Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit

12308

INSTRUCTIONS FOR USE

Ver. EN20230328

CONTENT

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.

Components

Components No	Name	12309ES08	12309ES24	12309ES96
12308-A	Frag/Prime Buffer	80 μL	250 μL	930 μL
12308-B	1st Strand Enzyme Mix	16 μL	48 μL	192 μL
12308-C	Strand Specificity Reagent	50 μL	150 μL	580 μL
12308-D	2nd Strand Buffer (dNTP)	240 μL	720 μL	2×1440 μL
12308-E	2nd Strand Buffer (dUTP)	240 μL	720 μL	2×1440 μL
12308-F	2nd Strand Enzyme Master Mix	40 μL	120 μL	480 μL
12308-G	Ligation Enhancer	240 μL	720 μL	2×1440 μL
12308-H	Novel T4 DNA Ligase	40 μL	120 μL	480 μL
12308-I	2×Super Canace™ II High-Fidelity Mix	200 μL	600 μL	2×1200 μL
12308-K	Nuclease Free H_2O	100 μL	300 μL	1000 μL

Specifications

Cat.NO.	12308ES08 / 12308ES24 / 12308ES96
Size	8T / 24T / 96T
Library type	RNA library
Strand-specificity	dNTP/dUTP
Total RNA input amount	10 ng-4 μg
Library preparation total time (hr)	~5h
Instrument compatibility	Illumina or MGI Platforms
Recommended application	Gene expression

Storage

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

Instructions

1. The workflow of RNA library construction kit

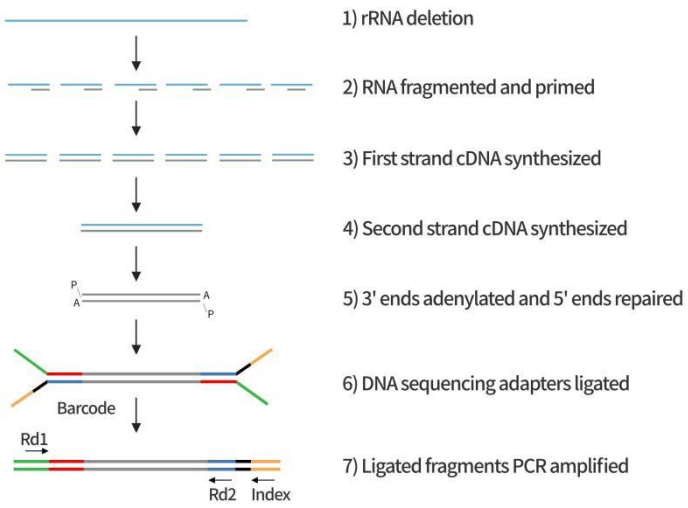

Figure 1. The workflow of RNA library construction kit

2. Other Materials

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

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

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

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

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

2.6 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).

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

2.8 Other materials: ethanol, Sterilized ddH2O, PCR tube, magnetic stand, thermal cycler etc.

2. 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.

2.1 Scheme A：mRNA Purification and Fragmentation

2.1.1 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 NGSTM 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.

2.1.2 Operations Steps

2.1.2.1. Take out the mRNA Capture Beads from 2-8°C, and equilibrate at room temperature for at least 30 min.

2.1.2.2. 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.

2.1.2.3. 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.

2.1.2.4. 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.

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

2.1.2.6. 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.

2.1.2.7. Repeat step 6.

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

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

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

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

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

2.1.2.13. 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 H2O equal volume mixing configuration, such as configuring a reaction system: 9.5 μL 2×Frag/Prime Buffer + 9.5 μL Nuclease Free H2O)

2.1.2.14. 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 1 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 1 The recommended time for mRNA fragmentation

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

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

2.2 Scheme B: rRNA Depletion and RNA Fragmentation

2.2.1 Sample requirements

Hieff NGS™ MaxUp Human rRNA Depletion Kit (rRNA & ITS/ETS) (Yeasen Cat#12257) 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.

2.2.2 Operation Steps

2.2.2.1 Probe Hybridization to RNA

2.2.2.1.1 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.

2.2.2.1.2 Prepare the following RNA/Probe hybridization reaction on ice according to Table 2.

Table 2 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

2.2.2.1.3 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.

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

Table 3 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

2.2.2.2 RNase H Digestion

2.2.2.2.1 Prepare the following RNase H digestion reaction on ice according to Table 4.

Table 4 RNase H digestion reaction

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

2.2.2.2.2 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.

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

2.2.2.3 DNase I Digestion

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

Table 5 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

2.2.2.3.2 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.

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

2.2.2.4 RNA Purification

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

2.2.2.4.2 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.

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

2.2.2.4.4 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.

2.2.2.4.5 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.

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

2.2.2.4.7 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.

2.2.2.4.8 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.

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

2.2.2.4.10 Transfer 17 μL of the supernatant to a nuclease-free tube for fragmentation according to Table 1. Table 6 recommends the fragmentation conditions of FFPE samples of different quality.

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

Table 6 Recommended FFPE RNA fragmentation conditions

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

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

3. mRNA library construction for Illumina platform

3.1 Synthesis of the first strand cDNA

3.1.1 Take the first-strand synthesis reagents from -20°C, invert and mix well, and then spin down briefly. As shown in Table 2, prepare the first-strand cDNA synthesis reaction.

Table 7 First-strand cDNA synthesis reaction system

Components	Volume (μL)
Fragmented mRNA from 3.1	17
Strand Specificity Reagent	6
1st Strand Enzyme Mix	2

3.1.2 Mix thoroughly by gently pipetting up and down at least 10 times, and then spin down briefly.

3.1.3 Incubate the sample in a preheated thermal cycler as shown in Table 8, and synthesize the first strand cDNA.

Table 8 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

3.1.4 Immediately, perform second strand cDNA synthesis reaction.

3.2 Synthesis of the second strand cDNA/end repair/dA-tailing addition

3.2.1 Take out the second-strand synthesis reagents from -20°C, thaw and mix thoroughly; prepare the second-strand cDNA synthesis/end repair/dA-tailing reaction as shown in Table 9.

Table 9 Second-strand cDNA synthesis reaction

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

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

3.2.2 Mix thoroughly by gently pipetting up and down at least 10 times, and then spin down briefly.

3.2.3 Incubate the sample in a preheated thermal cycler as shown in Table 10 to synthesize the second strand cDNA.

Table 10 Second-strand cDNA synthesis reaction program

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

3.3 Adapter Ligation

Specific Illumina adapters can be ligated into the products of end repair and dA-tailing addition in this step.

3.3.1 Refer to Table 29 and dilute the adapter to an appropriate concentration according to the amount of Input RNA.

3.3.2 Thaw the reagents in Table 6 and mix them upside down. Place them on ice.

3.3.3 Prepare the reaction mix shown in Table 6 in the PCR tube from step 3.2.

Table 11 Adapter Ligation reaction

Components	Volume (μL)
dA-tailed DNA	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 concentration of the adapter of YEASEN is 15 μM. Please dilute the adapter according to the input amount according to the tips in Table 29-1 to make the volume of the adapter fixed at 5 μL.

3.3.4 Mix thoroughly by gently pipetting up and down at least 10 times , and spin down briefly to collect all liquid from the sides of the tube.

3.3.5 Incubate the sample in a preheated thermal cycler as shown in Table 7 and perform the adapter connection reaction:

Table 12 Adapter Ligation reaction program

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

3.4 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):

3.4.1 Preparation: Take the Hieff NGS™ DNA Selection Beads from the 4°C and place it at room temperature for at least 30 minutes. Prepare 80% ethanol. Vortex or fully invert the magnetic beads.

3.4.2 Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to Adapter-ligated DNA and mix well on a vortex mixer or by pipetting up and down at least 10 times, and incubate at room temperature for 5 min. Quickly spin the tube in a microcentrifuge and place the tube on a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully discard the supernatant.

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

3.4.4 Repeat step 3.4.3 once for a total of 2 washing steps.

3.4.5 Keep the PCR tube on the magnetic stand at all times, completely remove the residual ethanol, and air-dry beads for 5 minutes while the tube is on the magnetic stand with the lid open

3.4.6 Remove the PCR tube from the magnetic stand, elute DNA target from the beads with 52 μl ddH2O. Mix well

on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature. Briefly spin the tube, and put the tube back in the magnetic stand until the solution is clear, carefully transfer 50 μL of supernatant to a new PCR tube, and perform another round of purification.

3.4.7 Add 40 μL Hieff NGS™ DNA Selection Beads (0.8×, Beads:DNA=0.8:1), vortex or pipette to mix, and incubate at room temperature for 5 min. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 3 minutes), carefully remove the supernatant.

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

3.4.9 Repeat step 3.4.8 once for a total of 2 washing steps.

3.4.10 Keep the PCR tube on the magnetic stand at all times, completely remove the residual ethanol, and ai- dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

3.4.11 Remove the PCR tube from the magnetic stand, Elute DNA target from the beads with 21 μl ddH2O. Mix well on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature. Briefly spin the tube, and put the tube back in the magnetic stand until the solution is clear, carefully transfer 20 μL of supernatant to a new PCR tube for PCR amplification.

3.5 Library Amplification

This step will carry out PCR amplification and enrichment on the adapter-ligated DNA after purification or size selection.

3.5.1 Thaw the reagents in Table 8 and mix them upside down. Put them on ice for later use.

3.5.2 Prepare the reaction mixture shown in Table 13 in a sterile PCR tube.

Table 13-A adapter-ligated DNA PCR reaction with short adapter

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 13-B Adapter-ligated DNA PCR reaction with long adapter

Components	Volume(μL)
2×Super Canace™ II High-Fidelity Mix	25
Primers 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) in needed.

3.5.3 Vortex or pipette to mix, and spin the tube briefly to collect all liquid from the sides of the tube.

3.5.4 Incubate the sample in a preheated thermal cycler as shown in Table 14, and perform PCR amplification.

Table 14 PCR amplification reaction

Temperature	Time	Cycle
98°C	1 min	1
98°C	10 sec	11~15 *
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 and other conditions for library construction. See Table 26 for details.

3.6 Clean Up Post Amplification

3.6.1 Preparation: Take out the Hieff NGS™ DNA Selection Beads from the 4°C and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol.

3.6.2 Vortex or fully invert the magnetic beads.

3.6.3 Add 45 μL Hieff NGS™ DNA Selection Beads (0.9×, Beads:DNA=0.9:1) to Adapter-ligated DNA and mix well on a vortex mixer or by pipetting up and down at least 10 times, and incubate at room temperature for 5 min.

3.6.4 Spin the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully discard the supernatant.

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

3.6.6 Repeat step 3.6.5 once for a total of 2 washing steps.

3.6.7 Keep the PCR tube on the magnetic stand at all times, completely remove the residual ethanol, and air-dry beads for 5 minutes while the tube is on the magnetic stand with the lid open (no more than 5 minutes).

3.6.8 Remove the PCR tube from the magnetic stand, Elute DNA target from the beads with 52 μl ddH2O. Mix well on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature. Briefly spin the tube, and put the tube back on the magnetic stand until the solution is clear, carefully transfer 50 μL of supernatant to a new PCR tube for library quantification and quality analysis.

3.7 DNA Library Quality Control

Generally, the quality of the constructed library can be evaluated by concentration detection and length distribution detection. Please refer to Note 5 for details.

4. mRNA library construction for MGI platform

4.1 Synthesis of the first strand cDNA

4.1.1 Take the first-strand synthesis reagents from -20°C, invert and mix well, and then leave in a flash. As shown in Table 15, prepare the first-strand cDNA synthesis reaction solution.

Table 15 First-strand cDNA synthesis reaction system

Components	Volume (μL)
Fragmented mRNA	17
Strand Specificity Reagent	6
1st Strand Enzyme Mix	2

4.1.2 Use a pipette to mix gently, and then centrifuge the reaction solution to the bottom of the tube.

4.1.3 Place the above PCR tube in a thermal cycler, set up the reaction program as shown in Table 12, and synthesize the first strand cDNA. Immediately after the reaction, the second-strand cDNA synthesis was performed.

Table 16 First-strand cDNA synthesis reaction program

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

4.2 Synthesis of the second strand cDNA/end repair/dA-tailing addition

4.2.1 Remove the second-strand synthesis reagents from -20°C, thaw and mix them upside down; follow Table 17 to prepare the second-strand cDNA synthesis/end repair/dA-tailing addition reaction solution.

Table 17 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

[Note]: *For construction a normal mRNA library, please use a buffer containing dNTP; for construction a strand-specific mRNA library, please use a buffer containing dUTP.

4.2.2 Use a pipette to mix gently, and then centrifuge the reaction solution to the bottom of the tube.

4.2.3 Place the PCR tube in a PCR machine and set up the reaction program as shown in Table18 to synthesize the second strand cDNA.

Table 18 Second-strand cDNA synthesis reaction program

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

4.3 Adapter Ligation

In this step, specific MGI adaptors can be connected to the ends of the end repair and dA-tailing products.

4.3.1 Refer to Table 29 and dilute the Adapter to an appropriate concentration according to the amount of Input RNA.

4.3.2 Thaw the reagents in Table 15 and mix them upside down. Place them on ice for later use.

4.3.3 Continue to prepare the reaction system shown in Table 19 in the PCR tube after step4.2.

Table 19 Adapter Ligation system

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

[Note]: *Ligation Enhancer should be turned upside down, shaken, mixed thoroughly and centrifuged briefly before use.

**The original concentration of the adapter of our company is 10 μM. Please dilute the adapter according to the input amount according to the tips in Table 29-2 to make the volume of the adapter fixed at 5 μL.

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

4.3.5 Place the PCR tube in the thermal cycler, set the reaction program shown in Table 20 and perform the adapter connection reaction:

Table 20 Adapter Ligation reaction program

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

4.4 Post Ligation Clean Up

This scheme is suitable for fragments <200 bp, to remove the linker residue in the system by two purifications; when the inserted fragments are ⩾200 bp, refer to the sorting scheme in Appendix III to obtain the target length by purification, sorting or direct sorting library.

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

4.4.1 Preparation: Take out the Hieff NGS™ DNA Selection Beads magnetic beads from the refrigerator and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol. Vortex or fully invert the magnetic beads to ensure adequate mixing.

4.4.2 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. Centrifuge the PCR tube briefly and place it in a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully remove the supernatant.

4.4.3 Keep the PCR tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, carefully remove the supernatant. Repeat step 4.4.2 for the twice rinsing.

4.4.4 Keep the PCR tube on the magnetic stand, open the lid and air-dry the magnetic beads until cracks just appear (no more than 5 minutes).

4.4.5 Remove the PCR tube from the magnetic stand, add 52 μL ddH_2O, vortex or pipette gently to mix well, and let it stand at room temperature for 5 minutes. Centrifuge the PCR tube briefly and place it on a magnetic stand. After the solution is clarified (about 3 minutes), carefully transfer 50 μL of supernatant to a new PCR tube, and perform another round of purification.

4.4.6 Pipette 40 μL Hieff NGS™ DNA Selection Beads (0.8×, 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. Centrifuge the PCR tube briefly and place it in a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 3 minutes), carefully remove the supernatant.

4.4.7 Keep the PCR tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, carefully remove the supernatant.

4.4.8 Repeat step 3.4.7 for a total of two washes.

4.4.9 Keep the PCR tube on the magnetic stand, open the lid and air-dry the magnetic beads until cracks just appear (no more than 5 minutes).

4.4.10 Take the PCR tube out of the magnetic stand, add 21 μL ddH2O, vortex or pipette gently until fully mixed, and let it stand at room temperature for 5 minutes. Centrifuge the PCR tube briefly and place it on a magnetic stand. After the solution clarifies (about 3 minutes), carefully transfer 20 μL of supernatant to a new PCR tube for PCR amplification.

4.5 Library Amplification

This step will carry out PCR amplification and enrichment on the adapter ligation products after purification or size sorting.

4.5.1 Thaw the reagents in Table 17 and mix them upside down. Place them on ice for later use.

4.5.2 Prepare the reaction system shown in Table 17 in a sterile PCR tube.

Table 21 PCR reaction system for products connected with short adapter

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

【Note:】Primer Mix for MGI is not included in this kit, and Hieff NGS™ Primer Mix for MGI (Yeasen Cat#13334) is needed.

4.5.3 Use a pipette to gently pipette or shake to mix, and centrifuge briefly to collect the reaction solution to the bottom of the tube.

4.5.4 Place the PCR tube in the PCR machine, set the reaction program shown in Table 18, and perform PCR amplification.

Table 22 PCR amplification reaction program

Temperature	Time	Cycle
98°C	1 min	1
98°C	10 sec	11~15 *
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 Table 30 for details.

4.6 Post Amplification Clean Up

4.6.1 Preparation: Take out the Hieff NGS™ DNA Selection Beads magnetic beads from the refrigerator and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol.

4.6.2 Vortex or fully invert the magnetic beads to ensure adequate mixing.

4.6.3 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.

4.6.4 Centrifuge the PCR tube briefly and place it in a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully remove the supernatant.

4.6.5 Keep the PCR tube on the magnetic stand, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, carefully remove the supernatant.

4.6.6 Repeat step 4.6.5 for a total of two washes.

4.6.7 Keep the PCR tube on the magnetic stand, open the lid and air-dry the magnetic beads until cracks just appear (no more than 5 minutes).

4.6.8 Take the PCR tube out of the magnetic stand, add 21 μL ddH2O, vortex or pipette gently to mix well, and let it stand at room temperature for 5 minutes. Centrifuge the PCR tube briefly and place it in a magnetic stand. After the solution clarifies (about 3 minutes), carefully transfer 20 μL of supernatant to a new PCR tube for library quantification and quality inspection.

4.7 DNA Library Quality Control

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

Appendix Ⅰ: Demonstration of mRNA Fragmentation Effects

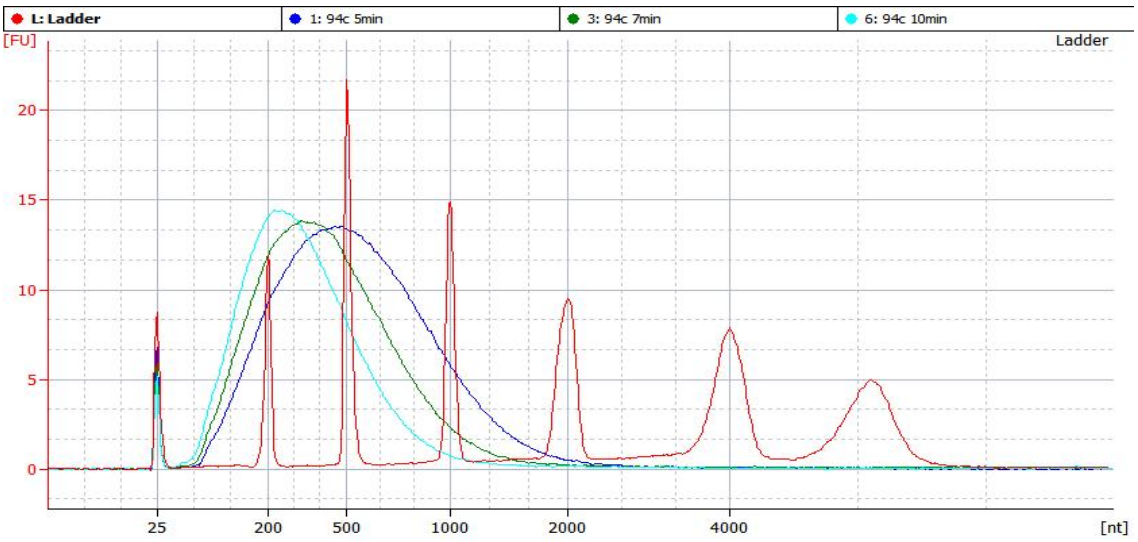

Figure 2. The range of RNA fragments corresponding to different fragmentation time of mRNA. They were treated at 94°C for 10 min, 94°C for 7 min and 94°C for 5 min, respectively. After that, mRNA was purified by 2.2x magnetic beads and detected by Agilent 2100 Bioanalyzer. [Note]: The RNA used in this result is Agilent's Universal Human Reference RNA. Had better optimize the fragmentation time if other sources of RNA is used.

Appendix II : Explanation of Size Selection Conditions for Illumina platform

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 purification

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

1. Preparation: Take the Hieff NGS™ DNA Selection Beads from the 4°C and place it at room temperature for at least 30 minutes. Prepare 80% ethanol.

2. Vortex or fully invert the magnetic beads.

3. Add 60 μL Hieff NGS™ DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to adapter ligation DNA and mix well on a vortex mixer or by pipetting up and down at least 10 times, and incubate at room temperature for 5 min.

4. Spin the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully discard the supernatant.

5. Keep the PCR tube on the magnetic stand at all times, add 200 μL of freshly prepared 80% ethanol to the tube, incubate at room temperature for 30 sec, carefully remove the supernatant.

6. Repeat step.5 once for a total of 2 washing steps.

7. Keep the PCR tube on the magnetic stand at all times, completely remove the residual ethanol, and air dry beads for 5 minutes while the tube is on the magnetic stand with the lid open.

8. Remove the PCR tube from the magnetic stand, elute DNA target from the beads with 102 μl ddH2O, Mix well on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature. Briefly spin the tube, and put the tube back in the magnetic stand until the solution is clear, carefully transfer 100 μL of supernatant to a new PCR tube for size selection

Adaptor-ligated DNA.

[Note]: The high concentration of PEG contained in Ligation Enhancer will affect the size selection of magnetic beads, so it must go through one round of purification before performing size selection.

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)

For the short adapter (small Y adapter), Hieff NGS™ RNA 384 CDI Primer Kit for Illumina, Set 1~Set 2 (Cat#12414~Cat#12415) is recommended. Size selection conditions for library construction refer to Table 19.For the long adapter (large Y adapter), Hieff NGS™ Complete Adapter Kit for Illumina, Set 1~Set 4 (Cat#12615~Cat#12618) is recommended., Size selection conditions for library construction refer to Table 20.

(1) Resuspend the Magnetic Beads thoroughly by inverting or vortexing.

(2) According to the insert DNA size, add 65 μL (0.65×) magnetic beads to the above 100 μL DNA, vortex or pipette 10 times to mix.

(3) Incubate at room temperature for 5 minutes.

(4) Spin the PCR tube briefly and place it on a magnetic stand. Wait until the solution is clear (about 5 minutes), carefully transfer the supernatant to a clean centrifuge tube, leaving 1-2 μL of solution at the bottom of the tube.

(5) Add 15 μL (0.15×) of magnetic beads to the supernatant refer to Table 19.

(6) Vortex to mix or pipette 10 times to mix, incubate at room temperature for 5 minutes.

(7) Spin the PCR tube briefly and place it on a magnetic stand. Wait until the solution is clear (about 3 minutes), carefully remove the supernatant.

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

(9) Repeat step (8).

(10) Keep the PCR tube on the magnetic stand, completely remove the residual ethanol, and air-dry beads for 3 minutes while the tube is on the magnetic stand with the lid open.

(11) Remove the PCR tube from the magnetic stand, Elute DNA target from the beads with 21 μl ddH2O, mix well on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature.

(12) Spin the PCR tube briefly and place it back in the magnetic stand until the solution is clear (about 3 minutes), carefully transfer 20 μL of supernatant to a clean PCR tube.

Table 23 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 Beads in the 1st round (μL)	80 (0.8×)	75 (0.75×)	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×)

Table 24 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 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 19 and Table 20 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 300 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.

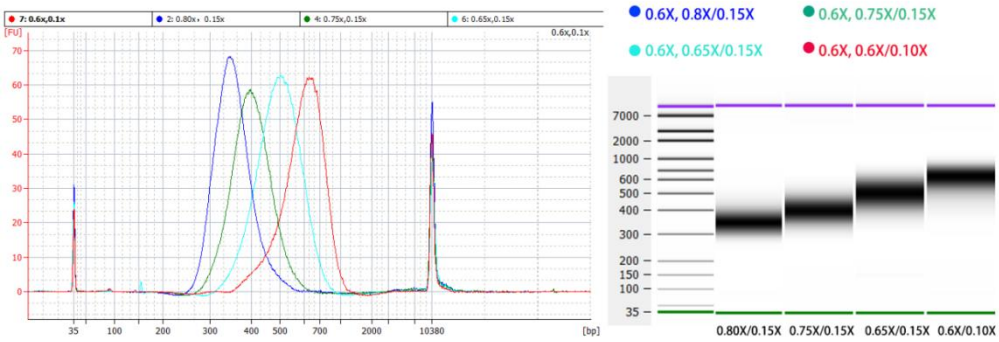

Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°C for 10 min, 94°Cfor 7 min and 94°Cfor 5 min using different bead/DNA ratios as indicated in Table 19

Plan B: Size Selection of Adapter-ligated DNA Adapter-ligated DNA (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)

Size selection was recommended to perform directly for mRNA capture from total RNA(≥500ng) and then library construction. The reaction mixture is thick and needs to be added carefully. There may be adapter residues in the sample for the RNA of poor quality.For the short adapter (small Y adapter), Hieff NGS™ RNA 384 CDI Primer Kit for Illumina, Set 1~Set 2 (Cat#12414~Cat#12415) is recommended. Size selection conditions for library construction refer to Table 21.For the long adapter (large Y adapter), Hieff NGS™ Complete Adapter Kit for Illumina, Set 1~Set 4 (Cat#12615~Cat#12618) is recommended., Size selection conditions for library construction refer to Table 22.

(1) Resuspend the Magnetic Beads thoroughly by inverting or vortexing.

(2) add 20 μL (0.20×) magnetic beads to the above 100 μL adapter-ligated DNA, vortex or pipette 10 times to mix. Incubate at room temperature for 10 min.

(3) Spin the PCR tube briefly and place it on a magnetic stand. When the solution is clear (about 5 minutes), carefully transfer 100μL of the supernatant to a clean PCR tube.

(4) Add 10 μL (0.10×) of magnetic beads to the supernatant

(5) Vortex or pipette 10 times to mix, incubate at room temperature for 10 minutes.

(6) Spin the PCR tube briefly and place it on a magnetic stand. Wait until the solution is clear (about 3 minutes), carefully remove the supernatant.

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

(8) Repeat step （7）.

(9) Keep the PCR tube on the magnetic stand, completely remove the residual ethanol, and air-dry beads for 3 minutes while the tube is on the magnetic stand with the lid open.

(10) Remove the PCR tube from the magnetic stand, elute DNA from the beads with 21 μl ddH2O. Mix well on a vortex mixer or by pipetting up and down, and incubate for 5 minutes at room temperature.

(11) Spin the PCR tube briefly and place it back in the magnetic stand until the solution is clear (about 3 minutes), carefully transfer 20 μL of supernatant to a clean PCR tube.

Table 25 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×)	25 (0.25×)	20 (0.2×)	18 (0.18×)
Volume of Beads in for the 2nd round	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

Table 26 Recommended size selection conditions for libraries with long 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×)	18 (0.18×)	20 (0.2×)	18 (0.18×)
Volume of Beads in for the 2nd round (μL)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

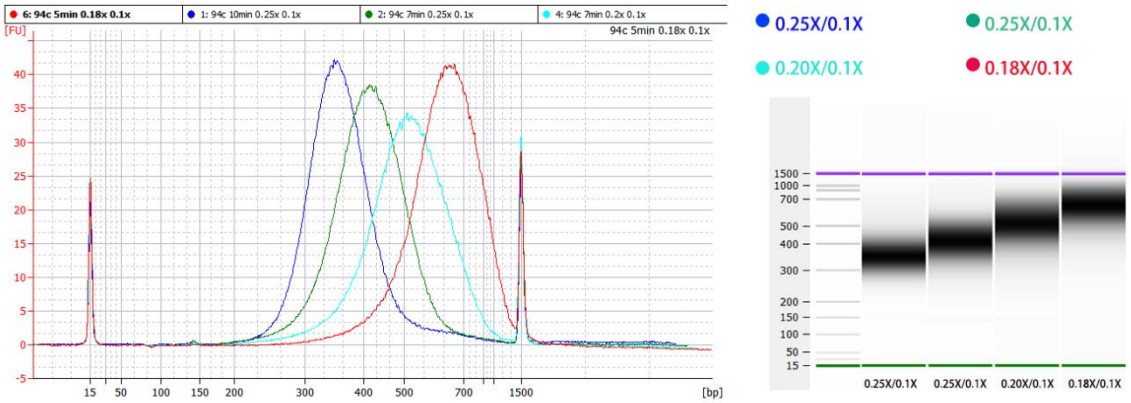

Figure 4. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°C for 7 min and 94°C for 5 min using different bead/DNA ratios as indicated in Table 21

Appendix III: Explanation of Sorting Conditions for MGI platform

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

Plan A: Sorting after purification of the linker ligation product

0.6×Hieff NGS™ DNA Selection Beads adapter ligation product

(1) Preparation: Take out the Hieff NGS™ DNA Selection Beads magnetic beads from the refrigerator and equilibrate at room temperature for at least 30 minutes. Prepare 80% ethanol.

(2) Vortex or fully invert the magnetic beads to ensure adequate 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 on a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 5 minutes), carefully remove the supernatant.

(5) Keep the PCR tube in the magnetic stand at all times, add 200 μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, carefully remove the supernatant.

(6) Repeat step 5 for a total of two washes.

(7) Keep the PCR tube on the magnetic stand at all times, open the lid and air dry the magnetic beads until cracks just appear (no more than 5 minutes).

(8) Take the PCR tube out of the magnetic stand, add 102 μL ddH2O, vortex or pipette gently to mix well, and let it stand at room temperature for 5 minutes. Centrifuge the PCR tube briefly and place it in a magnetic stand. After the solution clarifies (about 5 min), carefully transfer 100 μL of supernatant to a new PCR tube, ready to perform double rounds of sorting.

Two-round sorting (94°C, 7 min interruption, the size of the sorting library is 380 bp~480 bp as an example, other library sizes are sorted by magnetic beads according to the recommended ratio)

(1) Please vortex or fully invert the magnetic beads to ensure uniform mixing.

(2) According to the DNA fragment length requirements, add 65 μL (0.65×) of the first round of sorting magnetic beads to the above 100 μL DNA, vortex or pipette 10 times to mix.

(3) Incubate at room temperature for 5 minutes.

(4) Centrifuge the PCR tube briefly and place it on a magnetic stand. After the solution is clarified (about 5 minutes), carefully transfer the supernatant to a clean centrifuge tube, leaving 1-2 μL of solution at the bottom of the tube.

(5) add 15 μL (0.15×) of the second round of sorting magnetic beads to the supernatant.

(6) Vortex to mix or pipette 10 times to mix, and incubate at room temperature for 5 minutes.

(7) Centrifuge the PCR tube briefly and place it in a magnetic stand. After the solution is clear (about 3 minutes), carefully remove the supernatant.

(8) Keep the PCR tube on the magnetic stand, 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.

(9) Repeat step 8.

(10) Keep the PCR tube in the magnetic stand, open the lid and dry the magnetic beads until cracks just appear (about 3 minutes).

(11) Take the PCR tube out of the magnetic stand, add 21 μL ddH2O, vortex or pipette gently to mix well, and let it stand at room temperature for 5 minutes.

(12) Centrifuge the PCR tube briefly and place it in a magnetic stand to separate the magnetic beads and liquid. After the solution is clear (about 3 minutes), carefully transfer 20 μL of supernatant to a clean tube.

Table 27 Recommended ratio of magnetic beads for complete adapter library selection

Inserted DNA library size (bp)	**200~300**	**250~350**	**350~450**	**450~550**
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 (μL)	70 (0.7×)	65 (0.65×)	58 (0.58×)	50 (0.5×)
Volume of Beads in for the 2nd round (μL)	20 (0.2×)	15 (0.15×)	15 (0.15×)	15 (0.15×)

[Note]: The recommended two-round sorting ratio in this table was suit for Hieff NGS™ DNA Selection Beads; "×" 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 μL after the short connector connection, the volume of magnetic beads used in the first round of separation is 0.65×100 μL=65 μL, and that of magnetic beads used in the second round is 0.15×100 μL=15 μL.

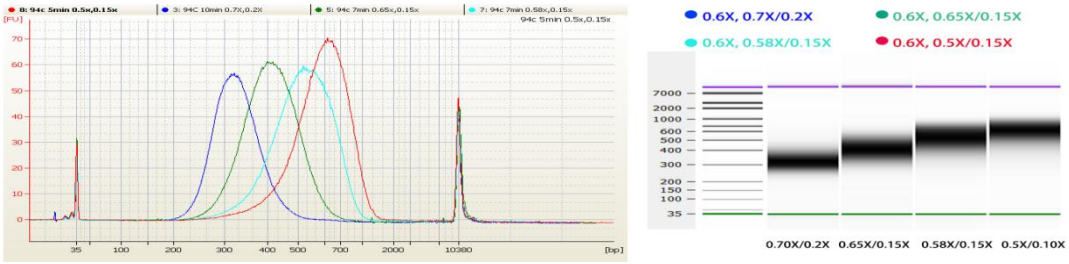

Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min

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)

It is recommended to sort directly for total RNA of 500 ng or more which used for mRNA capture and library construction. The regents are viscous and needs to be added carefully. Samples with slightly poor RNA quality may have residual adapters.

(1) Please vortex or fully invert the magnetic beads to ensure uniform mixing.

(2) According to the DNA fragment size requirements, add 20 μL (0.20×) of the first round of sorting magnetic beads to the above 100 μL ligation system, vortex or pipette 10 times to mix. Incubate at room temperature for 10 min.

Table 28 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 1st round (μL)	25 (0.25×)	20 (0.20×)	15 (0.15×)	15 (0.15×)
Volume of Beads in for the 2nd round (μL)	10 (0.1×)	10 (0.1×)	10 (0.1×)	10 (0.1×)

[Note]: The recommended two-round sorting ratio in this table was suit for Hieff NGS™ DNA Selection Beads; "×" 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 μL after the short connector connection, the volume of magnetic beads used in the first round of separation is 0.20×100 μL=20 μL, and that of magnetic beads used in the second round is 0.1×100 μL=10 μL.

(3) Centrifuge the PCR tube briefly and place it in a magnetic stand. After the solution is clarified (about 5 minutes), carefully transfer 100 μL of the supernatant to a clean centrifuge tube.

(4) add 10 μL (0.20×) of magnetic beads for the second round of sorting to the supernatant.

(5) Vortex to mix or pipette 10 times to mix, and incubate at room temperature for 10 minutes.

(6) Centrifuge the PCR tube briefly and place it on a magnetic stand. After the solution is clarified (about 3 minutes), carefully remove the supernatant.

(7) Keep the PCR tube on the magnetic stand, 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 stand, open the lid and dry the beads until cracks just appear (about 3 minutes).

(10) Take the PCR tube out of the magnetic stand, add 21 μL ddH2O, vortex or pipette gently to mix thoroughly, and let it stand at room temperature for 5 minutes.

(11) Centrifuge the PCR tube briefly and place it in a magnetic stand to separate the magnetic beads and liquid.

After the solution is clear (about 3 minutes), carefully transfer 20 μL of supernatant to a clean tube.

Notes

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.

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. Application

2.1 For research use only!

2.2 This kit is suitable for high-quality total RNA from eukaryotes such as animals, plants, and fungi with a starting input of 10ng-4 μg (volume≤50 μL). If the initial RNA concentration is low and the volume exceeds 50 μL, it is recommended to condensed the DNA 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.

2.3 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.

2.4 The library prepared by this kit can be applied to a variety of RNA-Seq, including:

- Gene expression
- Single nucleotide variation discovery
- Gene fusion identification
- Splice variant analysis

3. Adapter Ligation

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

3.2 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.

3.3 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.

3.4 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 25 lists the recommended adapter amount for different amounts of input RNA.

Table 29-1 The recommended Illumina adapter amount for different input RNA

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

Table29-2 The recommended MGI adapter amount for different input RNA

Input Total RNA	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.

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 Amplification

5.1 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.

5.2 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 26 lists the recommended cycle numbers for PCR amplification.

Table 30 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.

6. Other Materials

6.1 DNA purification magnetic beads: Hieff NGS™ DNA Selection Beads (Yeasen Cat#12601) or AMPure® XP Beads (A63880) or other equivalent products.

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

6.3 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).

6.4 Library quality analysis: Agilent 2100 Bioanalyzer DNA 1000 Chip/ High Sensitivity Chip or other equivalent products; library quantitative reagents.

6.5 Other materials: absolute ethanol, sterile ultrapure water, low retention pipette tips, PCR tube, magnetic stands, thermal cycler, etc.

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

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