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DNA Library Prep Kit PLUS for Illumina

Universal library preparation kit for Illumina high-throughput sequencing platforms

Component	Cat#	M4401.0024	M4401.0096	Colour code of cap
FEA Buffer		120µL	480µL	
FEA Enzyme Mix		240µL	960µL	
Rapid Ligation Buffer		600µL	4 x 600µL	
Rapid DNA Ligase		120µL	480µL	
HiFi Amplification Mix		600µL	4 x 600µL	
PCR Primer Mix		120µL	480µL	
Neutralization Buffer		120µL	480µL	
Control DNA (264bp, 50ng/µL)		10µL	10µL	

Description

Genaxxon's DNA Library Prep Kit PLUS for Illumina is an advanced solution meticulously designed to elevate your sequencing experience on Illumina high-throughput platforms. This kit introduces enzyme-based fragmentation tailored exclusively for Illumina sequencing.

This kit streamlines the DNA library preparation process by seamlessly integrating DNA fragmentation, end repair, and dA-tailing into a single step. Further simplifying the workflow, adapter ligation, library amplification, and size selection can be carried out directly without the need for additional purification steps. Whether working with 100 pg or 1 µg of template DNA, this kit effortlessly transforms it into a library compatible with Illumina high-throughput sequencing platforms.

Genaxxon's DNA Library Prep Kit PLUS is compatible with DNA from different sources and of different input amounts, while you only need to adjust the fragmentation time based on the target insert size to obtain a library with the desired fragment size.

Features and Applications

Discover the top features of Genaxxon's DNA Library Prep Kit PLUS for Illumina:

- Enhanced Reliability: The kit has the ability to significantly reduce the proportion of Artificial Invert Chimera Reads in DNA libraries of FFPE samples. Simultaneously, it enhances the reliability of detecting SNV and other biomarkers.
- Wide Compatibility: The kit is suitable for various species and compatible with FFPE.
- Simple and fast: The optimized kit eliminates mechanical disruption of the genome, simplifies library preparation, and reduces operating time.

The kit is suitable for:

- Whole-genome sequencing
- Whole-exome sequencing or other targeted capture sequencing
- Metagenome and Methylation sequencing

This product is for research use only.

Storage and shipment

Shipment: on wet ice. Store at -30°C to -15°C.

Required additional material

- Magnetic beads (Cat# S5352)
- DNA Adapters (Cat# M4402, M4403)
- Absolute ethanol
- ddH_2O

Product Use Limitations

DNA Library Prep Kit PLUS for Illumina is developed, designed, and sold for research purposes only. It is not to be used for human, diagnostic or drug purposes or to be administered to humans unless expressly cleared for that purpose by the Food and Drug Administration in the USA or the appropriate regulatory authorities in the country of use. All due care and attention should be exercised in the handling of many of the materials described in this manual.

Safety information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate material safety data sheets (MSDSs). These are available online as a pdf-file or upon request (info@genaxxon.com).

Quality control

Genaxxon bioscience DNA Library Prep Kit PLUS for Illumina undergoes stringent quality controls and functional testing to ensure optimal stability and repeatability.

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Protocol Part

Important notes before getting started

The protocol serves as a guideline for library preparation. Multiple factors such as the sample, protocol, equipment, and operation may vary. Thus, it may be necessary to adjust the parameters of the library preparation procedures. Please read the following notes carefully to obtain a high-quality library. In case of any questions, please contact Genaxxon: info@genaxxon.com.

1. Input DNA and Fragmentation

• Starting material: 100pg to 1µg input DNA; please use high-quality input DNA (A260/A280 = 1.8-2.0) whenever possible. Please find recommended amounts of input DNA for different applications in Table 1.

Table 1: Recommended amounts of input DNA for various applications.

Application	Sample type	Recommended amount of input DNA
Whole-genome sequencing	Complex gDNA	50ng - 1µg
Targeted capture sequencing	Complex gDNA	10ng - 1µg
Whole-genome/targeted capture sequencing	FFPE DNA	≥50ng
Whole-genome sequencing	Microbial genome	1ng - 1µg
Whole-genome sequencing (PCR-free library)	Complex/Simple Genome	≥50ng (no size selection) ≥200ng (size selection)

Note: Table 1 presents the cycle number measured when using approximately 200 bp of high-quality input DNA. When the DNA quality is poor and the library fragment length is long, it is necessary to increase the number of cycles as appropriate to obtain a sufficient library.

Note: If size selection has been performed during library preparation, Library Amplification should be performed with the higher number of cycles; otherwise, the lower number of cycles is feasible.

• It is recommended to use ddH_2O to dissolve DNA samples. Since FEA Enzyme Mix is sensitive to EDTA, the EDTA concentration must be examined. For example, if the final EDTA concentration in the end repair reaction solution is greater than 0.1 mM, pre-treat DNA samples (see Experimental Process section of this manual).

2. DNA Adapters

• Genaxxon's DNA Library Prep Kit PLUS is compatible with a variety of non-index, single-index and dual-index adapters.

• The quality and amounts of adapters directly affect the preparation efficiency and library quality. Table 2 lists the recommended adapter usage for different input DNA amounts.

Table 2: Recommended adapter concentration for 100pg to 1µg input DNA.

Input DNA	Adapter: Input molar ratio	Adapter Working concentration
500ng to 1µg	10:1 to 20:1	10µM
100ng to 500ng	20:1 to 100:1	10µM
25ng to 100ng	40:1 to 200:1	5µM
5ng to 25ng	50:1 to 200:1	1µM
100pg to 5ng	60:1 to 3000:1	0.1 to 0.2µM

Note: A too high input of adapters may lead to residual adapters or adapter dimer, while an insufficient input may affect the ligation efficiency and reduce library yield.

• It is recommended to use 0.1x TE-buffer to dilute adapters according to the above table. This ensures that the adapters are used with a fixed volume (5µL) during the library preparation process, avoiding incorrect loading volume.

• The adapter quality directly affects the molar ratio of adapter and input DNA, which in turn affects the ligation efficiency and library yield. High-quality adapters should be used; 0.1 × TE-buffer should be used to dilute and store the adapter solution. Repeated freeze-thaw cycles should be avoided.

• Increasing the amount of adapters used can improve library yields to some extent. However, it is important to note that increasing the adapter concentration may increase the adapter residue in the library, resulting in wasted sequencing data.

3. Adapter ligation product purification

• After adapter ligation, the remaining adapters must be removed before library amplification (PCR library) or direct sequencing (PCR-free library). The default purification condition of 0.6 × (100µL products and 60µL magnetic beads) is applicable to most cases. To obtain libraries with larger insert size, you can reduce the amount of magnetic beads to reduce the content of small DNA fragments. However, this adjustment can only roughly change the position of the main peak of the library. If accurate control of the library size distribution is required, size selection can be performed after this purification process.

• If library size selection is being performed later, the recommended elution volume is 105µL; otherwise, the elution volume is recommended to be 22.5µL.

• If the data shows that the purification products are heavily contaminated with unwanted adapters or adapter dimers, they can be re-purified using magnetic beads: Make up the volume of the initial purified product to 50µL using ddH_2O, and add 50µL magnetic beads (1×) for the second purification. This can significantly reduce the residual adapters or adapter dimers, especially for PCR-free library. Sometimes, the amount of adapters used also needs to be reduced to completely eliminate the residual adapters or adapter dimers.

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4. Magnetic beads

It is recommended to use magnetic beads (Cat# S5352) for purification.

• The amount of magnetic beads used is indicated by the usual multiplier "×", expressed as a multiplier of the original sample volume.

• The amount of magnetic beads used directly affects the lower limit of the length of DNA that can be purified. The higher the multiplier, the shorter the purified DNA length is, and vice versa.

• The magnetic beads should be brought to room temperature at least 30min prior to use; otherwise, decreased yield and poor size selection effect may occur.

• The magnetic beads should be mixed well by shaking thoroughly, vortexing or pipetting up and down before each use.

• After the sample and magnetic beads are thoroughly mixed, place the mixture on the magnetic stand for separation. When the solution becomes completely clear, pipette the supernatant, 2 – 3μL supernatant should be left. Avoid disturbing the magnetic beads during pipetting; otherwise, the yield will decrease, the effect of size selection will be poor, and even the subsequent enzymatic reaction will be affected. In this case, the magnetic beads can be mixed and placed on the magnetic stand again for separation. Given the varying performance of the magnetic stand, a longer separation time may be needed to separate the magnetic beads from the liquid completely.

• Magnetic beads should be rinsed using 80% ethanol that is freshly prepared and brought to room temperature. During rinsing, always keep the EP tube on the magnetic stand without disturbing the magnetic beads.

• Dry the magnetic beads at room temperature before elution. Insufficient drying may lead to absolute ethanol residue which will affect subsequent reactions. Excessive drying may cause cracking on the surface of beads and thereby reduce the purification yield. To get higher purification yield, incubation time may be doubled as appropriate. In general, magnetic beads can be fully dried through air-drying at room temperature for 5 - 10 min. Do not dry by heating.

• It is generally recommended to use an elution buffer (10 mM Tris-HCl, pH 8.0 – pH 8.5; e.g. TE-buffer Cat# M3091) for product elution, which is more conducive to the stable preservation of the product. However, if targeted capture of the library is required later, product elution should be performed with ddH_2O to facilitate drying and concentration of the library before capture and to prevent any impact on subsequent capture reactions.

5. Size Selection

• If the Input DNA distribution range is wide, size selection is usually required during library preparation to control the final library size distribution. Two Rounds Beads selection using magnetic beads are recommended. Size selection can also be performed by gel-based size selection technique.

• Size selection can be carried out before DNA Damage Repair and End Preparation, after Adapter Ligation, or after Library Amplification. No size selection step is included in the experiment procedure. If such step is required, refer to Appendix I: Two Rounds Beads Selection.

• There is a large amount of DNA loss involved in size selection. Sometimes it is necessary to choose between the library size distribution (with size selection) and the library complexity (no size selection). When the amount of input DNA is low, it must be guaranteed that the size selection stage occurs only once. Two or more size selections can lead to a significant reduction in library complexity and yield.

• Over-amplification typically results in trailing band or tail peak appearing at the high molecular weight position. The corresponding products are mostly non-complementary strand cross-annealing products (refer to 6. Library Amplification). The recommended solution is to adjust the number of amplification cycles to avoid over amplification. It is not recommended to remove trailing or tailing peaks by size selection.

• The high-concentration PEG contained in Rapid Ligation Buffer has a significant impact on the Two Rounds Beads Selection and gel extraction. Therefore, if size selection is performed after Adapter Ligation, the ligation product purification steps cannot be omitted, and the purified product should be eluted into a suitable volume of elution buffer, followed by Two Rounds Beads Selection or gel-based size selection technique. If size selection must be performed after Adapter Ligation, the size selection conditions need to be adjusted separately. If size selection is performed before End Preparation or after Library Amplification, the initial purification step can be directly replaced by Two Rounds Beads Selection or gel-based size selection technique.

6. Library Amplification

• PCR Primer Mix is designed to be used for amplification of the Illumina high-performance sequencing platform library with full length adapter. Replace the amplification primers for short adapters or other platform libraries, and the recommended amplification concentration for each primer is 5 - 20 μM.

• During the late stage of PCR, primers are usually depleted before dNTP. At this point, too many cycles can cause non-specific annealing after the amplification products unwinding, resulting in non-complementary chain cross-annealing products. These products migrate lower and are diffused in higher molecular weight region in electrophoresis-based analysis. They are made up of single-strand libraries that have the correct length, which can be cyclized and sequenced after denaturation. The existence of these products has a decisive impact on the library's quantitative methods. When library quantification is performed by using fluorescent dyes that recognize double-stranded DNA, the quantification results are lower than the actual values. But when library quantification is performed by using the qPCR-based library quantification methods, the quantification results are lower than the actual values.

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• The Library Amplification step requires strict control of the number of amplification cycles. An insufficient number of cycles will lead to an insufficient library yield, while an excessive number of cycles will lead to various adverse effects such as over-amplification, increased amplification bias, PCR duplicates, chimeric products, and amplification mutations. Table 3 specifies the recommended number of amplification cycles to obtain a 100 ng or 1 µg library when using 100 pg - 1 µg of high-quality input DNA.

Table 3: Recommended number of amplification cycles for 100pg – 1µg input DNA.

Input DNA	Number of cycles required to generate	
	100 ng	1 µg
100pg	13-15	16-18
1ng	9-11	13-15
10ng	4-6	9-11
50ng	2-4	5-8
100ng	0-2	4-6
250ng	/	3-5
500ng	/	2-3
1µg	/	0-2

Note: Table 3 shows the number of cycles measured for high-quality mouse gDNA which was fragmented for 8 min at 37℃. When DNA quality is poor, the number of cycles must be adjusted to obtain sufficient library.

Note: If size selection has been performed during library preparation, Library Amplification should be performed with the higher number of cycles; otherwise, the lower number of cycles is feasible

• If complete adapters are used during Adapter Ligation, and the library yield meets the application requirement, the Library Amplification step may be exempted to obtain PCR-free libraries.

• When incomplete adapters are used during Adapter Ligation, a minimum number of amplification cycles (at least 2) is required to complete adapter sequences.

7. Library Quality Control

Generally, the quality of the prepared libraries is evaluated through size distribution and concentration analysis.

• Library size distribution analysis: The size distribution of libraries can be determined by devices based on the electrophoretic separation principle such as LabChip GX, GXII, GX Touch (PerkinElmer); Bioanalyzer, Tapestation (Agilent Technologies); Fragment Analyzer (Advanced Analytical).

• Conventional Adapters suitable for Illumina platform are "Y" type adapters with single chain bifurcation area at the end. Therefore, there were single-strand bifurcation regions at both ends of the unamplified PCR-free library after Adapter Ligation. When the length distribution of library is detected, the bifurcation structure of single chain will slow down the migration rate of library, and lead to some problems such as longer detection results, wider distribution range and abnormal peak type. In order to accurately detect the size distribution of the library, a small number of PCR-free libraries should be used for moderate PCR amplification, and the amplification products should be taken for detection to reflect the size distribution of the PCR-free library.

• Library concentration analysis: There are two common methods for determining library concentration: one uses fluorescent dsDNA-binding dyes. The other is based on qPCR-based absolute quantification. Although the former is simple and easy to operate, the qPCR-based absolute quantification method is recommended for the following reasons:

  ○ When complete adapters are used and Adapter Ligation is completed, the qPCR-based absolute quantification method can quantify library concentration of products at any step. Thus, this method enables monitoring the efficiency of Adapter Ligation, magnetic bead-based purification/size selection, and Library Amplification, to provide useful data for system optimization and analysing the causes of abnormalities in library preparation.

  ○ Since PCR-free libraries are not subject to Library Amplification, the prepared library contains a certain proportion of products with single-end adapters or without adapters. For methods using fluorescent dsDNA-binding dyes for concentration determination, these products cannot be effectively distinguished. However, qPCR absolute quantification is based on the principle of PCR amplification, and only quantifies libraries with an adapter at both ends in the sample (i.e., sequenceable libraries), which can rule out the interference of unsequenceable libraries with single-end adapters or without adapters. Thus, the concentration of PCR-free libraries can only be determined through the qPCR-based absolute quantification method.

  ○ Methods using fluorescent dsDNA-binding dyes, are not applicable to overamplified libraries since they contain a large number of incomplete double-stranded structures. However, over-amplification does not impact qPCR-based absolute quantification.

8. Other notes

• The size and distribution range of DNA fragments are determined by a time dependent enzyme-based reaction; thus the fragmentation reaction should be carried out on ice.

• Thaw all the components at room temperature before use. After thawing, mix thoroughly and centrifuge briefly before putting them on ice.

• To avoid cross-contamination of samples, it is recommended to use filter tips and change the pipette tip after each sample.

• It is recommended to use a PCR instrument with a heating lid. Pre-heat the PCR instrument to a temperature close to the reaction temperature before use.

• Improper operations make PCR products prone to occur aerosol contamination and thereby affect the accuracy of experiment results. Thus, it is recommended to physically separate the PCR reaction system preparation area from the PCR product purification and experiment area, use dedicated equipment (e.g., pipettes), and regularly clean each experiment area (wipe with 0.5% sodium hypochlorite or 10% bleach) to ensure clean experiment environments.

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Workflow

The construction principle and the workflow of library preparation using Genaxxon's DNA Library Prep for Illumina is presented in figure 1 and figure 2 below.

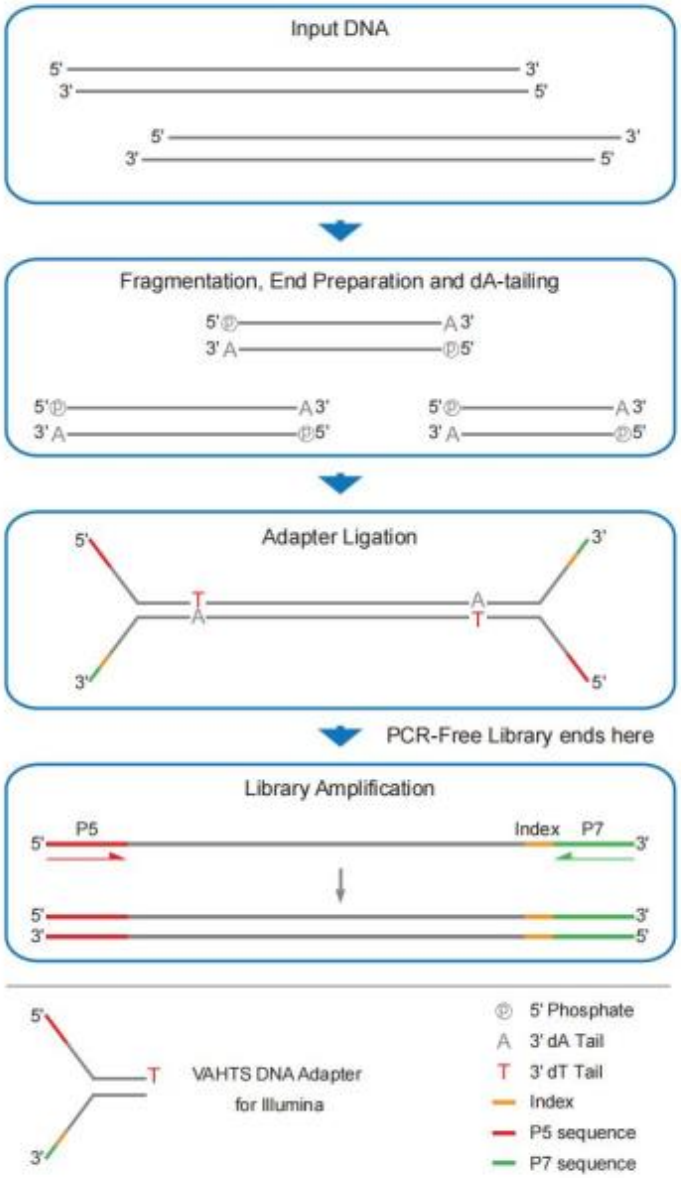

Figure 1: Construction Principle for DNA Library Prep Kit PLUS for Illumina

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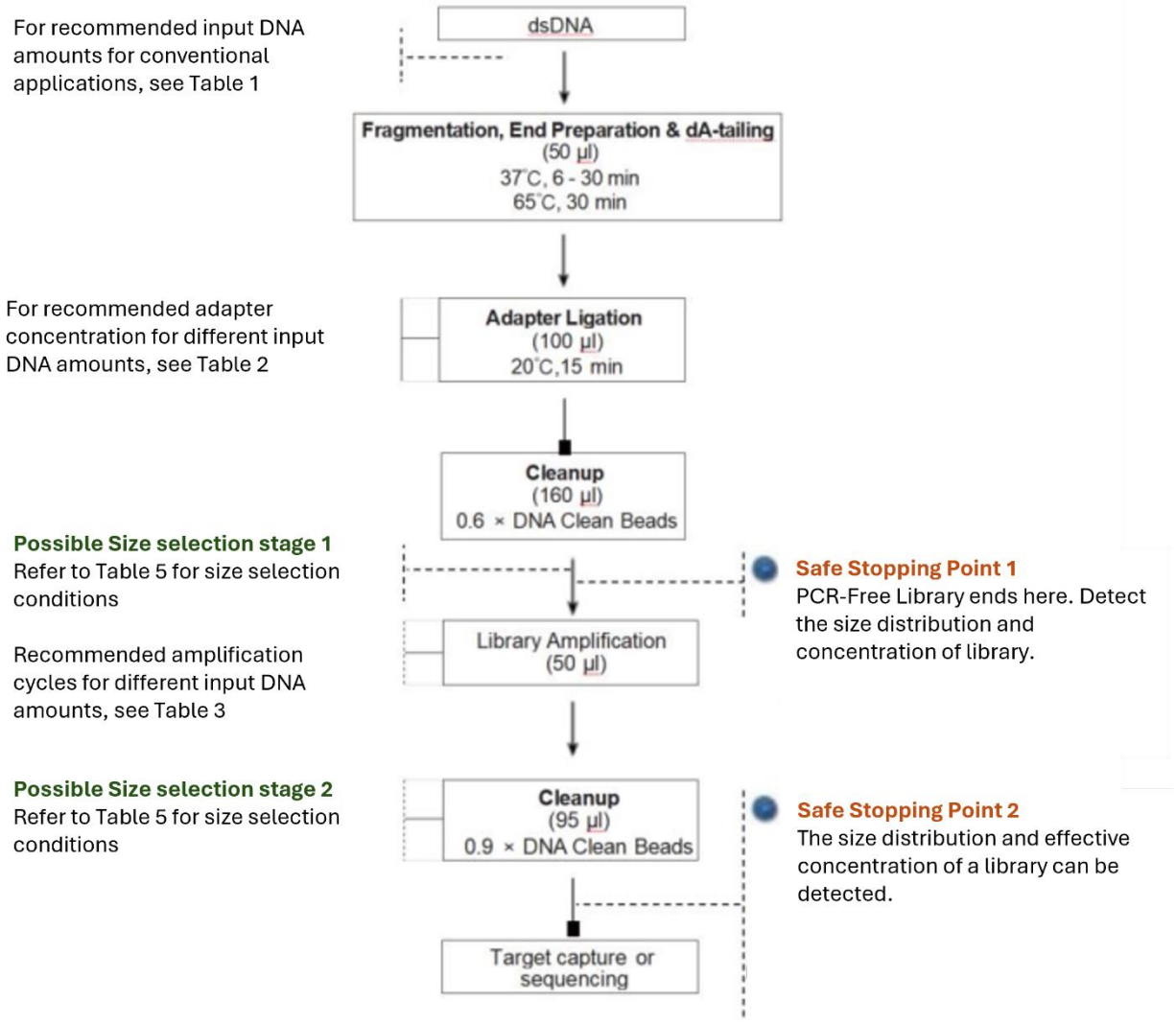

Figure 2: Workflow for DNA Library Prep Kit PLUS for Illumina

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Experimental Process

Please read the following notes carefully to obtain a high-quality library. In case of any questions, please contact Genaxxon: info@genaxxon.com.

1. Fragmentation, End preparation and dA Tailing

This step is used to fragment the input DNA while simultaneously repairing the fragmented DNA end, as well as the 5' end phosphorylation and the 3' end dA tailing.

• Before starting the experiment, confirm which solvent the template DNA is dissolved in (ddH_2O is recommended) and whether the solvent contains EDTA. If it does not contain EDTA, proceed directly to Step 3. If it does contain EDTA, pre-treat the sample according to Step 2.

• If the solvent contains EDTA, the template DNA can be purified using 2.2 × beads and eluted with ddH_2O. Alternatively, the corresponding amount of neutralization buffer can be added according to the final concentration of EDTA in the fragmentation solution, in order to neutralize the EDTA.

EDTA final concentration of fragmentation solution	Volume of neutralization buffer
1mM	5μL
0.8mM	4μL
0.6mM	3μL
0.5mM	2.5μL
0.4mM	2μL
0.2mM	1μL
0.1mM	0.5μL
<0.1mM	0μl

Note: EDTA final concentration of fragmentation solution = EDTA concentration in DNA solution × amount of DNA used/50 μL. For example, if the DNA is dissolved in TE containing 1 mM of EDTA and 10 μL is used for library preparation, the EDTA final concentration will be 1 mM × 10 μL/50 μL = 0.2 mM.

• Thaw the FEA Buffer and FEA Enzyme Mix. Mix gently and centrifuge briefly and put them on ice before use. All of the following steps are performed on ice.

• Prepare the reaction solution in a sterile PCR tube as follows:

Component	Volume
Input DNA	XμL
Neutralization Buffer	YμL
FEA Buffer	5μL
ddH_20	Up to 40μL

Note: If the solvent does not contain EDTA, there is no need to add neutralization buffer. Too much neutralization buffer can cause overreaction during fragmentation.

Note: When there are a large number of samples and the samples contain EDTA, different amounts of neutralization buffer must be calculated and added, which is relatively complicated during actual operation. See Appendix 3.

• Add 10μL of FEA Enzyme Mix to each sample, pipetting up-and-down or vortexing to mix, and centrifuge to collect the reaction solution to the bottom of the tube and place it in the PCR instrument immediately for reaction!

Please note: Fragmentation reaction is a time-dependent enzyme-based reaction, and the size of the fragment product depends on the reaction time. It is therefore recommended to add the FEA Enzyme Mix to the reaction solution separately at the end. Mix immediately, and then carry out the follow-up reaction.

Please note: Fragmentation reactions are sensitive to oxidation, so the FEA Buffer and FEA Enzyme Mix caps should be tightly screwed on as soon as possible after use, and then store them at -20°C.

• Place the PCR tube into the PCR instrument and perform the following program:

Temperature	Time
Hot lid 105°C	On
37°C	Refer to the table below
65°C	30min
4°C	Hold

Please note: Fragmentation time depends on the size of input DNA and target fragment:

Expected insert size	Fragmentation time
150bp	20 to 30min
250bp	15 to 20min
350bp	10 to 15min
550bp	6 to 10min

Note: The recommended time above was calculated using high-quality human placenta gDNA as a template. Using high-quality human placenta gDNA for library preparation, different inputs within the recommended range (100 pg - 1 μg) with the same reaction time resulted in little variation in the distribution of fragmentation of product. The distribution range is essentially consistent, but the main peak position may vary slightly. If the input DNA quality is poor or the fragment size is outside the predicted range, increase or decrease the fragmentation duration by 2 - 5 minutes. There is no need to change the fragmentation time for FFPE DNA samples based on their integrity if the size of the inserted fragments in the library is not strictly required. The FFPE DNA samples with varying integrity were fragmented for 15 - 20 minutes, yielding insertion fragments ranging from 150 to 250 base pairs. Appendix II contains information about other segmented time experiments.

2. Adapter Ligation

This step is used to ligate adapters to the previous products.

• Dilute the Adapter to the appropriate concentration specified in Table 2.

• Thaw the Rapid Ligation Buffer and Rapid DNA Ligase, mix thoroughly by turning upside down several times and centrifuge briefly before placing it on ice.

• Prepare the following reaction solution:

Component	Volume
Product from previous step	50μL
Rapid Ligation Buffer	25μL
Rapid DNA Ligase	5μL
ddH_20	15μL
DNA Adapter X	5μL

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• Mix well by gently pipetting up and down (do not vortex) and centrifuge briefly.

• Place the PCR tube into the PCR instrument and perform the below reaction:

Temperature	Time
Hot lid 105°C	On
20°C	15min
4°C	Hold

Note: When the amount of input DNA is low, the ligation time may be doubled as appropriate. However, longer reaction time may increase adapter dimers. The concentration of adapters needs to be adjusted if necessary.

• Purify the reaction products using DNA Clean Beads:

I. Mix DNA Clean Beads well by vortexing after equilibrating the magnetic beads to room temperature.

II. Pipette 60µL of DNA Clean Beads into 100µL of the Adapter Ligation solution and mix well by gently pipetting up and down 10 times.

III. Incubate at room temperature for 5 min.

IV. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully remove the supernatant.

V. Keep the PCR tube on the magnetic stand. Add 200µL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.

VI. Repeat step V. rinse twice in total.

VII. Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 3 to 5 min until there is no residual ethanol. Please note: The surface of the magnetic beads changes from dark brown to brown, and the surface can be eluted without reflection. If the magnetic beads are excessively dry, the elution experience and efficiency will be affected.

VIII. Remove the PCR tube from magnetic stand for elution:

○ If the purification products do not undergo Two Rounds Beads Selection: Add 22.5µL of eluent (10mM Tris-HCl, pH 8.0 to 8.5) or ddH_2O for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5 min), carefully transfer 20µL of supernatant to a new EP tube. Do not disturb the magnetic beads.

○ If the purification products undergo Two Rounds Beads Selection: Add 105µL of eluent (10 mM Tris-HCl, pH 8.0 to 8.5) or ddH_2O for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5min), carefully transfer 100µL of supernatant to a new EP tube. Do not disturb the magnetic beads. Perform size selection according to the two rounds beads selection criteria in Table 5. The samples can be stored for one week at 4°C, and for a longer time at -20°C. Repeated freeze-thaw cycles should be avoided.

3. Library Amplification

This step is for PCR amplification of the Adapter Ligation products after purification or size selection. Whether to proceed with this step depends on the amount of input DNA, whether adapters are in complete length, and downstream application. If adapters are not in complete length, this step is necessary. If adapters are in complete length, for input DNA <50ng, library amplification is recommended. Skip this step and proceed directly to Library Quality Control, if input DNA is ≥50 ng or there is no need for library amplification.

• Thaw PCR Primer Mix for Illumina and HiFi Amplification Mix, mix thoroughly by turning upside down and centrifuge briefly. Prepare the following reaction in a sterile PCR tube (on ice):

Component	Volume
Purified or selected Adapter Ligation products	20µL
PCR Primer Mix for Illumina	5µL
HiFi Amplification Mix	25µL
Total	50µL

• Mix gently by gentle pipetting up and down (do not vortex) and centrifuge briefly.

• Place the PCR tube into the PCR instrument and perform the below reaction:

Temperature	Time	Number of cycles
95°C	3min	1
98°C 60°C 72°C	20sec 15sec 30sec	For number of cycles see Table 3
72°C	5min	1
4°C	Hold	

• In case of size selection, refer to Appendix I: Two Rounds Beads Selection. If no size selection is required, purify the reaction products using DNA Clean Beads:

I. Mix DNA Clean Beads well by vortexing after equilibrating the magnetic beads to room temperature.

II. Pipette 45µL of DNA Clean Beads into 50µL of the Library Amplification solution and mix well by gently pipetting up and down 10 times.

III. Incubate at room temperature for 5 min.

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IV. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5 min), and carefully remove the supernatant.

V. Keep the PCR tube on the magnetic stand. Add 200µL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30 sec, and carefully remove the supernatant.

VI. Repeat step V., rinse twice in total.

VII. Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 5 to 10 min until there is no residual ethanol.

VIII. Remove the PCR tube from magnetic stand for elution:

- Add 22.5µL of eluent (10 mM Tris-HCl, pH 8.0 to 8.5) or ddH_2O (ddH2O elution must be used if targeted capture is required later) for elution, vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2min. Briefly centrifuge the PCR tube and place it on the magnetic stand. Once the solution is clear (approximately 5min), carefully transfer 20µL of supernatant to a new EP tube. Do not disturb the magnetic beads.

The samples can be stored for one week at 4°C, and for a longer time at -20°C. Repeated freeze-thaw cycles should be avoided.

8. Library Quality Control

Check the quality of your library as described in the previous section of this manual.

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Appendix I: Two Rounds Beads Selection

In order to meet the needs of different application scenarios, Two Rounds Beads Selection are usually required during library preparation to control the library insert size distribution. Table 4 lists the time points for size selection and the advantages and disadvantages of each time point. **It must be guaranteed that the selection process is performed only once. Two or more selections can lead to a significant reduction in library complexity and yield!**

Table 4: Choosing the stage of size selection

Time Point for Size Selection	Applicable conditions	Advantage	Disadvantage	Examples of Applicable Samples
After adapter ligation	Input DNA distribution is suitable and adequate	Reduce the loss of short input DNA	Cannot accurately evaluate the library distribution	Proper fragmentation of genomic DNA or FFPE DNA with wider distribution range
After library amplification	Low input DNA amount	Reduce the loss of input DNA during library preparation and increase the complexity of the library	Library size distribution range is slightly broad	
No size selection during library preparation	Input DNA distribution range meets the library preparation requirements; input DNA amount is low	Reduce the loss of input DNA during library preparation and increase the complexity of the library	Library insert size cannot be controlled	Proper fragmentation of genomic DNA

Note: The effect of Two Rounds Beads Selection is affected by the state of the DNA end. The single-stranded part of the input DNA end and the adapter non-complementary region will lead to a wider distribution of selected product length.
Note: If the input DNA amount is ≥100ng, it is recommended to perform the size selection after Adapter Ligation. If the input DNA amount is <100ng or the sample copy number is limited, perform size selection after Library Amplification.

Two Rounds Beads Selection is made by controlling the amount of beads used to perform DNA size selection. The basic principle is that the first round of beads bind to DNA with a larger molecular weight and this kind of DNA is removed when the beads are discarded; while the second round of beads binds to DNA with a larger molecular weight in the remainder of the products and the smaller-sized DNA is removed by discarding the supernatant. Many components in the initial sample interfere with the two rounds beads selection effect. Therefore, when the stage of the size selection is different, the amount of beads used for two rounds selection would be different. Select the most appropriate selection parameter according to Table 5 based on the expected library insert size and the stage of the size selection.

Table 5: Library Size Selection

Stage and conditions for performing size selection	Purification rounds	Expected Library Insert Size (bp)							
		150	200	250	300	350	400	450	500
After adapter ligation (sample volume 100μL)	1st Round X(μL)	78	68	65	59	56	53	51	50
	2nd Round Y(μL)	20	20	15	15	12	12	10	10
After library amplification (fill sample volume up to 100μL)	1st Round X(μL)	78	70	63	55	50	46	45	44
	2nd Round Y(μL)	20	20	20	20	20	20	20	15

If adapters are not in complete length, please refer to the following table to choose the volume of beads according to expected insert size and selection points.

Stage and conditions for performing size selection	Purification rounds	Expected Library Insert Size (bp)							
		150	200	250	300	350	400	450	500
After Adapter Ligation (sample volume 100μL)	1st Round X(μL)	100	90	75	65	60	55	53	50
	2nd Round Y(μL)	20	20	20	20	20	20	20	18

Note: When magnetic beads are used for size selection, the larger Insert Size, the broader size distribution. However, beads cannot select DNA with insert when the size is greater than 700 bp, two rounds purification with magnetic beads has almost no size-selection effect. In this case, it is recommended to carry out size selection through gel extraction.
Note: The volume ratio of samples and beads is important for size selection. Please ensure the accuracy of initial sample volume and pipetting volume.

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Sample Pretreatment (Important!)

• If size selection takes place after Adapter Ligation products purification, the sample volume should be 100μL. If not, the sample should be filled up to 100μL with ddH_2O.

• If size selection takes place after Library Amplification, the sample volume should be 100μL. If not, the sample should be filled up to 100μL with ddH_2O.

• If the sample is not pretreated by volume, the beads amount can also be adjusted in proportion to the actual volume of the sample. However, if the sample volume is too small, this will increase pipetting errors, which in turn affect the accuracy of size selection. Therefore, direct size selection of samples <50μL is not recommended.

Selection Protocol (refer to Table 5)

I. Mix DNA Clean Beads thoroughly by vortexing after equilibrating the magnetic beads to room temperature.

II. Pipette X μL of DNA Clean Beads into 100μL of the solution and mix well by gently pipetting up and down 10 times. If the solution is less than 100μL, fill up to 100μL with ddH_2O.

III. Incubate at room temperature for 5min.

IV. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5min), and carefully transfer the supernatant to a new PCR tube and discard the magnetic beads.

V. Pipette Y μL of DNA Clean Beads into the supernatant and mix well by gently pipetting up and down 10 times.

VI. Incubate at room temperature for 5min.

VII. Centrifuge the PCR tube briefly and place it on a magnetic stand to separate the magnetic beads from the solution. Wait until the solution becomes clear (about 5min), and carefully remove the supernatant.

VIII. Keep the PCR tube on the magnetic stand. Add 200μL of freshly prepared 80% ethanol to rinse the magnetic beads, incubate at room temperature for 30sec, and carefully remove the supernatant.

IX. Repeat step VIII, rinse twice in total.

X. Keep the PCR tube on the magnetic stand. Uncap the tube and air-dry the magnetic beads for 3 to 5 min until there is no residual ethanol.

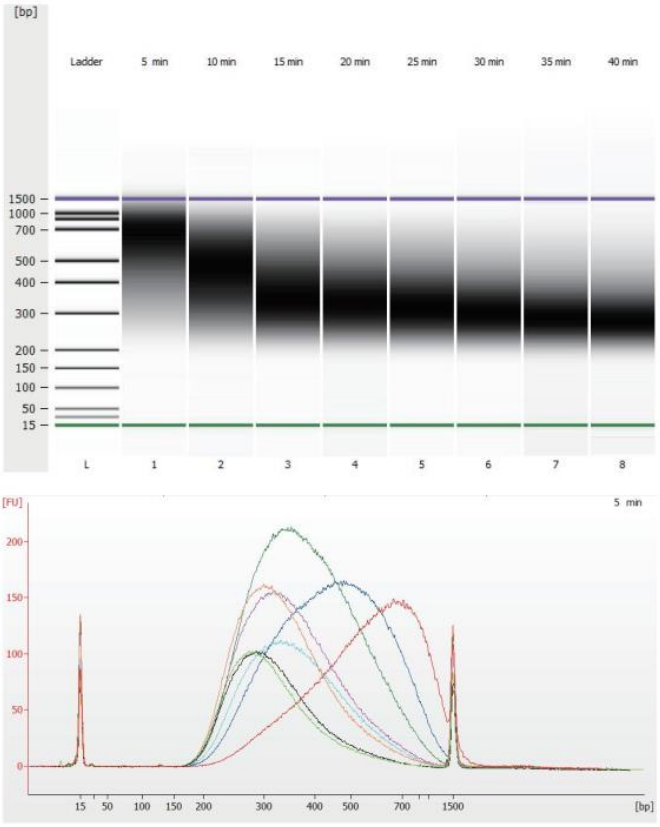
XI. Remove the PCR tube from magnetic stand for elution:

○ Add 22.5 μL of eluent (10 mM Tris-HCl, pH 8.0 to 8.5) or ddH_2O (ddH_2O elution must be used if targeted capture is required later). Vortex or gently pipette up and down to mix thoroughly and place it at room temperature for 2min. Briefly centrifuge the PCR tube and place it on the magnetic stand. After the solution is clear (approximately 5 min),carefully transfer 20μL of supernatant to a new EP tube. Do not disturb the magnetic beads.

Appendix II: Experiments Example

Experiments example with different fragmentation time:

Using 100ng human genome DNA as template, the kit was used to construct the library. The fragmentation conditions were 37℃ for 5/10/15/20/25/30/35/40 min, respectively. PCR amplification lasted for 4 cycles, and the final library distribution was shown as follows:



Appendix III: Solution for Fragmentation of Multiple Samples

When there is a large number of samples and the samples contain EDTA, different amounts of neutralization buffer must be calculated and added, which is relatively complicated during actual operation. At this point, you can dilute the sample to the same concentration using the same solvent, ensuring that multiple samples are added in the same volume and that an equal volume of neutralization buffer is added. As shown in the following table, the reaction solution mixture is prepared, mixed and divided into appropriate volumes in each tube. Quickly proceed to PCR to prevent great difference of fragmentation between different samples due to the long loading time.

• For example, for each 10μL of DNA sample, 2.5μL of Neutralization Buffer must be added by means of calculation and dilution. Dilute the DNA samples according to the calculated results and arrange sequentially in 8-pipe or 96-well plate.

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• Thaw the FEA Buffer, FEA Enzyme Mix and Neutralization Buffer, Mix gently and centrifuge briefly. Put them on ice before use. All of the following steps are performed on ice:

Components	Single reaction volume	96-reaction mixture volume
Neutralization buffer	2.5µL	250µL
FEA Buffer	5µL	500µL
FEA Enzyme Mix	10µL	1000µL
ddH_20	22.5µL	2250µL

Note: When preparing multiple reaction mixtures, it is recommended to prepare them with 1.1 times the volume of the actual number required in order to ensure there is a sufficient amount for dispensing.
Note: Fragmentation reaction mixture should be freshly prepared and used and should not be stored for a long time.

• Mix gently and centrifuge briefly.

• Distribute the reaction mixture into reaction tubes or 96-well plates, with 40µL in each well.

• Using a pipette or an automated workstation, add 10µL of the DNA sample to each reaction well as quickly as possible, Mix thoroughly and centrifuge briefly. Then immediately proceed to PCR.
Please note: Fragmentation reaction is a time-dependent enzyme-based reaction, and the size of the fragment products depends on time. Therefore, operate quickly to minimize the difference between different samples. Mix immediately after adding and perform the follow-up reaction.