iGeneTech
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For Illumina® and DNBSEQ® platform.
Generate WGS library.

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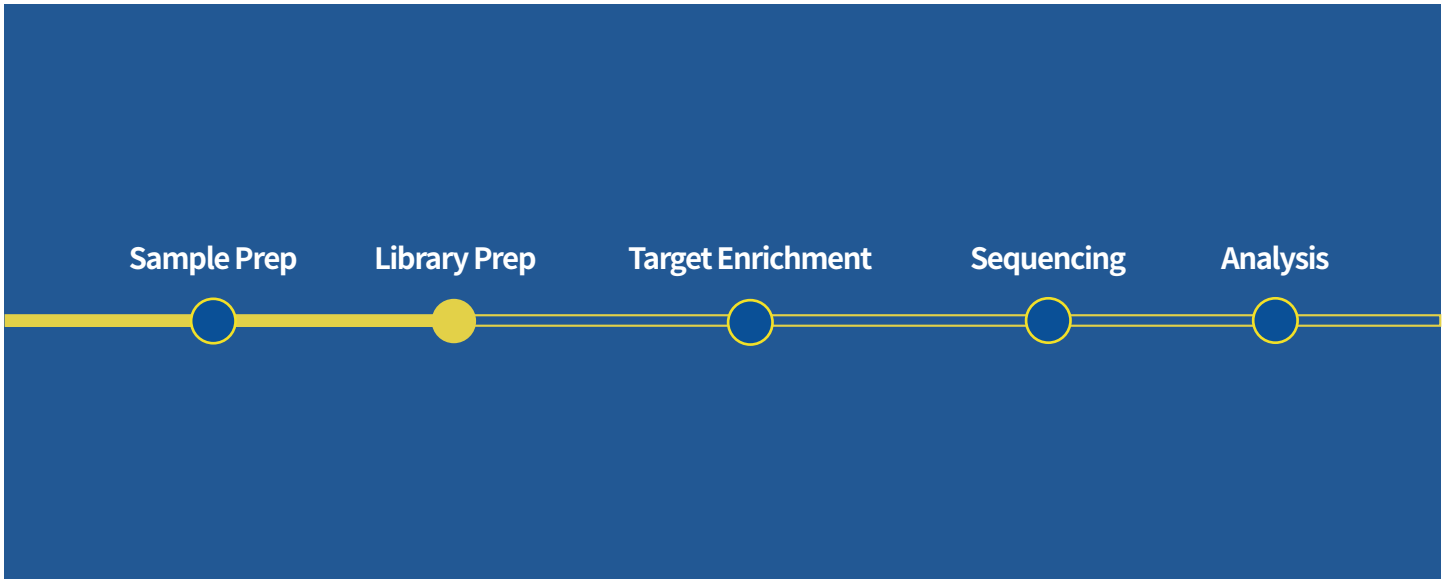
IGT ™ Fast Library Prep Kit v2.0

Protocol

For 2022 New Product System

English Version
Version: C.1(E), June 2022
Document Number: PROT226008



Version Notes

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Protocol Notes

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

Product Notes

IGT ™ Fast Library Prep Kit v2.0 is a universal DNA library preparation kit based on the TA cloning ligation principle and generates sequencing libraries of Illumina® and DNBSEQ® platform. The kit produces whole genome sequencing libraries for target capture sequencing with input DNA from 1 ng to 1 μg. It can be used with unique molecular identifier (UMI) technology for low frequency mutation detection of cfDNA.

The kit is suitable for various sample types: gDNA (extracted from blood, Saliva, Oropharyngeal swabs, fresh tissue and frozen tissue and cell), FFPE DNA and et al.

Revision history

Version	Date	Description
C.1(E)	June, 2022	Minor correction
C.0(E)	June, 2022	First release in English
C.0	June, 2022	Optimization for new product system
B.0	April, 2022	Optimization of format
1.0	February, 2022	Initial release

Materials from iGeneTech

IGT ™ Fast Library Prep Kit v2.0 and IGT ™ Adapter & Primer are needed for library constrcution.

IGT ™ Fast Library Prep Kit v2.0

Screw Cap	Components	Amount		Storage
		16 rxn	96 rxn	
Yellow	End Repair & A-Tailing Buffer	124 µL	744 µL	-20°C ± 5°C
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	PCR Master Mix	450 µL	2*1350 µL	

IGT ™ Adapter & Primer

Please select one of the following IGT ™ Adapter & Primer according to requirement.

IGT ™ Adapter & Single-Indexed Primer (for MGI)

Screw Cap	Components	Amount	Storage
		96*1 rxn	
Blue	Adapter (15 µM, for MGI SI)	540 µL	-20°C ± 5°C
White	TPE 1.0 Primer (20µM, for MGI)	264 µL	
/	TPE 2.0 Indexed Primer N (20µM, for MGI)*	4 µL each	

*N is the index number.

IGT ™ Adapter & UDI Primer (for Illumina/MGI)

Screw Cap	Components	Amount		Storage
		96*1 rxn	96*10 rxn	
Blue	Adapter (15 µM)	540 µL	4*1350 µL	-20°C ± 5°C
/	UDI Primer N (10 µM each)*	8 µL each	96*75 µL	

*N is the index number.

IGT ™ UMI Adapter & UDI Primer (for Illumina/MGI)

Screw Cap	Components	Amount		Storage
		96*1 rxn	96*10 rxn	
Blue	Insert UMI Adapter (15 µM)	540 µL	4*1350 µL	-20°C ± 5°C
/	UDI Primer N (10 µM each)*	8 µL each	96*75 µL	

*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)

*Choose one of the recommended reagents.

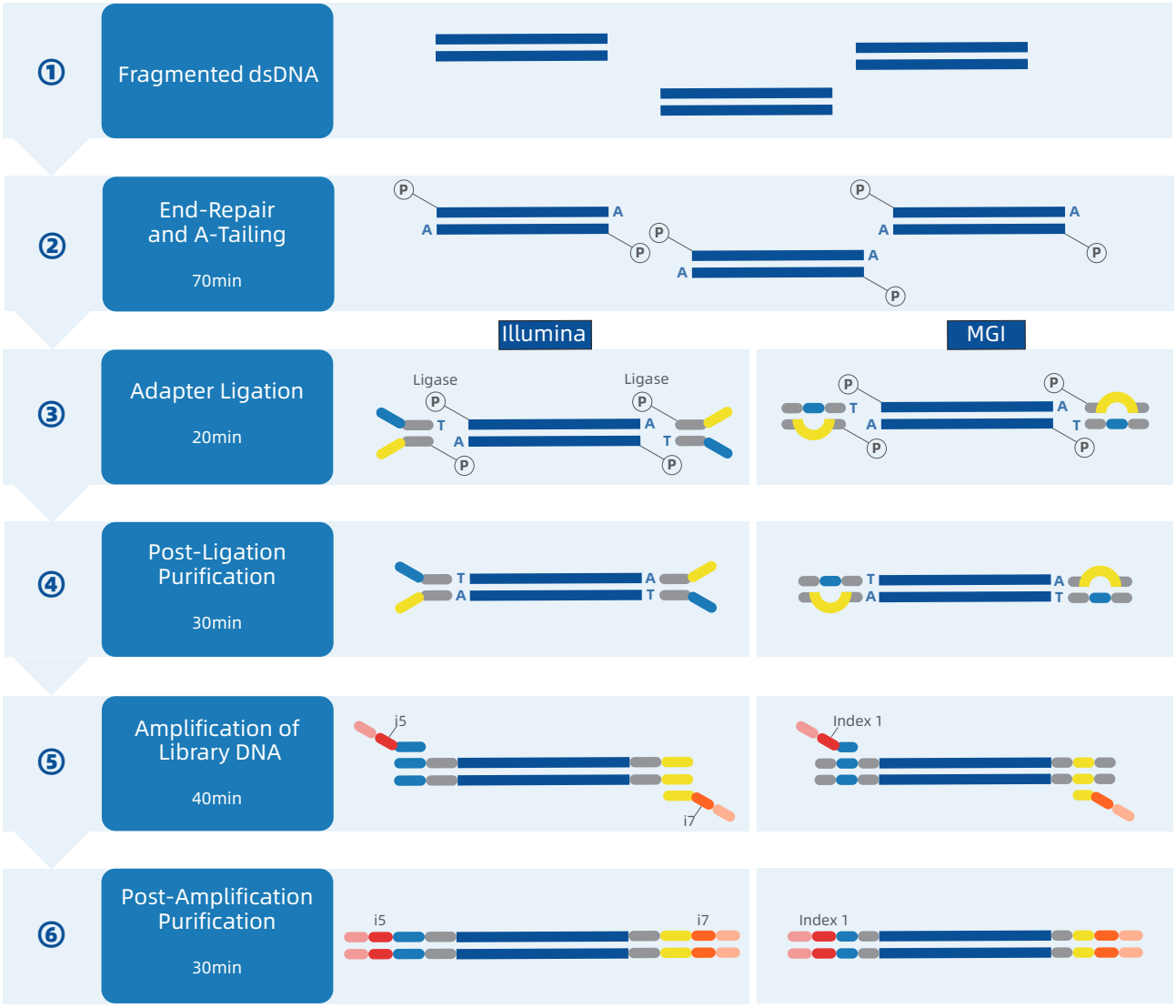
Equipments

Item	Recommended equipments	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)
Thermal Mixer, 0.2 mL block	Eppendorf ThermoMixer® C	Eppendorf (5382000015)
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
Pipette tips, 10 µL	Varies	Varies
Pipette tips, 200 µL	Varies	Varies

Workflow


①
Fragmented dsDNA
②
End-Repair and A-Tailing
70min
Illumina
MGI
③
Adapter Ligation
20min
Ligase
Ligase
④
Post-Ligation Purification
30min
⑤
Amplification of Library DNA
40min
i5
i7
Index 1
⑥
Post-Amplification Purification
30min
i5
i7
Index 1

Before You Begin

To start, please confirm the following requirements are met.

- ☐ 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 cfDNA amounts is more than 20 ng.
- ☐ Please preparing reaction mixture on ice or ice block.
- ☐ The 0.2 mL magnetic stand will be used.
- ☐ Agilent 2100 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 one.

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

STEP 1 End-Repair and A-Tailing

Experiment Notes

Regents required:

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

Equipments required:

- Thermal Cycler

1.1 Before next step, the genomic DNA or FFPE DNA needs to be fragmented to a size of 150-200 bp. Please refer to Appendix 1 for DNA fragmentation methods. Fragmentation is not required if the input DNA is cfDNA or FFPE DNA that is severely degraded to very small fragments.

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°C
30°C	30 min
65°C	30 min
4°C	**Hold**

1.7 Proceed to STEP 2 immediately when the program finishes.

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

STEP 2 Adapter Ligation

Experiment Notes

Regents required:

- Ligation Buffer
- DNA Ligase
- PCR Master Mix
- Nuclease-Free Water
- Adapter (15 μM)

Equipments required:

- Thermal Cycler

2.1 Thaw Adapter (15 μM) on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

2.2 Dilute the Adapter (15 μM) according to the table below.

DNA input	Adapter concentration	Dilution times
50 ng to 1 μg	15 μM	/
25 ng	7.5 μM	2
10 ng	3 μM	5
5 ng	1.5 μM	10
2.5 ng	750 nM	20
1 ng	300 nM	50

Avoid adding excessive Adapter (15 μM) to generate adapter self-ligation and less to reduce libraries 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
Sample from STEP 1	60 μL
Adapter (diluted)	5 μL
Ligation Buffer	30 μL
DNA Ligase	5 μL
Nuclease-Free Water	10 μL
Total Volume	**110 μL**

If handling many samples at the same time, prepare the reaction mixture without Adapter. It is suggested to mix the Adapter and the products from STEP 1 first, and then add the reaction mixture to 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, increase the ligation reaction time to 4 h or overnight at 4℃. However, excessive ligation time will also increase adapter self-ligation.

2.8 Proceed to STEP 3 immediately when the program finishes.

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

STEP 3 Post-Ligation Purification

Experiment Notes

Regents required:

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

Equipments required:

- Magnetic Stand

IGT ™ Pure Beads and Agencourt AMPure XP are the recommended magnetic beads in this protocol. Please chose the suitable volume according to the library type. Please assess the volume of beads used for purification via pre-experiments if not using the recommended one.

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, and equilibrate the Magnetic Beads to room temperature for 30 min.

3.3 Add 88 µL (0.8×) of Magnetic Beads to each ligated sample 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 or plate 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 or plate containing beads 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 or plate containing beads 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 Spin briefly on a mini centrifuge. Place the PCR tube or plate containing beads 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 or plate containing beads on the magnetic stand at room temperature for 3 to 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 or plate containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

3.10 Spin briefly on a mini centrifuge. Place the PCR tube or plate containing beads on a magnetic stand and allow the beads to fully separate from the supernatant.

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

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

STEP 4 Amplification of Library DNA

Experiment Notes

Regents required:

- PCR Master Mix
- UDI Primer or TPE Primer (based on kit selected)

Equipments required:

- Thermal Cycler

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

4.2 Thaw UDI Primer or TPE Primer on ice. Mix thoroughly and centrifuge briefly. Place back on ice.

4.3 Prepare PCR reaction mixture on ice as indicated below.

Component	Volume per reaction with Unique Dual Identifier	Volume per reaction with Single Index
Sample from STEP 3	20 µL	20 µL
PCR Master Mix	25 µL	25 µL
UDI Primer N (10 µM each)	5 µL	/
TPE 1.0 Primer (20µM, for MGI)	/	2.5 µL
TPE 2.0 Indexed Primer N (20µM, for MGI)	/	2.5 µL
Total Volume	**50 µL**	**50 µL**

Make sure the index number N of indexed primer are marked down.

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°C		
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 (100 ng library output)	PCR cycles N (1 µg library output)
1 µg	3~4	3~4
500 ng	3~4	3~4
250 ng	3~4	4~6
100 ng	3~4	6~7
50 ng	4~5	7~8
10 ng	6~7	9~10
5 ng	7~8	10~12
2.5 ng	9~11	13~15

The recommended PCR cycle of cfDNA is 10 to 12.

4.6 Proceed to the STEP 5 when the program finishes.

Amplified libraries of STEP 4 can be stored at -20°C temporary. It is recommended to proceed to STEP 5 in the same day.

STEP 5 Post-Amplification Purification

Regents required:

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

Equipments required:

- Magnetic Stand

5.1 Vortex the Magnetic Beads for 30 sec to mix well. Equilibrate the Beads to room temperature for 30 min.

5.2 Add 50 µL (1.0×) of Magnetic Beads to each amplified sample from STEP 4. Pipet or vortex to mix well. Incubate at room temperature for 5 min.

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

5.4 Keep the PCR tube or plate containing beads 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.

5.5 Keep the PCR tube or plate containing beads 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.

5.6 Spin briefly on a mini centrifuge. Place the PCR tube or plate containing beads on a magnetic stand and allow the beads to fully separate from the supernatant. Remove all traces of supernatant using a 10 µL pipette.

5.7 Keep the PCR tube or plate containing beads on the magnetic stand at room temperature for 3 to 5 min to dry the bead pellet. Do not overdry the bead pellet.

5.8 Add 30 µL of Nuclease-Free Water. Remove the PCR tube or plate containing beads from the magnetic stand. Vortex for 10 sec or pipet to mix well. Incubate at room temperature for 2 min.

5.9 Spin briefly on a mini centrifuge. Place the PCR tube or plate containing beads on a magnetic stand and allow the beads to fully separate from the supernatant.

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

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

Experiment Notes

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 genomic DNA of high molecular weight. 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 to 1 μg of genomic DNA to a 0.6 mL centrifuge tube, add Nuclease-Free Water to a total volume of 35 μL, mix well, centrifuge instantaneously and place on ice for use.
2. Ture on the Bioruptor® Pico in advance and turn on the cold cycle until the circulating water temperature drop to 4°C. Before working on the Bioruptor® Pico sonicator, make sure that the circulating water temperature is around 4°C to prevent excessive temperatures.
3. Set the cycle conditions, time ON for 30 sec, time OFF for 30 sec for 1 cycle, run 10 cycles for 3 times. Keep the the temperature of the cooler at 4°C all the way before next time.
4. Analyze the fragment size of shearing DNA by fragment analyzer and the main DNA peak size is around 150 to 200 bp.

[Method 2] DNA fragmentation by Covaris®

1. Add fresh deionised water to the sink to FILL 10 to 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°C.
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°C.
3. Dilute the genomic DNA to 35 μL in 1×Low TE Buffer in a centrifuge tube and add 35 μL of DNA to the Covaris® microTUBE to 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 may 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 to 8℃

5. Analyze the fragment size of shearing DNA by fragment analyzer and the main DNA peak size around 150 to 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 that purify sample before library prep. Insteadly, KAPA Frag Conditioning Solution is used when EDTA is present in the DNA.
3. After the enzyme fragmentation reaction, purify the DNA fragments to remove the enzyme and Buffer from the reaction system to avoid affectiong the next step of the reaction.

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

Appendix 2: Final Library Peak size Quality Check for cfDNA

cfDNA quality control

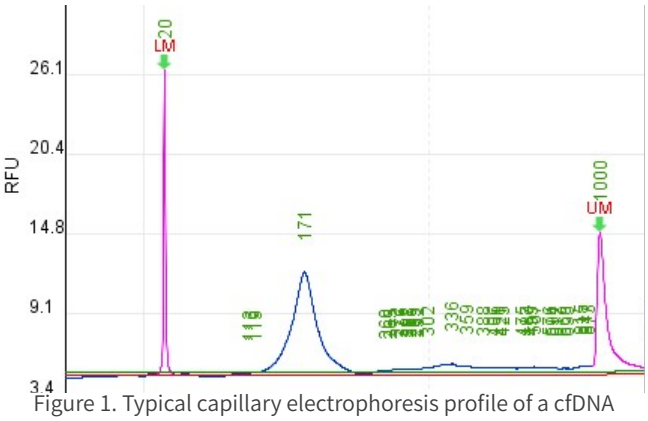


Figure 1. Typical capillary electrophoresis profile of a cfDNA sample

When cfDNA is analyzed by capillary electrophoresis, the primary peak is around 166 bp and there may be a secondary peak at around 332 bp and occasionally another peak at around 498 bp. Extraction of high quality cfDNA should be carried out to avoid haemolysis and contamination of genomic DNA (>2000 bp).

Pre-library library quality control

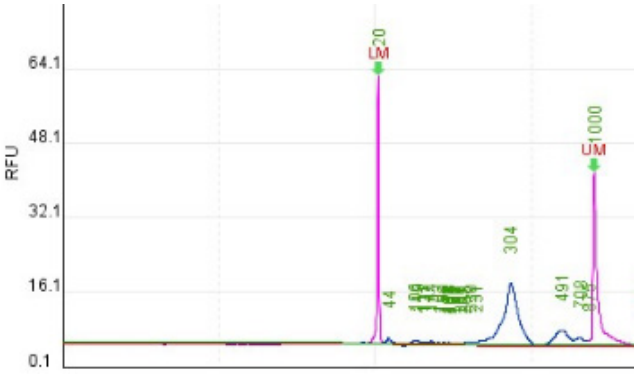


Figure 2. Typical capillary electrophoresis profile of a cfDNA sample

Capillary electrophoresis analysis of the pre-library constructed after cfDNA ligation, with a primary peak at around 300 bp. In addition, there are two secondary peaks at > 300 bp. High quality libraries should have no or minimal self-ligation, with the main peak around 150 bp.

Hybridization capture library quality control

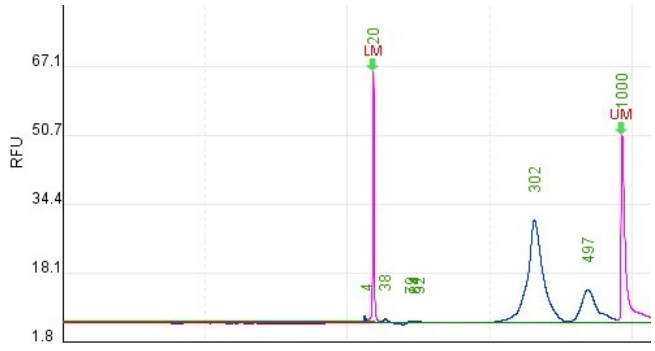


Figure 3. Typical capillary electrophoresis profile of Cap-library

The final captured library constructed from high quality cfDNA has a primary peak at around 300 bp, with no splice self-ligation dimers. There are several secondary peaks at >300 bp.

Appendix 3: Troubleshooting

Type	Issue	Possible cause	Suggested solution
Sample	cfDNA with residual gDNA	Low plasma quality	Plasma separation is carried out as soon as possible after collecting to avoid hemolysis.
			Ensure adequate centrifugation for effective separation of plasma.
			When collecting plasma after centrifugation, avoid aspirating the brownish-yellow layer of blood sediment.
			If plasma is stored temporarily at -20°C or -80°C, it will need to be thawed and centrifuged again to remove any remaining cellular debris.
Library building issues	Adapter self-ligation after Pre-PCR	Improper connection of connectors	Ensure that the reagents are added in the recommended order to avoid Adapter self-ligation of the connectors.
		Low input cfDNA	Ensure accurate quantification of cfDNA.
			Put in more cfDNA.
			Ensure that there is no gDNA in the cfDNA.
		Too much pure beads	Ensure that the volume of purified beads put in is in the right proportion to the sample volume.
	Abnormal library fragment analysis after Pre-PCR	Presence of large library fragments	Ensure that there is no gDNA in the cfDNA.
			Select a suitable fragment selection method to remove large fragments library.
			Over-amplification leads to large fragments library and it is recommended to reduce the number of cycles.
	Low yield of libraries after Pre-PCR	Inefficient ligation	Put in the right proportion of cfDNA and connectors.
			Extend the ligation time appropriately.
		Low PCR amplification efficiency	Avoid a high proportion of adapter self-ligation fragments, which can affect effective fragment amplification.

Note

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