Hieff NGS™ EvoMax RNA Library Prep Kit (dUTP) (Premix and Sealing Version)

12340ES

INSTRUCTIONS FOR USE

Ver. EN20240620

CONTENT

Product description

Hieff NGS™ EvoMax RNA Library Prep Kit (dUTP) is a premixed, actinomycin D free and strand-specific total RNA sequencing library prep kit compatible with Illumina and MGI platforms. This product is available in two types: tube or sealing kit, and this kit is more convenient whether by Automated Liquid Handling Device or manual manipulation . The kit contains RNA fragmentation reagents, reverse transcription reagents, strand-specific ds-cDNA synthesis reagents, and library amplification reagents. It can be linked with the mRNA purification kit or the rRNA removal kit to do mRNA or lncRNA research. This product optimizes the reverse transcription module to obtain a high chain specificity library without actinomycin D, which ensures the safety of the experimenters to a greater extent. All reagents were subjected to strict quality control and functional validation to maximize the stability and repeatability of the library preparation.

Specifications

Cat.No.	12340ES24/12340ES96/12340ES97/12340ES98
Size	24 T / 96 T / 96 T (automation) / 96 T (plate)

Components

Components No.	Name	12340ES24	12340ES96	12340ES97 (automation)	12340ES98 (Plate)**
12340-A	Frag/Prime Buffer	450 µL	2×900 µL	2×1064 µL	8×266 µL
12340-B	1st Reaction Module 2.0	192 µL	768 µL	960 µL	8×120 µL
12340-C	2nd Reaction Module(dUTP)	840 µL	3×1120 µL	3×1280 µL	8×480 µL
12340-D	Ligation Reaction Module	840 µL	3×1120 µL	3×1280 µL	8×480 µL
12340-E	2×Super Canace® II High-Fidelity Mix	600 µL	2×1200 µL	2×1360 µL	8×340 µL
*	Primer Mix*	/	/	/	/

Note: * The notation indicates that the component is not included in the kit. Primer mix is required if the complete adaptors are used, otherwise it isn’ t. This kit is compatible with Illumina and MGI platforms, but need additional primer mix (Cat # 13334 Primer Mix for MGI™ and Cat # 13335 Primer Mix for Illumina®) for Illumina® or MGI platform if the complete adaptors are used.

** See the following diagram for the layout of plate reagent group.

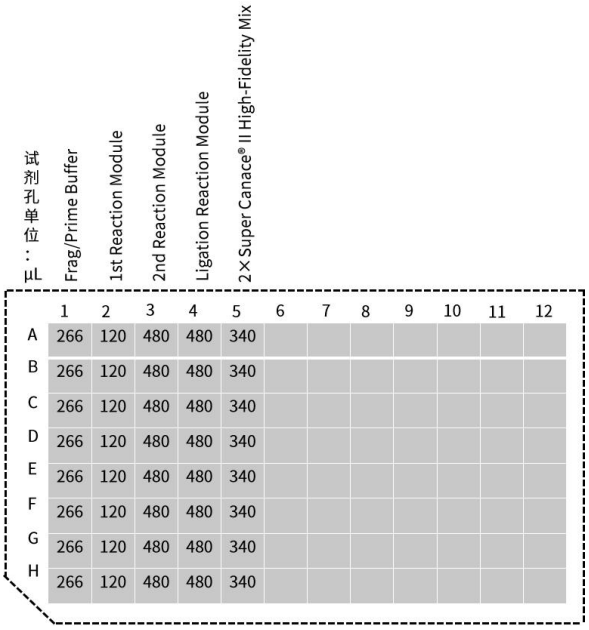


Figure: reagent layout of sealing-plate library kit

The left figure shows the layout of the sealing-plate library kit, with each component in one column, and each reagent well for 12 library reactions.

When using the sealing-plate library kit with automatic instrument, the sealing-plate library kit (column 6-12) can add magnetic beads, adaptor and other reagents before the experiment according to the experimental requirements.

Storage

This product should be stored at -25~-15℃ for 1 years.

Notes

1. Operation

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

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

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.

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.

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.

6) This reagent is for one-time use. Multiple use is strictly prohibited.

7) This product is for research use only.

2. Adapter Ligation

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

2) 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) 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.

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

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

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

Table 1-2 The recommended MGI adapter amount for different input RNA

Input Total RNA	MGI® Adapter stock concentration
10~99 ng	1 μM
100~499 ng	2 μM
500~4000 ng	5 μM

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

3. Library Amplification

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.

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 2 lists the recommended cycle numbers for PCR amplification.

3) The recommended number of cycles recommended in Table 2 can meet the vast majority of library prep requirements. If your sample is of poor quality (such as FFPE samples with severe degradation), the number of cycles can be increased appropriately, according to the actual situation.Pay attention to note that mRNA varies from different species and tissues, the number of amplification cycles should be adjusted.

Table 2 Input Total RNA volume and amplification cycles recommended Table *

Input Total RNA	Number of cycles
10 ng	16
100 ng	14
500 ng	12
1 μg	11

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

4. Bead-based DNA Cleanup and Size Selection

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.

2) The magnetic bead should be balanced to room temperature before use, otherwise the yield will decrease and the size selecting effect will be affected.

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

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

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

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.

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

5. Library Quality Analysis

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

6. Other Material

1) mRNA enrichment kit: Hieff NGS® mRNA Isolation Master Kit V2 (Yeasen Cat # 12629).

2) rRNA removal kit: Hieff NGS® MaxUp Human rRNA Depletion Kit (rRNA & ITS / ETS) (Yeasen Cat # 12257) or other rRNA removal kit.

3) RNA purification of magnetic beads: Hieff NGS® RNA Cleaner (Yeasen Cat # 12602) or other equivalent products.

4) DNA purified magnetic beads: Hieff NGS® DNA Selection Beads (Yeasen 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® use (Cat # 13519-13520 or other equivalent) or Complete Adapter for MGI® use (Cat # 13360-13362 or other equivalent).

7. Library quality inspection

Agilent 2100 Bioanalyzer DNA 1000 Chip / High Sensitivity Chip or other equivalent products; library quantification reagents.

8. Other materials

Anhydrous ethanol, sterile ultrapure water, low adsorption gun head, PCR tube, magnetic frame, PCR instrument, etc.

Database flow chart

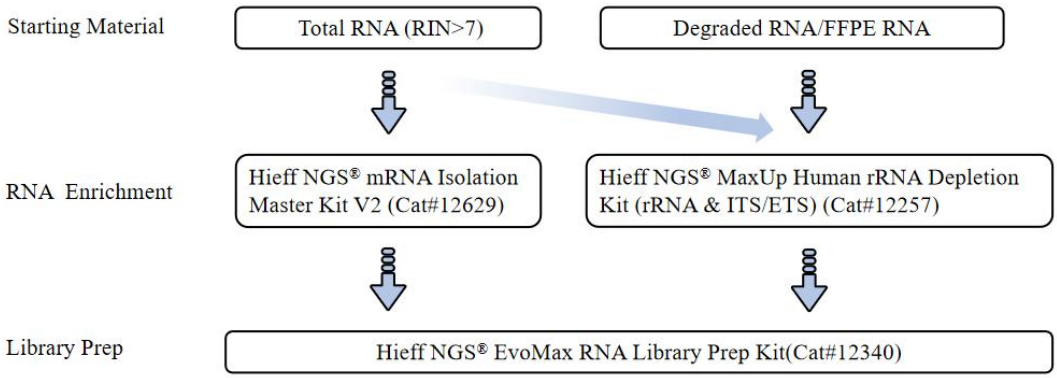


Figure 1: RNA library building process

Instructions

Part I: Enrichment and fragmentation of the target RNA

This step is the target RNA preparation before library prepration, and mRNA Isolation protocol (Scheme A) or rRNA Depletion scheme (Scheme B) can be selected according to the library prepration requirements. Yeasen Cat # 12340 Library prep kit does not contain the reagents used in this step. Please prepare the corresponding reagents according to the requirements.

Scheme A: mRNA purification and fragmentation

1. Sample requirements

Hieff NGS® mRNA Isolation Master Kit V2 (Yeasen Cat # 12629) is used. Suitable for total RNA from high quality eukaryotes such as animals, plants and fungi with starting templates of 10 ng-4 μg (volume 50 μL). If the initial RNA concentration is low and the volume exceeds 50 μL, Hieff NGS® RNA Cleaner (Yeasen Cat # 12602) magnetic beads are recommended. To ensure that the mRNA has a complete poly(A) tail structure, RNA should be detected by Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico chip. Oligo (dT) magnetic beads was applied in the mRNA isolation Master 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. Operation steps

1) Take out the mRNA Capture Beads2.0 from 2-8°C, and equilibrate at room temperature for at least 30 min.

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

3) Mix the magnetic beads by inverting upside down or vortexing. Add 50 μL of the magnetic beads into 50 μL total RNA sample and pipette 6 times to mix well. Spin down briefly to the bottom of the tube.

4) Incubate the mixture of magnetic beads and RNA in a thermal cycler and run the following program: 65°C, 5 min; 25°C, 5 min; 25°C, hold.

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

6) Remove the tube from the magnetic stand and resuspend the magnetic beads with 200 μL Beads Wash Buffer2.0. Pipette the entire volume up and down 6 times to mix thoroughly. Place the tube on a magnetic stand for 5 min, and carefully remove the supernatant.

7) Repeat Step 6.

8) Remove the tube from the magnetic stand. Add 50 μL Tris Buffer2.0 to resuspend the magnetic beads and pipette 6 times to mix thoroughly.

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

10) Remove the sample from the thermal cycler. Add 50μL Beads Binding Buffer2.0 and pipette repeatedly 6 times to mix thoroughly.

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

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

13) Remove the tube from the magnetic stand, resuspend the magnetic beads with 200 μL Beads Wash Buffer2.0, 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.

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 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 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

Insertion fragment 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；

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-step 1).

Scheme B: rRNA Depletion and RNA Fragmentation

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. Operation steps

1) Probe hybridization

a. Thaw the probe and hybridization Buffer on ice.

b. Prepare RNA samples: 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.

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

Table 4 RNA/Probe hybridization reaction

Components	Volume (μL)
Hybridization Buffer	3
Human Probe Mix (rRNA & ITS/ETS)	2
Total RNA	10 (100 ng~1 μg)
Total	15

d. 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.

e. Place tube in a thermocycler and run the following Table 5 program with the heated lid set to 105°C .

Table 5 Reaction program of RNA/Probe hybridization

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

2) RNase H digestion

a. Reaction program of RNA/Probe hybridization Table 6.

Table 6 RNase H digestion reaction

Components	Volume (μL)
RNase H Buffer	3
RNase H	2
The last step product	15
Total	20

b. 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.

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

3) DNase I digestion

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

Table 7 DNase I digestion reaction

Components	Volume (μL)
DNase I Buffer	27.5
DNase I	2.5
Last step product	20
Total	50

b. 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.

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

4) RNA Purification

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

b. 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.

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

d. 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.

e. 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.

f. Repeat Step e once for a total of two washes.

g. 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.

h. 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.

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

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

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

Table 8 FFPE RNA The fragmentation conditions are recommended

DV200*	Fragmentation program
>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；
<20% (risk building)	65°C, 8 min，4°C，hold；

* The sample quality of degraded RNA is judged using the DV 200 index, as described in Appendix III

Part II: RNA library prepration of the Illumina & MGI platform

1. Synthesis of the first-strand cDNA

This step synthesizes the first-stranded cDNA from a target enriched /fragmented RNA. mRNA isolation or rRNA depletion protocol were done based on the requirements and sample conditions, as described in Part I.

1)Take the first-strand synthesis reagents from -20°C, invert and mix well, and then spin down briefly. As shown in Table 9, prepare the first-strand cDNA synthesis reaction .

Table 9 First-strand cDNA synthesis reaction system

Components	Volume (μL)
Frag/Prime Buffer with Fragmented RNA	17
1st Reaction Module 2.0	8
Total	25

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

3) Incubate the sample in a preheated thermal cycler as shown in Table 10, and synthesize the first 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

2. 2nd Strand Synthesis / dA-Tailing

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 11.

Table 11 Second-strand cDNA synthesis reaction

Components	Volume (μL)
1st Strand cDNA	25
2nd Reaction Module (dUTP)*	35
Total	60

[Note]: * This kit is a strand-specific RNA library. If need a non stand-specific mRNA library, please use Yeasen Cat # 12341.

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

3) Incubate the sample in a preheated thermal cycler as shown in Table 12 to synthesize the second strand cDNA.

Table 12 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. Adapter Ligation

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

1) Referring to Table 1 in Note 2, and dilute the adapter to an appropriate concentration according to the amount of Input RNA.

2) Thaw the reagents in Table 13 and mix them upside down. Place them on ice.

Table 13 Adapter Ligation reaction

Components	Volume (μL)
dA-tailed DNA	60
Ligation Reaction Module	35*
DNA Adapter**	5***
Total	100

[Note]: * Ligation Reaction Module should be mixed thoroughly followed by a quick spin prior to use.

** For the experiment, if a short adaptor is used, the DNA Adapter is PE adapter.

*** 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) 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.

4) Incubate the sample in a preheated thermal cycler as 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

4. Post Ligation Clean Up

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)

Note: During the experiment, ensure that the sample RNA fragment is less than 200 nt.

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.

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) 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.

4) Repeat step 3) once for a total of twice washing steps.

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.

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.

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.

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.

9) Repeat step 8) once for a total of twice washing steps.

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.

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.

5. Library Amplification

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

1) Thaw the reagents in Table 15 and mix them upside down. Put them on ice for later use.Prepare the reaction mixture shown in Table 13 in a sterile PCR tube.

Table 15-A adapter-ligated DNA PCR reaction with short adapter for Illumina platform

Components	Volume (μL)
2×Super Canace® II High-Fidelity Mix	25
Universal Primer/ i5 Primer*	2.5
Index Primer/ i7 Primer*	2.5
Adapter Ligated DNA	20
Total	50

Table 15-B Adapter-ligated DNA PCR reaction with long adapter for Illumina platform

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 # 13519~Cat # 13520), commonly known as long adapter (big Y adapter), Hieff NGS™ Primer Mix for Illumina (Yeasen Cat#13335) in needed.

Table 15-C PCR reaction with long adaptor for MGI platform

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

[Note]: * This primer mix for MGI is not contained in this kit and can be needed with the Hieff NGS™ Primer Mix for MGI® in Cat # 13334.

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

3) Incubate the sample in a preheated thermal cycler as shown in Table 16, and perform PCR amplification.

Table 16 PCR amplification reaction

Temperature	Time	Cycle
Hot lid 105°C	on	-
98°C	1 min	1
98°C	10 sec	11~16 cycles*
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 Note 3 for details..

6. Post Amplification Clean Up

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.

2) Vortex or fully invert the magnetic beads.

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.

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, 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 twice 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 (no more than 5 minutes).

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.

7. 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

Appendix I: Demonstration of mRNA Fragmentation Effects

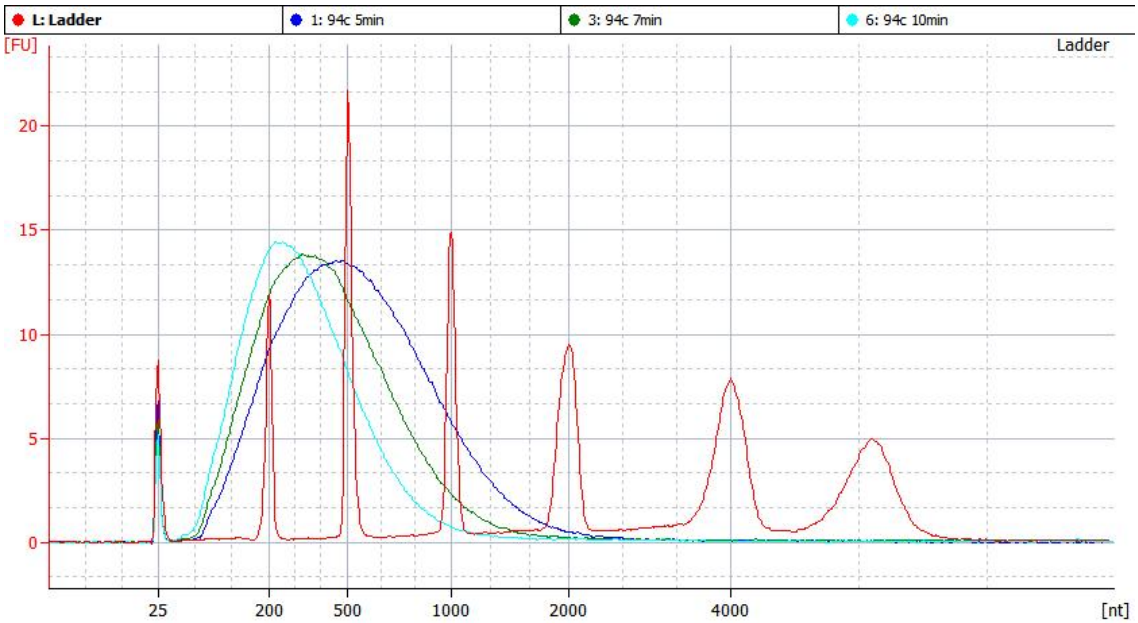


Figure 2. The range of RNA fragments corresponding to different fragmentation time of mRNA. They were treated at 94°C for 10 min, 94°C for 7 min and 94°C for 5 min, respectively. After that, mRNA was purified by 2.2x magnetic beads and detected by Agilent 2100 Bioanalyzer.

[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 prepration 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:

Size selection after adapter-ligated DNA purification

1. 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 twice 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.

2. Double-round sorting (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 17.For the long adapter (large Y adapter), Hieff NGS™ Complete Adapter Kit for Illumina, Set 1~Set 2 (Cat#12319~Cat#12320) is recommended., Size selection conditions for library construction refer to Table 18.

Sorting of 0.65X/0.15X Hieff NGS™ DNA Selection Beads

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 17.

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 17 Recommended size selection conditions for libraries with short adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Library Length (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 18 Recommended size selection conditions for libraries with complete adapter

Inserted DNA size (bp)	200~300	250~350	350~450	450~550
Library Length (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 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.

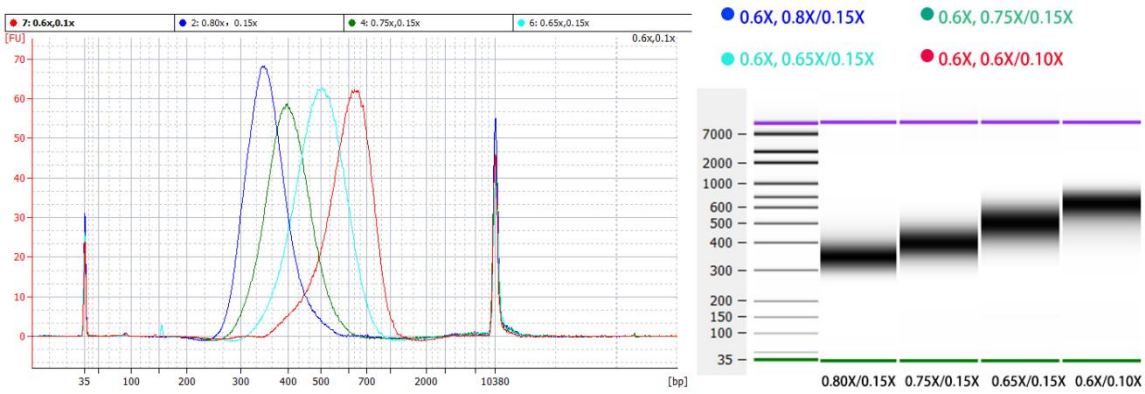


Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°C for 10 min, 94°Cfor 7 min and 94°Cfor 5 min using different bead/DNA ratios as indicated in Table 17

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:

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

1) Preparation: Take out the Hieff NGSTM 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 once for a total of twice washing steps.

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.

[Note]: High concentration of PEG contained in Ligation Enhancer will have an impact on the double round sorting of magnetic beads, so the double round sorting must be conducted after one round of purification.

2. **Double-round sorting** (**The protocol below is for libraries with a 380–480 bp insert size with fragmentation at 94° C for 7 min. For libraries with different size fragment inserts, refer 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 Table 21, 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.

[Note]: The recommended two-round sorting ratio in this table applies 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.

3) Incubate at room temperature for 5 min.

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 21 Recommended ratio of magnetic beads for complete adapter library selection

Inserted DNA size (bp)	200~300	300~400	400~500	500~600
Library Length (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 Beads in the 1st round (μL)	70（0.7×）	65（0.65×）	58（0.58×）	50（0.5×）
Volume Beads in the 2nd round (μL)	20（0.2×）	15（0.15×）	15（0.15×）	15（0.15×）

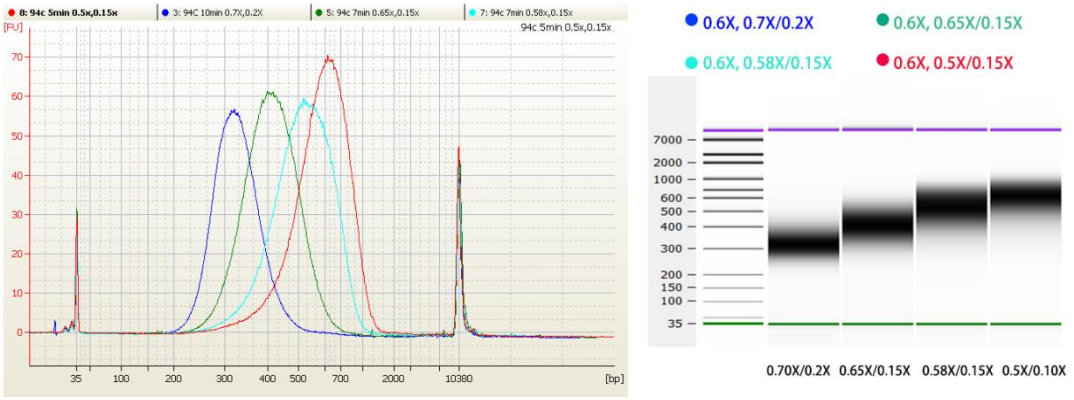


Figure 5. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min.

Appendix IV: Description of FFPE samples

1. FFPE RNA Quality evaluation

The rRNA deletion library prepration scheme can be used for low-quality total RNA samples such as FFPE. It is needed to evaluate DV200 to select different fragmentation condition.DV200 Indicates the proportion of RNA fragments greater than 200 nt in the sample, and for the DV 200 value improves the quality of the reaction sample. The DV 200 is calculated as follows:

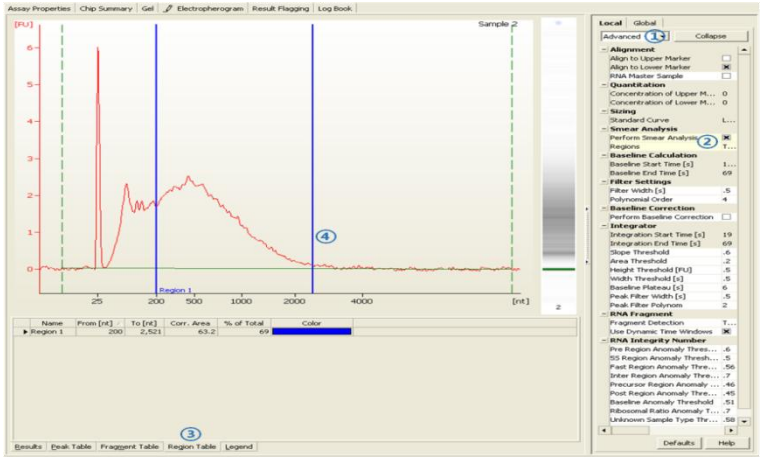


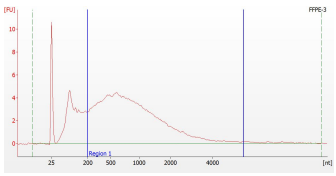
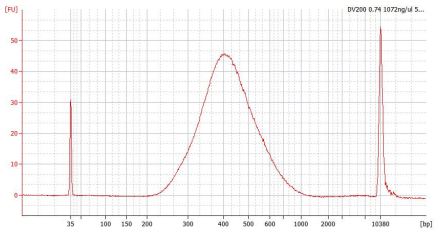
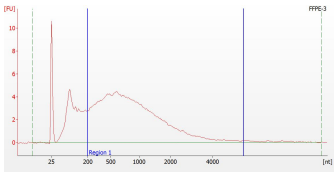
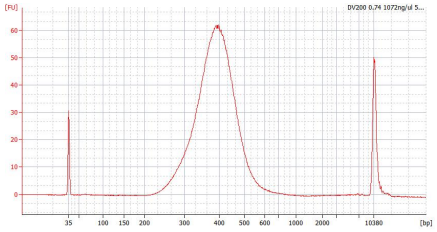
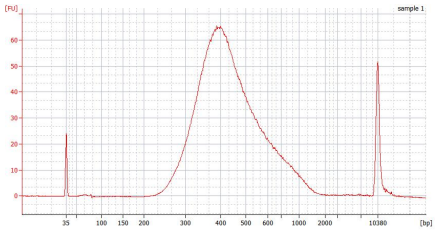
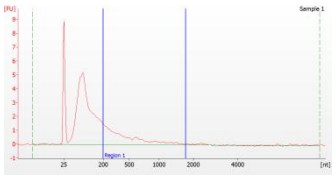
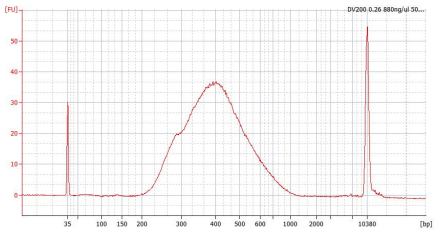
Figure 6. Proportion of DV 200

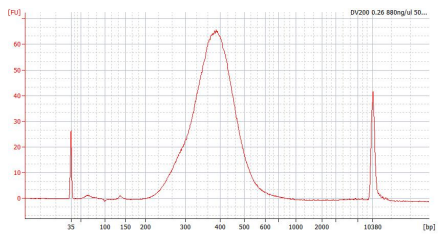
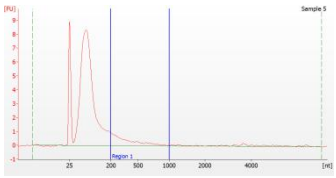
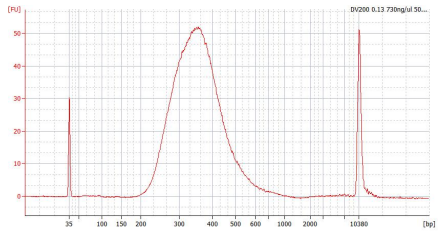
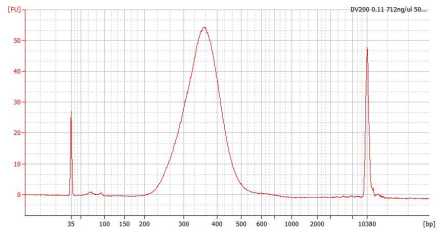
In a completed Agilent 2100 Bioanalyzer result diagram, select Advanced under Local/Region Table page, right click the mouse, select Add Region to adjust the range of the indicator line to get the proportion of the selected segment range.

3. RNA Library prep for different quality FFPE samples

Table Table 23 shows the peak patterns of different quality FFPE samples. For severely degraded FFPE RNA (DV 200 <50%) and low input samples, we recommend a two-purification protocol after adapter ligation to reduce library loss.

Table 23 Peak patterns of different quality FFPE samples

QC for varies RNA sample	Construction of storage conditions	Library distribution quality control
 RIN=2.2; DV200=74%	Input RNA: 500 ng Segmentation conditions: 94°C, 7 min Two-purification protocol after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 717.2 ng	
 RIN=2.2; DV200=74%	Input RNA: 500 ng Segmentation conditions: 94°C, 7 min Purification / sorting: 0.6 ×; 0.7 × / 0.15× Strand-specific library amplification: 13 cycles Library yield: 437.8 ng	
RIN=2.2; DV200=74%	Input RNA: 100 ng Segmentation conditions: 94°C, 7 min Two-purification protocol after adapter ligation: 0.6×; 0.8× Chain-specific library amplification: 15 cycles Library yield: 206.8 ng	
 RIN=2.5; DV200=26%	Input RNA: 500 ng Segmentation conditions: 85°C, 8 min Two-purification protocol after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 207 ng	

	Input RNA: 500 ng Segmentation conditions: 85°C, 8 min Purification and sorting: 0.6×; 0.7× / 0.15× Strand-specific library amplification: 13 cycles Library yield: 98.56 ng	
 RIN=2.5; DV200=11%	Input RNA: 500 ng Segmentation conditions: 65°C, 8 min Two-purification protocol after adapter ligation: 0.6×; 0.8× Strand-specific library amplification: 12 cycles Library yield: 354.2 ng	
	Input amount: 500 ng Segmentation conditions: 65°C, 8 min Purification and sorting: 0.6×; 0.7× / 0.15× Strand-specific library amplification: 13 cycles Library yield: 172.48 ng	

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