MGIEasy

stLFR Library Prep Kit User Manual

Item Number: 1000005622

Kit Version: V1.0

User Manual Version: A0

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Contents

Chapter 1 Product Description

1.1 Introduction

MGIEasy stLFR Library Prep Kit is a human genome re-sequencing kit compatible with MGI high-throughput sequencing platforms. This kit can be used to label a single long DNA fragment (length greater than 40 kb) is paired with a barcode, then subsequently construct a library. After sequencing, the long fragment DNA is reconstructed using the paired DNA/barcode read. This enables identification of haplotype and structural variants present in the long DNA fragment. All reagents provided within this kit have passed stringent quality control and functional verification procedures, ensuring performance stability and reproducibility.

1.2 Applications

The MGIEasy stLFR Library Preparation Kit is suitable for human genome resequencing and can identify haplotype and structural variants.

1.3 Platform Compatibility

Constructed libraries are compatible with
BGISEQ-500RS High-throughput Sequencing Set (stLFR) (Part Number 1000011543);
MGISEQ-2000RS High-throughput Sequencing Set (stLFR) (Part Number 1000011545).

1.4 Contents

The MGIEasy stLFR Library Prep Kit is composed of 2 boxes and is designed for a total of 16 reactions. The kit information and storage conditions are listed in Table 1.

Table 1. MGIEasy stLFR Library Prep Kit (16 RXN) (Cat. No: 1000005622)

Kit information	Components	Cap Colour	Spec & Quantity
MGIEasy stLFR Library Prep Kit (Box 1) Cat. No. 1000005622 (Storage Conditions: -25℃ to -15℃)	TI Buffer	Green	160 μL/Tube × 1 Tube
	TI Enzyme	Green	8 μL/Tube × 4 Tubes
	DNA Ligase	Red	224 μL/Tube × 1 Tube
	Ligation Buffer I	Red	416 μL/Tube × 1 Tube
	Digestion Enzyme	Black	80 μL/Tube × 1 Tube
	Digestion Buffer I	Black	1520 μL/Tube × 1 Tube
	Pre Ligation Enzyme	Black	64 μL/Tube × 1 Tube
	Pre Ligation Buffer	Black	320 μL/Tube × 1 Tube
	Ligation Buffer II	Orange	768 μL/Tube × 1 Tube
	Adapter	Orange	288 μL/Tube × 1 Tube
	PCR Enzyme	Blue	36 μL/Tube × 1 Tube
	PCR Buffer	Blue	1182 μL/Tube × 2 Tubes
MGIEasy stLFR Library Prep Kit (Box 2) Part Number: 1000005622 (Storage Conditions: 2℃ to 8℃)	Capture Beads	Yellow	480 μL/Tube × 1 Tube
	Capture Buffer	Yellow	800 μL/Tube × 1 Tube
	DNA Clean Beads	White	1760 μL/Tube × 1 Tube
	Wash Buffer I	White	800 μL/Tube × 1 Tube
	Wash Buffer II	White	5067 μL/Tube × 3 Tubes
	TIS Buffer	White	176 μL/Tube × 1 Tube
	TE Buffer	White	2080 μL/Tube × 1 Tube

1.5 Storage Conditions and Shelf Life

MGIEasy stLFR Library Prep Kit (Box 1)

- Storage Conditions: -25°C to -15°C.
- Production Date and Expiration Date: refer to label
- Transportation Conditions: Dry Ice*

MGIEasy stLFR Library Prep Kit (Box 2)

- Storage Conditions: 2℃ to 8℃
- Production Date and Expiration Date: refer to label
- Transportation Conditions: Ice Pack

* Dry Ice Shipments: Please ensure that an abundance of dry ice remains after transportation.

* Performance of products is guaranteed until the expiration date under appropriate transport, storage, and usage conditions. Unexpected freezing or thawing may lead to failure.

1.6 Equipment and Materials Required but not Provided

Table 2. Equipment and Materials Required but not Provided

Equipment	Vortex—Genie 2, SI-0246
	Microcentrifuge（Baygene, BG-Qspin）
	Pipettes
	2100 Bioanalyzer (AGILENT)
	Thermocycler
	DynaMag™-2 Magnet (Thermo Fisher, Cat. No. 12321D) or equivalent
	DynaMag™-96 Side Magnet (Thermo Fisher, Cat. No.12331D) or equivalent
	Lab incubator capable of holding Tube Revolver/Rotator
	Tube Revolver/Rotator (Thermo Fisher, Cat. No. 88881001) or equivalent
	Qubit® 3.0 (Thermo Fisher, Cat. No. Q33216)
Reagents	MagAttract HMW DNA Kit (QIAGEN, Cat No./ID: 67563) or RECOVEREASE DNA ISOLATION KIT (Agilent Technologies, Catalog No.720203)
	Nuclease free water (Ambion, Cat. No. AM9937)
	100% Ethanol
	Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)
	Qubit® dsDNA HS Assay Kit (Invitrogen, Cat. No. Q32854)
	Agilent 2100 High Sensitivity DNA Kit
	BGISEQ-500RS High-throughput Sequencing Set (stLFR) (Item Number 1000011543) or MGISEQ-2000RS High-throughput Sequencing Set (stLFR) (Item Number 1000011545)
Materials	Pipette Tips
	200 μL Clear Wide Bore Tips (Axygen, T-205-WB-C-R-S)
	1.5 mL EP Tube (Axygen, Cat. No. MCT-150-C)
	0.2 mL PCR Tube (Axygen, Cat. No. PCR-02-C) or 0.2 mL 8-strip Tube (Axygen, Cat. No. PCR-0208-CP-C) or equivalent
	Qubit® Assay Tubes (Invitrogen，Cat. No. Q32856) or equivalent

1.7 Precautions and Warning

a) This user guide aims to provide a standard protocol for making an stLFR library for human whole genome sequencing purposes only. Changes can be made for different applications, but performance will not be guaranteed.

b) Before step 3.5 (Termination Reaction), all liquid containing target DNA fragments should be treated as gently as possible. Wide-bore tips are highly recommended during these steps and vigorous vortexing should be avoided. For mixing purposes, flicking the bottom of the tube or inverting the tube at very low speed is

sufficient.

c) Prepare reagents for each step in advance. Place all enzymes on ice immediately after centrifugation. Place the buffers and other reagents on ice after thawing, vortexing, and centrifugation.

d) Set the tube rotator speed at 20 rpm and place it in an incubator. Set the incubator temperature during the following steps: Capture, Ligation Reaction 1, Digestion Reaction 1, Termination Reaction, Pre-Ligation Reaction 2, and Ligation Reaction 2. Tubes should be placed on the rotator when the incubator is at the correct temperature.

e) During the Ligation Reaction 1, Termination Reaction, and Ligation reaction 2 steps, the room temperature range should be 20 ° C to 25 ° C.

f) Always keep the Capture Beads wet and **do not** let them dry completely at any time. Add the reaction mix immediately after disposing of bead wash buffer.

g) Change pipette tips between samples avoid sample cross-contamination. Tips with filters are highly recommended.

h) Avoid touching the Capture Beads and their solution when pipetting the reaction mix into the sample tube.

i) Check the lid of the sample tube after every reaction and make sure there are no residual beads. If there are residual beads, use a small volume of the Wash Buffer II to wash the beads off the lid and collect them into the sample tube.

j) A thermocycler with a heated lid is highly recommended, and the reaction volume of the thermocycler should not be less than 100 µL when doing the PCR step.

k) The PCR reaction is on Beads PCR. It is recommended to preheat the heated lid to 105 ° C, mix the sample tube, then place into the PCR machine to ensure that the beads are completely suspended at the beginning of the reaction.

l) Pre-PCR steps and Post-PCR steps should be performed in pre-PCR and post-PCR rooms, respectively.

m) All libraries should be sequenced using the matching sequencing kit (Item Number. 1000011543 or 1000011545).

n) If you have other questions, please contact MGI technical support MGI-service@genomics.cn

Chapter 2 Sample Requirements and Preparation

2.1 Sample Requirements

2.1.1 For DNA isolation/purification, either QIAGEN MagAttract HMW DNA Kit (Cat No./ID: 67563) or Agilent Technologies RECOVEREASE DNA ISOLATION KIT (Catalog No.720203) are highly recommended.

2.1.2 The following criteria are recommended for best performance after sample extraction: sample concentration should be 1 to 3 ng/μL, the A260/A280 value of the DNA sample should be 1.6 to 2.2, and the mean length of the initial DNA fragments should be longer than 40 Kb with no degradation below 40 Kb. DNA fragments with longer length will produce better results.

2.1.3 Store isolated DNA samples at 4℃. Samples can also be stored at -20℃, but frequently freezing and thawing the DNA should be avoided. DNA Sample can be stored at 4℃ for 6 months or -20℃ for 1 year (samples must be stored separately and removed no more than twice. Avoid physical fragmentation.) Use nuclease-free reagents and consumables.

2.1.4 Protein contamination and/or high concentration of salts and other contaminants may lead to failure of the library construction process.

2.1.5 All samples must meet the conditions listed above for library construction. Failure to meet the requirements may result in failure to build a database and yield unsatisfactory data analysis results.

2.2 DNA Sample Quantification Preceding Library Construction

2.2.1 Use the Qubit® dsDNA HS Assay Kit for quantitation.

2.2.2 Gently and slowly invert the extracted gDNA to mix the gDNA. Take 3 μL each of the gDNA sample from top, middle and bottom part of the tube, and quantitate them with Qubit® dsDNA HS Assay Kit. If the replicates differ by over 10%, continue to gently and slowly invert the extracted gDNA to mix the gDNA and repeat the quantification step until the the replicates differ by less 10% and the concentration range is 1 to 3 ng/μL.

2.2.3 If the concentration of gDNA is >3 ng/μL, dilute the DNA sample to 1 to 3 ng/μL in TE Buffer.

⚠ **Note 1: The long-fragment DNA and its dilutions should be stored at 4°C to prevent freeze-and-thaw cycles. DNA mixing should be performed slowly and gently to avoid physical fragmentation.**

2.2.4 10 ng of DNA are needed for Step 3.1 (Transposon Insertion), 15% of transposon-inserted product (~1.5 ng) are required for Step 3.2 (Capture).

Chapter 3 Library Construction Protocol

3.1 Transposon Insertion

⚠ Preparation before proceeding to Transposon Insertion steps:

a) Remove the TI Enzyme from -20°C and immediately place on ice. Return to -20℃ immediately after use.

b) Remove the TI Buffer from -20℃ and immediately place on ice.

c) Remove the TE Buffer and Nuclease Free Water from 4℃ and place at room temperature.

d) Preheat the incubator to 60℃.

⚠ **Note 1: Remove the long-fragment gDNA from 4°C and store on ice. Pipetting of DNA should be performed slowly and gently to avoid physical fragmentation.**

3.1.1 Transfer 10 ng long-fragment gDNA gently into a 0.2 mL PCR tube. Without mixing, add **Nuclease Free Water** to a total volume of 38 μL. Collect and dispense long fragment DNA slowly (the process should take >10 s.) each time when pipetting.

3.1.2 Dilute the TI Enzyme 16-fold with TE Buffer. Add 6 μL TE Buffer into a new 0.2 mL PCR tube and then transfer 2 μL TI Enzyme to the tube. Vortex intermittently for 4 times (2s each) to mix. Label it as "4× dilution TI Enzyme".

3.1.3 Add 18 μL TE Buffer into a new 0.2 mL PCR tube and transfer 6 μL of the 4× dilution TI Enzyme into the tube. Vortex intermittently for 4 times (2s each) to mix the tube. Label it as "TI Enzyme (Working Mix)." TI Enzyme (Working Mix) can be used for 12 reactions.

⚠ **Note 2: Keep all materials on ice. The TI Enzyme dilution must be freshly prepared.**

3.1.4 Prepare the Transposon Insertion Reaction Mix on ice as shown in Table 3. Vortex intermittently for 4 times (2s each) to mix.

Table 3. Transposon Insertion Reaction Mix preparation

Reagents	Volume (1×)
TI Buffer	10 μL
TI Enzyme (Working Mix)	2 μL
Total	12 μL

3.1.5 Transfer 12 μL Transposon Insertion Reaction Mix to the DNA sample from step 3.1.1(total volume is 50 μL). Mix by very gently pipetting 10 times using wide-bore tip. Briefly centrifuge the tube. Transfer the tube to the thermocycler and start the Transposon Insertion Reaction Program as shown in Table 4.

Table 4. Transposon insertion Reaction Program

Temperature	Time
Lid 60℃	On
55℃	10 minutes
4℃	Hold

3.1.6 Store the tubes on ice after the transposon insertion step has finished.

⚠**Note 3: Only 15% of transposon-inserted product from step 3.1.6 is used for Capture (Step 3.2).**

3.1.7 Pipette 42.5 µL TE buffer to a new 0.2 mL tube then transfer 7.5 µL transposon-inserted product (from step 3.1.6) to this new 0.2 mL tube. Mix by inverting the tube very gently and collect liquid to the bottom of the tube by briefly centrifuging (1 second) on a microcentrifuge. Label this new tube as the sample tube.

3.2 Capture

⚠ Preparation before proceeding to the Capture steps:

a) Set the rotator to 20 rpm at 60℃ in the incubator.

b) Remove the Capture Beads from 4°C and mix by vortexing. Store at room temperature.

c) Remove the Wash Buffer I and Capture Buffer from 4℃. and mix by vortexing. Store at room temperature.

3.2.1 Vortex Capture Beads to mix thoroughly before use. Pipette 30 µL Capture Beads per sample to the tube*.

* If there are multiple samples, pipette $n \times 1.1 \times 30$ µL Capture Beads to the same tube, where n = number of samples. If $n \leq 3$, pipette Capture Beads to the 0.2 mL PCR tube. If $n > 3$, pipette Capture Beads to a 1.5 mL EP tube.

3.2.2 Place the tube on a magnetic separation rack. Once the liquid is clear, carefully collect and discard the supernatant

3.2.3 Pipette 50 µL Wash Buffer I per sample into the 0.2 mL PCR tube or 1.5 mL EP tube. Ensure that Wash Buffer I covers all of the Capture Beads*.

* If there are multiple samples, pipette $n \times 50$ µL Wash Buffer I to the 0.2 mL PCR tube or 1.5 mL EP tube, where n = number of samples. Ensure that all of the Capture Beads are completely immersed in Wash Buffer I.

3.2.4 Rotate the tube 180 degrees within the rack such that the beads are forced to pass through the Wash Buffer I. Repeat the tube rotation once. Carefully remove and discard the supernatant once the liquid in

the tube is clear. Pipette 50 µL Capture Buffer per sample to resuspend the Capture Beads*.

* If there are multiple samples, pipette $n \times 1.1 \times 50$ µL Capture Buffer to resuspend the Capture Beads, where n = number of samples.

⚠**Note 1: Due to the high sedimentation rate of Capture Beads, please mix the Capture Beads from step 3.2.4 every 30 seconds.**

3.2.5 Transfer 50 µL Capture Beads from step 3.2.4 to the sample tube from step 3.1.7 and mix thoroughly by inverting gently at least 10 times.

⚠**Note 2: To ensure a proper capture reaction, mix all components in the tube by gently inverting upside-down several times, followed by brief centrifugation (1s). DO NOT VORTEX at this step. Ensure that Capture Beads are homogeneously resuspended (Figure 1). Incomplete resuspension of the Capture Beads (Figure 2) may cause poor library performance.**

⚠**Note 3: Sample tubes placed on the rotator should be used in the incubator for uninterrupted rotating during different steps (Capture, Ligation Reaction 1, Digestion Reaction 1, Termination Reaction, Pre-Ligation Reaction 2, Ligation Reaction 2).**

3.2.6 Centrifuge the product from step 3.2.5 for 1 second and place on the rotator in the incubator. Immediately start rotating the sample. Perform the capture using the conditions listed in Table 5.

Table 5. Capture Incubation Conditions

Temperature	Time
60℃	10 minutes
45℃	50 minutes

⚠**Note 4: After the first 10 minutes of incubation at 60℃, switch the temperature of the incubator to 45℃. Open the door of the incubator to accelerate cooling, then close the door of the incubator and start the 50 minutes countdown once the temperature drops to 48℃.**

Figure 1. Homogenous suspension of Capture Beads

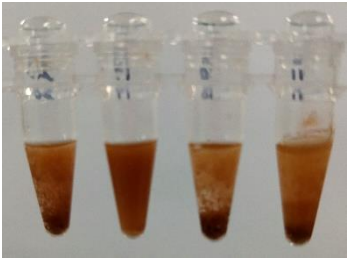

Figure 2. Incomplete suspension of Capture Beads

⚠ **Note 5: Capture Beads may aggregate after the reaction (Figure 3), which is a normal phenomenon.**

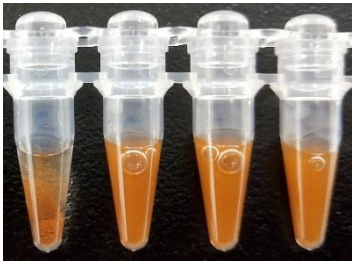

Figure 3. Aggregation, seen in the tube on the far left, may occur after the reaction

3.3 Ligation Reaction 1

⚠ Preparation before proceeding to Ligation Reaction 1 steps:

a) Set the rotator to 20 rpm at 25℃ in incubator or at room temperature (20℃ to 25℃).

b) Remove the DNA Ligase and Ligation Buffer I from -20℃ and mix by vortexing. Store on ice.

c) Remove Wash Buffer II from 4℃. Store at room temperature.

3.3.1 Prepare the Ligation Reaction 1 Mix on ice following Table 6.

Table 6. Ligation Reaction 1 Mix

Reagents	Volume (1×)
Ligation Buffer I	26 μL
DNA Ligase	4 μL
Total	30 μL

3.3.2 Centrifuge the product from step 3.2.6 and allow the product to cool to room temperature.

⚠ **Note 1: DNA Ligase will be inactivated at high temperature. Ensure the product has cooled to room temperature before transferring the Ligation Reaction 1 Mix.**

3.3.3 Ensure the product has cooled to room temperature, then transfer 30 µL Ligation Reaction 1 Mix to the 100 µL sample. Mix by inverting the tubes gently at least 10 times then briefly centrifuge (1s). Place the sample tube on the rotator and turn it on.

⚠ **Note 2: Mix all components in the tube by gently inverting upside down several times followed by instantaneous centrifugation. DO NOT VORTEX at this step. Ensure that Capture Beads are homogeneously resuspended (Figure 1).**

3.3.4 Perform Ligation Reaction 1 with the incubation conditions in Table 7.

Table 7. Ligation Reaction I Incubation Conditions

Temperature	Time
25℃ or Room Temperature (20℃ to 25℃)	60 minutes

⚠ **Note 3: A small amount of aggregation of Capture Beads is normal after the reaction (see Figure 3).**

3.3.5 After incubation, centrifuge the sample and place it on the magnetic separation rack. Carefully remove and discard the supernatant once the liquid is clear.

3.3.6 Pipette 180 µL of Wash Buffer II into the sample tube. Rotate the tube 180 degrees while on the magnetic separation rack to let the beads move through the Wash Buffer II. Repeat the tube rotation once. Carefully remove and discard the supernatant once the liquid is clear. Keep the Capture Beads in the Wash Buffer II until the Digestion Reaction Mix 1 (step 3.4.3) is ready.

⚠ **Note 4: Digestion Reaction 1 MUST be carried out immediately after discarding Wash Buffer II. Capture Beads can be stored in Wash Buffer II for up to 5 minutes until Digestion Reaction 1 Mix is prepared.**

3.4 Digestion Reaction 1

⚠ Preparation before proceeding to the Digestion Reaction 1 steps:

a) Set the rotator to 20 rpm at 37℃ in the incubator.

b) Remove the Digestion Enzyme from -20℃ and mix by vortexing. Store on ice.

c) Remove the Digestion Buffer I from -20℃, thaw at room temperature, then mix by vortexing. Store on ice.

3.4.1 Prepare Digestion Reaction 1 Mix according to Table 8.

Table 8. Digestion Reaction 1 Mix

Reagents	Volume (1×)
Digestion Buffer I	95 µL
Digestion Enzyme	5 µL
Total	100 µL

3.4.2 Transfer 100 µL Digestion Reaction 1 Mix to the sample tube from step 3.3.6. Mix by inverting the tube gently at least 10 times followed by an instantaneous centrifugation (1s).

⚠ **Note 1: Strictly control the reaction time of Digestion Reaction 1 to avoid excessive digestion. Keep the sample tube on ice before and after Digestion Reaction 1.**

⚠ **Note 2: Mix all components in the tube by gently inverting upside down several times followed by instantaneous centrifugation. DO NOT VORTEX at this step. Ensure that Capture Beads are homogeneously resuspended (Figure 1).**

3.4.3 Place the sample tube on the rotator and turn it on. Perform the Digestion Reaction 1 incubation using the conditions listed in Table 9. Remove the sample tube from the incubator at the end of the reaction. Immediately add TIS Buffer.

Table 9. Digestion Reaction 1 Incubation Conditions

Temperature	Time
37℃	10 minutes

3.5 Termination Reaction

⚠ Preparation before proceeding to Termination Reaction steps:

a) Set the rotator to 20 rpm at 25℃ in the incubator or at room temperature.

b) Note that the TIS Buffer will crystallize at 4℃. Remove the TIS Buffer from 4°C in advance and thaw until crystals dissolve (around 5 to 10 minutes). Mix by vortexing, then centrifuge briefly. Store at room temperature.

c) Remove Wash Buffer II from 4℃ and centrifuge briefly. Store at room temperature.

⚠ **Note 1: Keep the sample tube at room temperature and add TIS Buffer to sample/s at room temperature within 1 minute. Cold temperatures may lead to failure of the Termination Reaction.**

3.5.1 Remove the sample from 37℃, centrifuge briefly, and store at room temperature. Immediately add 11 µL

TIS Buffer to each sample from step 3.4.3.

⚠ **Note 2: After adding TIS Buffer, all subsequent steps can be mixed by vortexing as the long fragment DNA has been completely fragmented.**

3.5.2 Ensure the sample tube is sealed tightly. Mix the sample tube by vortexing at medium speed for 3 to 5 seconds to make sure the beads are fully resuspended. Centrifuge the tube for 1 second and place on the rotator. Start the rotator and perform the incubation according to the condition in Table 10.

Table 10. Incubation Conditions of the Termination Reaction

Temperature	Time
25℃ or Room Temperature (20℃ to 25℃)	10 minutes

3.5.3 After incubation, centrifuge the sample and place on the magnetic separation rack. Carefully remove and discard the supernatant once the liquid is clear.

⚠ **Note 3: All enzymes will be denatured after adding TIS buffer. Therefore slight ivory discoloration of the solution is normal.**

3.5.4 Keep tube on the magnetic separation rack and pipette 150 µL Wash Buffer II into the sample tube. Mix the beads by vortexing for 5 seconds. Centrifuge briefly and place the tube back onto the magnetic rack for 2 minutes. Carefully remove and discard the supernatant once the liquid becomes clear.

3.5.5 Repeat step 3.5.4 twice. Once washing is completed, ensure that there is no residue in Wash Buffer II. If using the eight tube-separated PCR strip tubes, please replace the PCR tube cap.

⚠ **Note 4: Completely remove residual TIS Buffer as this will inhibit future steps. After adding Wash Buffer II, mix by vortexing at high speed for several seconds to fully rinse all parts of the tube with Wash Buffer II.**

⚠ **Note 5: You may keep the beads in Wash Buffer II for up to 5 minutes until Pre-Ligation 2 Reaction Mix (step 3.6.2) is ready. Pre-Ligation 2 Reaction MUST be carried out immediately after discarding the Wash Buffer II.**

✓ **Safe Stopping Point: Capture Beads can be stored in Wash Buffer II at 4℃ for up to 24 h.**

3.6 Pre-Ligation Reaction 2

⚠ Preparation before proceeding to the Pre-Ligation 2 Reaction steps:

a) Set the rotator to 20 rpm at 37℃ in the incubator.

b) Remove the Pre-Ligation Enzyme from -20℃, briefly centrifuge, and store on ice. Return to -20℃

immediately after use.

c) Remove the Pre-Ligation Buffer from -20℃, equilibrate to room temperature, and mix by vortexing. Centrifuge briefly and store on ice.

3.6.1 Prepare Pre-Ligation 2 Reaction Mix according to the instructions in Table 11. Keep all reagents on ice.

Table 11. Pre-Ligation 2 Reaction Mix

Reagents	Volume (1×)
Pre-ligation Buffer	20 μL
Pre-ligation Enzyme	4 μL
Total	24 μL

3.6.2 Pipette 24 μL Pre-Ligation 2 Reaction mix into the sample tube from step 3.5.5. Mix the sample tube by vortexing for 3-5 seconds to ensure the beads are fully resuspended. Centrifuge the tube for 1 second and place on the rotator stored in the 37℃ incubator. Perform the incubation according to the conditions in Table 12.

⚠**Note 1: Make sure the sample, reagents, and Capture Beads are homogeneously resuspended followed by instantaneous centrifugation. Incomplete resuspension of the Capture Beads (Figure 2) may cause poor library performance.**

Table 12. The Incubation Conditions of Pre-Ligation Reaction

Temperature	Time
37℃	30 minutes

3.6.3 When the Pre-Ligation 2 Reaction is complete, remove the product from incubator immediately and centrifuge briefly. Keep it at room temperature and proceed to next step.

3.7 Ligation Reaction 2

⚠ Preparation before proceeding to the Ligation Reaction 2 steps:

a) Set the rotator to 20 rpm at 25℃ in the incubator or at room temperature（20℃ to 25℃）.

b) Remove the DNA Ligase from -20°C, briefly centrifuge, and store on ice. Return to -20℃ immediately after use.

c) Remove the Ligation Buffer II and Adapter from -20℃, equilibrate to room temperature, and mix by vortexing. Centrifuge briefly and store on ice

3.7.1 Prepare the Ligation Reaction 2 Mix according to the instructions in Table 13. Keep the reagents on ice.

Please note that the Ligation Buffer II is viscous. Pipette slowly. Mix the Ligation Reaction 2 Mix by vortexing.

Table 13. Ligation Reaction 2 Mix

Reagents	Volume (1×)
Ligation Buffer II	48 µL
Adapter	18 µL
DNA Ligase	10 µL
Total	76 µL

⚠ **Note 1: Centrifuge the product from step 3.6.3 and keep the product at room temperature. Transfer the Ligation Reaction 2 Mix after the products cool to room temperature.**

3.7.2 Pipette all 76 µL Ligation Reaction 2 mix into the sample tube from step 3.6.3 for a total volume of 100µL. Mix the sample tube by vortexing for 10 seconds. Centrifuge the tube for 1 second to make sure beads are fully resuspended as shown in figure 1.

⚠ **Note 2: To ensure proper Ligation Reaction 2, mix all components in the tube by vortexing several times followed by brief centrifugation (1s). Esure that Capture Beads are homogeneously resuspended (Figure 1).**

3.7.3 Place the tube on rotator and perform the incubation according to Table 14.

Table14. Incubation Conditions of Ligation Reaction 2

Temperature	Time
25℃ or Room Temperature (20℃ to 25℃)	120 minutes

⚠ **Note 3: A small amount of aggregation of Capture Beads is normal after the reaction (Figure 3).**

3.7.4 After incubation, centrifuge the sample and pipette 80 µL Wash Buffer II into the sample tube. Place the sample tube on the magnetic separation rack for 2 minutes. Carefully remove and discard the supernatant when the liquid becomes clear.

3.7.5 Keep the sample tube on the magnetic separation rack and pipette 180 µL Wash Buffer II into the sample tube. Rotate the tube 180 degrees within the magnetic separation rack to allow the beads to move through Wash Buffer II. Repeat once. Carefully remove and discard the supernatant when the solution becomes clear. Make sure Wash Buffer II is completely removed.

⚠**Note 4: Keep the beads in Wash Buffer II for up to 5 minutes until the PCR Reaction Mix (step 3.8.1) is**

ready. PCR MUST be carried out immediately after discarding the Wash Buffer II.

✓ Safe Stopping point: Beads can be stored in Wash Buffer II at 4℃ for up to 24 h.

3.8 PCR

3.8.1 Prepare the PCR mix according to Table 15.

Table 15. PCR Mix

Reagents	Volume (1×)
PCR Buffer	147.75 µL
PCR Enzyme	2.25 µL
Total	150 µL

3.8.2 Pipette 150 µL of the PCR mix into sample tube from step 3.7.5. Use the pipet to mix the beads until fully resuspend. Transfer 75 µL of the sample to a different 0.2 mL tube.

⚠Note 1: Ensure that there is no Wash Buffer II residue in the tube from step of 3.7.5. Wash Buffer II will inhibit the reaction.

⚠Note 2: Samples, reagents, and Capture Beads must be thoroughly mixed to ensure complete capture of the Capture Beads. After mixing, place the reaction tube in a low-speed centrifuge, tap the centrifuge button, and centrifuge at low speed to ensure that no liquid remains on the tube cover. Ensure that the mix is fully suspended.

⚠Note 3: Due to the high sedimentation rate of Capture Beads, please heat the lid of thermocycler to 105 ℃, mix the sample tube again, centrifuge at low speed, then start the PCR Process. Make sure that Capture Beads are homogeneously resuspended.

3.8.3 Place all samples on the thermocycler. Set up the PCR program as shown in Table 16. Make sure the beads are fully resuspended before starting.

Table 16. PCR Process

Temperature	Time	Cycle
Lid 105℃	on	
98℃	3 minutes	1 Cycle
95℃	30 seconds	
58℃	30 seconds	8 Cycles
72℃	2 minutes	
72℃	10 minutes	1 Cycle
4℃	Hold	

3.8.4 Centrifuge the sample and place on the magnetic separation rack. Transfer all the supernatant of the two PCR tube from the same sample into a new 1.5mL EP tube and mix together.

3.8.5 After confirming complete recovery of the supernatant, discard the original PCR tube.

✓ **Safe Stopping Point: PCR products can be stored at -20℃ for up to 24 hours**

3.9 PCR Product Purification

⚠ **Note:** Please read Appendix B: About Magnetic Beads and Purification before operation.

3.9.1 Remove the DNA Clean beads from 4℃ and equilibrate to room temperature for at least 30 minutes before use. Vortex at full speed for 10 seconds to ensure the beads are completely resuspended.

3.9.2 Measure the volume of the PCR product from step 3.8.4. Add 0.8-fold DNA Clean beads to the PCR product. Vortex the tube to mix the beads with the sample.

* e.g., add 112 µL DNA Clean beads to the PCR product with a volume of 140 µL.

3.9.3 Incubate the sample at room temperature for 10 minutes.

3.9.4 Centrifuge the tube and place on the magnetic separation rack. Wait for 2 minutes or until the solution is clear. Discard the supernatant.

3.9.5 Keep the sample tube on the magnetic separation rack and transfer 500 µL 80% (v/v) ethanol into the tube. Let stand for 30 seconds. Carefully remove and discard the supernatant.

3.9.6 Repeat step 3.9.5 once more.

3.9.7 Keep the sample tube on the magnetic separation rack, open the cap of tube and air-dry the beads for 3 to 5 minutes until no wetness is observed (the surface of the beads will dim). Do not over dry the beads as this will significantly decrease the elution efficiency (cracks can be observed on pellet).

3.9.8 Remove the sample tube from the magnet and add 33 µl TE Buffer for elution. Vortex for 3 seconds to

resuspend the beads, then briefly centrifuge.

3.9.9 Incubate the sample at room temperature for 5 minutes.

3.9.10 Place the sample tube on the magnetic separation rack and wait for 2 minutes or until the supernatant is clear. Transfer 31 μL of supernatant from the sample tube to a new 1.5 mL EP tube. Do not disturb or pipette the beads.

3.9.11 Remove 1 μL of the sample for quantification with the Qubit® dsDNA HS Assay Kit. The typical concentration of a successful run should be ≥ 1.5 ng/μL. If the concentration is < 1.5 ng/μL, it is recommended to re-prepare the library.

3.9.12 The Agilent 2100 Bioanalyzer system is the preferred method for estimating the size range of the PCR products. Traces should resemble the overall shape of the sample electropherogram shown in Figure 4.

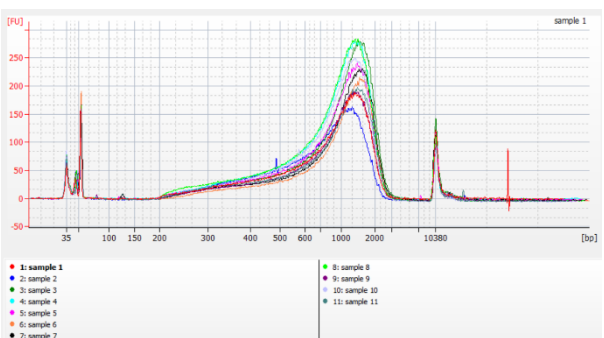

Figure 4. Agilent 2100 Bioanalyzer image of PCR products

✓ Stopping Point: Store the sample at 4℃ for up to 72 hours or at -20℃ for up to 6 months. High-throughput Sequencing Set (stLFR) can be used for DNB preparation and sequencing.

Chapter 4 Sequencing and Analysis

4.1 The library is optimized for sequencing on the BGISEQ-500RS or the MGISEQ-2000RS sequencing platforms with the following reagent kits:

BGISEQ-500RS High-throughput Sequencing Set (stLFR) (Part Number 1000011543);

MGISEQ-2000RS High-throughput Sequencing Set (stLFR) (Part Number 1000011545).

4.2 stLFR analysis software is used for data analysis after sequencing.

Appendix

Appendix A Capture Reaction and on Beads Reaction

- Due to the high sedimentation rate of Capture Beads, please mix the Capture Beads every 30 seconds during step 3.2.5.
- Mix sample, reagent, and Capture Beads by inverting the tubes gently at least 10 times followed by brief (1s) centrifugation before step 3.5. DO NOT VORTEX.
- Sample tubes placed on a rotator should be used in an incubator for uninterrupted rotating during different steps (Capture, Ligation Reaction 1, Digestion Reaction 1, Termination Reaction, Pre-Ligation Reaction 2, Ligation Reaction 2).
- Rotators placed in an incubator or a rotator with temperature control systems are feasible.

Appendix B Magnetic Beads and Purification Procedures

For magnetic bead-based purification, we recommend using DNA Clean Beads included in the MGIEasy stLFR Library Prep Kit (MGI, Cat. No. 1000005622). If you choose Magnetic Beads from other sources, please optimize the cleanup conditions before getting started.

Before You Use

- To ensure capture efficiency of the Magnetic Beads, remove beads from 4°C refrigerator storage and equilibrate to room temperature (expected time: 30 minutes). Vortex and mix thoroughly before use.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed before each use.
- The volume of the beads determines the lower limit of fragment size that can be purified.

Operation Notes

- If the sample volume decreases due to evaporation during incubation, add additional TE buffer to reach the designated volume before using the beads to purify. It ensures that the correct ratio for the beads is used.
- During the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process takes approximately 2-3 minutes. Consider the different magnetic strength of your specific Separation Plate / Rack and allow enough time for the solution to turn completely clear.
- Avoid contacting the beads with pipette tips when pipetting. 2-3 μL of fluid can be left in the tube to avoid contact. In the event of contact between the beads and the pipette tip, expel all of the solution and beads back into the tube and restart the separation process.
- Use freshly prepared 80% ethanol (at room temperature) to wash the beads twice. Keep the centrifuge tube on the Magnetic Separation Rack when washing with ethanol. Do not shake or disturb the beads in any way.
- After the 2nd bead wash with ethanol, try to remove all of the liquid within the tube. You may centrifuge briefly to collect any remaining liquid at the bottom. Separate beads magnetically and remove the remaining liquid by using a small volume pipette.
- After washing twice with ethanol, air-dry the beads at room temperature. Drying takes approximately 2-5 minutes depending on your specific lab environment. Watch closely until the pellet appears sufficiently dry with a matte appearance, then continue to the elution step with TE Buffer.
- During the elution step, do not touch the beads with the pipette tips when removing the supernatant. Contamination of DNA by the beads may affect subsequent purification. Therefore, the total volume of TE buffer and the beads should be 2 μL more than the volume of the supernatant.

- Pay attention when opening and closing the lids of centrifuge tubes on the Separation Rack. Strong vibrations may cause sample loss by spilling liquid or beads from the tubes. Secure the tubes before opening the lids.

MGI Wechat

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