PluS4PiGs: Cell tools for pig genetics

PluS4PiGs – Protocols

PluS4PiGs

Getting true Pluripotent Stem Cells in Pigs: a key step for large scale ex-vivo "Genotype to Phenotype" studies

Protocol 7
Production and sequencing of bulk stranded mRNA-seq using BGI services

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Version: 1.0

Submission date to FAANG:	**22/10/2024**

https://api.faang.org/files/protocols/experiments/INRAE_SOP_PLUS4PIGS_mRNAseq_LIBRARIES_PROTO7_20241022.pdf

Project-ANR-19-CE20-0019
Duration of the project: 01 February 2020 – 31 July 2024, 54 months

This project has received funding from the French National Research Agency (ANR) under grant agreement No ANR-19-CE20-0019

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1 Summary

To address the current challenges of animal breeding, a better knowledge of the link between genotype and phenotype is necessary. The use of pluripotent stem cells is an interesting solution because these cells can be driven *in vitro* to differentiate towards all cell lineages and their genome can be easily manipulated. These characteristics make PSCs powerful tools to assess the causality of genetic variants associated with cellular intermediate phenotypes. PSCs can be derived either from the pluripotent stem cells of the embryo, also named embryonic stem cells (ESCs) or by reprogramming somatic cells to the pluripotent state to get induced pluripotent stem cells (iPSCs). We propose, within the framework of the PluS4PiGs project, to use the numerical dimension from multi-omics data at the single-cell and tissue scale to predict the molecules necessary and sufficient to maintain porcine pluripotency and transfer this knowledge for the production and use of porcine embryonic stem cell lines (pESCs), for animal and human health applications.

We generated bulk mRNAseq from four porcine embryonic stem cells (ESC) and from their differentiated products.

Metadata related to pESC lines are available on BioSamples (https://www.ebi.ac.uk/biosamples/). Protocols for pig Embryonic stem cells derivation, maintenance and in vitro differentiation are available on the FAANG Data Portal using the following URLs:

We describe here briefly the procedures used to produce mRNAseq libraries from total RNA extracted from pESC lines and from differentiated pESCs.

2 Protocol description

2.1 Required reagents and instruments

- A fully equipped molecular biology room
- Vortex mixer
- Mini-centrifuge
- Eppendorf DNA LoBind tubes 1.5ml and 2.0ml
- Eppendorf PCR Tubes 0.2ml 8-tubes strips
- Nuclease free water
- Ethanol pure
- Phosphate Buffer Saline (PBS)
- EB buffer (Qiagen)
- Agilent BioAnalyzer
- Nanodrop or any other type of spectrophotometer
- Macherey Nagel Nucleospin RNA mini kit
- YEASEN Kit：Hieff NGS® Ultima Dual-mode mRNA Library kit (#13462ES96)

2.2 Summary of the produced libraries

Library name	Sequencing strategy	Sequencing machine
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW7.1	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.1	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.5	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.8	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW7.1_EB2	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW7.1_EB5	PE150	DNBseq-G400
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW9.5_EB6	PE150	DNBseq-G400

2.3 Extraction of total RNA from pESC lines and differentiated pESCs

Total RNA was extracted from frozen cell pellets (around 1million cells per pellet) using the NucleoSpin RNA mini kit from Macherey-Nagel. On column DNase treatment was performed for each sample.

Total RNA was then quantified using Qbit and RNA integrity was checked using a BioAnalyzer (Agilent).

2.4 Production of stranded mRNAseq libraries using the Hieff NGS® Ultima Dual-mode mRNA Library kit from MGI®

Libraries have been produced by BGI using the Hieff NGS® Ultima Dual-mode mRNA Library kit and the following workflow:

2.4.1 mRNA Isolation

A certain amount of RNA samples are denatured at suitable temperature to open their secondary structure, and mRNA is enriched by oligo (dT) -attached magnetic beads.

2.4.2 mRNA Fragmentation

The reaction system is configured. After reacting at the suitable temperature for a fixed period of time, RNAs are fragmented.

2.4.3 cDNA Synthesis

The first-strand synthesis reaction system is added to the interrupted mRNA, and the reaction procedure is set up to synthesize the first-strand cDNA. The second-strand synthesis reaction system (including dUTP) is prepared, and the reaction procedure is set up to synthesize the second-strand cDNA.

2.4.4 End Repair, Add 'A' and Adaptor Ligation

After the reaction system and program are configured and set up, double-stranded cDNA fragments are subjected to end-repair, and then a single 'A' nucleotide is added to the 3' ends of the blunt fragments. The reaction system and program for adaptor ligation are subsequently configured and set up to ligate adaptors with the cDNAs.

2.4.5 PCR

The PCR reaction system and program are configured and set up to amplify the cDNAs.

2.4.6 Library QC

The corresponding library quality control protocol will be selected depending upon product requirements.

2.4.7 Circularization

Single-stranded PCR products are produced via denaturation. The reaction system and program for circularization are subsequently configured and set up. Single-stranded cyclized products are produced, while uncyclized linear DNA molecules are digested.

2.4.8 Sequencing

Single-stranded circle DNA molecules are replicated via rolling cycle amplification, and a DNA nanoball (DNB) which contain multiple copies of DNA is generated. Sufficient quality DNBs are then loaded into patterned nanoarrays using high-intensity DNA nanochip technique and sequenced through combinatorial Probe-Anchor Synthesis (cPAS).

2.5 Sequencing of the libraries

Each mRNAseq library has been sequenced by BGI services on a DNBseq sequencing machine.

Library name	Sequencing strategy	Sequencing date	Number of raw reads
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW7.1	PE150	2023-05	48M
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.1	PE150	2023-05	48M
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.5	PE150	2023-05	48M
SSC_INRAE_Plus4PigS_cell_lines_RNAseq_pESC_LW9.8	PE150	2023-05	48M
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW7.1_EB2	PE150	2023-07	48M
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW7.1_EB5	PE150	2023-07	48M
SSC_INRAE_Plus4PigS_embryoid_bodies_RNAseq_ESC_LW9.5_EB6	PE150	2023-07	48M

3 Annexes

3.1 Hieff NGSTM Ultima Dual-mode RNA Library Prep Kit for MGI®

Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit for MGI®

Cat# 13333

INSTRUCTION FOR USE

Yeasen Biotechnology (Shanghai) Co., Ltd.

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Product Information

Product Name	Cat#	Specification
Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit for MGI ®	13333ES24	24 T
	13333ES96	96 T

Product Description

Hieff NGS™ Ultima Dual-mode RNA Library Prep Kit for MGI® is an RNA sequencing library construction kit for the 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 by connecting the mRNA purification kit or rRNA removal kit. The two-strand synthesis module is equipped with two buffers, and customers can build a library according to their needs. Among them, dTTP is replaced with dUTP in the strand-specific two-strand synthesis Buffer, so that dUTP is incorporated into the second strand of cDNA, and the high-fidelity DNA polymerase used in this kit cannot amplify the DNA template containing uracil, achieving strand specificity. All reagents provided have undergone strict quality control and functional verification, ensuring the stability and reproducibility of library construction to the greatest extent.

Product Components

Components			13333ES24	13333ES96
13333-A		Frag/Prime Buffer	450 μL	2×900 μL
13333-B		1st Strand Enzyme Mix	48 μL	192 μL
13333-C		Strand Specificity Reagent	150 μL	580 μL
13333-D		2nd Strand Buffer (dNTP)	720 μL	2×1440 μL
13333-E		2nd Strand Buffer (dUTP)	720 μL	2×1440 μL
13333-F		2nd Strand Enzyme Master Mix	120 μL	480 μL
13333-G		Ligation Enhancer	720 μL	2×1440 μL
13333-H		Novel T4 DNA Ligase	120 μL	480 μL
13333-I		2×Super Canace™ II High-Fidelity Mix	600 μL	2×1200 μL
13333-J		Primer Mix For MGI	120 μL	480 μL

Shipping and Storage

All the components are shipped with dry ice and can be stored at -20°C for one year.

Cautions

1 Operation

1.1 For your safety and health, please wear lab coats and disposable gloves for operation.

1.2 Thaw components at room temperature. Once the components are thawed, mix thoroughly by vortexing, spin the tube briefly and place on ice for later use.

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

1.4 Please use consumables that are free of RNase contamination and clean the experimental area regularly. It is recommended to use ThermoFisher's RNA*Zap*™ high-efficiency nucleic acid removal spray to remove RNase contamination.

1.5 Improper operations may very likely cause carry-over contaminations through aerosols, impacting the experiment's accuracy. It is highly recommended to divide the experiment environment into the pre-PCR and post-PCR regions, with separate sets of devices and disposables in each area. Perform routine cleaning for each area (It is recommended to use ThermoFisher's DNA*Zap*™ high-efficiency nucleic acid removal spray).

2 Adapter Ligation

2.1 Currently, MGI only has two kinds of serial adapters:1-128 and 501-596. User Requirement Specification can be seen or known from MGI or Yeasen. In addition, MGI states: Due to the different design process of the two joints, it is forbidden to use them together, otherwise the sequencing data cannot be separated!

2.2 We recommend using high-quality commercial joints. If self-made adapters are used, please entrust a company with experience in NGS primer synthesis, and note that strict pollution control is required. In addition, when performing joint annealing operations, please complete it in a clean bench. Only operate one type of connector at a time to prevent cross-contamination.

2.3 The concentration of the adapter directly affects the ligation efficiency and library yield. In this kit, the volume of the adapter is fixed at 5 μL. Please dilute the adapter for the different amounts of input RNA according to Table 1. 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 The recommended adapter amount for Input RNA

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

* Adapter usage can be adjusted according to different types of Total RNA samples and inputs

3 Library Amplification

3.1 The library amplification components in this kit are composed of the company's second-generation high-fidelity DNA polymerase. On the basis of the first-generation, the uniformity of amplification is greatly enhanced, even for low-copy genes also can amplify without preference.

3.2 The library amplification step requires strict control of the number of amplification cycles. Insufficient number of cycles will result in low library yield; too many cycles will lead to increased library preference, repetition and chimeric products. Table 2 lists the recommendations for library amplification using this kit, the amount of Input total RNA and the number of corresponding amplification cycles.

Table 2 The recommended number of cycles to generate RNA library

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

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

4 Bead-based DNA Cleanup and Size Selection

4.1 There are multiple steps that require the use of DNA purification magnetic beads. We recommend using Hieff NGSTM DNA Selection Beads (Yeasen Cat#12601) or AMPureTM XP magnetic beads (Beckman Cat#A63880) for DNA purification and sorting.

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

4.3 The magnetic beads should be mixed well by vortexing or pipetting before use.

4.4 When transferring the supernatant, do not touch the magnetic beads, even a small amount of residue will affect the quality of the subsequent library.

4.5 The 80% ethanol used for magnetic bead rinsing 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 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 the purified or size selected DNA needs to be stored, it can be eluted with 0.1×TE Buffer. The solution can be stored at 4°C for 2 days, and at -20°C for 1 month.

5 Library Quality Analysis

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

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

5.3 Unavailable ibrary concentration detection: methods based on spectral detection, such as NanoDrop™, etc.

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

5.5 Library length distribution detection can be performed by equipment based on capillary electrophoresis or micro-control flow principle such as Agilent Bioanalyzer 2100.

6 Materials not included

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

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

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

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

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

6.6 Adapters：Consult MGI or Yeasen..

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

6.8 Other materials: ethanol, Sterilized ddH_2O, PCR tube, low adsorption pipette tips, magnetic stand, thermocycler etc..

Operation flowchart

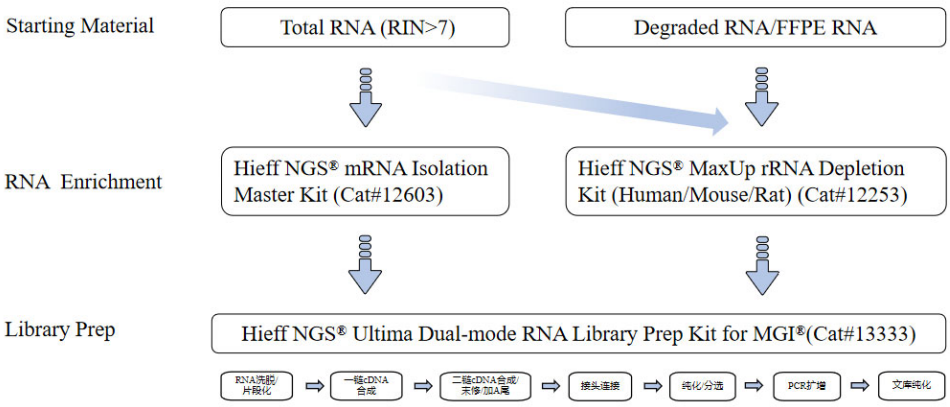


Figure 1 RNA library construction flowchart

Instructions

Part 1：Enrich and fragment target RNA

This step is the preparation of target RNA before library construction. According to the requirements of library construction, either Poly(A) mRNA Isolation protocol (Scheme A) or rRNA Depletion protocol (Scheme B) is able to be chosen. Yeasen Cat#13333 library building module does not include the reagents used in this step, please prepare the corresponding reagents according to the library building needs.

Scheme A：mRNA Purification and Fragmentation

Sample criterias

This protocol uses the Hieff NGS™ mRNA Isolation Master Kit (Yeasen Cat#12603) for mRNA enrichment. It is suitable for high-quality total RNA of eukaryotic organisms such as animals, plants and fungi with a starting template amount of 10 ng-4 μg (volume ≤50 μL). If the initial RNA concentration is low and the volume exceeds 50 μL, use Hieff NGS™ RNA Cleaner (Yeasen Cat#12602) magnetic beads for concentration. RNA needs to be detected by the Agilent 2100 Bioanalyzer RNA 6000 Nano/Pico chip, and the RIN value must be greater than 7, to ensure that the mRNA has a complete poly(A) tail structure. The mRNA isolation module of this kit uses oligo (dT) magnetic beads, 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. cannot be applied to this kit. In addition, the mRNA in the FFPE sample is severely degraded and usually does not have a complete poly(A) tail structure, so this kit cannot be used for library construction.

Steps

1. Take out the mRNA Capture Beads from 2-8°C and equilibrate at room temperature for at least 30 min.
2. Dilute 10 ng-4 μg of total RNA with Nuclease-free Water to a final volume of 50 μL in a PCR tube and place on ice for later use.
3. Mix the magnetic beads by inversion or vortexing, add 50 μL of the magnetic bead suspension to tube, pipette 6 times to mix well.
4. Place tube in a thermocycler at 65°C, 5 min; 25°C, 5 min; 25°C, hold, to complete the combination of RNA and capture magnetic beads.
5. Place the tube in a magnetic stand and let it stand at room temperature for 5 minutes to separate mRNA from total RNA. Carefully remove the supernatant.
6. Remove the tube from the magnetic stand, add 200 μL Beads Wash Buffer to resuspend the magnetic beads, pipette repeatedly 6 times to mix thoroughly. Place the sample in a magnetic stand and let it stand at room temperature for 5 min. Carefully remove the upper clear liquid.
7. Repeat step 6 for a total of two washes.
8. Remove the tube from the magnetic stand, add 50 μL Tris Buffer to resuspend the magnetic beads, and mix by pipetting 6 times to mix thoroughly.
9. Place the tube in a thermocycler at 80°C for 2 min; 25°C, hold, to elute the mRNA.
10. Take the tube out of the thermocycler, add 50 μL Beads Binding Buffer, pipette repeatedly 6 times to mix thoroughly.
11. Incubate at room temperature for 5 min to allow the mRNA to bind to the magnetic beads.
12. Place the tube onto magnetic stand. When the solution is clear (about 5 min), aspirate the supernatant and discard.。
13. Remove the tube from the magnetic stand. Add 200 μL Beads Wash Buffer to resuspend the magnetic beads, pipette repeatedly 6 times to mix thoroughly. Put the sample back in the magnetic stand, incubate at room temperature for 5 min, and aspirate all the supernatant.

Note:：You need to use a 10 μL pipette to suck up the remaining liquid.

14. Remove the tube from the magnetic stand. Add 18.5 μL Frag/Prime Buffer to resuspend the magnetic beads, pipette 6 times to mix thoroughly. Place the tube in the thermocycler (Pre-set to 94℃). However, the samples of different species have different fragmentation effects. A gradient of fragmentation time test can be process according to their own situation, such as 94℃, 5 min on Agilent 2100, to analyze the mRNA purified product size.

Table 3 The reaction programs for mRNA Fragmentation

Inserted DNA (bp)	Program

200-300	94°C, 10 min
300-400	94°C, 7 min
400-500	94°C, 5 min

15. After the fragmentation, place the tube in the magnetic stand immediately. After the solution is clear, transfer 17 μL of the upper clear liquid to a new Nuclease Free centrifuge tube, and process the first-strand synthesis reaction at once(Part 2-Step 1).

Scheme B: rRNA Depletion and RNA Fragmentation

Sample criterias

This protocol uses the Hieff NGS™ MaxUp rRNA Depletion Kit (Human/Mouse/Rat) (Yeasen Cat#12253) to remove rRNA from Total RNA. Suitable for 1 ng~1 μg (volume ≤ 11 μL) total RNA samples from humans, mice, and rats; both complete and partially degraded RNA (such as FFPE RNA) samples.

Steps

Step 1 Probe Hybridization to RNA

1.1 Taking out of the probe and hybrid Buffer from -20 °C, thawed, mixed upside down, and placed on ice for use.

1.2 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.

3.3 Assemble the following RNA/Probe hybridization reaction **on ice** according to Table 4.

Table 4 RNA/Probe hybridization reaction

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

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

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

Table 5 Reaction program of RNA/Probe hybridization

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

Step 2 RNase H Digestion

2.1 Assemble the following Rnase H digestion reaction **on ice** according to Table 6.

Table 6 Rnase H digestion reaction

Components	Volume (μL)
Rnase H Buffer	3
Rnase H	2
Hybridized RNA (Step 1.5)	15
Total	20

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

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

Step 3 DNase I Digestion

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

Table 7 DNase I digestion reaction

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

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

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

Step 4 RNA Purification

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

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

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

4.4 Place the tube on a magnetic stand to separate the beads from the supernatant. When the solution is clear (about 5 mins), discard the supernatant. Be careful not to touch the beads with the pipette tips.

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

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

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

4.8 Remove the tube from the magnetic stand. Elute the RNA from the beads by adding 18.5 μL of Frag/Prime buffer. Mix thoroughly by pipetting up and down at least 5 times and briefly spin the tube.

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

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

11. After fragmentation, please put it on ice immediately and enter the first-chain synthesis reaction (Part 2-Step 1).

Table 8 Recommended FFPE RNA fragmentation conditions

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

*The quality of degraded RNA samples was determined by DV200 index, as described in Appendix III

Part 2：RNA library construction

Step 1 1st Strand Synthesis

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

Table 9 1st Strand cDNA synthesis reaction

Components	Volume (μL)

Frag/Prime Buffer with Fragmented RNA	17
Strand Specificity Reagent	6
1st Strand Enzyme Mix	2
Total	25

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

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

Table 10 Reaction program of 1st Strand Synthesis

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

Step 2 2nd Strand Synthesis/dA-Tailing

2.1 Assemble the following 2nd Strand Synthesis/dA-Tailing digestion reaction according to Table 11.

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

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

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

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

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

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

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

Step 3 Adapter Ligation

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

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

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

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

Table 13 The reaction system for Adapter Ligation

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

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

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

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

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

Table 14 The reaction programs for Adapter Ligation

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

Step 4 Post Ligation Clean Up

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

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

4.15 Spin the tube briefly and place it on magnetic stand. When the solution is clear (about 5 min), transfer 20 μL of supernatant to a

new PCR tube carefully without touching the magnetic beads.

Step 5 Library Amplification

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

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

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

Table 15 PCR reaction system for adapter ligation

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

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

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

Table 16 Reaction programs for PCR amplification

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

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

Step 6 Cleanup and Size Selection of PCR product

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

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

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

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

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

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

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

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

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

Step 7 DNA Library Quality Control

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

Addendum 1:Fragment mRNA display

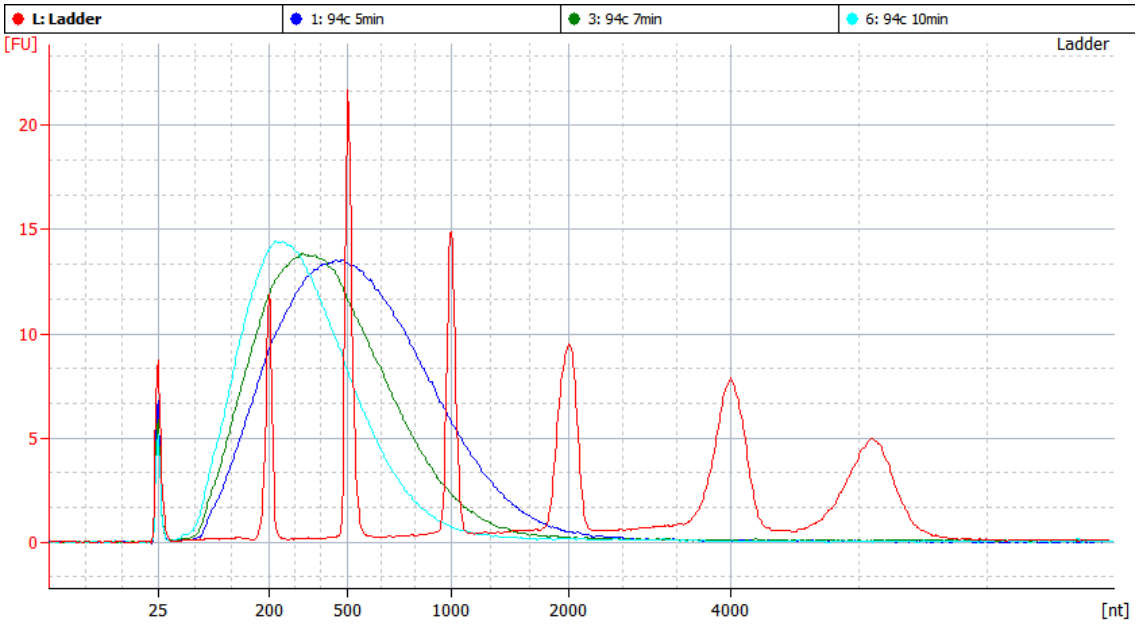


Figure 2 The range of RNA fragments corresponding to different interruption times of mRNA.

Note: Treat at 94°C, 10 min, 94°C, 7 min and 94°C, 5 min. After fragmentation, mRNA was purified by 2.2X magnetic beads and detected by Agilent 2100 Bioanalyzer.

** The RNA used in this study was Universal Human Reference RNA from Agilent. If RNA from other sources was used, it would be better to optimize the fragmentation time.*

Addendum 2: Illustrate for library size selection

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

Scheme 1: Selection after purification of the linker ligation product

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

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

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

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

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

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

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

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

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

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

2 Two-round sorting. (Take the 380-480bp library which undergo the 94°C, 7min fragmentation l as an example. Other library

sizes are sorted by magnetic beads according to the recommended ratio.）

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

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

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

2.3 Incubate at room temperature for 5 min.

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

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

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

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

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

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

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

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

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

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

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

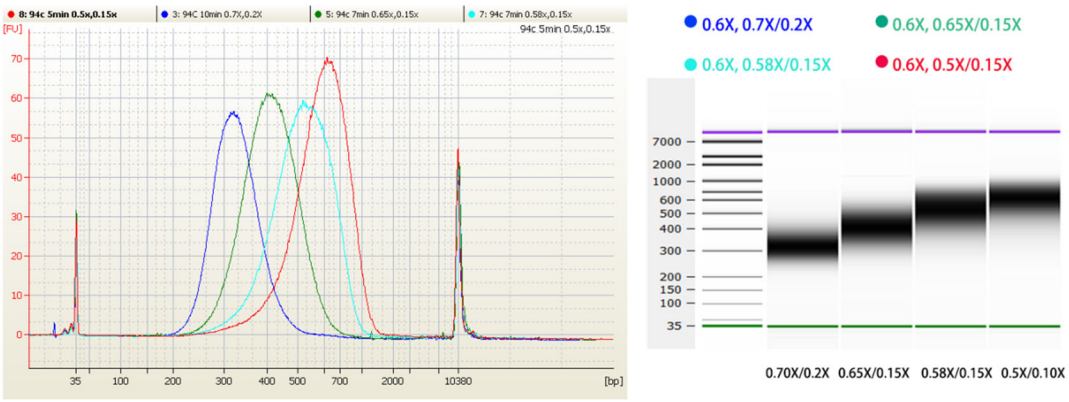


Figure 3. Library size of 1 μg 293 total RNA after fragmentation at 94°Cfor 10 min, 94°Cfor 7 min and 94°Cfor 5 min, according to the recommended ratio of magnetic beads in Table 17

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

The total RNA of 500 ng or more is used for mRNA capture and then the library is built. It is recommended to sort directly. The

system is relatively viscous and needs to be added carefully. Samples with slightly poor RNA quality may have residual adapters.

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

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

2.3 Incubate at room temperature for 5 min.

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

2.5 Refer to Table 18, add 10 μL (0.10×) of magnetic beads for the second round of sorting to the supernatant.

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

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

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

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

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

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

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

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

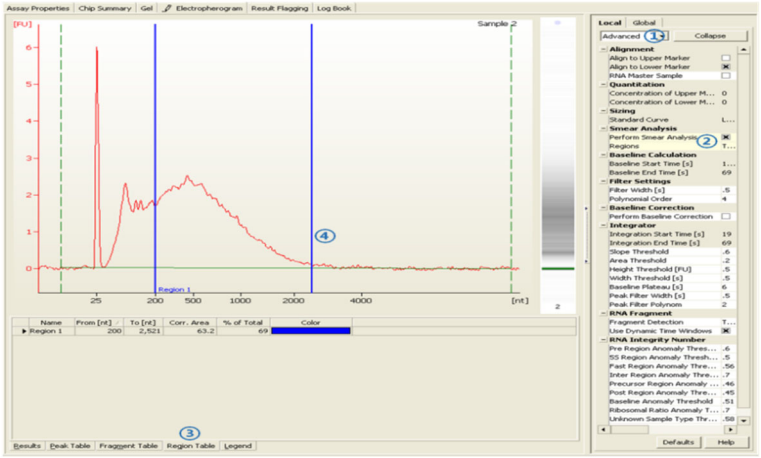
Inserted DNA library size (bp)	200~300	300~400	400~500	500~600
Final DNA library size (bp)	280~380	380~480	480~580	580~680
Fragmentation	94℃ 10min	94℃ 7min	94℃ 7min	94℃ 5min
1st volume ratio（Beads:DNA）	25 (0.25×)	20 (0.2×)	15 (0.15×)	15 (0.15×)
2nd volume ratio（Beads:DNA）	10 (0.1×)	10 (0.1×)	10 (0.1×)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

Addendum 3: FFPE sample library

1 FFPE RNA quality control

The rRNA removement library construction program can be used for low-quality total RNA samples such as FFPE, but due to the large quality gap of different FFPE samples, the library construction conditions need to be adjusted according to the sample situation. The conventional parameter for evaluating the quality of RNA samples is the RIN value, but for degraded samples such as FFPE, the RIN value cannot be used to accurately measure the quality of the sample. At this time, the DV200 indicator is also needed. DV200 represents the proportion of RNA fragments larger than 200 nt in the sample. For severely degraded FFPE samples, the DV200 value can better reflect the quality of the sample.

DV200 calculation method：



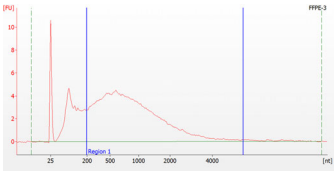
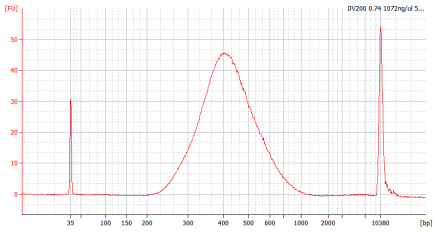
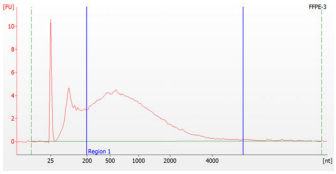
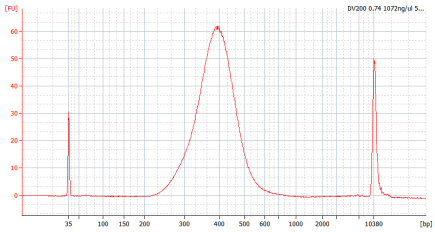
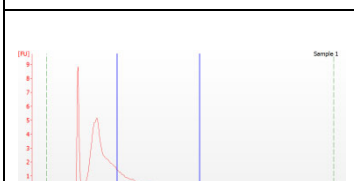
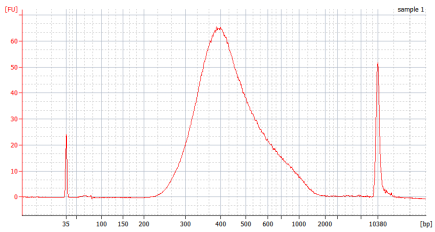
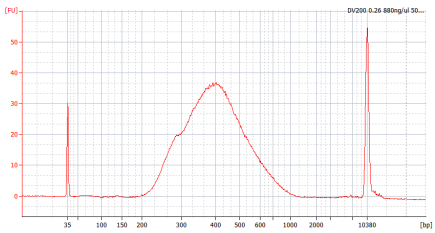
① Select “Advanced” under “Local”

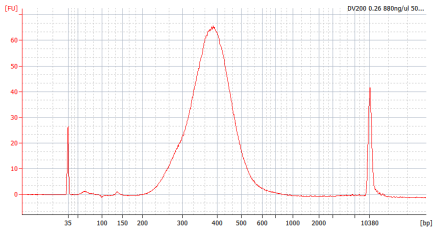
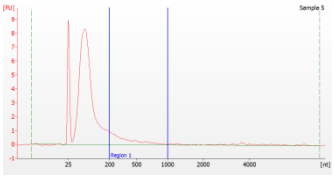
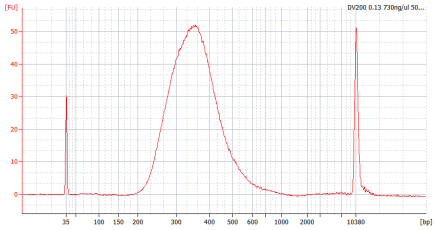
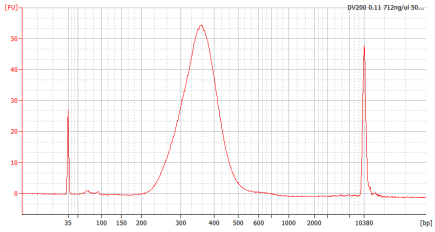
② Check the “Perform Smear Analysis” option under “Smear Analysis”

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

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

2 FFPE RNA library example

RNA sample quality control	conditions	Library distribution quality control
 RIN=2.2; DV_{200}=74%	Input RNA：500 ng Fragmentation conditions：94℃，7 min Two purifications after connection of the adapter：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：717.2 ng	
 RIN=2.2; DV_{200}=74%	Input RNA：500 ng Fragmentation conditions：94℃，7min Purification/sorting after adapter connection：0.6×；07×/0.15× Chain-specific library amplification：13cycles Library yield：437.8 ng	
 RIN=2.5; DV_{200}=26%	Input RNA：100 ng Fragmentation conditions：94℃，7 min Purification/sorting after adapter connection：0.6×；0.8× Chain-specific library amplification：15cycles Library yield： 206.8ng	
	Input RNA：500 ng Fragmentation conditions：85℃，8 min Purification/sorting after adapter connection：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：207 ng	

	Input RNA：500 ng Fragmentation conditions：85℃，8 min Purification/sorting after adapter connection：0.6×；0.70×/0.15× Chain-specific library amplification：13cycles Library yield：98.56 ng	
 RIN=2.5; DV_{200}=11%	Input RNA：500 ng Fragmentation conditions：65℃，8 min Purification/sorting after adapter connection：0.6×；0.8× Chain-specific library amplification：12cycles Library yield：354.2 ng	
	Input RNA：500 ng Fragmentation conditions：65℃，8 min Purification/sorting after adapter connection：0.6×；0.70×/0.15× Chain-specific library amplification：13cycles Library yield：172.48 ng	

Memo：

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