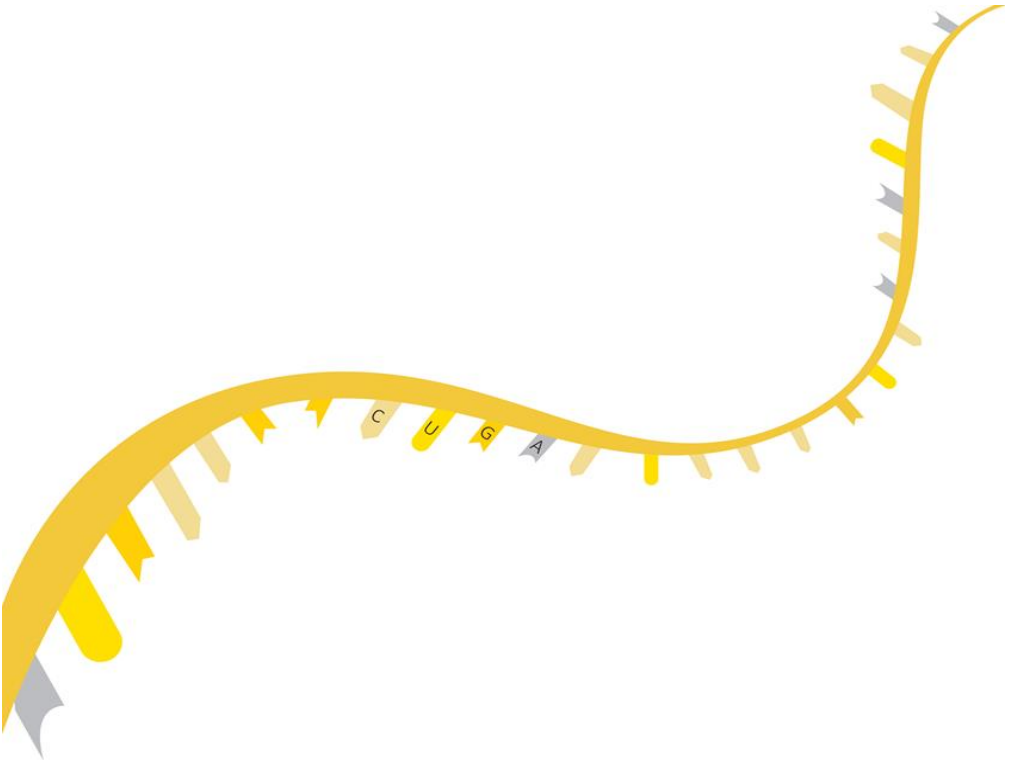
Stranded mRNA-seq Lib Prep Module for Illumina®

RK20349



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Version: N17H18v1.2

Table of Contents

1. Product Overview 1
2. Kit Components 2
3. Storage 3
4. Additional Materials Required 3
5. Workflow Diagram 4
6. Precautions 6
7. Protocol 10
8. Appendix 24
9. Appendix Table 31

1. Product Overview

- The kit is compatible with the Illumina sequencing platform.
- The kit is applicable to total RNA samples or purified mRNA samples from eukaryotes, including animals, plants, and fungi.
- The total initial RNA input is 10 ng-1 μg.
- The Stranded mRNA-seq Lib Prep Module for Illumina® contains the First Strand Synthesis Module，Second Strand Synthesis Module，DNA Lib Prep Module with UDG.
- The kit incorporates dUTP into the second strand cDNA during synthesis; the labeled strand is degraded with uracil-DNA glycosylase (UDG) before PCR-based library enrichment. This ensures that the final sequencing data comes exclusively from the first strand cDNA, maintaining mRNA strand specificity for stranded mRNA-seq.
- All reagents provided in the kit have undergone rigorous quality control. Each lot of the kits has been verified through library preparation and sequencing to ensure stable performance.

2. Kit Components

Tube Name and Color	24 rxns	96 rxns
2X Frag/Elute Buffer	144 µL	576 µL
RT Strand Specificity Reagent	192 µL	768 µL
First Strand Synthesis Enzyme Mix	48 µL	192 µL
Second Strand Synthesis Reaction Buffer with dUTP	192 µL	768 µL
Second Strand Synthesis Enzyme Mix	96 µL	384 µL
Nuclease-free Water	1 mL × 2	8 mL
End-prep Buffer	240 µL	960 µL
End-prep Enzyme Mix	72 µL	288 µL
Ligation Buffer	396 µL	1,584 µL
Ligase Mix	72 µL	288 µL
2X PCR Mix	600 µL	1,200 µL X 2
UDG Enzyme	12 µL	48 µL
Low EDTA TE	1 mL X 3	10 mL

denotes the color of the tube cap.

The kit needs to be used with truncated adapters, so PCR is a must to ensure an intact library structure and a unique index must be added to each sample. The kit can be combined with the following adapter modules for Illumina as needed:

Kit Name	Cat. No.
Unique Dual Index for Illumina MiniSet (8 indices)	RK21622
Unique Dual Index for Illumina MidiSet (24 indices)	RK21623
Unique Dual Index for Illumina Set_A (48 indices)	RK21624
Unique Dual Index for Illumina Set_B (48 indices)	RK21625
Unique Dual Index for Illumina Set_C (48 indices)	RK21626
Unique Dual Index for Illumina Set_D (48 indices)	RK21627

3. Storage

-20°C.

4. Additional Materials Required

- RNA enrichment kit:

  poly(A) mRNA Capture Module (ABclonal, Cat. No. RK20340).

  rRNA Depletion Module (H/M/R) (ABclonal, Cat. RK20348).

- Adapters:

  Truncated adapters for Illumina (ABclonal, Cat. No. RK21622~RK21627).

- Magnetic beads for purification:

  AFTMag NGS DNA Clean Beads (ABclonal, Cat. No. RK20257), Agencourt RNAClean XP Beads (Beckman Coulter, Inc., #A63987), or other equivalent magnetic beads for nucleic acid purification.

- RNA quality control:

  Qubit fluorometer.

  Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32855).

  Nanodrop.

  Agilent RNA 6000 Pico Chip (Agilent #5067-1513).

- Library quality control:

  Qubit fluorometer.

  ABQubit dsDNA Quantitation Kit (ABclonal, Cat. No. RK30140).

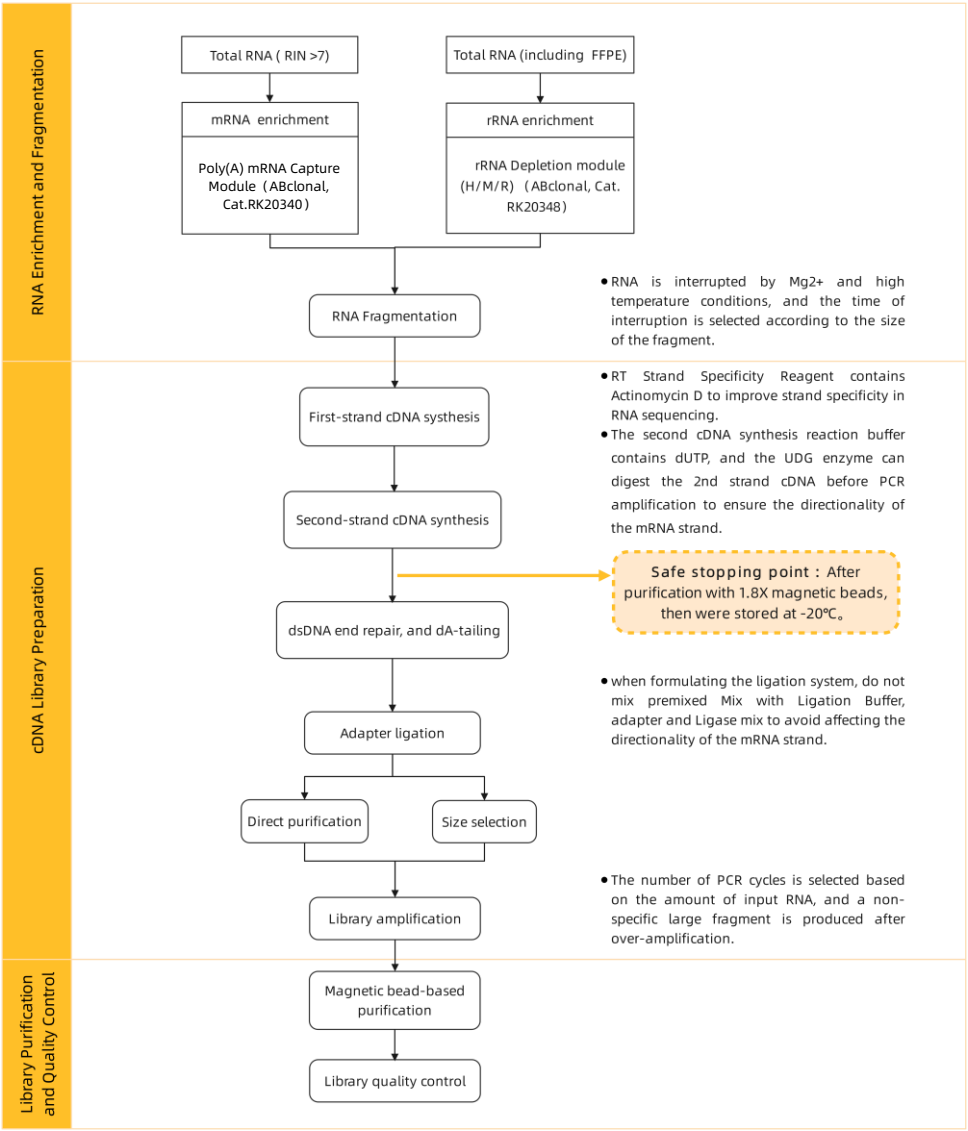
  Agilent High Sensitivity DNA Chips (Agilent #5067-4626).

  Agilent DNA 1000 Chip (Agilent #5067-1504).

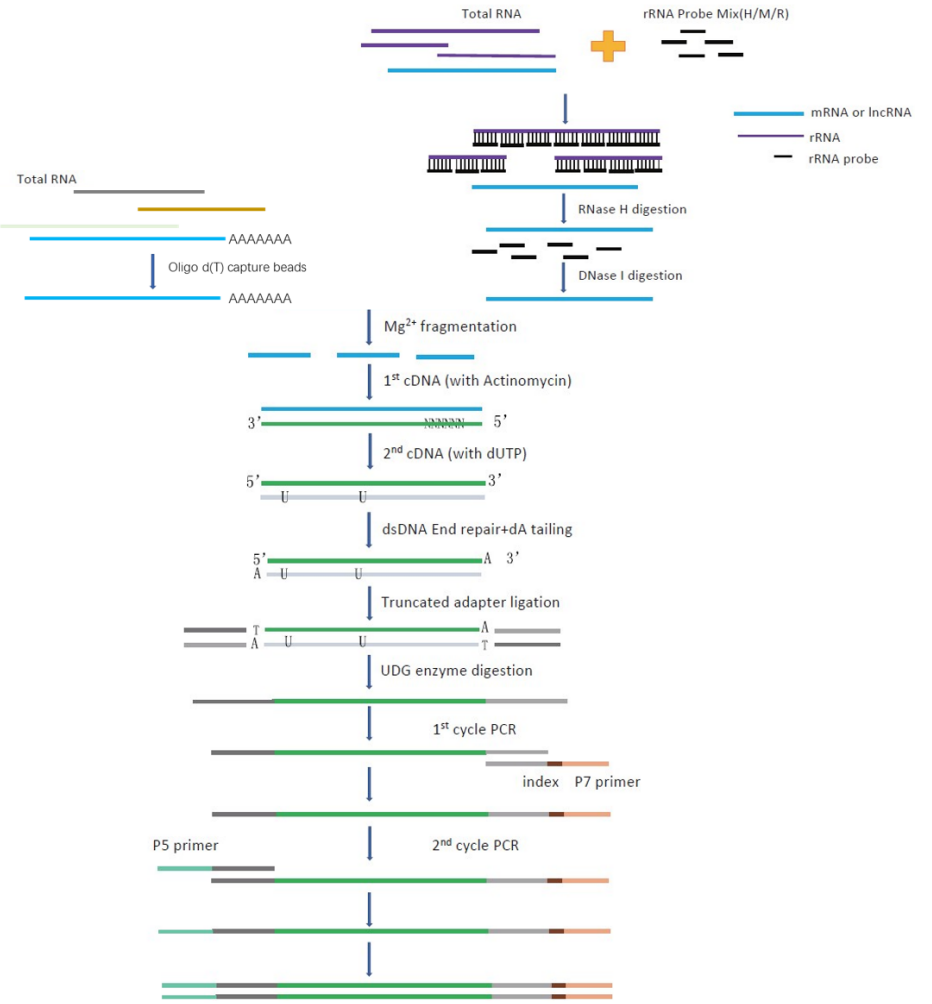
- Other reagents: freshly prepared 80% ethanol.
- Other instruments: magnetic rack, PCR system, etc.

5. Workflow Diagram

Flow diagram:



Schematic diagram:



6. Precautions

1. Quality Control of RNA Samples

◆ It is recommended to quantify the total RNA using the Qubit RNA HS Assay Kit (Thermo Fisher Scientific, Cat. No. Q32855). A lower RNA input may affect library preparation.

◆ The total initial RNA input is 10-1,000 ng, and RNA RIN is ≥ 7. It is recommended to combine the kit with a poly(A) mRNA capture kit for mRNA capture. If the RNA quality is poor, the rRNA Depletion Module series can be used for mRNA enrichment.

◆ The degraded RNA samples show poor mRNA integrity and can be fragmented under the conditions recommended in Table 1. It is recommended to choose direct purification after adapter ligation. If size selection is done after adapter ligation, the library yield will be compromised. In this case, PCR cycles can be increased by 2-3 cycles to improve the library yield.

Table 1. Fragmentation Conditions for RNA Samples of Varying Quality

RNA RIN	Fragmentation Condition
> 7	94°C 15 min
2-6	94°C 7 min
< 2	65°C 5 min

◆ For plant or other eukaryote cell RNA samples, if RNAs are degraded but their electrophoresis with the agarose gel shows the separate presence of 28S and 18S bands, it is recommended to increase the total RNA input and appropriately increase PCR cycles, which can also obtain desired libraries.

2. Use of Magnetic Beads

◆ The magnetic beads should be taken out half an hour in advance and brought to room temperature.

◆ The Oligo $(dT)_{25}$ Capture Beads and AFTMag NGS DNA Clean Beads should not be stored at -20°C. If they are frozen, they will agglomerate and cannot be separated. Therefore, the frozen magnetic beads cannot be used any longer and new ones need to be purchased.

◆ During the purification with AFTMag NGS DNA Clean Beads, the Low EDTA TE must be added for elution after complete alcohol volatilization, namely when the bead color changes from bright brown to frosted brown. Incomplete alcohol volatilization or excessive drying (leading to surface cracks) of beads may reduce the library yield.

3. Library Quality Control

◆ The library is deemed conforming if there is no burr-like peak, no detectable peak at 130 bp (adapter dimer), or no fragment peak with a large peak area at the right side of the library peak.

◆ The possible reason why library preparation with conforming total RNA (RIN > 7) fails and the solutions are as follows:

The RIN of total RNA is a metric for total RNA quality assessment and cannot definitely indicate the abundance and integrity of poly(A) RNAs. For some samples, the total RNA is intact, but many mRNAs are degraded. The degradation causes a large loss during poly(A) RNA purification. Thus, library preparation fails. In this case, the abundance and integrity of purified poly(A) RNAs can be assessed as follows: After Step 1.12, add 6 μL of Tris Buffer, heat the solution at 80°C for 2 minutes, place the centrifuge tube on the magnetic rack until the solution becomes

clear, and pipette 1 μL of the supernatant (namely intact poly(A) RNAs) for analysis with the Agilent 2100 Bioanalyzer and Agilent RNA 6000 Pico Chip.

If analysis results show that the mRNA abundance is low, increase the total RNA input. If the mRNA integrity is poor, adjust the mRNA fragmentation time.

4. Adapters for Library Preparation

◆ The kit is compatible with Illumina Truncated Adapter (as detailed in Appendix Table). Clients can select appropriate adapters as needed.

◆ The adapter quality and concentration directly affect the ligation efficiency and library yield. Excessive adapters may produce many adapter dimers, while fewer adapters may compromise the ligation efficiency and library yield. Table 2 lists the recommended adapter dilution factors for different RNA input.

Table 2. Recommended Adapter Dilution Factors

Input RNA	Dilution Factor
1 μg	1
100 ng	5
10 ng	10

5. Operating Procedures

◆ Masks and gloves should be worn during mRNA library preparation.

◆ After magnetic beads are added, all poly(A) mRNA enrichment steps should be conducted at room temperature.

◆ The RNA sample should be placed on ice and used for the next operation as soon as possible to avoid RNA degradation.

◆ The conditions for mRNA fragmentation and subsequent size selection should be determined according to the recommended parameter ranges in the Instructions for Use. Otherwise, the library size and yield will be compromised.

◆ During purification with magnetic beads, the supernatant should be pipetted cautiously to avoid disturbing the magnetic beads. Otherwise, the library fragment size and yield will be compromised.

◆ The PCR Index should be used cautiously to avoid cross contamination between the reagent and the sample.

◆ The reagent for each step can be pre-mixed in advance, and its volume should be 1.1 times the sample volume to make up for the natural loss.

7. Protocol

1 mRNA Capture and Fragmentation

Select one of the following RNA enrichment and fragmentation methods based on the experiment objective: Protocol 1: Poly(A) mRNA capture and fragmentation; Protocol 2: rRNA depletion and fragmentation.

Protocol 1: Poly(A) mRNA Capture and Fragmentation

1.1 Thaw RNA on ice, dissolve 10-1,000 ng of total RNA in 50 μL of nuclease-free water, and place the solution on ice for later use.

1.2 Vortex-mix 2X Oligo (dT)25 Capture Beads after returning to room temperature. Add 50 μL of the beads to the RNA solution, and mix by pipetting.

1.3 Incubate the mixture in the PCR system (heating lid temperature ≥ 75°C).

Temperature	Time
65°C	5 min
25°C	5 min

1.4 After incubation, place the centrifuge tube on the magnetic rack for about 2 minutes until the solution becomes clear, and remove and discard the supernatant.

1.5 Add 200 μL of Washing Buffer, and mix the solution by pipetting. Let the centrifuge tube stand on the magnetic rack until the solution becomes clear, and remove and discard the supernatant..

1.6 Take down the centrifuge tube from the magnetic rack, add 50 μL of Tris Buffer, mix the solution well by pipetting, and incubate the mixture in the PCR system (heating lid temperature 105°C).

Temperature	Time
80°C	2 min

1.7 After the solution reaches room temperature, add 50 µL of mRNA Binding Buffer, mix the solution well by pipetting, and let the mixture stand at room temperature for 5 minutes.

1.8 place the centrifuge tube on the magnetic rack for about 2 minutes until the solution becomes clear, and remove and discard the supernatant.

1.9 Take down the centrifuge tube from the magnetic rack, add 200 µL of Washing Buffer, and mix the solution by pipetting. place the centrifuge tube on the magnetic rack until the solution becomes clear, and remove and discard the supernatant.

1.10 Gently centrifuge the tube after capping, place the centrifuge tube on the magnetic rack, and remove all the residual supernatant with a 10 µL pipette and discard it.

1.11 Prepare the Frag/Elute Buffer according to the following table:

Reagent	Volume
2X Frag/Elute Buffer*	6 µL
Nuclease-free Water*	6 µL
Total volume	12 µL

* : The pre-mix can be prepared in advance based on the number of samples. No additional amount is needed.

1.12 Add 11 µL of Frag/Elute Buffer, mix the solution well by pipetting, and elute and fragment RNAs as per the following table (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-300 nt	94°C 15 min, 4°C hold
300-450 nt	94°C 10 min, 4°C hold
400-700 nt	94°C 5 min, 4°C hold

1.13 After cooling to 4°C, take out the tube, centrifuge it instantaneously, and place the tube on the magnetic rack until the solution becomes clear. Transfer 10 μL of the supernatant into another PCR tube, and immediately use it for the first strand cDNA synthesis.

Protocol 2: rRNA Depletion and Fragmentation

1.1 Probe/rRNA Hybridization

1.1.1 Take 10 ng-1 μg of total RNA, dilute to 12 μL with nuclease-free water, and place on ice for later use.

1.1.2 Thaw the Probe Hybridization Buffer on ice and prepare the probe hybridization pre-mix according to the following:

Reagent	Volume
Probe Hybridization Buffer	2 μL
rRNA Probe Mix (H/M/R)	1 μL
Total Volume	**3 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.1.3 Add 3 μL of the probe hybridization pre-mix into 12 μL of prepared RNA solution, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.1.4 Load the sample into the PCR system (**heating lid temperature 105°C**) to hybridize the probes to rRNA:

Temperature	Time
95°C	2 min
95°C-22°C	Ramp down to 22°C at 0.1°C/sec
22°C	5 min

1.1.5 After hybridization, transfer the sample from the PCR system onto ice, and proceed immediately with RNase H digestion.

1.2 RNase H Digestion

1.2.1 Thaw the 10X RNase H Buffer on ice and prepare the RNase H digestion pre-mix according to the following:

Reagents	Volume
10X RNase H Buffer	2 µL
RNase H	2 µL
Nuclease-free Water	1 µL
Total Volume	**5 µL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.2.2 Add 5 µL of the RNase H digestion pre-mix into the solution prepared in Step 1.1.5 to make a reaction system of 20 µL, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.2.3 Load the reaction system into the PCR system (heating lid temperature ≥ 45°C) for RNase H digestion:

Temperature	Time
37°C	30 min

1.2.4 After RNase H digestion, transfer the sample from the PCR system onto ice, and proceed immediately with DNase I digestion.

1.3 DNase I Digestion

1.3.1 Thaw the 10X DNase I Buffer on ice and prepare the DNase I digestion pre-mix according to the following:

Reagent	Volume
10X DNase I Buffer*	5 μL
DNase I*	2.5 μL
Nuclease-free Water*	22.5 μL
Total Volume	**30 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

1.3.2 Add 30 μL of the DNase I digestion pre-mix into the solution prepared in Step 1.2.4 to make a reaction system of 50 μL, mix well by gently pipetting up and down, and centrifuge instantaneously.

1.3.3 Load the reaction system into the PCR system (**heating lid temperature ≥ 45°C**) for DNase I digestion:

Temperature	Time
37°C	30 min

1.3.4 After DNase I digestion, transfer the sample from the PCR system onto ice, and proceed immediately with RNA purification.

1.4 Purification of rRNA-depleted RNA

1.4.1 Take Agencourt RNAClean XP Beads out of the 2-8°C environment in advance, let it stand for 30 min until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

1.4.2 After DNase I digestion, add 110 μL of Agencourt RNAClean XP Beads (2.2X) into each reaction tube, and mix them well by pipetting.

1.4.3 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

1.4.4 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

1.4.5 Repeat Step 1.4.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

1.4.6 Dry the magnetic beads for 2-3 min, add 7 μL of nuclease-free water after the ethanol has fully evaporated, and mix them well by pipetting.

1.4.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 5 μL of the supernatant into a new centrifuge tube.

1.4.8 Add 5 μL of 2X Frag/Elute Buffer, mix the solution well by pipetting, and fragment the RNA as per the following table (heating lid temperature 105°C):

Target Fragment Size	Fragmentation Condition
200-300 nt	94°C 15 min, 4°C hold
300-450 nt	94°C 10 min, 4°C hold
400-700 nt	94°C 5 min, 4°C hold

Notes: For FFPE RNA samples or highly degraded samples (RIN < 6), fragmentation time can be shortened. Refer to Appendix 2 for specific durations.

1.4.9 After cooling to 4°C, centrifuge the tube instantaneously and then place it on ice. Proceed immediately with the first strand cDNA synthesis.

2 First Strand cDNA Synthesis

2.1 Thaw RT Strand Specificity Reagent at room temperature, and prepare the following system on ice:

Reagent	Volume
Fragmented mRNA**	10 μL
RT Strand Specificity Reagent*	8 μL
First Strand Synthesis Enzyme Mix*	2 μL
Total Volume	**20 μL**

* : The pre-mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

** : The 2X Frag/Elute Buffer contains the Random Primer required for the First strand cDNA synthesis. Ensure that the 2X Frag/Elute Buffer is added when performing this step.

2.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system.

Temperature	Time
25°C	10 min
42°C	15 min
70°C	15 min
4°C	Hold

3 Second Strand cDNA Synthesis

3.1 Take Second Strand Synthesis Reaction Buffer with dUTP out of the refrigerator, thaw it on ice, and add the reagents listed in the table below.

Reagents	Volume
First strand cDNA	20 μL
Second Strand Synthesis Reaction Buffer with dUTP*	8 μL
Second Strand Synthesis Enzyme Mix*	4 μL
Nuclease-free Water*	48 μL
Total Volume	**80 μL**

* : The mix of Second Strand Buffer, Second Strand Enzyme Mix, and nuclease-free water can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

3.2 Mix the prepared system well by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled).

Temperature	Time
16°C	60 min

3.3 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

3.4 Add 144 μL of AFTMag NGS DNA Clean Beads (1.8X) into each incubated sample, and mix them well by pipetting.

3.5 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

3.6 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

3.7 Repeat Step 3.6, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

3.8 Dry the magnetic beads for 2-3 min, add 39 μL of Low EDTA TE after the ethanol has fully evaporated, and mix them well by pipetting.

3.9 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 37 μL of the supernatant into a new centrifuge tube.

- **The double-stranded cDNA elute can be stored at -20°C for no more than 24 hours.**

4 End Repair

4.1 Take the End-prep Buffer out of the refrigerator, thaw it on ice, and prepare the systems listed in the table below.

Reagent	Volume
Double-stranded cDNA (Step 3.9)	37 μL
End-prep Buffer*	10 μL
End-prep Enzyme Mix*	3 μL
Total Volume	**50 μL**

* : The mix of End-prep Buffer and End-prep Enzyme Mix can be prepared in advance based on the number of samples (recommended 1.1 times the required volume) and aliquoted into sample tubes.

4.2 Mix the prepared system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system according to the table below.

Temperature	Time
20°C	30 min
65°C	30 min
4°C	Hold

5 Adapter Ligation

5.1 Thaw the Ligation Buffer and Truncated Adapter on ice and prepare the adapter ligation system on ice.

Reagent	Volume
End-prep DNA (Step 4.2)	50 μL
Ligation Buffer*	16.5 μL
Truncated Adapter*	2.5 μL
Ligase Mix	3 μL
Total Volume	**About 70 μL**

* Ligation Buffer is viscous as it contains PEG, so it needs to be pipetted slowly to avoid volume errors that may affect the subsequent size selection.

** The adapter is a truncated one and is therefore not applicable to PCR-free library preparation, so the ligation products must be amplified.

Note: Do not pre-mix the Ligase Mix and Truncated Adapter when preparing the ligation system. Otherwise, adapter dimers will be produced, thus affecting the ligation efficiency.

5.2 Mix the ligation system by pipetting, centrifuge it instantaneously, and incubate it in the PCR system (with the heating lid disabled).

Temperature	Time
22°C	15 min

6 Size Selection

After the end of ligation, the ligation products can be purified with two options: direct purification or size selection:

When the total RNA input < 100 ng, direct purification is preferred.

When the total RNA input ≥ 100 ng, size selection is preferred according to the following table.

Fragmentation Condition	94°C 15 min	94°C 10 min	94°C 5 min
RNA fragment size	200-300 nt	300-450 nt	400-600 nt
Library fragment size	320-420 bp	420-570 bp	520-720 bp
Proportion of beads (1st round)	0.35X (35 μL)	0.3X (30 μL)	0.25X (25 μL)
Proportion of beads (2nd round)	0.2X (20 μL)	0.2X (20 μL)	0.15X (15 μL)

Notes: Refer to the Appendix for more size selection options (Options 1, 2, and 3). Both Options 1 and 2 are cautiously performed in Ligation Buffer. The fragments obtained with Option 2 are slightly larger and have a narrower size distribution range compared with those in Option 1. Option 3 is implemented in the aqueous phase. In this option, the ligation products are purified first (Step 5.2) before size selection (refer to the Appendix for specific procedures).

Direct Purification of Ligation Products

6.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

6.2 After ligation, add 56 μL of AFTMag NGS DNA Clean Beads (0.8X) into the ligation products, and mix them well by pipetting.

6.3 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.4 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.5 Repeat Step 6.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

6.6 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

6.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 μL of the supernatant into a new PCR tube for later use.

Size Selection of Ligation Products (fragmentation conditions: 94°C 10 min)

6.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

6.2 **Add 30 μL of nuclease-free water into the ligation system to get a 100 μL volume.**

6.3 Add 30 μL of AFTMag NGS DNA Clean Beads (0.30X), and mix the solution well by pipetting.

6.4 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear (**Do not discard the supernatant)**.

6.5 Transfer the supernatant into a new centrifuge tube, add 20 μL of AFTMag NGS DNA Clean Beads (0.2X), and mix them well by pipetting.

6.6 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

6.7 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

6.8 Repeat Step 6.7, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

6.9 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (***when the bead color changes from bright brown to frosted brown***), and mix them well by pipetting.

6.10 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 19.5 μL of the supernatant into a new PCR tube for later use.

7 PCR Amplification

7.1 After purification of ligation products, enrich the library with the Adapter Kit.

Reagent	Volume
Purification products	19.5 μL
2X PCR Mix	25 μL
UDG Enzyme	0.5 μL
UDI Primer*	5 μL
Total Volume	**50 μL**

*: UDI primer is premixed P5 and P7 index labeled primer. Caution is required when using UDI Primer. Both need to be pipetted very cautiously. Each pipette tip is for single use to avoid cross contamination between samples and reagents.

7.2 Mix the reaction system by pipetting, microcentrifuge the tube, and incubate it in the PCR system according to the table below (heating lid temperature 105°C).

Temperature	Time	Cycles
37°C	10 min	1
98°C	1 min	1
98°C	10s	
60°C	15s	8-16*
72°C	30s	
72°C	1 min	1
4°C	Hold	

* : Recommended PCR cycles:

Total RNA Input	Direct Purification PCR Cycles	Size Selection PCR Cycles
10 ng	15-16	-
100 ng	12-13	14-15
1 μg	8-9	10-11

7.3 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

7.4 After the end of reaction, add 40 μL of AFTMag NGS DNA Clean Beads (0.8X) into each reaction tube, and mix them well by pipetting.

7.5 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

7.6 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

7.7 Repeat Step 7.6, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

7.8 Dry the magnetic beads for 2-3 min, add 31 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

7.9 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 30 μL of the library into a new centrifuge tube for later use.

8. Appendix

1. Size Distribution of RNA Fragments

Fragment mRNAs purified from 1 μg of mouse tissue total RNA using 1X Frag/Elute Buffer at 94°C for 5, 10, and 15 minutes, respectively, place them on the magnetic rack, pipette the supernatant, and use Agencourt RNAClean XP Beads (2.2X volume) for purification. Use the Agilent RNA 6000 Pico Chip to analyze the fragment size distribution.

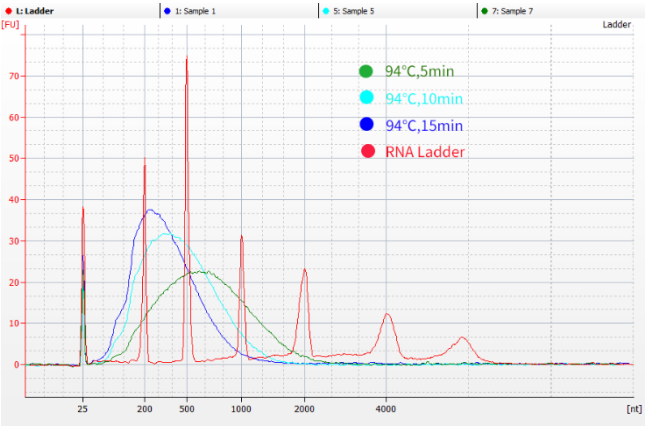


Figure 1. Size Distribution of mRNA Fragments (Agilent 2100 Bioanalyzer)

2. Library Size of Different RNA Fragmentation Time and Size Selection Programs

Different size selection options can help obtain fragments with varying main-peak patterns and those with varying size distribution ranges. ABclonal provides multiple size selection options to meet different needs. Options 1 and 2 are carried out in Ligation Buffer. The fragments obtained with Option 2 have smaller size distribution ranges and narrower library peak patterns compared with Option 1.

Option 3 is implemented in the aqueous phase, and library fragments selected with this option show stable size distribution ranges. Refer to Appendix 2.3 for specific procedures.

2.1 Size Selection of Adapter Ligation Products (Option 1)

Option 1. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 μL)	0.15X (15 μL)	520-720
94°C 10 min	0.30X (30 μL)	0.2X (20 μL)	420-570
94°C 15 min	0.35X (35 μL)	0.2X (20 μL)	320-420

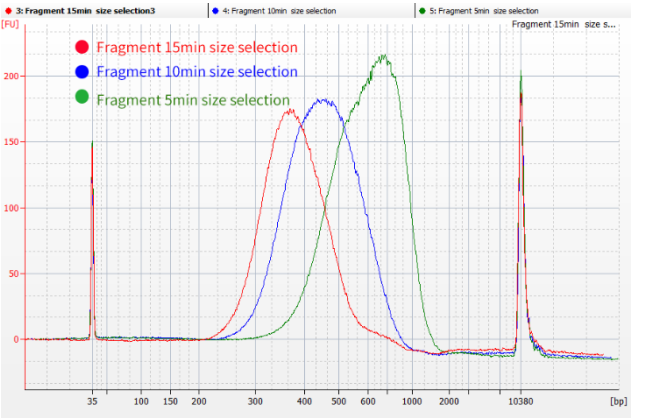


Figure 2. Library Fragment Size Distribution of Option 1 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and analyze them using Agilent High Sensitivity DNA Chips and the Agilent 2100 Bioanalyzer.

2.2 Size Selection of Adapter Ligation Products (Option 2)

Option 2. Bead Proportions for Size Selection and Library Size Distribution

mRNA Fragmentation Condition	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library Fragment Size (bp)
94°C 5 min	0.25X (25 μL)	0.1X (10 μL)	500-700
94°C 10 min	0.3X (30 μL)	0.1X (10 μL)	450-550
94°C 15 min	0.35X (35 μL)	0.1X (10 μL)	350-450

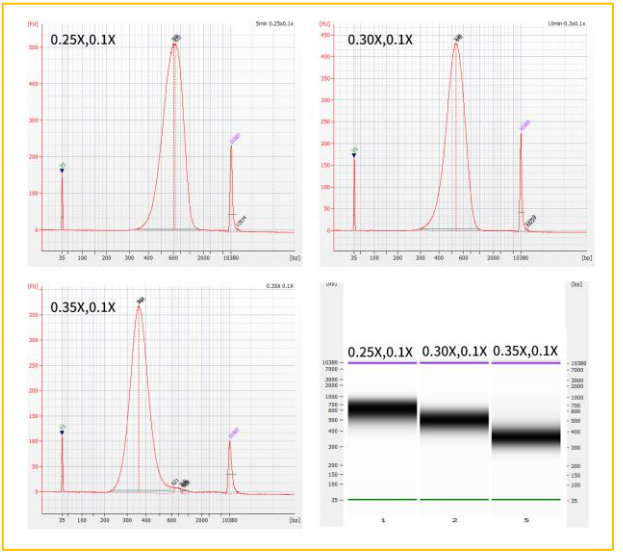


Figure 3. Library Fragment Size Distribution of Option 2 (Agilent 2100 Bioanalyzer)

Use 1 μg of mouse cell total RNA to obtain ligation products, select target fragments using different size selection conditions in Option 2, and amplify the selected fragments by 10 PCR cycles to obtain libraries. Dilute the libraries to 2 ng/μL and analyze their peak patterns using the Agilent 2100 Bioanalyzer.

2.3 Size Selection of Adapter Ligation Products (Option 3)

During adapter ligation, PEG contained in Ligation Buffer enables very sensitive size selection. Thus, a bead volume error tends to cause fragment size deviations. If high-quality fragments are required, it is recommended to conduct size selection in the aqueous phase. Specifically, purify the ligation products with 1.0X magnetic beads after ligation, elute them with 103 μL of water, and take 100 μL of the elution product for size selection as per the following table.

Option 3. Bead Proportions for Size Selection and Library Size Distribution

Fragmentation Condition	Purification of Ligation Products	Proportion of Beads (1st Round)	Proportion of Beads (2nd Round)	Library fragment size (bp)
94°C 5 min	Purify with 1.0X magnetic beads	0.55X (55 μL)	0.1X (10 μL)	600-720
94°C 10 min		0.6X (60 μL)	0.1X (10 μL)	500-600
		0.65X (65 μL)	0.1X (10 μL)	420-500
94°C 15 min		0.75X (75 μL)	0.1X (10 μL)	360-420
		0.8X (80 μL)	0.1X (10 μL)	320-360

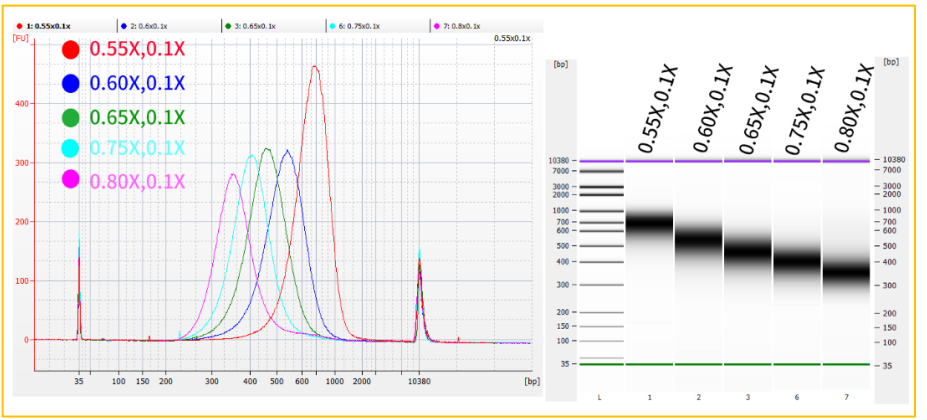


Figure 4. Library Fragment Size Distribution of Option 3 (Agilent 2100 Bioanalyzer)

Option 3: Operating Procedures for Size Selection in Aqueous Phase

2.3.1 Take AFTMag NGS DNA Clean Beads out of the 2-8°C environment in advance, let it stand until it reaches room temperature, and mix well by vortexing or oscillation prior to use.

2.3.2 After ligation, add 70 μL of AFTMag NGS DNA Clean Beads (1.0X) into the ligation products, and mix them well by pipetting.

2.3.3 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and carefully remove and discard the supernatant.

2.3.4 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.5 Repeat Step 2.3.4, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

2.3.6 Dry the magnetic beads for 2-3 min, add 102.5 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.7 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 100 μL of the supernatant into a new PCR tube for size selection.

2.3.8 Add 65 μL of AFTMag NGS DNA Clean Beads (0.65X 100 μL) into the purified ligation products, and mix them well by pipetting.

2.3.9 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear (Do not discard the supernatant).

2.3.10 Transfer 160 μL of the supernatant into a new centrifuge tube, add 10 μL of AFTMag NGS DNA Clean Beads (0.1X 100 μL), and mix them well by pipetting.

2.3.11 Let the mixture stand at room temperature for 5 min and then on the magnetic rack for another 5 min until the solution becomes clear, and cautiously remove and discard the supernatant.

2.3.12 Hold the centrifuge tube on the magnetic rack, add 200 μL of 80% ethanol, let it stand for 30s, and remove and discard all the supernatant.

2.3.13 Repeat Step 2.3.12, wash the magnetic beads once with 80% ethanol, and remove all the residual liquid with a 10 μL pipette.

2.3.14 Dry the magnetic beads for 2-3 min, add 22 μL of Low EDTA TE after the ethanol has fully evaporated (when the bead color changes from bright brown to frosted brown), and mix them well by pipetting.

2.3.15 Let the mixture stand at room temperature for 2 min and then on the magnetic rack for another 1 min until the solution becomes clear, and cautiously transfer 20 μL of the supernatant into a new PCR tube for later use.

2.4 The above results are internal test data. The ligation system is sensitive and closely associated with the size selection system, so the operating habits of operators or pipette errors may cause fragment size deviations.

- When larger library fragments are obtained, it is recommended to increase the volume of beads in the first round.
- When smaller library fragments are obtained, it is recommended to reduce the volume of beads in the first round.
- The bead proportion can be adjusted by 0.01X-0.05X based on the size deviations.

9. Appendix Table

Table 2. List of Truncated adapters for Illumina Kits

Type	Product Name	catalog
Unique Dual Index (8-base)	Unique Dual Index for Illumina MiniSet (8 indices)	RK21622
	Unique Dual Index for Illumina MidiSet (24 indices)	RK21623
	Unique Dual Index for Illumina Set_A (48 indices)	RK21624
	Unique Dual Index for Illumina Set_B (48 indices)	RK21625
	Unique Dual Index for Illumina Set_C (48 indices)	RK21626
	Unique Dual Index for Illumina Set_D (48 indices)	RK21627

* Note: Primers in the ABclonal Illumina truncated adapters kit can be used in this kit.

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