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DNA Library Prep Kit for Illumina

Universal library preparation kit for Illumina high-throughput sequencing platforms

Component	Cat#	M4400.0024	M4400.0096	Colour code of cap
End Prep Buffer		168µL	672µL	
End Prep Enzyme		72µL	288µL	
Rapid Ligation Buffer		720µL	4 x 720µL	
Rapid DNA Ligase		240µL	4 x 240µL	
HiFi Amplification Mix		600µL	4 x 600µL	
PCR Primer Mix		120µL	480µL	
Control DNA (264bp, 50ng/µL)		10µL	10µL	

Description

Genaxxon's DNA Library Prep Kit for Illumina is a universal solution to prepare libraries for sequencing on Illumina high-throughput sequencing platforms. This kit converts fragmented input DNA ranging from 100pg to 1µg into libraries suitable for Illumina high-throughput sequencing.

The kit is optimized to feature a high conversion rate of low-quality templates and a low library duplication rate with an optimized end-repair module, ligation module, and library amplification module. It is widely applicable to PCR or PCR-free library preparation from diverse sample types and is compatible with the targeted capture process.

Features and Applications

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

- Wide Compatibility: The kit is compatible with FFPE and cfDNA samples of low quality, in addition to conventional samples.
- High Efficiency: The kit demonstrates a superior library conversion rate.
- Accurate Detection: The kit enables precise identification of low-frequency mutations.

The kit is suitable for:

- Whole-genome sequencing
- Whole-exome sequencing or other targeted capture sequencing
- Amplicons sequencing
- ChIP sequencing
- Metagenome sequencing
- 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
- TE-Buffer, pH 8.0 (Cat# M3091)

Product Use Limitations

DNA Library Prep Kit 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 pdf-file or by request (info@genaxxon.com).

Quality control

Genaxxon bioscience DNA Library Prep Kit 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/targeted capture sequencing	cfDNA/ctDNA	≥100pg
Whole-genome sequencing	Microbial genome	1ng – 1µg
Whole-genome sequencing (PCR-free library)	Complex/Small Genome	≥50ng (no size selection) ≥200ng (size selection)
ChIP sequencing	ChIP DNA	≥100pg
Targeted sequencing	Amplicon	≥100pg

Note: Table 1 presents recommendations on the amount of input DNA for high-quality DNA samples. If the quality of input DNA is low, the appropriate amount should be increased.

• Input DNA refers specifically to DNA used in the End Preparation step. If the DNA sample has been purified or subjected to size selection following fragmentation, its pre-fragmentation amount cannot be directly used as the amount of input DNA, and its concentration should be redetermined. Otherwise, the library yield may be low due to insufficient amplification cycles.

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

• If high-concentration metal ion chelators or other salts are introduced during input DNA preparation, the efficiency of the End Preparation step may be affected. When fragmentation is performed using mechanical methods, and library preparation is performed without product purification or size selection, DNA should be diluted in 0.1xTE buffer (Cat# M3091) instead of ddH_2O for fragmentation. When fragmentation is performed using enzyme digestion, and library preparation is performed without product purification or size selection, ensure that no excess metal ion chelators are introduced in stop buffer. If the above conditions are not met, fragmentation products should be purified or subjected to size selection, and then dissolved in 0.1xTE buffer (Cat# M3091) or ddH_2O(≤50µL) before library preparation.

2. Adapters

• The quality and amounts of adapters directly affect the preparation efficiency and library quality. The recommended molar ratio of adapter: input DNA ranges from 10:1 to 200:1. Table 2 lists the recommended adapter usage for different amount of input DNA.

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

Input DNA	Adapter: Input molar Ratio	Adapter Working concentration
1µg	10:1	10µM
100ng	30:1	5 – 10µM
10ng	100:1	2µM
1ng	200:1	0.5µM
100pg	300:1	0.1µM

Note: Using too high of an 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 pre-dilute the adapter with 0.1× TE buffer according to table 2. This ensures that the adapters are used at a fixed volume (5µL) during library preparation, thus avoiding pipetting errors.

• The adapter quality directly affects the molar ratio of adapter: 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, especially when the input DNA is ≤25ng. When the preparation efficiency needs to be optimized, the adapter input may be increased (by 2 - 10 folds, preferably) under the recommended conditions in table 2.

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 0.8 × (110µL products and 88µL 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.

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• 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 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.

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 30 min. prior to use; otherwise, decreased yield and poor size selection effect may occur.

• The magnetic beads should be mixed well by shaking thoroughly 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, the 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.

• The eluent can be stable at +2°C to +8°C for one week. Store at -20°C for long-term preservation. Repeated freeze-thaw cycles should be avoided.

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 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.

• The amount of DNA loss involved in size selection is about 60% - 95%. Sometimes it is necessary to choose between the library size distribution (with size selection) and the library complexity (no size selection), especially when input DNA is limited. Please note that two or more size selections can lead to a significant decrease in library complexity and yield.

• Over-amplification typically results in trailing band or tail peak appeared 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 suitable for amplifying libraries containing complete adapters for the Illumina high-throughput sequencing platform. Amplification primers need to be replaced for incomplete-length Adapters or libraries on other platforms, and the recommended final amplification concentration for each primer is 5 - 20µM.

During the late stage of PCR, primers are usually depleted before dNTP. In this case, excessive cycles can cause non-specific annealing of the amplified product after unwinding, resulting in non-complementary strand cross-annealing products. These products migrate slowly in electrophoresis-based assays and diffuse in the high molecular weight region. These products are prepared from single-stranded libraries with the correct length and can bind to the Flow Cell normally and be sequenced after denaturation. Therefore, their presence or absence has no significant impact on sequencing. However, the presence of such products has a decisive impact on how libraries are quantified. As the product is not a complete double-stranded structure, when a

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fluorochrome-based assay for double-stranded DNA (dsDNA) quantitation is used for library quantification, the results will be lower than the actual values. However, qPCR-based library quantification systems include a denaturation process, so that such over-amplified libraries can still be accurately quantified.

• The Library Amplification step requires strict control of the number of amplification cycles. Insufficient number of cycles will lead to insufficient library yield; excessive number of cycles will lead to over amplification, increased amplification bias, increased duplicates, increased chimeric products, cumulative amplification mutation, and other adverse consequences. Table 3 lists the recommended number of amplification cycles to obtain approximately 1µg of amplified library when using 100pg - 1µg of high-quality input DNA.

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

Input DNA (Into End Preparation)	Number of cycles required to generate (1µg)
1µg	3-5
100ng	6-8
10ng	10-13
1ng	13-15
100pg	17-19

Note: Table 3 shows 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

• 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).

• 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

• Thaw all components at room temperature before use. After thawing, fully mix the components by turning upside down several times, centrifuge briefly, and place them on the ice for later use.

• It is recommended to thoroughly mix the reaction solution by pipetting up and down at each step (vigorous upside-down shaking may decrease library yields).

• 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 procedural operations will lead to cross contamination thereby affecting the accuracy of the 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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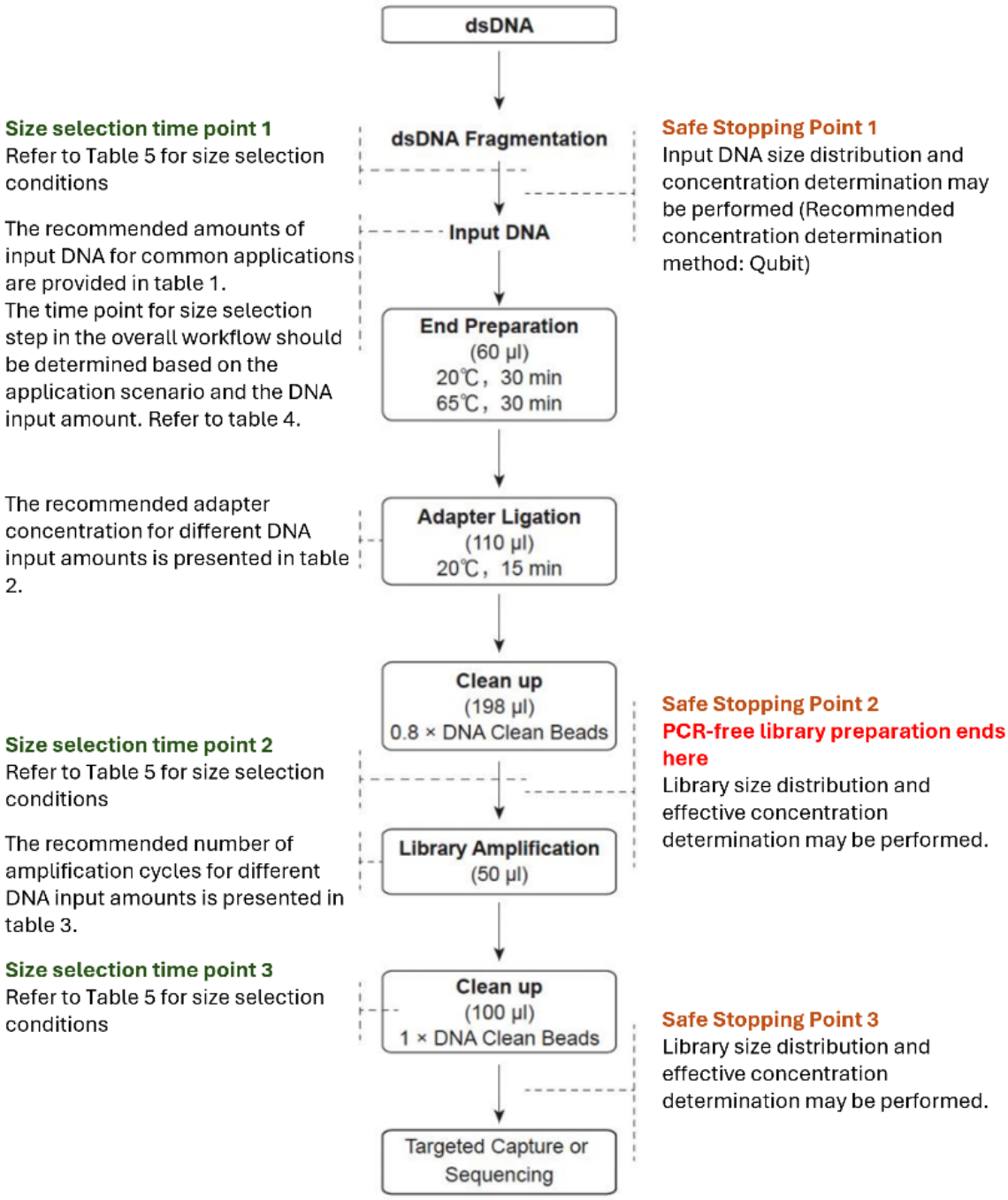
Workflow

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



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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. End preparation

This step is used to repair the input DNA end and to add a dA-tail at the 3'end.

• Thaw the End Prep Buffer, fully mix it with End Prep Enzyme by turning upside down, and prepare the following reaction in a sterile PCR tube (on ice):

Component	Volume
Input DNA	x µL
End Prep Buffer	7µL
End Prep Enzyme	3µL
ddH_2O	to 60µL

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

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

Temperature	Time
Heating lid at 105°C	On
20°C	30min
65°C	30min
4°C	Hold

2. Adapter Ligation

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

• Dilute the Adapter to the appropriate concentration based on the amount of input DNA as specified in Table 2.

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

• Prepare the following reaction solution in a PCR tube at the End Preparation step (on ice):

Component	Volume
End Preparation Product	60µL
Rapid Ligation Buffer	30µL
Rapid DNA Ligase	10µL
DNA Adapter X	5µL
ddH_20	5µL

Note: The pre-mixture of Rapid Ligation Buffer and Rapid DNA Ligase can be stored at 4°C or up to 24 h.

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

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

Temperature	Time
Heating lid at 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 88µL of DNA Clean Beads into 110µL of the Adapter Ligation products 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 5 - 10 min until there is no residual ethanol.

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

- If the purified products are not subject to Two Rounds Beads Selection: Elute with 22.5µL elution buffer (10 mM Tris-HCl, pH 8.0 - pH 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 20µL of the supernatant into a new EP tube without disturbing the magnetic beads.

- If the purified products are subject to Two Rounds Beads Selection: Elute with 105µL elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 100µL of the supernatant into a new EP tube without disturbing the magnetic beads. Perform size selection according to 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.

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3. Library Amplification

This step is to amplify the purified or size-selected Adapter Ligation products. Whether this step is required depends on factors such as the amount of input DNA, incomplete-length adapter or not, and the application scenarios. This step is required if an incomplete-length adapter is used. If a complete adapter is used, Library Amplification is recommended when input DNA is less than 50ng; this step may be skipped (proceed directly to 4. Library Quality Control instead) when input DNA is more than 50ng 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 prepare the following reaction in a sterile PCR tube (on ice):

Component	Volume
Purified or size-selected adapter ligation product	20µL
PCR Primer Mix for Illumina	5µL
Hi-Fi Amplification Mix	25µL

• Mix well 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
98°C	45sec	1
98°C	15sec	Please refer to table 3 for the number of cycles
60°C	30sec	
72°C	30sec	
72°C	1min	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 50µL of DNA Clean Beads into 50µL of the Library Amplification products 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 5 - 10 min until there is no residual ethanol.

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

- If no subsequent targeted capture is performed: Elute with 22.5µL elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5), mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear about 5 min), and carefully pipette 20µL of the supernatant into a new EP tube without disturbing the magnetic beads.

- If subsequent targeted capture is performed: Elute with 22.5µL ddH_2O. Mix well by gently pipetting up and down, and place the mixture at room temperature for 5 min; centrifuge the PCR tube briefly and place it on a magnetic stand without shaking; wait until the solution is clear (about 5 min), and carefully pipette 20µL of the supernatant into a new EP tube without disturbing the magnetic beads.

The samples can be stored for one week at +2°C to +8°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.

Table 4: Choosing the stage of size selection

Time Point for Size Selection	Application Scenarios	Advantage	Disadvantage	Examples of Applicable Samples
Before End Preparation	Sufficient input DNA, with broad distribution or inconsistency between the main peak size and the expected library insert size; low input DNA purity	Centralized size distribution of size selection products; accurate amount of input DNA; increased input DNA purity to improve the success rate of library preparation	High DNA loss; slightly broad library distribution	Under- or over-fragmented genomic DNA
After Adapter Ligation (Recommended)	Sufficient input DNA with appropriate distribution	Reduced loss of short DNA fragments; applicable to most cases	Slightly broad library distribution	Moderately fragmented genomic DNA or FFPE DNA with broad distribution
After Library Amplification	Small amount of input DNA	Reduced loss of input DNA during library preparation and increased library complexity	Broad library distribution	cfDNA
No Size Selection	Small amount of input DNA with appropriate distribution for library preparation	Reduced loss of input DNA during library preparation and increased library complexity	Unable to control the library insert size	Multiple PCR products, highly fragmented FFPE DNA

Note: Size selection after Adapter Ligation is recommended when the amount of input DNA is ≥50ng; size selection after Library Amplification is recommended when the amount of input DNA is <50 ng or the number of copies of samples is limited.

Note: For other size selection time points, the library distribution will be further centralized after the library amplification.

The Two Rounds Beads Selection select DNA fragments of expected length by controlling the amount of magnetic beads used. The basic principle is, in the first round, DNA with larger molecular weight binds to magnetic beads and is discarded with these beads; in the second round, magnetic beads bind to DNA with larger molecular weight among the remaining products, and DNA with smaller molecular weight is discarded with the supernatant. Many components in the initial sample would interfere with the result of the Two Rounds Beads Selection. Therefore, the amount of magnetic beads used in the Two Rounds Beads Selection varies across the time points for size selection. Please refer to Table 5 to choose the optimal size selection parameters based on the expected library insert size and the time point for size selection.

Table 5: Library Size Selection

Time Point and Condition for Size Selection (Sample volume is made up to 110μL	Amount of Magnetic Bead Per Round	Expected Library Insert Size (bp)								
		200	250	300	350	400	450	500	550	700
Before End Preparation	1st Round X(μL)	98	88	77	66	60	57	55	53	47
	2nd Round Y(μL)	22	22	22	22	22	17	17	17	13
After Adapter Ligation	1st Round X(μL)	75	72	65	62	58	56	55	/	/
	2nd Round Y(μL)	22	17	17	13	13	11	11	/	/
After Library Amplification	1st Round X(μL)	77	69	61	55	51	50	48	/	/
	2nd Round Y(μL)	22	22	22	22	22	22	17	/	/

When incomplete length adapters are used, the size selection after ligation is performed under different conditions compared with complete adapters. Please refer to the following table to choose the optimal size selection parameters:

Time Point and Condition for Size Selection (Sample volume is made up to 110μL	Amount of Magnetic Bead Per Round	Expected Library Insert Size (bp)						
		200	250	300	350	400	450	500
After Adapter Ligation	1st Round X(μL)	99	83	72	66	60	58	55
	2nd Round Y(μL)	22	22	22	22	22	22	20

In case of size selection using magnetic beads, the larger the Insert Size, the broader the final product distribution. When the Insert Size is >700 bp, Two Rounds Beads Selection make little difference. In this case, gel extraction is recommended for size selection.
The volume ratio of the sample to the magnetic beads is critical for size selection. Please ensure the accuracy of the initial volume and pipetting volume of the sample.

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Sample Pretreatment (Important!)

• If size selection is performed before End Preparation, the sample volume should be 110µL, and if it is not, ddH_2O can be used to fill the gap.

• If size selection is performed after purification of the Adapter Ligation products, the sample volume should be 110µL, and if it is not, ddH_2O can be used to fill the gap.

• If size selection is performed after Library Amplification, the sample volume should be 110µL, and if it is not, ddH_2O can be used to fill the gap.

• If the sample is not pretreated by volume, please adjust the amount of magnetic beads in equal proportion to the actual sample volume. However, a too small sample volume may lead to less accurate pipetting and then less accurate size selection. Therefore, it is not recommended select sample sizes of <50µL directly without pretreatment.

• **Size 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 110µL of the above products and mix well by gently pipetting up and down 10 times.

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 5 - 10 min until there is no residual ethanol.

XI. Remove the PCR tube from magnetic stand for elution:
If no subsequent targeted capture is performed: Elute with 22.5µL elution buffer (10 mM Tris-HCl, pH 8.0 - 8.5). Mix well by gently pipetting up and down and place the mixture at room temperature for 5min. Centrifuge the PCR tube briefly and place it on a magnetic stand without shaking. Wait until the solution becomes clear (about 5 min), and carefully pipette 20µL of the supernatant into a new EP tube without disturbing the magnetic beads.

○ If subsequent targeted capture is performed: Elute with 22.5µL ddH_2O. Mix well by gently pipetting up and down and place the mixture at room temperature for 5min. Centrifuge the PCR tube briefly and place it on a magnetic stand without shaking. Wait until the solution becomes clear (about 5min), and carefully pipette 20µL of the supernatant into a new EP tube without disturbing the magnetic beads.

Appendix II:

cfDNA Library Preparation

Cell-free DNA (cfDNA) is highly fragmented DNA (about 180bp) in blood with low content. cfDNA is of great value in non-invasive prenatal testing (NIPT) and liquid biopsy (ctDNA detection). Genaxxon's DNA Library Prep Kit for Illumina provides a highly simplified and optimized library preparation solution for cfDNA.

Please note:

• Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50µL.

• cfDNA is highly fragmented and does not require Fragmentation.

• To ensure the quality of libraries, it is recommended to determine the size distribution (2100 Bioanalyzer) and concentration (Qubit) of cfDNA templates.

Library preparation procedure

I. End Preparation (refer to section "End Preparation"); amount of input DNA: 100pg - 100ng.

II. Adapter Ligation (refer to section "Adapter Ligation"); Adapter: Pre-dilute according to Table 2. Clean up: Purify with 0.8 × magnetic beads, elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant for the next step.

III. Library Amplification (refer to section "Library Amplification"); Number of cycles: Refer to Table 3 (adjustable based on library yield requirements). Clean up: Whether to do size-selection of cfDNA library depends on the sample condition and data analysis requirements:

○ If the amplified products are not subject to Two Rounds Beads Selection: Purify with 1 × magnetic beads, elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant into a new EP tube and store at -20°C.

○ If the amplified products are subject to Two Rounds Beads Selection: Size select with 0.73 ×/0.25 × magnetic beads, elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant into a new EP tube and store at -20°C.

IV. Library Quality control; Library concentration determination: Fluorescent dye-based (Qubit or PicoGreen) or qPCR-based absolute quantification methods are recommended for library concentration determination.
Library size distribution determination: Library size distribution is determined using Agilent 2100 Bioanalyzer.

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Appendix III:

FFPE DNA Library Preparation

FPE DNA is obtained from Formalin-Fixed and Paraffin-Embedded (FFPE) sections, which is characterized by difficult extraction (close cross-linking with histones) and low quality (serious degradation). FFPE samples are easy to store with a wide range of origins and are of great applicable value in the medical field. Genaxxon's DNA Library Prep Kit for Illumina provides a highly simplified and optimized library preparation solution for FFPE DNA.

Please note:

• Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50µL.

• The quality of extracted FFPE DNA is different due to tissue difference, embedding quality, storage time and other factors. When low-quality FFPE DNA is used for library preparation, the amount of Input DNA or the number of amplification cycles should be increased as appropriate.

• To ensure the quality of libraries, it is recommended to determine the size distribution (Agilent 2100 Bioanalyzer) and concentration (Qubit) of FFPE DNA templates. Templates may also be pre-tested using the qPCR-based FFPE DNA quality evaluation system.

• If the FFPE DNA degradation degree is insufficient and the average molecular weight is large, fragmentation should be conducted before library construction.

Library preparation procedure

I. End Preparation (refer to section "End Preparation"); amount of input DNA: ≥50 ng.

II. Adapter Ligation (refer to section "Adapter Ligation"); Adapter: Pre-dilute according to Table 2. Clean up: Purify with 0.8 × magnetic beads.

- If the purified products are not subject to Two Rounds Beads Selection: Elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant.
- If the purified products are subject to Two Rounds Beads Selection: Elute DNA with 105µL elution buffer, pipette 100µL of supernatant, and size-select the libraries based on the conditions for Two Rounds Beads Selection listed in Table 5.

III. Library Amplification (refer to section "Library Amplification"); Number of cycles: Refer to Table 3 (adjustable based on library yield requirements). Clean up:

- If the amplified products are not subject to Two Rounds Beads Selection: Purify with 1 × magnetic beads, elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant into a new EP tube and store at -20°C.
- If the amplified products are subject to Two Rounds Beads Selection: Add ddH_2O to make up the volume to 11µL, and size-select the libraries based on the conditions for Two Rounds Beads Selection listed in Table 5.

IV. Library Quality control; Library concentration determination: Fluorescent dye-based (Qubit or PicoGreen) or qPCR-based absolute quantification methods are recommended for library concentration determination. Library size distribution determination: Library size distribution is determined using Agilent 2100 Bioanalyzer.

Appendix IV:

Targeted Capture Library Preparation

In the capture process of NimbleGen SeqCap EZ, Genaxxon's DNA Library Prep Kit for Illumina can be used to prepare pre-capture libraries.

Please note:

• Input DNA refers specifically to the DNA added to End Preparation with a volume of ≤50µL.

• Input DNA should range from 180 - 220 bp in length.

• To ensure the quality of libraries, it is recommended to determine the size distribution (Agilent 2100 Bioanalyzer) and concentration (Qubit) of Input DNA.

Library preparation procedure

I. End Preparation (refer to section "End Preparation"); amount of input DNA: refer to table 1 and based on the sample type.

II. Adapter Ligation (refer to section "Adapter Ligation"); Adapter: Pre-dilute according to Table 2. Clean up: Purify with 0.8 × magnetic beads, elute DNA with 105µL elution buffer, and pipette 100µL of supernatant for double rounds of size selection using magnetic beads (0.68 ×/0.2 ×), elute DNA with 22.5µL elution buffer, and pipette 20µL of supernatant for the next step.

III. Library Amplification (refer to section "Library Amplification"); Number of cycles: Refer to Table 3. It is recommended to use the upper limit of the number of cycles, which is enough for a library yield of ≥1µg. If sample pooling is performed prior to capture, please ensure that the library yield is ≥1 µg/n (n=number of samples) for each sample. In this case, amplification cycles per sample can be reduced to increase the library complexity and decrease duplication rates. Clean up: Purify with 1× magnetic beads, elute DNA with 22.5µL ddH_2O, and pipette 20 µL of supernatant into a new EP tube.

IV. Library Quality Control; Refer to SeqCap EZ Library SR User's Guide v5.1 (Roche document number 06588786001, 09/15) to perform library quality control.

V. Targeted Enrichment; Refer to Chapters 5 - 8 of SeqCap EZ Library SR User's Guide v5.1 to complete the targeted capture process.