Remove the PCR tube from the magnetic rack, add 21 μ L ddH₂O, vortex or use a pipette to gently blow to mix thoroughly, and let it stand at room temperature for 5 min.
12. Centrifuge the PCR tube briefly and place it in the magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 3 min), carefully transfer 20 μ L of supernatant to a clean tube.

Table 17 Recommended magnetic bead ratios for Illumina library with short adapter

Insert DNA size (bp)	200~300	250~350	350~450	450~550
Library size (bp)	260~360	310~410	410~510	510~610
fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume Beads in the 1st round (μ L)	80 (0.8 $\times$)	75 (0.75 $\times$)	65 (0.65 $\times$)	60 (0.6 $\times$)
Volume Beads in the 2nd round (μ L)	15 (0.15 $\times$)	15 (0.15 $\times$)	15 (0.15 $\times$)	10 (0.1 $\times$)

Table 18 Recommended magnetic bead ratios for Illumina library with complete adapter

Insert DNA size (bp)	200~300	250~350	350~450	450~550
Library size (bp)	320~420	370~470	470~570	570~670
fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume Beads in the 1st round (μL)	75 (0.75×)	70 (0.7×)	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 size selection conditions in Table 17 and Table 18 is applicable to Hieff NGS™ DNA Selection Beads; “×” indicates the volume of sample DNA. For example, when the range of peak of the required library insert is 350 bp, if the adapter-ligated DNA volume with short adapter is 100 μL, the volume of the magnetic beads used in the first round of size selection is 0.65×100 μL=65 μL, and the volume of magnetic beads in the second round is 0.15×100 μL=15 μL; if the adapter-ligated DNA with long adapter, the volume of the 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.

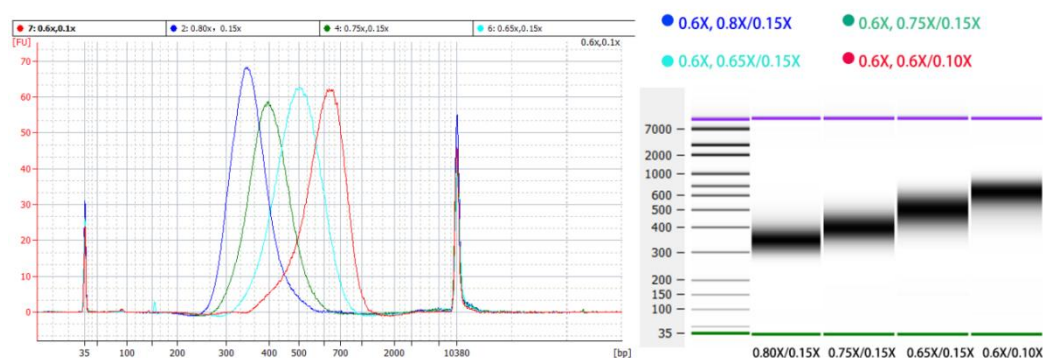


Figure 3. Library sizes obtained with 1 μg 293 total RNA after fragmentation at 94°C, 10 min, 94°C, 7 min, and 94°C, 5 min, according to the bead ratios recommended in Table 17.

Scheme 2: Direct size selection of adapter ligation products (using 94°C, 7 min fragmentation, and sorting library size of 410 bp~510 bp as an example, other library sizes are sorted by magnetic beads according to the recommended ratio)

Direct size selection is recommended for mRNA capture and library preparation after more than 500 ng of total RNA. The system is relatively viscous and needs to be added carefully. Samples with slightly poor RNA quality may have adapter residues.

When short adapters (small Y adapters) are selected for post-ligation purification, use the double-ended 384 Index Primers: Hieff NGS™ RNA 384 CDI Primer Kit for Illumina, Set 1~Set 2 (Cat#12414-12415) or Hieff NGS™ Stubby UDI Primer Kit for Illumina, PE adapter plus, set1~set 4(Cat#12327-12330) for RNA library preparation, and DNA size selection conditions for library preparation refer to Table 19. refer to Table 19 for size selection ratio. When long adapters (large Y adapters) are selected, use Indexed Adapters: Hieff NGS™ Complete Adapter Kit for Illumina, Set 1~Set 4 (Cat#13519~Cat#13520) or Hieff NGS™ Full UDI Adapter Kit for Illumina, set1~set4 (Cat#12333~12336) for post-ligation purification, and size selection conditions for library preparation refer to Table 20.

1. Please vortex or invert the beads to ensure mixing.

2. According to the DNA fragment length requirements, refer to Table 19, add 20 μL (0.20 $\times$) of the first round of sorting beads to the above 100 μL ligation system, and vortex or pipette 10 times to mix. Incubate at room temperature for 10 min.
3. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 5 min), carefully transfer 100 μL of the supernatant to a clean centrifuge tube.
4. Refer to Table 19 to add 10 μL (0.10 $\times$) of the second round of magnetic beads to the supernatant.
5. Vortex or pipette 10 times to mix, and let stand at room temperature for 10 min.
6. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 3 min), carefully remove the supernatant.
7. Keep the PCR tube in the magnetic rack, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
8. Repeat step 7.
9. Keep the PCR tube in the magnetic rack, open the lid and dry the magnetic beads until they just crack (about 3 min).
10. Remove the PCR tube from the magnetic rack, add 21 μL ddH₂O, vortex or use a pipette to mix thoroughly, and let stand at room temperature for 5 min.
11. Centrifuge the PCR tube briefly and place it on the magnetic rack to separate the beads and liquid. After the solution is clear (about 3 min), carefully transfer 20 μL of the supernatant to a clean tube.

Table 19 Recommended size selection conditions for libraries with short adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Library size (bp)	260~360	310~410	410~510	510~610
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume of Beads for the 1st round (μL)	25 (0.25 $\times$)	25 (0.25 $\times$)	20 (0.2 $\times$)	18 (0.18 $\times$)
Volume of Beads in for the 2nd round	10 (0.1 $\times$)	10 (0.1 $\times$)	10 (0.1 $\times$)	10 (0.1 $\times$)

Table 20 Recommended size selection conditions for libraries with complete adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Library size (bp)	320~420	370~470	470~570	570~670
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume of Beads for the 1st round (μL)	25 (0.25 $\times$)	18 (0.18 $\times$)	20 (0.2 $\times$)	18 (0.18 $\times$)
Volume of Beads in for the 2nd round (μL)	10 (0.1 $\times$)	10 (0.1 $\times$)	10 (0.1 $\times$)	10 (0.1 $\times$)

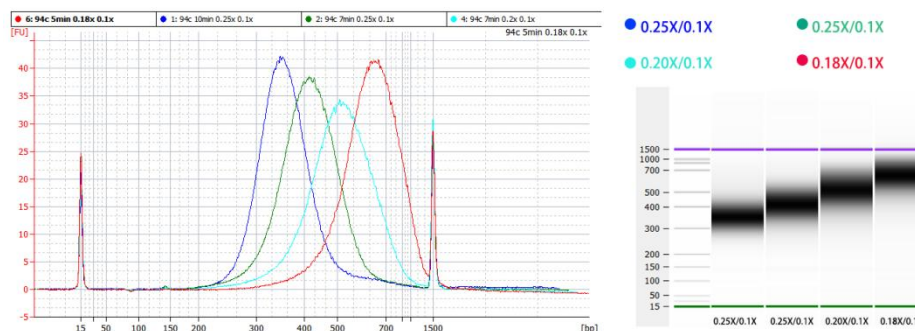


Figure 4. Library sizes obtained with the recommended bead ratios in Table 19 for 1 µg 293 total RNA after fragmentation at 94°C for 10 min, 94°C for 7 min, and 94°C for 5 min.

Appendix II: DNA size selection for the MGI platform

The sorting scheme is suitable for RNA fragmentation at 94°C, 10 min, 94°C, 7 min, and 94°C, 5 min. It can obtain libraries with insert fragments larger than 200 bp:

Scheme 1: Purification of adapter ligation products and size selection

Purification of 0.6X Hieff NGS™ DNA Selection Beads adapter ligation products

1. Preparation: Take the Hieff NGS™ DNA Selection Beads magnetic beads out of the refrigerator and equilibrate at room temperature for at least 30 min. Prepare 80% ethanol.
2. Vortex or invert the magnetic beads to ensure sufficient mixing.
3. Pipette 60 µL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) into the Adapter Ligation product, vortex or pipette to mix, and incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it in a magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 5 min), carefully remove the supernatant.
5. Keep the PCR tube in the magnetic rack, add 200 µL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
6. Repeat step 5 for a total of two rinses.
7. Keep the PCR tube in the magnetic rack, open the lid and air dry the magnetic beads until they just crack (no more than 5 min).
8. Remove the PCR tube from the magnetic rack, add 102 µL ddH₂O, vortex or use a pipette to gently blow until fully mixed, and let it stand at room temperature for 5 min. Centrifuge the PCR tube briefly and place it in a magnetic rack to stand. After the solution is clear (about 5 min), carefully transfer 100 µL of supernatant to a new PCR tube and prepare for double-round size selection.

[Note]: The high concentration of PEG contained in Ligation Enhancer will affect the double-round sorting of magnetic beads, so it is necessary to go through a round of purification before double-round DNA size selection.

Double round DNA size selection (using 94°C, 7min RNA fragmentation, and selection library size of 380 bp~480 bp as an example. Other library sizes are sorted by magnetic beads according to the recommended ratio)

1. Please vortex or invert the beads to ensure mixing.
2. According to the DNA fragment length requirements, refer to Table 21, add 65 µL (0.65×) of the first round of sorting beads to the above 100 µL DNA, and vortex or pipette 10 times to mix.

[Note]: The two-round sorting ratio recommended in this table is applicable to Hieff NGS™ DNA Selection Beads; "x" in the table represents the sample DNA volume. If the main peak of the required library insert fragment is 300 bp, if the sample DNA volume is 100 µL after the short linker is connected, the volume of the first round of sorting beads is 0.65×100 µL=65 µL, and the volume of the second round of sorting beads is 0.15×100 µL=15 µL.

3. Incubate at room temperature for 5 min.
4. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 5 min), carefully transfer the supernatant to a clean centrifuge tube, leaving 1-2 µL of solution at the bottom of the tube.
5. Refer to Table 21 and add 15 µL (0.15×) of the second round of sorting magnetic beads to the supernatant.
6. Vortex or pipette 10 times to mix, and let stand at room temperature for 5 min.

7. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 3 min), carefully remove the supernatant.
8. Keep the PCR tube in the magnetic rack, add 200 μ L of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
9. Repeat step 8.
10. Keep the PCR tube in the magnetic rack, open the lid and dry the magnetic beads until they just crack (about 3 min).
11. Remove the PCR tube from the magnetic rack, add 21 μ L ddH₂O, vortex or use a pipette to gently blow to mix thoroughly, and let stand at room temperature for 5 min.
12. Centrifuge the PCR tube briefly and place it on the magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 3 min), carefully transfer 20 μ L of the supernatant to a clean tube.

Table 21 Recommended magnetic bead ratio for MGI library with complete adapter

Inserted DNA library size (bp)	200~300	300~400	400~500	500~600
Library size (bp)	280~380	380~480	480~580	580~680
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume of Beads for the 1st round (μ L)	70 (0.7 $\times$)	65 (0.65 $\times$)	58 (0.58 $\times$)	50 (0.5 $\times$)
Volume of Beads in for the 2nd round (μ L)	20 (0.2 $\times$)	15 (0.15 $\times$)	15 (0.15 $\times$)	15 (0.15 $\times$)

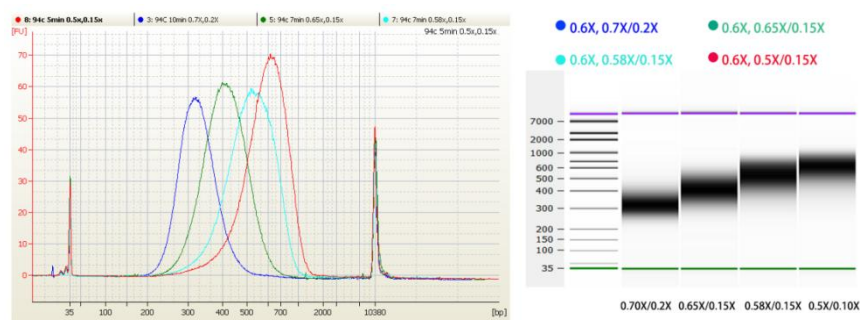


Figure 5 Library sizes obtained with 1 μ g 293 total RNA after fragmentation at 94°C, 10 min, 94°C, 7 min, and 94°C, 5 min, according to the magnetic bead ratios recommended in Table 21.

Scheme 2: Direct size selection of adapter ligation products (using 94°C, 7 min fragmentation, and sorting library size of 410 bp~510 bp as an example, other library sizes are sorted by magnetic beads according to the recommended ratio)

Direct size selection is recommended for mRNA capture and library preparation after more than 500 ng of total RNA. The system is relatively viscous and needs to be added carefully. Samples with slightly poor RNA quality may have adapter residues.

1. Please vortex or fully invert the magnetic beads to ensure uniform mixing.
2. According to the DNA fragment size requirements, add 20 μ L (0.20 $\times$) of the first round of sorting magnetic beads to the above 100 μ L ligation system, vortex or pipette 10 times to mix. Incubate at room temperature for 10 min.

[Note]: The recommended two-round sorting ratio in this table was suit for Hieff NGS™ DNA Selection Beads; "x" in the table represents sample DNA volume. For example, the main peak of the required library insertion fragment is needed to be 300 bp, if the sample DNA volume is 100 μ L after the short connector connection, the volume of magnetic beads used in the first round of separation

is $0.20 \times 100 \mu\text{L} = 20 \mu\text{L}$, and that of magnetic beads used in the second round is $0.1 \times 100 \mu\text{L} = 10 \mu\text{L}$.

3. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 5 min), carefully transfer $100 \mu\text{L}$ of the supernatant to a clean centrifuge tube.
4. Refer to Table 22 to add $10 \mu\text{L}$ ($0.10 \times$) of the second round of sorting magnetic beads to the supernatant.
5. Vortex or pipette 10 times to mix, and let stand at room temperature for 10 min.
6. Centrifuge the PCR tube briefly and place it in a magnetic rack. After the solution is clear (about 3 min), carefully remove the supernatant.
7. Keep the PCR tube in the magnetic rack, add $200 \mu\text{L}$ of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.
8. Repeat step 7.
9. Keep the PCR tube in the magnetic rack, open the lid and dry the magnetic beads until they just crack (about 3 min).
10. Remove the PCR tube from the magnetic rack, add $21 \mu\text{L}$ ddH₂O, vortex or use a pipette to gently blow to mix thoroughly, and let stand at room temperature for 5 min.
11. Centrifuge the PCR tube briefly and place it on the magnetic rack to separate the magnetic beads and liquid. After the solution is clear (about 3 min), carefully transfer $20 \mu\text{L}$ of the supernatant to a clean tube.

Table 22 Recommended ratio of magnetic beads for complete adapter library selection

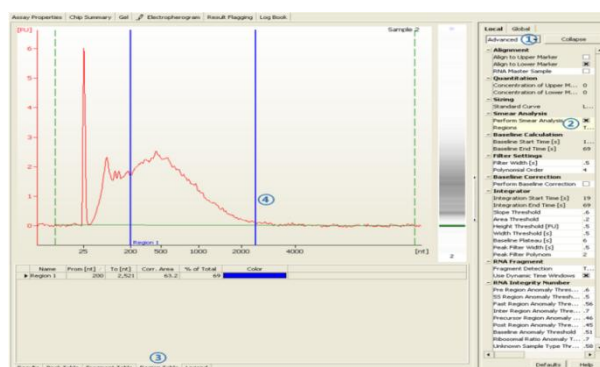
Inserted DNA library size (bp)	200~300	300~400	400~500	500~600
Library size (bp)	280~380	380~480	480~580	580~680
Fragmentation	94°C 10 min	94°C 7 min	94°C 7 min	94°C 5 min
Volume of Beads for the 1 st round (μL)	25 ($0.25 \times$)	20 ($0.20 \times$)	15 ($0.15 \times$)	15 ($0.15 \times$)
Volume of Beads in for the 2 nd round (μL)	10 ($0.1 \times$)	10 ($0.1 \times$)	10 ($0.1 \times$)	10 ($0.1 \times$)

Appendix IV: FFPE sample library preparation instructions

1. FFPE RNA quality evaluation

The rRNA removal library preparation scheme can be used for low-quality Total RNA samples such as FFPE, but due to the large quality differences between different FFPE samples, the library preparation conditions need to be adjusted according to the sample conditions. The conventional parameter for evaluating RNA sample quality is the RIN value, but for degraded samples such as FFPE, the RIN value cannot be used to accurately measure the quality of the sample. At this time, the DV200 indicator is also needed. DV200 indicates the proportion of RNA fragments greater than 200nt in the sample. For severely degraded FFPE samples, the DV200 value can better reflect the quality of the sample.

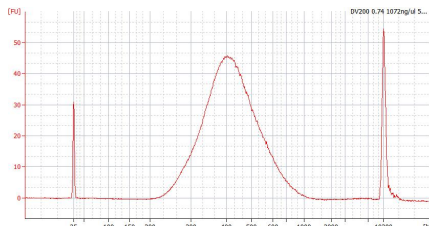
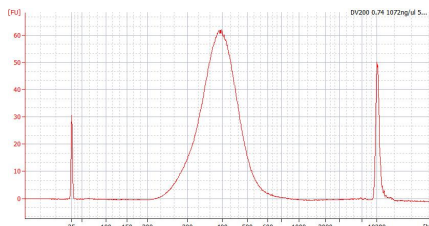
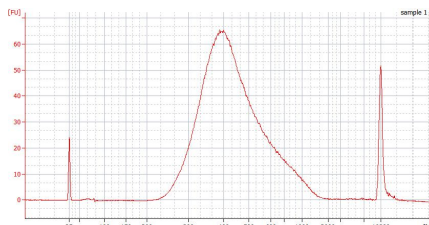
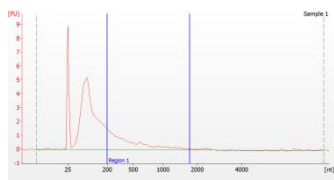
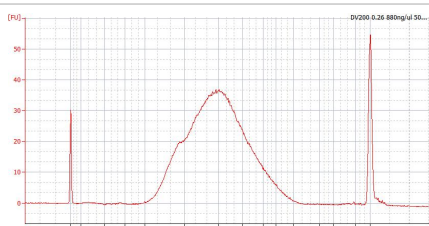
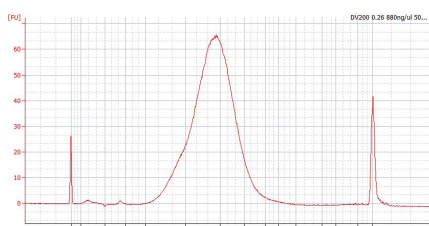
The DV200 calculation method is as follows:

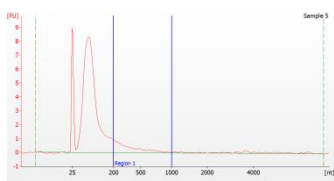
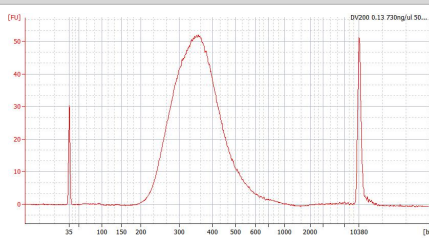
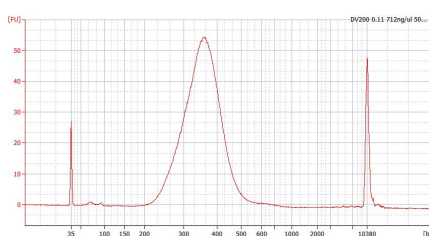


- ① In a completed Agilent 2100 Bioanalyzer result graph, select Advanced under Local
- ② Check the Perform Smear Analysis option under Smear Analysis
- ③ Select the Region Table page, right-click, and select Add Region
- ④ Adjust the range of the indicator line to get the percentage of the selected fragment range % of Total

2. FFPE RNA library preparation example

The following table shows the library distribution of FFPE samples of different quality under different library preparation conditions for reference. For library preparation with severely degraded FFPE RNA (DV200<50%) and low starting amount, we recommend using a double purification scheme after adapter ligation to reduce library loss.

RNA sample quality control	Library preparation conditions	Library distribution quality control
 RIN=2.2; DV200=74%	Input DNA: 500 ng Fragmentation conditions: 94°C, 7 min Purification twice after adapter ligation: 0.6×; 0.8×; Strand-specific library amplification: 12cycles Library yield: 717.2 ng	
	Input DNA: 500 ng Fragmentation conditions: 94°C, 7 min Purification&size selection after adapter ligation: 0.6×; 0.7×/0.15× Strand-specific library amplification: 13cycles Library yield: 437.8 ng	
	Input DNA: 100 ng Fragmentation conditions: 94°C, 7 min Purification twice after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 15cycles Library yield: 206.8ng	
 RIN=2.5; DV200=26%	Input DNA: 500 ng Fragmentation conditions: 85°C, 8 min Purification twice after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12cycles Library yield: 207 ng	
	Input DNA: 500 ng Fragmentation conditions: 85°C, 8 min Purification&size selection after adapter ligation: 0.6×; 0.70×/0.15× Strand-specific library amplification: 13cycles Library yield: 98.56 ng	

RNA sample quality control	Library preparation conditions	Library distribution quality control
 RIN=2.5; DV200=11%	Input DNA: 500 ng Fragmentation conditions: 65°C, 8 min Purification twice after adapter ligation: 0.6× ; 0.8× Strand-specific library amplification: 12cycles Library yield: 354.2 ng	
	Input DNA: 500 ng Fragmentation conditions: 65°C, 8 min Purification twice after adapter ligation: 0.6× ; 0.70×/0.15× Library yield: 13 cycles Library yield: 172.48 ng	



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Together to make a healthier and brighter world**