iGeneTech
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For Research Use Only.
For Illumina and MGI platform.
Generate library for WGBS or targeted methyl sequencing

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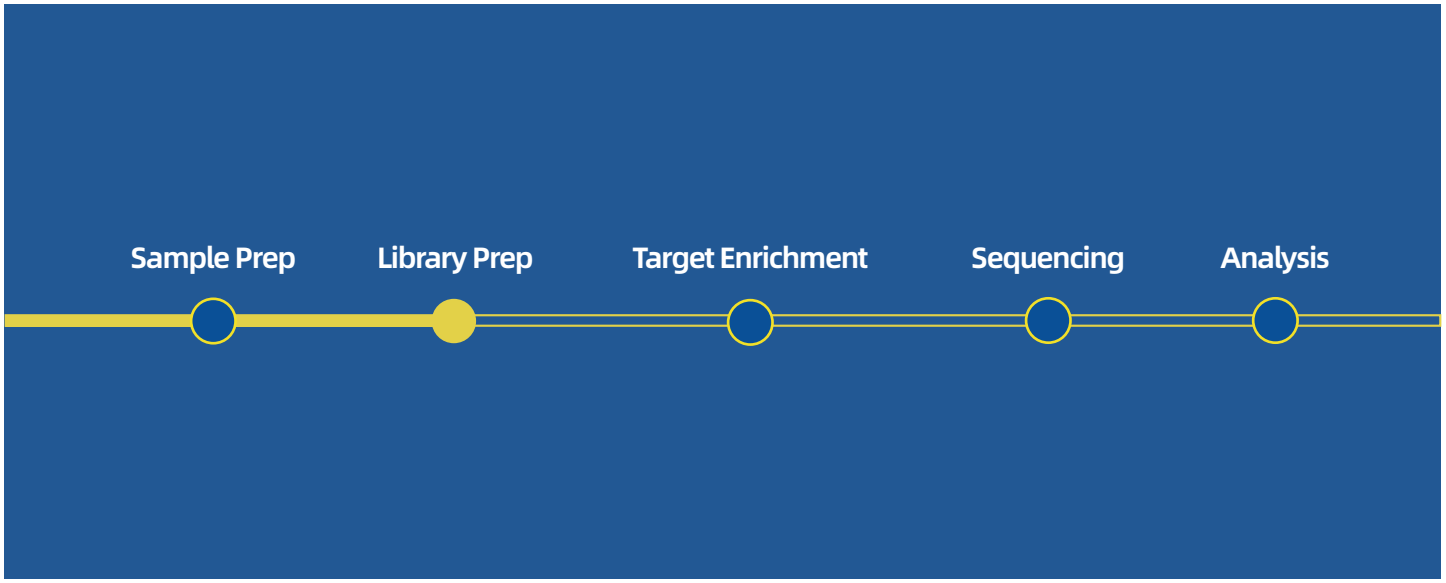
IGT® Methyl-Seq Library Prep Workflow

Protocol

For use with:

- IGT® Methyl Fast Library Prep Kit v2.0
- IGT® Methyl Adapter & UDI Primer

Version: A.0(E), March 2024
Document Number: PROT240301



Version Notes

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Protocol Notes

This protocol provides an instruction for library construction using IGT® Methyl Fast Library Prep Kit v2.0. Before you begin, please read this protocol carefully, and strictly follow the protocol for experiment.

Product Notes

IGT® Methyl Fast Library Prep Kit v2.0 is a universal DNA library preparation kit for whole genome busulfite sequencing (WGBS) or targeted methylation sequencing with DNA input from 20 ng to 1 μg. Based on the TA cloning ligation principle and specific methyl adapters & indexed primers, after bisulfite conversion, the kit could generate sequencing libraries for Illumina and MGI platform accordingly.

Revision history

Version	Date	Description
A.0(E)	March, 2024	Initial release

Materials from iGeneTech

The complete library preparation workflow requires modules of library construction, adapter & indexed primer, and bisulfite conversion. Please choose the appropriate adapter & indexed module according to your needs. The bisulfite conversion kit shall be purchased on your own.

IGT® Methyl Fast Library Prep Kit v2.0

Screw Cap	Component	Amount		Storage
		16 rxn	96 rxn	
Yellow	End Repair & A-Tailing Buffer	124 μL	744 μL	-20℃ ± 5℃
Yellow	End Repair & A-Tailing Enzyme Mix	54 μL	320 μL	
Blue	Ligation Buffer	540 μL	2*1584 μL	
Blue	DNA Ligase	88 μL	540 μL	
White	U+ PCR Master Mix	450 μL	2*1350 μL	

IGT® Methyl Adapter & UDI Primer (for Illumina/MGI)

Screw Cap	Component	Amount		Storage
		96*1 rxn	96*10 rxn	
Blue	Methyl Adapter (10 μM)	540 μL	4*1350 μL	-20℃ ± 5℃
Plate or white	UDI Primer N (10 μM each) [1]	8 μL each	96*75 μL	

[1] N is the index number.

Materials Supplied by User

The materials below are recommended by iGeneTech. Please select the appropriate materials to start the experiment.

Reagents

Item	Recommended Reagents	Supplier (Cat #)
Ethanol Absolute	Varies	Varies
Nuclease-Free Water	Nuclease-Free Water	Ambion (AM9930)
Magnetic Beads	Agencourt AMPure XP Kit	Beckman Coulter (A63880)
	IGT® Pure Beads	iGeneTech (C80661)
Fragment Analyzer	Agilent DNA 1000 Kit	Agilent (5067-1504)
Nucleic Acid Quantification Assays	Qubit dsDNA HS Assay Kit	Thermo Fisher (C47257)
Bisulfite Conversion Kit	EpiTect Fast DNA Bisulfite Kit (50)	QIAGEN (59824)

Equipment

Item	Recommended Equipment	Supplier (Cat #)
96-well Magnetic Stand, 0.2 mL block	DynaMag-96 Side	Thermo Fisher (12331D)
Fragment Analyzer	Agilent 2100 Bioanalyzer system	Agilent (G2939AA)
Nucleic Acid Quantification Instrument	Qubit® 4.0 Fluorometer	Thermo Fisher (Q33238)
Centrifuge	Varies	Varies
Vortex Mixer	Varies	Varies
Mini Centrifuge	Varies	Varies
Ice Block	Varies	Varies
Thermal Cycler	Varies	Varies

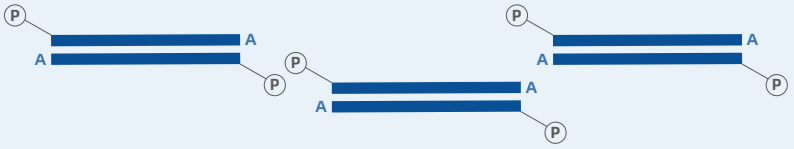
Consumables

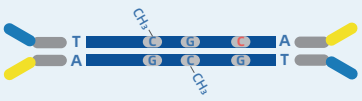
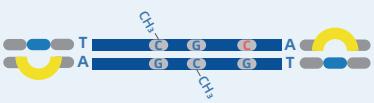
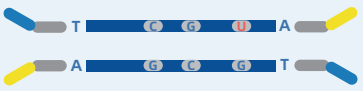
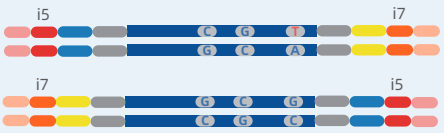
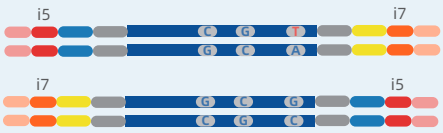
Item	Recommended consumables	Supplier (Cat #)
Qubit tubes, 0.5 mL	Qubit® assay tubes	Thermo Fisher (Q32856)
PCR tubes, 0.2 mL	Varies	Varies
8-tubes strip, 0.2 mL	Varies	Varies
Microcentrifuge tube, 1.5 ml	Varies	Varies
Pipette tips, 10 µL	Varies	Varies
Pipette tips, 200 µL	Varies	Varies

Workflow

① dsDNA Fragmentation — 30 min

② End-Repair and A-Tailing — 70 min



	Illumina	MGI
③ Methyl Adapter Ligation — 20 min		
④ Post-Ligation Purification — 30 min		
⑤ Bisulfite Conversion — 90 min		
⑥ Amplification of Library DNA — 40 min		
⑦ Post-Amplification Purification — 30 min		

Before You Begin

Please read the following precautions carefully before you begin.

- ☐ A260/A280 = 1.8 to 2.0 is generally accepted as "pure" for DNA. If the ratio is appreciably lower, it may indicate the presence of protein. If the A260/A230 ratio is lower than 2.0, it may indicate the presence of guanidine. If the purity of DNA is not as expected, purification using Magnetic Beads before library preparation is recommended.
- ☐ Qubit dsDNA High Sensitivity Assay Kit should be used to measure dsDNA concentration.
- ☐ The recommended input amount of cfDNA is above 20 ng.
- ☐ Please prepare reaction mixture on ice or ice block.
- ☐ The 0.2 mL magnetic stand will be used.
- ☐ Fragment Bioanalyzer is used to assess the fragment size of each library.
- ☐ IGT® Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please assess the volume of beads used for purification via pre-experiments if not using the recommended ones.
- ☐ EpiTect Fast DNA Bisulfite Kit is recommended for bisulfite conversion.

The experiment can be started if the conditions above are met.

STEP 1 End-Repair and A-Tailing

Experiment Notes

Reagents Required:

- End Repair & A-Tailing Buffer
- End Repair & A-Tailing Enzyme Mix
- Nuclease-Free Water

Equipment Required:

- Thermal Cycler
- Vortex Mixer
- Mini Centrifuge

1.1 Genomic DNA or FFPE DNA need to be fragmented to a size of 150-200 bp. Please refer to **Appendix 1** for DNA fragmentation methods. Fragmentation is not required if cfDNA or severely degraded FFPE DNA is used for library preparation.

1.2 Thaw End Repair & A-Tailing Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

1.3 Place End Repair & A-Tailing Enzyme Mix on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

1.4 Prepare reaction mixture on ice as indicated below.

Component	Volume
Fragmented DNA	X μL
Nuclease-Free Water	(50-X) μL
End Repair & A-Tailing Enzyme Mix	3 μL
End Repair & A-Tailing Buffer	7 μL
Total Volume	**60 μL**

1.5 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

1.6 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 75℃	
30℃	30 min
65℃	30 min
4℃	**Hold**

1.7 Proceed to STEP 2 immediately when the program finished.

This is not a stop point, please proceed to STEP 2 immediately.

STEP 2 Methyl Adapter Ligation

Experiment Notes

Reagents Required:

- Ligation Buffer
- DNA Ligase
- Nuclease-Free Water
- Methyl Adapter (10 μM)

Equipment Required:

- Thermal Cycler
- Vortex Mixer
- Mini Centrifuge

2.1 Thaw Methyl Adapter (10 μM) on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.2 Dilute the Methyl Adapter (10 μM) according to the table below.

DNA input	Adapter concentration	Dilution times
50 ng ~ 1 μg	10 μM	/
25 ng	10 μM	/
10 ng	5 μM	2
5 ng	2.5 μM	4
2.5 ng	1.25 μM	8
1 ng	625 nM	16

Excessive Methyl Adapter could cause adapter self-ligation, while insufficient Methyl Adapter may reduce library output.

2.3 Thaw Ligation Buffer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.4 Place DNA Ligase on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

2.5 Prepare reaction mixture on ice as indicated below.

Component	Volume
Product from STEP 1	60 μL
Methyl Adapter (diluted)	5 μL
Ligation Buffer	30 μL
DNA Ligase	5 μL
Nuclease-Free Water	10 μL
Total Volume	**110 μL**

If the sample number in a single experiment is large and a master mix is required, do not directly add the Methyl Adapter to the master mix. It is suggested to mix the diluted Methyl Adapter and the products from STEP 1 first, and then add the reaction mixture to effectively reduce adapter self-ligation.

2.6 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

2.7 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temperature	Time
	Shut down heat lid
22℃	15 min
4℃	**Hold**

To increase the efficiency of the ligation reaction, especially for low input samples, the ligation time could be considered to increase to 4 hours upmost, or overnight at 4℃. However, excessive ligation duration will also increase adapter self-ligation.

2.8 Proceed to STEP 3 immediately when the program finished.

This is not a stop point, please proceed to the STEP 3 immediately.

STEP 3 Post-Ligation Purification

Experiment Notes

Reagents Required:

- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment Required:

- Magnetic Stand
- Vortex Mixer
- Mini Centrifuge

IGT® Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please assess the volume of beads used for purification via pre-experiments if not using the recommended ones.

3.1 Prepare the 80% ethanol for purification. Place the 80% ethanol at room temperature.

3.2 Vortex the Magnetic Beads for 30 s to mix well. Equilibrate the Magnetic Beads to room temperature for 30 min.Votex to mix well before use.

3.3 Add 88 µL (0.8×) of Magnetic Beads to each ligated product from STEP 2. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

3.4 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

3.5 Keep the PCR tube on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

3.6 Keep the PCR tube on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

3.7 Close the lid of the PCR tube and spin briefly on a mini centrifuge. Place the PCR tube on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

3.8 Keep the PCR tube on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

3.9 Add 22 µL of Nuclease-Free Water. Remove the PCR tube from the magnetic stand. Vortex or pipet to mix well. Incubate at room temperature for 2 min.

3.10 Spin briefly on a mini centrifuge. Place the PCR tube on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 2 min).

3.11 Transfer 20 µL of clear supernatant containing each library to a clean PCR tube. Please proceed to STEP 4.

This is a safe stop point, and sample can be stored at -20℃ for up to one month.

STEP 4 Bisulfite Conversion

Experiment Notes

Reagents Required:

- EpiTect Fast DNA Bisulfite Kit
- Ethanol Absolute
- Nuclease-Free Water

Equipment Required:

- Thermal Cycler
- Centrifuge
- Mini Centrifuge

a. DNA Protect Buffer, Buffer BD and Spin Columns should be stored at 4℃. The others could be kept at room temperature.
b. Add Ethanol Absolute to Buffer BD and Buffer BW according to the requirement.
c. Dissolve the carrier RNA with Nuclease-Free Water to obtain a 1 μg/μL solution and keep the solution at -20℃ for storage.
d. Check if any precipitates present in the reagents. If some, pre-heat at 37℃ to dissolve.
e. The room temperature should be above 20℃ otherwise there's high possibility that the experiment fails.

4.1 Place Bisulfite solution and DNA Protect Buffer at room temperature. Mix well and centrifuge briefly.

4.2 Prepare bisulfite conversion reaction mixture as indicated below.

Component	Volume
Product from STEP 3	20 μL
Bisulfite Solution	85 μL
DNA Protect Buffer	35 μL
Total Volume	**140 μL**

4.3 DNA Protect Buffer should turn from green to blue after addition to the mixture. Pipet to mix well and separate each of the mixtures into 2 tubes (carefully labelled).

The total volume of the mixture may be too large for the reaction on a thermal cycler. Therefore, aliquot mixture into 2 PCR tubes is required.

4.4 Place the PCR tubes on the thermal cycler, and start the program as indicated below.

Temperature	Time
Heat lid temperature 105℃	
95℃	5 min
60℃	10 min
95℃	5 min
60℃	10 min
20℃	**Hold**

4.5 Purification:

4.5.1 After the program finished, remove the PCR tubes from thermal cycler and centrifuge briefly. Combine the products of the 2 PCR tubes to a new 1.5 ml microcentrifuge tube. Add 310 μl Buffer BL, 1 μL Carrier RNA (1 μg/μL) and 250 μL Ethanol Absolute to the tube. Votex to mix well and centrifuge briefly. Transfer the mixture to the spin column.

Carrier RNA is not necessary when using >200 ng DNA.

4.5.2 Incubate the spin column with collection tube at room temperature for 1 min and centrifuge at 12,000 rpm for 1 min. Transfer the flow-through in the collection tube to the spin column and place the column back to the collection tube. Incubate at room temperature for 1 min and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and place the spin column back into the collection tube.

4.5.3 Add 500 μl Buffer BW (ensure Ethanol Absolute has been added) to the spin column, and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and place the spin column back into the collection tube.

4.5.4 Add 500 µl Buffer BD (ensure Ethanol Absolute has been added) to the spin column and incubate for 15 min at room temperature. Centrifuge at 12,000 rpm for 1 min. Discard the flow-through and place the spin column back into the collection tube.

4.5.5 Add 500 µl Buffer BW (ensure Ethanol Absolute has been added) to the spin column and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and place the spin column back into the collection tube.

4.5.6 Repeat STEP 4.5.5 once.

4.5.7 Add 250 µl Ethanol Absolute to the spin column and centrifuge at 12,000 rpm for 1 min. Discard the flow-through and place the spin column back into the collection tube. Centrifuge the spin column at 12,000 rpm for 1 min to remove any residual liquid (please confirm there's no liquid left around the membrane in spin column).

4.5.8 Place the spin column into a clean 1.5 ml microcentrifuge tube. Add 22 µl Nuclease-Free Water directly onto the center of each spin-column membrane. Incubate the column for 2 min at room temperature and centrifuge at 12,000 rpm for 1 min. Transfer the flow-through in the collection tube to the spin column and place the column back to the collection tube. Incubate at room temperature for 1 min and centrifuge at 12,000 rpm for 1 min.

4.5.9 Discard the spin column. Label and keep the 1.5 ml microcentrifuge tube with bisulfite converted product. Proceed to STEP 5.

Experiment Notes

STEP 5 Amplification of Library DNA

Reagents Required:

- U+ PCR Master Mix
- UDI Primer

Equipment Required:

- Thermal Cycler
- Vortex Mixer
- Mini Centrifuge

5.1 Thaw PCR Master Mix on ice. Mix well by rotating and centrifuge briefly. Place back on ice.

5.2 Thaw UDI Primer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

5.3 Prepare PCR reaction mixture on ice as indicated below.

Component	Volume
Product from STEP 4	20 µL
U+ PCR Master Mix	25 µL
UDI Primer	5 µL
Total Volume	**50 µL**

4.4 Mix well by gently pipetting (do not vortex to mix) and centrifuge briefly.

4.5 Place the PCR tube on the thermal cycler, and start the program as indicated below.

Temprture	Time	PCR cycles
Heat lid temperature 105℃		
98℃	2 min	1
98℃	20 s	N cycles
60℃	30 s	
72℃	30 s	
72℃	1 min	1
4℃	Hold	1

DNA input	PCR cycles N (1 µg library output)
1 µg	7~8
500 ng	8~9
250 ng	10~11
100 ng	11~12
50 ng	12~13
10 ng	16~17
5 ng	18~19
2.5 ng	19~20

5.6 Proceed to the STEP 6 when the program finished.

This is not a stop point, please proceed to the STEP 6 immediately.

STEP 6 Post-Amplification Purification

Experiment Notes

Reagents Required:

- Magnetic Beads
- 80% Ethanol (freshly prepared)
- Nuclease-Free Water

Equipment Required:

- Magnetic Stand
- Vortex Mixer
- Mini Centrifuge

IGT® Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please assess the volume of beads used for purification via pre-experiments if not using the recommended ones.

6.1 Vortex the Magnetic Beads for 30 sec to mix well. Equilibrate the Beads to room temperature for 30 min. Votex to mix well before use.

6.2 Add 50 µL (1.0×) of Magnetic Beads to each amplified sample from STEP 5. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

6.3 Spin briefly on a mini centrifuge. Place the PCR tube containing beads on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 3 min).

6.4 Keep the PCR tube on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec.

6.5 Keep the PCR tube on a magnetic stand. Remove and discard the clear supernatant. Add 200 µL of 80% ethanol to the PCR tube. Incubate at room temperature for 30 sec. Remove and discard the clear supernatant.

6.6 Close the lid of the PCR tube and spin briefly on a mini centrifuge. Place the PCR tube on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

6.7 Keep the PCR tube on the magnetic stand at room temperature for 3 ~ 5 min to dry the bead pellet. Do not overdry the bead pellet.

6.8 Add 30 µL of Nuclease-Free Water. Remove the PCR tube from the magnetic stand. Vortex or pipet to mix well. Incubate at room temperature for 2 min.

6.9 Spin briefly on a mini centrifuge. Place the PCR tube on a magnetic stand and allow the beads to fully separate from the supernatant (approximately 2 min).

6.10 Transfer 28 µL of clear supernatant containing each library to a clean PCR tube. Library can be stored at -20℃ for up to one month.

6.11 Quantify each library using Qubit dsDNA High Sensitivity Assay Kit. Analyze the fragment size of each library using Agilent 2100 Bioanalyzer.

The experiment ends here.

Appendix 1: Genomic DNA Fragmentation

There are three methods for shearing DNA. cfDNA and FFPE DNA which are already severely fragmented is not suitable for this step. This kit does not contain reagents and consumables for fragmentation. Please refer to the supplier for use.

The sonication settings listed below are recommended guidelines for the shearing of intact genomic DNA. Some additional optimization may be required to determine the appropriate treatment for FFPE DNA or other sheared DNA. For FFPE DNA, repair of FFPE DNA may be introduced before DNA fragmentation and library constuction in order to improve library yield. However, repair of FFPE DNA may result in higher false positive rates for mutation detection.

[Method 1] DNA fragmentation by Bioruptor® Pico

1. Add 1 ng ~ 1 μg of genomic DNA to a 0.6 mL centrifuge tube, and add Nuclease-Free Water to a total volume of 35 μL. Mix well and centrifuge briefly and place on ice.
2. Ture on the Bioruptor® Pico in advance and turn on the cold cycle until the circulating water temperature drop to 4℃. Before working on the Bioruptor® Pico sonicator, make sure that the circulating water temperature is around 4℃ to prevent excess temperature.
3. Set the cycle conditions: time ON for 30 sec and time OFF for 30 sec as 1 cycle, where 10 cycles are 1 round and there're 3 rounds in total. Votex to mix well and centrifuge briefly after each round. If the circulating water temperature is high after each round, please wait until the temperature drop to 4℃.
4. Analyze the fragment size of by fragment analyzer and the main DNA peak size is around 150 ~ 200 bp.

[Method 2] DNA fragmentation by Covaris®

1. Add fresh deionised water to the sink to FILL 10 ~ 15, ensuring that the liquid level is above the glass part of the Covaris® microTUBE and set the temperature of the cooling unit to 4℃.
2. Start SonoLab software and vent the water by opening the vent button on the software interface and wait until the software interface shows that the water temperature in the sink has dropped to 5℃.
3. Dilute the genomic DNA to 35 μL in 1×Low TE Buffer in a centrifuge tube. Add 35 μL of DNA to the Covaris® microTUBE and avoid air bubbles.
4. Load the Covaris® microTUBE with samples in rack and set the conditions below for DNA shearing. When the "Run" button is green, the selected method could be started. Click the "Run" button to start.

Settings	Parameters
Duty Factor	10%
Peak Incident Power	175
Cycles per Burst	200
Treatment Time	360 s
Bath Temperature	4 ~ 8℃

5. Analyze the fragment size by fragment analyzer and the main DNA peak size is around 150 ~ 200 bp.

[Method 3] DNA fragmentation by fragmentase

1. Please use the appropriate enzyme fragmentation kit, e.g. KAPA Frag Kit for Enzyme Fragmentation. Set the conditions of the enzyme fragmentation reaction according to the required peak size and refer to the manufacturer's instructions.
2. If EDTA is present, we recommend to purify sample before library prep. For instance, use KAPA Frag Conditioning Solution and follow the instruction to purify DNA sample with EDTA.
3. After the enzyme fragmentation reaction, purify the DNA fragments to remove the enzymes and buffer from the reaction mixture to avoid any influence on next steps.

Fragmented DNA can be stored temporary at -20℃ if not proceed to next step immediately.

Note

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