HB211130

Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit for Illumina®

Cat#12301

INSTRUCTION FOR USE

Yeasen Biotechnology (Shanghai) Co., Ltd.

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Product Information

Product Name	Cat#	Specification
Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit for Illumina®	12301ES24	24 T
	12301ES96	96 T

Product Description

Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit for Illumina® is specially developed for mRNA transcriptome library construction by high-throughput sequencing platform. Compared with the traditional library construction method, this product combines cDNA second strand synthesis with Endprep and dA-tailing, which greatly reduces the time for library construction and simplifies the operation. The two types of buffers is provided in the cDNA synthesis module, which can be choosed for constructing strand-specific or conventional library as needed. This product is compatible with 10 ng-4 μg of input total RNA from eukaryotes. After mRNA isolation, fragmentation, double-stranded cDNA synthesis, end repair, dA-tailing, adaptor ligation, and library amplification, the total RNA sample is finally converted into a library suitable for sequencing on the Illumina® platform.

Hieff NGS® Ultima Dual-mode mRNA Library Prep Kit for Illumina® contains two independent modules. The core components of BOX-I is oligo (dT) magnetic beads required for mRNA purification. BOX-II contains mRNA fragmentation reagents, reverse transcription reagents, conventional and chain-specific ds-cDNA synthesis reagents, and others required for subsequent library construction. Therein dTTP is replaced with dUTP in the strand-specific two-strand synthesis Buffer, so that dUTP is incorporated into the second strand of cDNA. While the high-fidelity DNA polymerase used in this kit cannot amplify the DNA template containing uracil, achieving strand specificity. All reagents provided have undergone strict quality control and functional verification, ensuring the stability and reproducibility of library construction to the greatest extent.

Product Component

Component Number and Name				12301ES24	12301ES96
BOX-I	12603-A	○	mRNA Capture Beads	1.2 mL	4.8 mL
	12603-B	○	Beads Binding Buffer	1.2 mL	4.8 mL
	12603-C	◎	Beads Wash Buffer	15 mL	60 mL
	12603-D	○	Tris Buffer	1.2 mL	4.8 mL
BOX-II	12301-A	●	Frag/Prime Buffer	450 μL	2×900 μL
	12301-B	●	1st Strand Enzyme Mix	48 μL	192 μL
	12301-C	●	Strand Specificity Reagent	150 μL	580 μL
	12301-D	●	2nd Strand Buffer (dNTP)	720 μL	2×1440 μL
	12301-E	●	2nd Strand Buffer (dUTP)	720 μL	2×1440 μL
	12301-F	●	2nd Strand Enzyme Master Mix	120 μL	480 μL
	12301-G	●	Ligation Library length distribution detection	720 μL	2×1440 μL
	12301-H	●	Novel T4 DNA Ligase	120 μL	480 μL
	12301-I	◌	2×Super Canace® II High-Fidelity Mix	600 μL	2×1200 μL
	12301-J	◌	Primer Mix	120 μL	480 μL

Shipping and Storage

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

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

Cautions

1 Operation

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

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

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

1.4 Supplies free of RNase contamination and cleaning the experimental area regularly are necessary. ThermoFisher's RNAZap™ high-efficiency nucleic acid removal spray was recommended to remove RNase contamination.

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

2 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 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 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 Yeasen provides long adapter (Barcoded Adapter) kits and short adapter (also called small Y adapters, incomplete adapters) kits, customers can choose according to experimental needs. There are currently 48 Indexed Adapters: Hieff NGS® Complete Adapter Kit for Illumina®, Set 1~Set 4 (Cat#12615-Cat#12618); Double-ended Index Primers: Hieff NGS® RNA 384 CDI Primer Kit for Illumina®, Set 1~Set 2 (Cat#12414-12415).

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 adaptor volume added to the kit is fixed to 5ul. The adapters are recommended to be diluted with 0.1×TE buffer and the diluted adapters can be stored at 4°C for 48 hours. Table 1 lists the recommended adapter amount for different amounts of input RNA.

Table 1 The recommended 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

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 the product is eluted. Insufficient drying will easily cause residual ethanol to affect subsequent reactions; excessive drying will cause the magnetic beads tocrack 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 If Indexed Adapter (also known as long adapter or large Y adapter) is ligated to the target DNA, primer mix provided in this kit can be used for amplification; if "short adapter" or "small Y adapter" is used for DNA ligation, index primers are needed for amplification.

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

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

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

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: Long adapters with Index (Yeasen Cat#12615-12618) or short adapter without Index (Yeasen Cat#12414-12415).

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.

Instructions

1 Required Materials Not Included

1.1 Purification magnetic beads: Hieff NGS® DNA Selection Beads (Cat#12601) or AMPure XP Beads (Cat#A63880) or other equivalent products.

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

1.3 Adapters: barcoded adapter (long adapter) or short adapter without barcode.

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

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

2 Workflow

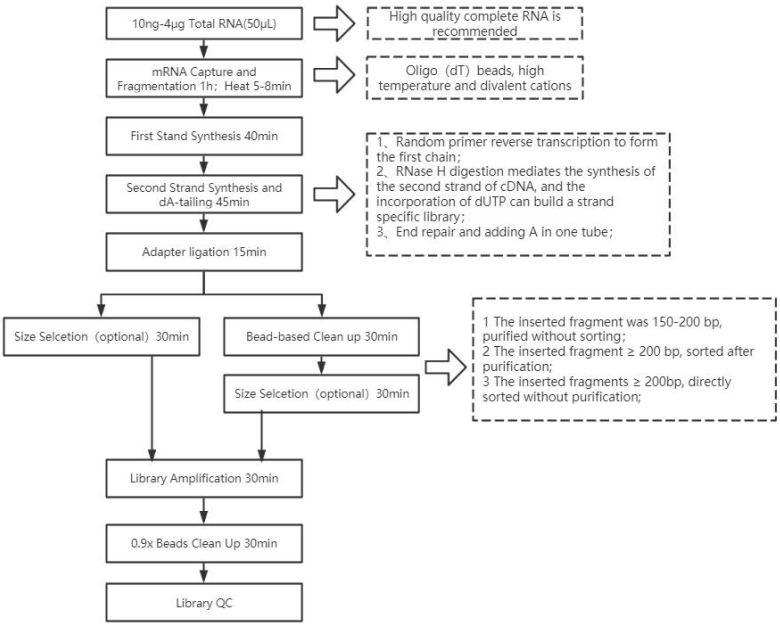

Figure 1 The workflow of mRNA library construction kit

3 Operation steps

3.1 mRNA Isolation and Fragmentation

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

3.1.2 Dilute 10 ng-4 μg of total RNA with Nuclease-free Water to a final volume of 50 μL in a nuclease-free 0.2ml PCR tube and keep on ice.

3.1.3 Mix the magnetic beads upside down or vortex. Aliquot 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.

3.1.4 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.

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

3.1.6 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 standfor 5 min, and carefully remove the supernatant.

3.1.7 Repeat step 3.1.6.

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

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

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

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

3.1.12Place the tube on the magnetic stand for 5 minutes, and carefully remove the supernatant.

3.1.13 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 needed to aspirate the remaining liquid.

3.1.14 Remove the tube from the magnetic stand and resuspend the magnetic beads with 18.5 μL Frag/Prime Buffer. Pipette 6 times to mix thoroughly and place the tube in the thermal cycler (preheated at 94°C). Table 3 lists the recommended time for mRNA fragmentation. (There are differences in the effect of fragmentation of different species, and customers may first make a gradient of fragmentation times, 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
300-400	94°C,7 min
400-500	94°C,5 min

3.1.15 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.

3.2 Synthesis of the first strand cDNA

3.2.1 Take the first-strand synthesis reagents from -20°C, invert and mix well, and then spin down briefly. As shown in Table 4, prepare the first-strand cDNA synthesis reaction.

Table 4 First-strand cDNA synthesis reaction system

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

3.2.2 Pipette gently, and then spin down briefly.

3.2.3 Incubate the sample in a preheated thermal cycler as shown in Table 5, and synthesize the first strand cDNA.

Table 5 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

3.2.4 Immediately, perform second strand cDNA synthesis reaction.

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

3.3.1 Fetch the second-strand synthesis reagents from -20°C, thaw and mix thoroughly; prepare the second-strand cDNA synthesis/end repair/dA-tailin reaction as shown in Table 6.

Table 6 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.

3.3.2 Pipette gently, and then spin down briefly.

3.3.3 Incubate the sample in a preheated thermal cycler as shown in Table 7 to synthesize the second strand cDNA.

Table 7 Second-strand cDNA synthesis reaction program

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

3.4 Adapter Ligation

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

3.4.1 Refer to Table 1 in Caution 3 and dilute the adapter to an appropriate concentration according to the amount of Input RNA.

3.4.2 Thaw the reagents in Table 8 and mix them upside down. Place them on ice.

3.4.3 Prepare the reaction mix shown in Table 8 in the PCR tube from step 3.3.

Table 8 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 YRASEN is 15 μM. Please dilute the adapter according to the input amount according to the tips in Table 1 of Caution 2 to make the volume of the adapter fixed at 5 μL.

3.4.4 Pipette gently, and spin down briefly to collect all liquid from the sides of the tube.

3.4.5 Incubate the sample in a preheated thermal cycler as shown in Table 9 and perform the adapter connection reaction:

Table 9 Adapter Ligation reaction program

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

3.5 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):

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

3.5.2 Add 60 μL Hieff NGS® DNA Selection Beads (0.6×, Beads:DNA=0.6:1) to Adaptor-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.

3.5.3 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.

3.5.4 Repeat step 3.5.3 once for a total of 2 washing steps..

3.5.5 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

3.5.6 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.

3.5.7 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.

3.5.8 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.

3.5.9 Repeat step 3.5.8 once for a total of 2 washing steps.

3.5.10 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.

3.5.11 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.

3.6 Library Amplification

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

3.6.1 Thaw the reagents in Table 10 and mix them upside down. Put them on ice for later use.

3.6.2 Prepare the reaction mixture shown in Table 10 in a sterile PCR tube.

Table 10-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 10-B Adaptor-ligated DNA PCR reaction with long adapter

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

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

**If you use an adapter with index(Cat#12615~ Cat#12618), commonly known as long adapter (big Y adapter), Primer Mix in the kit is selected for amplification.

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

3.6.4 Incubate the sample in a preheated thermal cycler as shown in Table 11, and perform PCR amplification.

Table 11 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 2 for details.

3.7 Clean Up Post Amplification

3.7.1 Preparation: Take the Hieff NGS® DNA Selection Beads from the 4℃ and place it at room temperature for at least 30 minutes. Prepare 80% ethanol.

3.7.2 Vortex or fully invert the magnetic beads.

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

3.7.4 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.

3.7.5 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.

3.7.6 Repeat step 3.7.5 once for a total of 2 washing steps.

3.7.7 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).

3.7.8 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 in 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.

3.8 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.

Appendix 1: Demonstration of mRNA Fragmentation Effects

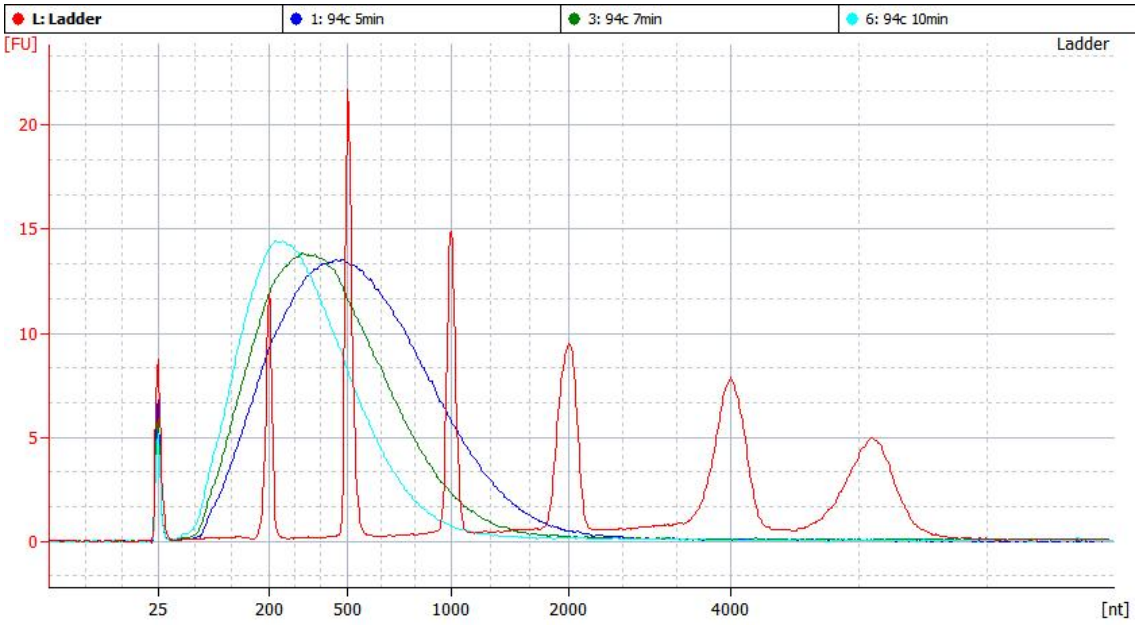

Figure 2. The range of RNA fragments corresponding to different fragmentation time of mRNA. They were treated at 94°C for 10 min, 94°C for 7 min and 94°Cfor 5 min, respectively. After that, mRNA was purified by 2.2x magnetic beads and detected by Agilent 2100 Bioanalyzer.

[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 were used.

Appendix 2: Explanation of Size Selection Conditions

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 Adaptor-ligated DNA purification

Adaptor-ligated DNA purification with 0.6×Hieff NGS® DNA Selection Beads

1. Preparation: Take the Hieff NGS® DNA Selection Beads from the 4℃ 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°Cfor 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 12.

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 13.

1. Suspend the Magnetic Beads thoroughly by inverting or vortex.
2. According to the insert DNA size refer to Table 12, add 65 μL (0.65×) magnetic beads to the above 100 μL DNA, vortex or pipette 10 times to mix.

[Note]: The recommended size selection conditions in Table 12 and Table 13 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.

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 12.
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 12 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 13 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 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×)

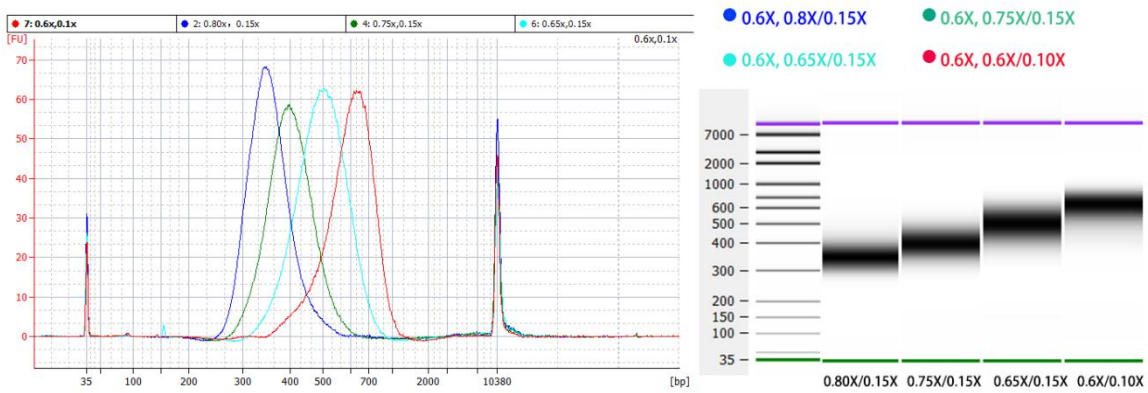

Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min using different bead/DNA ratios as indicated in Table 12

Plan B: Size Selection of Adaptor-ligated DNAAdaptor-ligated DNA (The protocol below is for libraries with a 410–510 bp insert size with fragmentation at 94°Cfor 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 14.

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 15.

1. Suspend the Magnetic Beads thoroughly by inverting or vortex.
2. According to the insert DNA size refer toTable 14, 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 100 μL of the supernatant to a clean PCR tube.
4. Add 10 μL (0.10×) of magnetic beads to the supernatant refer to Table 14.
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 14 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 15 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×)

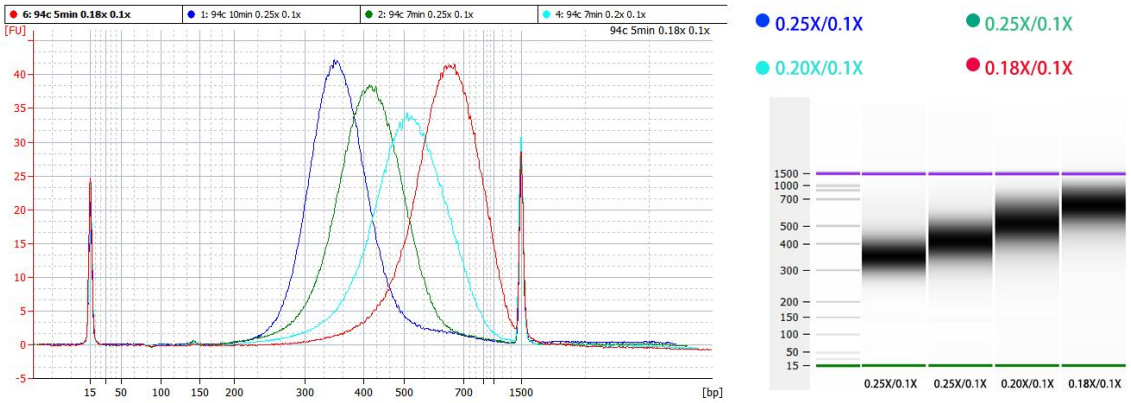

Figure 4. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min using different bead/DNA ratios as indicated in Table 14

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