*Trans*NGS® Fast RNA-Seq Library Prep Kit for Illumina®

Please read the datasheet carefully prior to use.

Version No. Version 5.0

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Cat. No. KP701

Storage: at -20°C for one year

Description

The kit is designed for the Illumina high-throughput sequencing platform to preparestrand-specific or non-strand-specific transcriptome libraries. The applicable sample is 0.1 ng-100 ng of processed RNA (mRNA captured by mRNA magnetic beads or RNA obtained after rRNA removal) to prepare a high-quality sequencing library with high yield and complete information. The kit **completes the second-strand cDNA synthesis, end repair and A-tailing in one step, without the purification steps**, which greatly simplifies the operation process and shortens the library preparation time, and can complete the preparation of high-quality sequencing libraries within 3 hours .

Features

• No actinomycin D, no need to avoid light, non-toxic operation, higher strand-specific ratio.

• High library conversion rate, high library yield, and high data quality.

Application

• Whole transcriptome sequencing

• Gene expression analysis

• Single nucleotide variation analysis

• Variable splicing testing

• Fusion genetic testing

• Analysis of non-coding RNA and RNA precursor

Kit Content

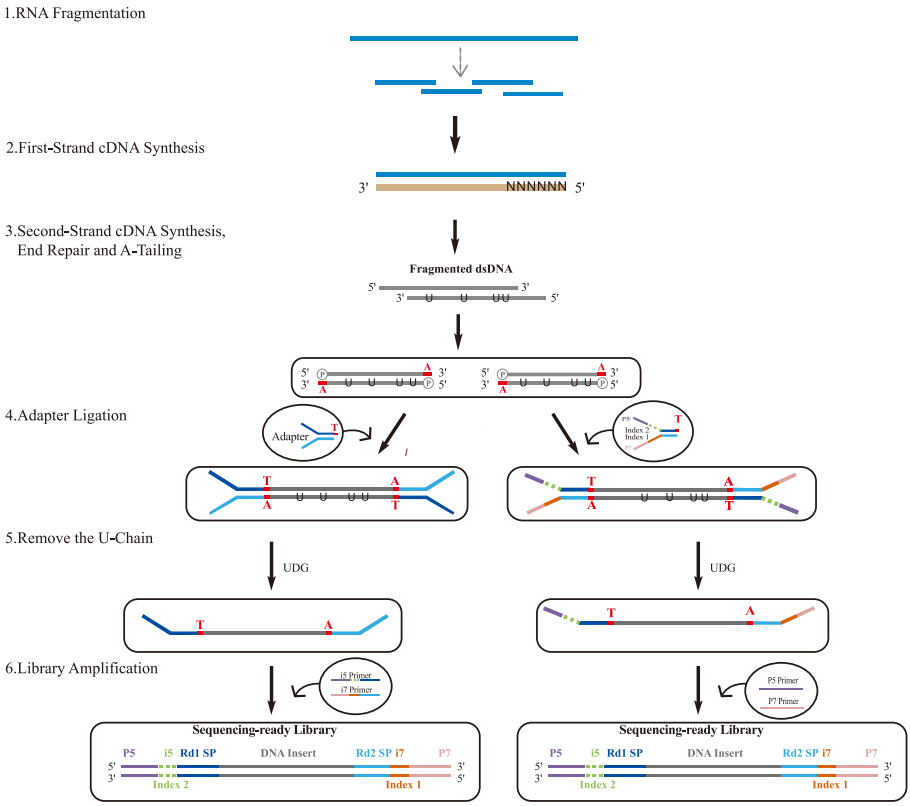
Component	KP701-01(12 rxns)	KP701-02(96 rxns)
1×RNA Fragmentation Buffer	230 µl	2×890 µl
Library First-Strand Buffer III	75 µl	580 µl
Library First-Strand Enzyme Mix II	24 µl	192 µl
Library SEA Buffer III (dUTP)	300 µl	4×600 µl
Library SEA Buffer III (dNTP)	300 µl	4×600 µl
Library SEA Enzyme Mix II	120 µl	960 µl
*Trans*NGS® Adapter for Illumina® (16 µM)	60 µl	480 µl
Adapter Dilution Buffer	600 µl	5 ml
Adapter Ligation Buffer IV	360 µl	4×720 µl
Adapter Ligation Enzyme III	60 µl	480 µl
*Trans*NGS® Library Amplification SuperMix (2×)	300 µl	4×600 µl
*Trans*NGS® Universal Primer for Illumina	60 µl	480 µl
Uracil-DNA Glycosylase	12 µl	96 µl
RNase-free Water	2×1 ml	3×5 ml

Note: The *Trans*NGS® Adapter for Illumina (16 µM) and *Trans*NGS® Universal Primer for Illumina do not contain an index and cannot be used together, and need to be used with anindex-containing primer (e.g. KI241/KI251) or an adapter containing an index (e.g. KI341/KI351), respectively.

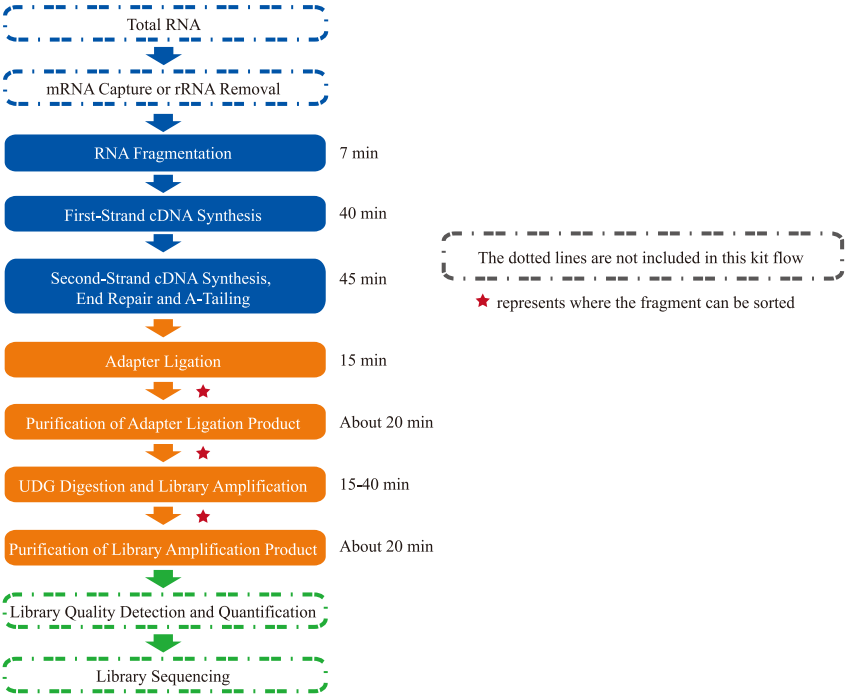
Input Sample Recommendations

For total RNA quantification, it is recommended to use a fluorescent dye method based on specific recognition of RNA, such as Qubit et al. To ensure the success rate of library construction, it is recommended to use Total RNA with RIN value ≥ 8 for mRNA capture (We recommend using *MagicPure*® mRNA Kit, Cat. No. EC511) or Total RNA with RIN value > 7 for rRNA Depletion (We recommend using *Trans*NGS® Ribo-Cap rRNA Depletion Kit (Bacteria/Animal/Plant), Cat. No. KD211/KD311/KD411).

Principle Chart and Flow Chart



Principle chart of strand-specific library construction
(No dUTP incorporation and UDG digestion steps in second-strand synthesis of non-strand-specific library)



Flow chart of strand-specific library construction
(No dUTP incorporation and UDG digestion steps in second-strand synthesis of non-strand-specific library)

Library Structure

If *Trans*NGS® Index Primers (384) Kit for Illumina®(Cat. No. KI241) or *Trans*NGS® UDI Primers (96) Kit for Illumina® (Cat. No. KI251) or *Trans*NGS® 96 UDI Indexed Adapter Kit for Illumina® (Cat. No. KI341), *Trans*NGS® 384 UDI Indexed Adapter Kit for Illumina® (Cat. No. KI351) are used, the library has the following sequences:

5' -AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT-XXXXXXXX-AGATCGGAAGAGCACACGTCTGAACTCCAGTCAC[i7]ATCTCGTATGCCGTCTTCTGCTTG-3'

i5：Index 2, 8 bases；i7：Index 1, 8 bases；-XXXXXXXX-：insert sequence.

Reagents not included in the kit:

- *MagicPure*® Size Selection DNA Beads (Cat. No. EC401).
- *Trans*NGS Index Primers (384) Kit for Illumina (Cat. No. KI241), or *Trans*NGS® UDI Primers (96) Kit for Illumina® (Cat. No. KI251), or *Trans*NGS 96 UDI Indexed Adapter Kit for Illumina (Cat. No. KI341), *Trans*NGS® 384 UDI Indexed Adapter Kit for Illumina® (Cat. No. KI351).
- Freshly prepared 80% ethanol.

Protocol

1.RNA Fragmentation

(1) After mRNA capture or rRNA removal, add 18.5 μl 1×RNA Fragmentation Buffer to the magnetic bead enrichment product of discarded supernatant, and vortex to mix.

(2) Place the tube in a thermal cycler and perform the following incubation steps (with heated lid set at 105°C). The RNA fragmentation procedure (insert size: 200 bp-350 bp) is as follows:

Temperature	Time
94°C	7 min
4°C	Hold

* For inserts of other sizes, the fragmentation conditions are as follows:

RNA insert sizes	Temperature	Time
150-250 bp	94°C	8-10 min
250-450 bp	94°C	3-7 min

(3) Remove the sample immediately after the sample temperature drops to 4°C. For fragmented products with magnetic beads, place the sample on a magnetic stand for 5 minutes, carefully pipet 17 μl of the supernatant into a new RNase-free PCR tube, and perform the first-strand cDNA synthesis. It is not recommended to pause.

2.First-Strand cDNA Synthesis

(1) Place the PCR tube completed in the previous step on ice, and add the following components:

Component	Volume
Fragmented RNA	17 μl
Library First-Strand Buffer III	6 μl
Library First-Strand Enzyme Mix II	2 μl
Total volume	25 μl

(2) Mix by pipetting several times, briefly centrifuge to collect the liquid on the wall of the tube.

(3) Place the tube in thermal cycler and perform the following incubation steps (with heated lid set at ≥85°C). The first-strand synthesis procedure is as follows:

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

3.Second-Strand cDNA Synthesis, End Repair and A-Tailing

(1) Place the PCR tube completed in the previous step on ice, and add the following components:

Component	Strand-specific library	Non-strand-specific library
First-Strand Product	25 µl	25 µl
Library SEA Buffer III (dUTP)	25 µl	-
Library SEA Buffer III (dNTP)	-	25 µl
Library SEA Enzyme Mix II	10 µl	10 µl
Total volume	60 µl	60 µl

(2) Mix by pipetting several times, briefly centrifuge to collect the liquid on the wall of the tube.

(3) Place the tube in thermal cycler and perform the following incubation steps (with heated lid set at ≥85°C). The second-strand synthesis procedure is as follows:

Temperature	Time
16°C	30 min
72°C	15 min
4°C	Hold

4.Adapter Ligation

(1) Thaw the Adapter with index (such as KI341/KI351) or the *Trans*NGS® Adapter for Illumina® (16 µM) without index on ice, dilute Adapter to appropriate concentration with Adapter Dilution Buffer referring to the table below.

RNA Input	Adapter Dilution Factor
1 µg - 5 µg	No dilution
200 ng - 999 ng	Diluted 3 times
50 ng - 199 ng	Diluted 10 times

* Select the Adapter according to the needs. If index is added in adapter ligation, use an Index-containing Adapter (such as KI341/KI351); if index is added in library amplification, use the *Trans*NGS® Adapter for Illumina without an index. The quality and input of Adapter directly affect the efficiency and quality of library construction. It is recommended to use TransGen Adapter. Increasing input of Adapter can increase the library output at a certain extent, but too high will lead to residues of Adapter and form dimers; insufficient input of Adapter will affect the ligation efficiency and lead to poor library quality.

(2) Place the PCR tube completed in the previous step on ice, and add the following components:

Component	Volume
ds-cDNA	60 µl
Appropriate concentration of Adapter	5 µl
Adapter Ligation Buffer IV	30 µl
Adapter Ligation Enzyme III	5 µl
Total volume	100 µl

* Do not mix the Adapter with Adapter Ligation Buffer IV and Adapter Ligation Enzyme III inadvance.

(3) Mix by pipetting several times, briefly centrifuge to collect the liquid on the wall of the tube.

(4) Place the tube in thermal cycler and perform the adapter ligation procedure (with hot lid closed):

Temperature	Time
20°C	15 min
4°C	Hold

5. Purification of Adapter Ligation Products

It is recommended to use 0.6×*MagicPure*® Size Selection DNA Beads (Cat. No. EC401) for purification of product with index; 0.8×*MagicPure*® Size Selection DNA Beads for purification of products without index.

* If the RNA input is small, or the RNA integrity is low, ligation products are recommended to sort directly (without purification), and the sorting conditions are shown in step 6.2.

(1) Take out the magnetic beads from 2-8°C and stay still for 30 minutes at room temperature before use.

(2) Vortex the magnetic beads to mix well, add 60 µl magnetic beads (0.6×) or 80 µl magnetic beads (0.8×) to the product of the previous step.

(3) Pipet to mix well, and incubate for 5 minutes at room temperature.

(4) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), discard the supernatant.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Make sure the beads settle to the magnet completely. Be careful not to disturb the beads, otherwise will affect the final yield.

(5) Add 200 µl of freshly prepared 80% ethanol to the tube on the magnetic stand, do not pipet the beads. Incubate at room temperature for 30 seconds. Discard the supernatant.

* Be sure to use freshly prepared ethanol, otherwise it will affect the experimental results.

(6) Repeat step (5) once.

(7) Air dry the beads at room temperature while the tube is on the magnetic stand.

* It is advisable to dry until the beads lose luster but are not cracked; do not heat to dry, otherwise the final yield will be affected.

(8) Remove the tube from the magnetic stand. Add 105 µl RNase-free Water. Mix the beads thoroughly by pipetting or vortexing. Incubate at room temperature for 2 minutes.

(9) Place the tube on the magnetic stand. Incubate at room temperature until the solution is clear (about 2 minutes). Make sure the beads settle to the magnet completely.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Incubation time can be extended to 5 minutes at room temperature. Make sure the beads settle to the magnet completely.

(10) Carefully transfer **100 µl** of the eluate to a clean PCR tube for sorting, or store at -20°C.

Note: DNA at low concentrations is unstable, it is recommended to sort and amplify the library immediately, and it is not recommended to store at -20°C.

6.1. Sorting after Purification of Adapter Ligation Products (Followed by Step 5)

It is recommended to use *MagicPure*® Size Selection DNA Beads (Cat. No. EC401). See the table below for sorting conditions.

Sorting conditions for long adapter ligation products				
Average product size(bp)	~ 380	~ 430	~ 480	~ 530
Insert size (bp)	~ 250	~ 300	~ 350	~ 400
First volume ratio (DNA Beads: DNA)	0.70×	0.65×	0.60×	0.55×
Second volume ratio (DNA Beads: DNA)	0.15×	0.15×	0.15×	0.15×

Sorting conditions for short adapter ligation products				
Average product size(bp)	~ 310	~ 360	~ 410	~ 460
Insert size (bp)	~ 250	~ 300	~ 350	~ 400
First volume ratio (DNA Beads: DNA)	0.75×	0.70×	0.65×	0.60×
Second volume ratio (DNA Beads: DNA)	0.15×	0.15×	0.15×	0.15×

Taking the sorting condition of library size ~480 bp as an example. (Long adapter libraries are recommended to use the following sorting conditions).

(1) Take out the magnetic beads from 2-8°C and incubate for 30 minutes at room temperature before use.

(2) Vortex the magnetic beads to mix well, pipet 60 μl beads (0.60×) to the 100 μl product of the previous step.

(3) Pipet to mix well, and incubate for 5 minutes at room temperature.

(4) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), pipet 155 μl supernatant into a clean PCR tube.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Make sure the beads settle to the magnet completely. Be careful not to disturb the beads, otherwise will affect the final selection effect.

(5) Add 15 μl beads (0.15×) to the supernatant again.

(6) Pipet to mix well, and incubate for 5 minutes at room temperature.

(7) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), discard the supernatant.

(8) Add 200 μl of freshly prepared 80% ethanol to the tube on the magnetic stand, do not pipet the beads. Incubate at room temperature for 30 seconds. Discard the supernatant.

* Be sure to use freshly prepared ethanol, otherwise it will affect the experimental results.

(9) Repeat step (8) once.

(10) Air dry the beads at room temperature while the tube is on the magnetic stand.

* It is advisable to dry until the beads lose luster but are not cracked; do not heat to dry, otherwise the final yield will be affected.

(11) Remove the tube from the magnetic stand. Add 22 μl RNase-free Water. Mix the beads thoroughly by pipetting or vortexing. Incubate at room temperature for 2 minutes.

(12) Place the tube on the magnetic stand. Incubate at room temperature until the solution is clear (about 3 minutes). Make sure the beads settle to the magnet completely.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Incubation time can be extended to 5 minutes at room temperature.

(13) Carefully transfer 19 μl or 20 μl of the eluate to a clean PCR tube for the next step of the library amplification or store at -20°C.

6.2. Direct Sorting of Adapter Ligation Products (Followed by Step 4)

With low RNA input, or low RNA integrity, direct sorting of the ligation product is recommended(without purification). There is no significant difference in the size of the main peak of the library, the yield is significantly increased, and the peak shape is slightly wider using this method. Taking the sorting condition of library size ~430 bp as an example.

(1) Take out the magnetic beads from 2-8°C and incubate for 30 minutes at room temperature before use.
(2) Vortex the magnetic beads to mix well, pipet 25 μl beads (0.25×) to the product of adapter ligation.
(3) Pipet to mix well, and incubate for 5 minutes at room temperature.
(4) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), pipet 122 μl supernatant into a new PCR tube, and discard the beads.
* Spin down briefly before put on magnetic stand if there is liquid on the wall. Make sure the beads settle to the magnet completely. Be careful not to disturb the beads, otherwise will affect the final selection effect.
(5) Add 15 μl beads (0.15×) to the supernatant again.
(6) Pipet to mix well, and incubate for 5 minutes at room temperature.
(7) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), discard the supernatant.
(8) Add 200 μl of freshly prepared 80% ethanol to the tube on the magnetic stand, do not pipet the beads. Incubate at room temperature for 30 seconds. Discard the supernatant.
* Be sure to use freshly prepared ethanol, otherwise it will affect the experimental results.
(9) Repeat step (8) once.
(10) Air dry the beads at room temperature while the tube is on the magnetic stand.
* It is advisable to dry until the beads lose luster but are not cracked; do not heat to dry, otherwise the final yield will be affected.
(11) Remove the tube from the magnetic stand. Add 22 μl RNase-free Water. Mix the beads thoroughly by pipetting or vortexing. Incubate at room temperature for 2 minutes.
(12) Place the tube on the magnetic stand. Incubate at room temperature until the solution is clear (about 2 minutes). Make sure the beads settle to the magnet completely.
* Spin down briefly before put on magnetic stand if there is liquid on the wall. Incubation time can be extended to 5 minutes at room temperature.
(13) Carefully transfer 19 μl or 20 μl of the eluate to a clean PCR tube for the next step of the library amplification or store at -20°C.

7. Library Amplification

(1) Prepare the library amplification system as needed. When using the adapter with index (such as KI341/351), add reagents as follows:

Component	Strand-specific library	Non-strand-specific library
Purified product	19 μl	20 μl
Uracil-DNA Glycosylase	1 μl	-
*Trans*NGS® Library Amplification SuperMix (2×)	25 μl	25 μl
*Trans*NGS® Universal Primer for Illumina	5 μl	5 μl
Total volume	50 μl	50 μl

When using the *Trans*NGS®Adapter for Illumina without index, it needs to be used with i5/i7 primer with index (such as KI241/KI251), add reagents as follows:

Component	Strand-specific library	Non-strand-specific library
Purified product	19 μl	20 μl
Uracil-DNA Glycosylase	1 μl	-
*Trans*NGS® Library Amplification SuperMix (2×)	25 μl	25 μl
i5 primer*	2.5 μl	2.5 μl
i7 primer*	2.5 μl	2.5 μl
Total volume	50 μl	50 μl

* *Trans*NGS® Index Primers (384) Kit for Illumina® (Cat. No. KI241) provides 16 i5 Primers and 24 i7 Primers; *Trans*NGS® UDI Primers (96) Kit for Illumina® (Cat. No. KI251) provides 96 kinds of UDI Primers, please choose according to your needs.

(2) Mix by pipetting several times, briefly centrifuge to collect the liquid on the wall of the tube.

(3) Perform the following amplification procedure in a thermal cycler (with the heated lid set at 105°C).

50°C	5 min*	
98°C	3 min	
98°C	10 sec	10-16 cycles**
60°C	30 sec	
72°C	30 sec	
72°C	3 min	
4°C	Hold	

* For strand-specific libraries, amplify after incubation; for non-strand-specific libraries, directly amplify without incubation.

** For different RNA inputs, the recommended number of amplification cycles is as follows. Adjust the number of cycles appropriately according to the species and RNA integrity.

Total RNA Input	Strand-specific library	Non-strand-specific library
1 μg	11-12 cycles	10-11 cycles
200 ng	13-14 cycles	12-13 cycles
50 ng	15-16 cycles	14-15 cycles

8. Purification of Library Amplification Products

It is recommended to use 0.9×*MagicPure*® Size Selection DNA Beads (Cat. No. EC401). Increase (to obtain libraries with shorter inserts) or decrease the beads ratio (to reduce primer residue) as needed. The specific steps of purification using 0.9× magnetic beads are as follows:

(1) Take out the magnetic beads from 2-8°C and incubate for 30 minutes at room temperature before use.

(2) Vortex the magnetic beads to mix well, add 45 μl magnetic beads (0.9×) to the product of the previous step.

(3) Pipet to mix well, and incubate for 5 minutes at room temperature.

(4) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), discard the supernatant.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Make sure the beads settle to the magnet completely. Be careful not to disturb the beads, otherwise will affect the final yield.

(5) Add 200 µl of freshly prepared 80% ethanol to the tube on the magnetic stand, do not pipet the beads. Incubate at room temperature for 30 seconds. Discard the supernatant.

* Be sure to use freshly prepared ethanol, otherwise it will affect the experimental results.

(6) Repeat step (5) once.

(7) Air dry the beads at room temperature while the tube is on the magnetic stand.

* It is advisable to dry until the beads lose luster but are not cracked; do not heat to dry, otherwise the final yield will be affected.

(8) Remove the tube from the magnetic stand. Add 22 µl RNase-free Water. Mix the beads thoroughly by pipetting or vortexing. Incubate at room temperature for 2 minutes.

* If the library needs to be stored for a long time, it is recommended to use 0.1×TE solution for elution.

(9) Place the tube on the magnetic stand. Incubate at room temperature until the solution is clear (about 2 minutes). Make sure the beads settle to the magnet completely.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Incubation time can be extended to 5 minutes at room temperature. Make sure the beads settle to the magnet completely.

(10) Carefully transfer 20 µl of the eluate to a clean 1.5 ml centrifuge tube. The product can be stored at -20°C.

9. Sorting of Library Amplification Products(Optional)

For low input or low quality RNA, fragment sorting after library amplification may also be considered. It is recommended to use *MagicPure*® Size Selection DNA Beads (Cat. No. EC401). According to the selection conditions (Table in step 6.1), taking the library size of ~480 bp as an example (Long adapter libraries are recommended to use the following sorting conditions).

(1) Take out the magnetic beads from 2-8°C and incubate for 30 minutes at room temperature before use.

(2) Vortex the magnetic beads to mix well, pipet 60 µl beads (0.60×) into a PCR tube, add 100 µl product from the previous step diluted with RNase-free Water.

(3) Pipet to mix well, and incubate for 5 minutes at room temperature.

(4) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), pipet 155 µl supernatant into a new PCR tube, and discard the beads.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Make sure the beads settle to the magnet completely. Be careful not to disturb the beads, otherwise will affect the final selection effect.

(5) Add 15 µl beads (0.15×) to the supernatant again.

(6) Pipet to mix well, and incubate for 5 minutes at room temperature.

(7) Place the tube on the magnetic stand and incubate at room temperature, make sure the beads settle to the magnet completely. After the solution is clear (about 5 minutes), discard the supernatant.

(8) Add 200 µl of freshly prepared 80% ethanol to the tube on the magnetic stand, do not pipet the beads. Incubate at room temperature for 30 seconds. Discard the supernatant.

* Be sure to use freshly prepared ethanol, otherwise it will affect the experimental results.

(9) Repeat step (8) once.

(10) Air dry the beads at room temperature while the tube is on the magnetic stand.

* It is advisable to dry until the beads lose luster but are not cracked; do not heat to dry, otherwise the final yield will be affected.

(11) Remove the tube from the magnetic stand. Add 22 μl RNase-free Water. Mix the beads thoroughly by pipetting or vortexing. Incubate at room temperature for 2 minutes.

(12) Place the tube on the magnetic stand. Incubate at room temperature until the solution is clear (about 3 minutes). Make sure the beads settle to the magnet completely.

* Spin down briefly before put on magnetic stand if there is liquid on the wall. Incubation time can be extended to 5 minutes at room temperature.

(13) Carefully transfer 20 μl of the eluate to a clean 1.5 ml centrifuge tube. Store at -20°C.

10. Library Quality Detection

It is recommended to use Qubit, etc. for concentration detection, and bioanalyzer (e.g. Agilent 2100, Qsep, etc.) for fragment size distribution detection. The main peak of a properly fragmented library should be between 350-470 bp, as shown in Figure 1. If there is a sharp peak around 135 bp (adapter-dimer contamination), the library product needs to be purified again with 0.8×*MagicPure*® Size Selection DNA Beads (Cat. No. EC401). After the library is diluted to 50 μl with RNase-free Water, the purification method referring to (2)-(10) in step 8, and assess peak shape.

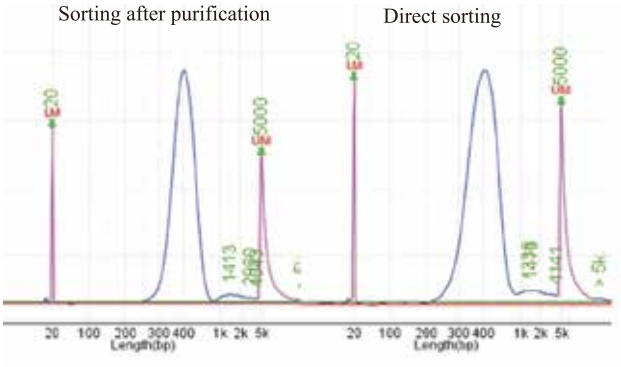


Figure 1 Library size distribution

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