

# MGIEasy

## RNA Exome Instructions

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Instruction Version: A3

Kit Version:

MGIEasy RNA Directional Library Prep Set ( 1000006385 ) V2.1

MGIEasy Exome Capture V5 Probe Set ( 1000007746 ) V1.0

MGIEasy Exome Capture Accessory Kit ( 1000007743 ) V1.0

## Revision History

| Manual Version | Date      | Description   |
|----------------|-----------|---|
| A3             | Jan. 2021 | <ul style="list-style-type: none"> <li>Update contact information.</li> </ul>   |
| A2             | Jul. 2020 | <ul style="list-style-type: none"> <li>Changed the specification of MGIEasy RNA Directional Library Prep Kit</li> </ul> |
| A1             | Sep. 2019 | <ul style="list-style-type: none"> <li>Add DNBSEQ series sequencing platform</li> </ul>                                 |
| A0             | Jun. 2019 | <ul style="list-style-type: none"> <li>Initial release.</li> </ul>  |

Note: Please download the latest version of the manual and use it with the corresponding kit.

Search manual by Cat. No. or product name from website:

<https://en.mgi-tech.com/download/files.html>

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# Chapter 1 Application Description

## 1.1 Introduction

The MGIEasy RNA Exome application is specifically designed for constructing RNA coding region libraries for the MGI high-throughput sequencing platform series. The reagents for this RNA capture method include MGIEasy RNA Directional Library Prep Set, MGIEasy Exome Capture V5 Probe Set and a MGIEasy Exome Capture Accessory Kit.

This user guide provides an RNA coding region capture method that has been optimized for poor quality and degraded RNA samples. 10 - 100 ng of degraded human total RNA can be converted into customized libraries for sequencing on an MGI sequencing platform listed below.

## 1.2 Application

This protocol is suitable for RNA derived from Human primary samples. It describes the process in which Total RNA is converted into stranded libraries and achieves the capture of the transcriptome coding regions using the MGIEasy Exome Capture V5 probe set.

## 1.3 Platform Compatibility

Constructed libraries are compatible with

BGISEQ-500 (PE100)

MGISEQ-2000 (PE100), DNBSEQ-G400 (PE100)

## 1.4 Reagents

The reagents for this RNA capture method include MGIEasy RNA Directional Library Prep Set, MGIEasy Exome Capture V5 Probe Set and a MGIEasy Exome Capture Accessory Kit. Further information on Cat. No., Components and Specifications are listed below.

Table 1 MGIEasy RNA Directional Library Prep Set (16 RXN) (Cat. No.: 1000006385)

| Modules & Cat. No.   | Components                       | Color-Coded Screw Cap | Spec& Quantity                    |
|--|----------------------------------|-----------------------|-----------------------------------|
| MGIEasy RNA Directional Library Prep Kit<br>Cat. No.: 1000005270 | Fragmentation Buffer             | Green                 | 93 $\mu$ L/tube $\times$ 1 tube   |
|  | Directional RT Buffer 1          | Green                 | 88 $\mu$ L/tube $\times$ 1 tube   |
|  | Directional RT Buffer 2          | Brown                 | 5 $\mu$ L/tube $\times$ 1 tube    |
|  | RT Enzyme Mix                    | Green                 | 24 $\mu$ L/tube $\times$ 1 tube   |
|  | Directional Second Strand Buffer | Yellow                | 470 $\mu$ L/tube $\times$ 1 tube  |
|  | Second Strand Enzyme Mix         | Yellow                | 78 $\mu$ L/tube $\times$ 1 tube   |
|  | ERAT Buffer                      | Orange                | 132 $\mu$ L/tube $\times$ 1 tube  |
|  | ERAT Enzyme Mix                  | Orange                | 55 $\mu$ L/tube $\times$ 1 tube   |
|  | Ligation Buffer                  | Red                   | 450 $\mu$ L/tube $\times$ 1 tube  |
|  | DNA Ligase                       | Red                   | 34 $\mu$ L/tube $\times$ 1 tube   |
| MGIEasy DNA Adapters-16 (Tube) Kit<br>Cat. No.: 1000005284       | PCR Enzyme Mix                   | Blue                  | 470 $\mu$ L/tube $\times$ 1 tube  |
|  | PCR Primer Mix                   | Blue                  | 90 $\mu$ L/tube $\times$ 1 tube   |
|  | UDG                              | Blue                  | 21 $\mu$ L/tube $\times$ 1 tube   |
|  | DNA Adapters                     | White                 | 10 $\mu$ L/tube $\times$ 16 tubes |
| MGIEasy DNA Clean Beads<br>Cat. No.: 1000005278                  | DNA Clean Beads                  | White                 | 8 mL/tube $\times$ 1 tube         |
|  | TE Buffer                        | White                 | 4 mL/tube $\times$ 1 tube         |
| MGIEasy Circularization Module<br>Cat. No.: 1000005260           | Splint Buffer                    | Purple                | 186 $\mu$ L/tube $\times$ 1 tube  |
|  | DNA Rapid Ligase                 | Purple                | 8 $\mu$ L/tube $\times$ 1 tube    |
|  | Digestion Buffer                 | White                 | 23 $\mu$ L/tube $\times$ 1 tube   |
|  | Digestion Enzyme                 | White                 | 42 $\mu$ L/tube $\times$ 1 tube   |
|  | Digestion Stop Buffer            | White                 | 120 $\mu$ L/tube $\times$ 1 tube  |

Table 2 MGIEasy Exome Capture V5 Probe Set (16 RXN) (Cat No.: 1000007746)

| Modules & Cat. No.   | Components             | Cap Color | Spec & Quantity                        |
|--|------------------------|-----------|--|
| MGIEasy Exome Capture<br>V5 Probe Kit                          | MGIEasy Exome V5 Probe | Black     | 80 $\mu$ L/tube $\times$ 1 tube        |
| Cat. No.: 1000007741   |                        |           |  |
| MGIEasy Exome Capture<br>Hybridization and Wash Kit<br>(Box 1) | Block 1                | Yellow    | 40 $\mu$ L/tube $\times$ 1 tube        |
|  | Block 2                | Yellow    | 40 $\mu$ L/tube $\times$ 1 tube        |
|  | Block 5                | Yellow    | 8 $\mu$ L/tube $\times$ 1 tube         |
| Cat. No.: 1000007742   | Hyb Buffer 3           | Green     | 64 $\mu$ L/tube $\times$ 1 tube        |
| MGIEasy Exome Capture<br>Hybridization and Wash Kit<br>(Box 1) | Hyb Buffer 1           | Green     | 160 $\mu$ L/tube $\times$ 1 tube       |
|  | Hyb Buffer 2           | Green     | 7 $\mu$ L/tube $\times$ 1 tube         |
|  | Hyb Buffer 4           | Green     | 90 $\mu$ L/tube $\times$ 1 tube        |
|  | Binding Buffer         | White     | 12800 $\mu$ L/bottle $\times$ 1 bottle |
| Cat. No.: 1000007742   | Wash Buffer I          | White     | 8000 $\mu$ L/bottle $\times$ 1 bottle  |
|  | Wash Buffer II         | White     | 24000 $\mu$ L/bottle $\times$ 1 bottle |

Table 3 MGIEasy Exome Capture Accessory Kit (16 RXN) (Cat. No.: 1000007743)

| Modules & Cat. No.   | Components          | Cap Color | Spec & Quantity                   |
|--|---------------------|-----------|-----------------------------------|
| MGIEasy Exome Capture<br>Accessory Kit<br>Cat. No.: 1000007743 | Post-PCR Enzyme Mix | Blue      | 800 $\mu$ L/ tube $\times$ 1 tube |
|  | PCR Primer Mix      | Blue      | 96 $\mu$ L/ tube $\times$ 1 tube  |
|  | Block 3             | Yellow    | 16 $\mu$ L/ tube $\times$ 1 tube  |
|  | Block 4             | Yellow    | 160 $\mu$ L/ tube $\times$ 1 tube |

## 1.5 Storage Conditions and Shelf Life

### MGEasy RNA Directional Library Prep Kit

- Storage Temperature: -25°C to -18°C
- Transport Conditions: transported on dry ice

### MGEasy DNA Adapters Kit

- Storage Temperature: -25°C to -18°C
- Transport Conditions: transported on dry ice

### MGEasy Circularization Kit

- Storage Temperature: -25°C to -18°C
- Transport Conditions: transported on dry ice

### MGEasy Exome Capture Accessory Kit

- Storage Temperature: -25°C to -18°C
- Transport Conditions: transported on dry ice

### MGEasy Exome Capture Hybridization and Wash Kit (Box 1)

- Storage Temperature: -25°C to -18°C
- Transport Conditions: transported on dry ice.

### MGEasy Exome Capture Hybridization and Wash Kit (Box 2)

- Storage Temperature: room temperature
- Transport Conditions: transported at room temperature

### MGEasy Exome Capture V5 Probe Kit

- Storage Temperature: -80°C
- Transport Conditions: transported on dry ice

### MGEasy DNA Clean Beads

- Storage Temperature: 2°C to 8°C
- Transport Conditions: transported with ice packs

\*Production Date and Expiration Date: refer to the label

\* Please ensure that an abundance of dry ice remains after transportation.

\* Under appropriate transport, storage, and usage conditions, performance of products is guaranteed up until the displayed expiration date.



## 1.6 Equipment and Materials Required but not Provided

Table 4 Equipment and Materials Required but not Provided

|             |  |
|-------------|--|
| Equipment   | <p>Vortex Mixer</p> <p>Desktop Centrifuge</p> <p>Thermocycler</p> <p>Magnetic rack DynaMag™-2 (Thermo Fisher Scientific™, Cat. No. 12321D) or equivalent</p> <p>Qubit™ 3 Fluorometer (Thermo Fisher Scientific™, Cat. No. Q33216)</p> <p>Agilent 2100 Bioanalyzer (Agilent Technologies™, Cat. No. G2939AA)</p> <p>Eppendorf Concentrator (Eppendorf, Cat. No. 5305000398)</p> <p>Thermomixer or water bath equipment</p> <p>Nutator or other nutating mixer/shaker</p> <p>Magnetic rack for 96-well plate (BioMag, Cat. No. BMB-96) or equivalent</p>   |
| Reagents    | <p>Nuclease free water (NF water) (Ambion, Cat. No. AM9937)</p> <p>RNase Zap (Ambion, Cat. No. AM9780)</p> <p>DNase I (NEB, Cat. No. M0303S) (optional)</p> <p>100% Ethanol (Analytical Grade)</p> <p>Qubit™ RNA HS Assay Kit (Invitrogen, Cat. No. Q32852) (optional)</p> <p>Qubit® ssDNA Assay Kit (Invitrogen, Cat. No. Q10212)</p> <p>Qubit® dsDNA HS Assay Kit (Invitrogen, Cat. No. Q32854)</p> <p>High Sensitivity DNA Kits (Agilent Technologies™, Cat. No. 5067-4626)</p> <p>Agilent DNA 1000 Kit (Agilent, Cat. No. 5067-1504)</p> <p>Agilent RNA 6000 Pico Kit (Agilent, Cat. No. 5067-1513) (optional)</p> <p>Dynabeads™ M-280 Streptavidin (Invitrogen, Cat. No. 112.06D)</p> |
| Consumables | <p>1.5 mL centrifuge tubes (Axygen, Cat. No. MCT-150-C)</p> <p>0.2 mL PCR tubes (Axygen, Cat. No. PCR-02-C) or 8-Strip PCR tubes (Axygen, Cat. No. PCR-0208-C) or 96-well plate (Axygen, Cat. No. PCR-96M2-HS-C)</p> <p>2.0 mL centrifuge tubes (Axygen, Cat. No. MCT-200-C) or equivalent</p> <p>8 Strip Domed Caps Fit 0.2 mL PCR Tube Strips (Axygen, Cat. No. PCR-02CP-C) or equivalent</p> <p>Qubit® Assay Tubes (Invitrogen, Cat. No. Q32856) or equivalent</p> <p>Filter Tips (Axygen, Cat. No. TF-100) or equivalent</p> <p>Clear Adhesive Film (ABI, Cat. No. 4306311)</p> <p>Blade or knife</p>  |

## 1.7 Precautions and Warning

- Instructions provided in this manual are intended for general use only and may require optimization for specific applications. We recommend adjusting according to the experimental design, sample types, sequencing application, and other equipment.
- Before RNA related processing, ensure the entire work area and equipment is cleaned using RNase Zap and 80% Ethanol. This includes cleaning all pipettes, tube racks, and small equipment. Wear mask, appropriate gloves and lab coats.
- Prior to starting, remove the reagents from storage, and prepare them for use: For enzymes, keep in storage until required, centrifuge briefly and place on ice until further use. For other reagents, first thaw at room temperature and invert several times to mix properly, then centrifuge briefly and place on ice until further use.
- To prevent cross-contamination, we recommend using filtered pipette tips. Use a new tip each time for pipetting different solutions.
- We recommend using thermocyclers with heated lids for reactions. Before placing the samples, preheat the thermal cycler and heated lid to the initial reaction temperature.
- Improper handling of samples and reagents may contribute to aerosol contamination of PCR Products and may decrease the accuracy of results. Therefore, we recommend physically separating two working areas in the laboratory for PCR reaction preparation and PCR product cleanup, respectively. Use designated equipment for each area and clean regularly to ensure a sterile working environment. (Use 0.5% Sodium Hypochlorite or 10% Bleach to clean working environment)
- If you have other questions, please contact MGI technical support [MGI-service@mgi-tech.com](mailto:MGI-service@mgi-tech.com)

# Chapter 2 Sample Preparation

## 2.1 Sample Compatibility and Requirement

This protocol is applicable for total RNA derived from human samples, particularly for total RNA from FFPE and fresh frozen tissues. It is recommended to use 10 – 100 ng total RNA as input.

## 2.2 Sample Quality Control

Before proceeding with library preparation, use an Agilent 2100 Bioanalyzer to perform quality control checks of RNA samples. DV<sub>200</sub> can be used for assessing the quality of RNA samples and determining the condition of library construction (Table 5). The DV<sub>200</sub> indicates the proportion of RNA fragments larger than 200 nt in the sample. Take the analysis result from Agilent 2100 Bioanalyzer trace below as an example for the DV<sub>200</sub> calculation. Detailed calculation is shown in Figure 1:

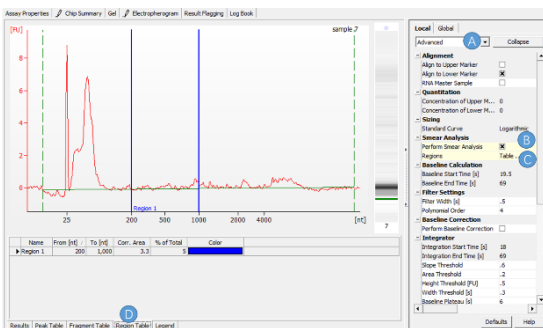


Figure 1 The Calculation of DV<sub>200</sub>

A: In the Agilent 2100 Bioanalyzer result figure, choose *Advanced* under *Local*

B: Check the *Perform Smear Analysis* option under *Smear Analysis*

C: Double-click on *Table* to enter the range of fragments to be calculated. The figure shows the example of *from 200 bp to 1000 bp*.

D: Obtain the proportion of selected fragment shown as *% of Total* in the *Region Table*

Table 5 Recommended Conditions for Library Construction from Different Quality Samples

| DV <sub>200</sub> Value | Recommended amount of total RNA input                       | Conditions of fragmentation | PCR cycles |
|-------------------------|---|-----------------------------|------------|
| > 70%                   | 10 ng   | 94°C, 8 min                 | 15         |
| 50-70%                  | 20-50 ng  | 94°C, 8 min                 | 15         |
| 30-50%                  | 50-100 ng   | 94°C, 6 min                 | 15         |
| <30%                    | 100 ng (with a risk of failure of NGS library construction) | No fragmentation            | 16         |

- RNA purity: OD<sub>260</sub>/280=1.8 to 2.0.
- If DNA contamination is found in RNA sample, perform DNase I digestion to remove DNA before proceeding to the next step in the procedure, please refer to Appendix A.
- While not recommended, processing <10 ng RNA as input into library preparation can be attempted, however this may lead to a decreased yield of PCR product and lower comparison rate in analysis result.

## Chapter 3 Library Construction Protocol

This protocol has been optimized using 50 ng total RNA (UHRR, Universal Human Reference RNA) with DV<sub>200</sub> > 70%. If a different quality of RNA input is used, please identify optimal input and fragment conditions by adjusting the recommended conditions in Table 5.

### 3.1 RNA Fragmentation



**Note:** In the following procedures, do not vortex. Please use pipette to mix solution.

- 3.1.1 Add 50 ng total RNA sample to a 0.2 mL PCR tube and add NF Water to a total volume of 10  $\mu$ L.
- 3.1.2 Add 4  $\mu$ L of Fragmentation Buffer to the 10  $\mu$ L RNA sample in step 3.1.1 and gently pipette mix 10 times. Briefly centrifuge and place the tube into the thermocycler. Follow Table 6 below for fragmentation conditions.

Table 6 The Recommended Condition of RNA Fragmentation

| Insert Size | Temperature of RNA Fragmentation | Time of RNA Fragmentation |
|-------------|----------------------------------|---------------------------|
| 150 bp      | 94°C                             | 8min                      |

- 3.1.3 When the reaction completes, immediately place the tube on ice for 2 minutes. Centrifuge for 10 seconds and immediately proceed to Reverse Transcription.

### 3.2 Reverse Transcription and Second Strand Synthesis



**Note:** In the following procedures, do not vortex. Please use pipette to mix solution.

- 3.2.1 Remove Directional RT Buffer 2 from -20°C storage and thaw it at room temperature. Vortex to mix and centrifuge briefly to collect the solution to the bottom of the tube. Make a 1:16 dilution of Directional RT Buffer 2, as shown in Table 7 below.

Table 7 Dilution (1:16) of Directional RT Buffer 2

| Components              | Volume     |
|-------------------------|------------|
| Directional RT Buffer 2 | 1 $\mu$ L  |
| NF water                | 16 $\mu$ L |
| Total                   | 17 $\mu$ L |



**Note:** The diluted Directional RT Buffer 2 should be used immediately. Please discard any unused diluent. Prepare fresh diluted Directional RT Buffer 2 for each library construction.

- 3.2.2 Remove Directional RT Buffer 1 from  $-20^{\circ}\text{C}$  and thaw it at room temperature. Invert several times to mix. Prepare the Reverse Transcription mixture on ice (see Table 8):

| Components                             | Volume          |
|--|-----------------|
| Directional RT Buffer 1                | 4 $\mu\text{L}$ |
| <b>Diluted</b> Directional RT Buffer 2 | 1 $\mu\text{L}$ |
| RT Enzyme Mix                          | 1 $\mu\text{L}$ |
| Total                                  | 6 $\mu\text{L}$ |

- 3.2.3 Transfer 6  $\mu\text{L}$  of Reverse Transcription Mixture to the RNA Fragmentation product in step 3.1.3. Pipette up and down 10 times to mix. Centrifuge briefly to collect the solution at the bottom of the tube.
- 3.2.4 Place the PCR tube in step 3.2.3 into the thermocycler and run the following program in Table 9:

| Temperature           | Time   |
|-----------------------|--------|
| Heated lid            | On     |
| 25 $^{\circ}\text{C}$ | 10 min |
| 42 $^{\circ}\text{C}$ | 30 min |
| 70 $^{\circ}\text{C}$ | 15 min |
| 4 $^{\circ}\text{C}$  | Hold   |

- 3.2.5 Once the reaction completes, place the product on ice and centrifuge for 10 seconds.
- 3.2.6 Remove Directional Second Strand Buffer from  $-20^{\circ}\text{C}$  and thaw at room temperature. Invert several times to mix. Prepare the Second Strand Synthesis mixture on ice (see Table 10):

| Components                       | Volume           |
|----------------------------------|------------------|
| Directional Second Strand Buffer | 26 $\mu\text{L}$ |
| Second Strand Enzyme Mix         | 4 $\mu\text{L}$  |
| Total                            | 30 $\mu\text{L}$ |

- 3.2.7 Transfer 30  $\mu\text{L}$  Second Strand Synthesis mixture to the Reverse Transcription product from step 3.2.5. Pipette 10 times to mix and centrifuge briefly to collect the solution at the bottom of the tube.

- 3.2.8 Place the PCR tube from step 3.2.7 into the thermocycler and run the following program in Table 11:

| Table 11 Reaction Conditions of Second Strand Synthesis |        |
|---|--------|
| Temperature   | Time   |
| Heated lid  | On     |
| 16°C  | 60 min |
| 4°C   | Hold   |

- 3.2.9 Once the reaction completes, place the product on ice and centrifuge for 10 seconds. Transfer all Second Strand Synthesis product to a new 1.5 mL tube and place it on ice for the next reaction.



**Stopping Point:** Second strand synthesis product can be stored at -20°C up to 16 hours.

### 3.3 Cleanup of Second Strand Synthesis Product



**Note:** Please read Appendix B carefully before you begin.

- 3.3.1 Remove DNA Clean Beads from the refrigerator and bring to room temperature for 30 min beforehand. Vortex and mix thoroughly before use.
- 3.3.2 Use pipette to transfer 75  $\mu$ L of DNA Clean Beads to the Second Strand Synthesis product in step 3.2.9. Gently pipette up and down at least 10 times to mix thoroughly. Ensure that all the solution and beads are expelled from the tip into the tube before discarding.
- 3.3.3 Incubate at room temperature for 5 minutes.
- 3.3.4 Centrifuge briefly and place the 1.5 mL tube on the Magnetic Separation Rack for 2-5 minutes until liquid becomes clear. Carefully remove and discard the supernatant using a pipette.
- 3.3.5 With the 1.5 mL tube on the Magnetic Separation Rack, add 200  $\mu$ L freshly prepared 80% Ethanol to the tube without disturbing the beads. Incubate 30 seconds. Carefully remove and discard the supernatant.
- 3.3.6 Repeat step 3.3.5 once more. Remove all liquid from the tube without disrupting the beads. Centrifuge briefly to collect any remaining liquid at the bottom, separate beads magnetically and then remove remaining liquid using a small volume pipette tip.
- 3.3.7 Keep the 1.5 mL tube on the Magnetic Separation Rack with the lid open and allow the beads to air dry until no wetness (reflectiveness) is visible but before the pellet over dries and cracks.
- 3.3.8 Remove the 1.5 mL tube from the Magnetic Separation Rack and add 42  $\mu$ L of TE Buffer to elute

the DNA. Gently pipette mix at least 10 times to mix thoroughly and centrifuge briefly.

3.3.9 Incubate at room temperature for 5 minutes.

3.3.10 Centrifuge briefly and place the 1.5 mL tube back onto the Magnetic Separation Rack for 2–5 minutes until liquid becomes clear. Transfer 40  $\mu$ L supernatant to a new 0.2 mL PCR tube.



**Stopping Point: Purified Second Strand Synthesis product can be stored at  $-20^{\circ}\text{C}$  overnight.**

### 3.4 End Repair and A-tailing

3.4.1 Prepare the End Repair and A-tailing mixture in a new 0.2 mL PCR tube on ice (see Table 12).

| Table 12 End Repair and A-tailing Mixture |             |
|---|-------------|
| Components                                | Volume      |
| ERAT Buffer                               | 7.1 $\mu$ L |
| ERAT Enzyme Mix                           | 2.9 $\mu$ L |
| Total                                     | 10 $\mu$ L  |

3.4.2 Transfer 10  $\mu$ L of the End Repair and A-tailing mixture to the 0.2 mL PCR tube from step 3.3.10. Vortex 3 times (3 s each) and briefly centrifuge to collect the solution at the bottom of the tube.

3.4.3 Place the 0.2 mL PCR tube from step 3.4.2 into the thermocycler and run the following program in Table 13.

| Table 13 End Repair and A-tailing Reaction Conditions |        |
|---|--------|
| Temperature   | Time   |
| Heated lid  | On     |
| 37°C  | 30 min |
| 65°C  | 15 min |
| 4°C   | Hold   |

3.4.4 Briefly centrifuge to collect the solution at the bottom of the tube.



**Warning: DO NOT STOP AT THIS STEP. Please continue to step 3.5.**



### 3.5 Adapter Ligation



**Note:** The amount of Adapter used in Adapter Ligation depends on the amount of RNA input. Please refer to Appendix C carefully before you proceed.

- 3.5.1 Following Table 14 below, make a 1:9 dilution of the Adapter. Mix and centrifuge briefly for further use.

| Table 14 Adapter Dilution |            |
|---------------------------|------------|
| Components                | Volume     |
| Adapter                   | 1 $\mu$ L  |
| TE Buffer                 | 9 $\mu$ L  |
| Total                     | 10 $\mu$ L |

- 3.5.2 Please refer to the instructions for MGIEasy DNA Adapters (see Appendix C). Add 5  $\mu$ L of diluted DNA Adapters to the PCR tube in step 3.4.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution to the bottom of the tube.

- 3.5.3 Prepare the Adapter ligation mixture in a new 0.2 mL PCR tube on ice (see Table 15).

| Table 15 Adapter Ligation Mixture |              |
|-----------------------------------|--------------|
| Components                        | Volume       |
| Ligation Buffer                   | 23.4 $\mu$ L |
| DNA Ligase                        | 1.6 $\mu$ L  |
| Total                             | 25 $\mu$ L   |

- 3.5.4 Pipette slowly to transfer 25  $\mu$ L of Adapter Ligation mixture to the PCR tube from step 3.5.2. Vortex 6 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.



**Note:** Due to the viscosity of the Adapter Ligation mixture, please pipette slowly and ensure that the correct amount has been used.

- 3.5.5 Place the 0.2 mL PCR tube from step 3.5.4 into the thermocycler and run the following program in Table 16.

Table 16 Adapter Ligation Reaction Conditions

| Temperature | Time   |
|-------------|--------|
| Heated lid  | On     |
| 23°C        | 30 min |
| 4°C         | Hold   |

- 3.5.6 Centrifuge briefly to collect the solution at the bottom of the tube.
- 3.5.7 Add 20  $\mu$ L TE Buffer, for a total volume of 100  $\mu$ L and transfer all of the solution to a new 1.5 mL tube.



**Stopping Point:** Adapter-ligated DNA can be stored at -20°C for up to 16 hours.

### 3.6 Cleanup of Adapter-Ligated DNA



**Note:** Please read Appendix B carefully before you begin.

- 3.6.1 Please refer to the instructions in Appendix B. Take out DNA Clean Beads from refrigerator and bring to room temperature for 30 minutes beforehand. Vortex and mix thoroughly before use.
- 3.6.2 Transfer 50  $\mu$ L DNA Clean Beads to the sample tube from step 3.5.7. Pipette up and down at least 10 times to mix thoroughly. Ensure that the solution and beads are fully dispensed from the pipette tip into the tube before proceeding.
- 3.6.3 Incubate at room temperature for 5 minutes.
- 3.6.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.6.5 Keep the tube on the Magnetic Separation Rack and add 200  $\mu$ L freshly prepared 80% Ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.
- 3.6.6 Repeat step 3.6.5 once more, remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom of the tube, separate the beads magnetically and then remove any remaining liquid using a small volume pipette.
- 3.6.7 Keep the tube on the Magnetic Separation Rack with the lid open and air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to over-dry and crack.
- 3.6.8 Remove the sample tube from the Magnetic Separation Rack and add 22  $\mu$ L TE Buffer to elute

the DNA. Pipette up and down at least 10 times to mix thoroughly.

3.6.9 Incubate at room temperature for 5 minutes.

3.6.10 Centrifuge briefly and place the sample tube back onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 20  $\mu$ L supernatant to a new 0.2 mL PCR tube.

✓ **Stopping Point: After cleanup, Adapter-ligated DNA can be stored at  $-20^{\circ}\text{C}$  up to 1 month.**

### 3.7 PCR Amplification

3.7.1 Prepare the PCR Amplification mixture on ice (see Table 17).

| Components     | Volume     |
|----------------|------------|
| PCR Enzyme Mix | 25 $\mu$ L |
| PCR Primer Mix | 4 $\mu$ L  |
| UDG            | 1 $\mu$ L  |
| Total          | 30 $\mu$ L |

3.7.2 Transfer 30  $\mu$ L of PCR amplification mixture to the PCR tube from step 3.6.10. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.

3.7.3 Place the PCR tube from step 3.7.2 into the thermocycler and run the following program in Table 18.

| Temperature | Time   | Cycles    |
|-------------|--------|-----------|
| Heated lid  | on     |           |
| 37°C        | 20 min |           |
| 95°C        | 3 min  |           |
| 95°C        | 30 s   | 15 cycles |
| 56°C        | 30 s   |           |
| 72°C        | 1 min  |           |
| 72°C        | 5 min  |           |
| 4°C         | Hold   |           |

3.7.4 Once PCR completion, centrifuge briefly to collect the solution at the bottom of the tube. Transfer all the solution to a new 1.5 mL tube.

### 3.8 Cleanup of PCR Product



**Note:** Please read Appendix B carefully before you begin.

- 3.8.1 Take DNA Clean Beads from refrigerator and bring to room temperature for 30 minutes beforehand. Vortex and mix thoroughly before use.
- 3.8.2 Transfer 50  $\mu$ L DNA Clean Beads to the tube from step 3.7.4. Pipette up and down at least 10 times to mix thoroughly. Ensure that all liquid and beads are expelled from the pipette tip into the tube before proceeding.
- 3.8.3 Incubate at room temperature for 5 minutes.
- 3.8.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.8.5 Keep the tube on the Magnetic Separation Rack and add 200  $\mu$ L freshly prepared 80% Ethanol to wash the beads and the walls of the tube. Incubate for 30 seconds, then carefully remove and discard the supernatant.
- 3.8.6 Repeat step 3.8.5 once more, remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, separate the beads magnetically, and remove any remaining liquid using a small volume pipette.
- 3.8.7 Keep the sample tube on the Magnetic Separation Rack with the lid open, and air-dry beads at room temperature until no wetness (reflectiveness) is observed but before the pellet begins to over-dry and crack.
- 3.8.8 Remove the sample tube from the Magnetic Separation Rack and add **32  $\mu$ L** TE Buffer to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.
- 3.8.9 Incubate at room temperature for 5 minutes.
- 3.8.10 Centrifuge briefly then place the sample tube back onto the Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Transfer **30  $\mu$ L** supernatant to a new 1.5 mL tube.



**Stopping Point:** After cleanup, purified PCR Products can be stored at  $-20^{\circ}\text{C}$ .

### 3.9 Quality Control of PCR Product

- 3.9.1 Quantify the purified PCR products with dsDNA Fluorescence Assay Kits such as Qubit® dsDNA HS Assay Kit or Quant-iT™ PicoGreen® dsDNA Assay Kit. The required yield for final PCR products is  $\geq 250$  ng.
- 3.9.2 Assess the fragment size distribution of purified PCR products with electrophoresis-based equipment such as a Bioanalyzer, Tapestation (Agilent Technologies), LabChip® GX, GXII, GX Touch (PerkinElmer), or Fragment Analyzer™ (Advanced Analytical). Figure 2 shows the final size distribution is 150 bp - 300 bp.

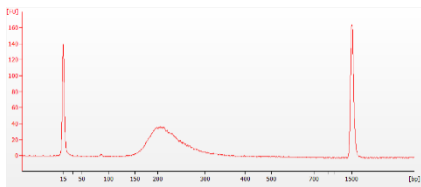


Figure 2 Agilent 2100 Bioanalyzer Fragment Size Distribution Results of the Purified PCR Product

### 3.10 Pre-Hybridization Preparation



**Note: A 96-well PCR plate is recommended for the hybridization reaction. Other steps can use PCR tubes or 8-Strip PCR tubes depending on the number of samples.**

- 3.10.1 If only one sample per hybridization reaction, transfer 250 ng of PCR products according to the concentrations measured in the last step. If multiplexing is performed to hybridization, 250 ng of each sample is required, and the total amount of PCR products should be between 500ng and 1000 ng. A 4-plex is the maximum pooling possible with this kit. **If starting with highly degraded FFPE samples, only one sample per hybridization reaction is recommended to ensure the hybridization efficiency is maximized.** For adapter and pooling strategies, please refer to Appendix C.
- 3.10.2 Prepare the Block mixture on ice (see Table 19).

Table 19 Block Mixture

| Components | Volume      |
|------------|-------------|
| Block 1    | 2.5 $\mu$ L |
| Block 2    | 2.5 $\mu$ L |
| Block 3    | 1 $\mu$ L   |
| Block 4    | 10 $\mu$ L  |
| Total      | 16 $\mu$ L  |



**Note: Block 3 and Block 4 are reagents from the MGIEasy Exome Capture Accessory Kit, which are designed exclusively for the MGISEQ /DNBSEQ platform.**

- 3.10.3 Transfer 16  $\mu$ L of Block mixture to each of the PCR products from step 3.10.1 to prepare the Pre-Hybridization Mixture. Pipette up and down at least 10 times to mix thoroughly. Uncap and place the tubes containing the mixture on the concentrator, set the temperature to 65°C, vacuum and spin time for around 30min, until the final concentrated volume is 9  $\mu$ L. If the final volume is less than 9  $\mu$ L, add NF water into the concentrated product to reach a final volume of 9  $\mu$ L.
- 3.10.4 Place the 9  $\mu$ L of Pre-Hybridization Mixture from step 3.10.3 into the thermocycler and run the program in Table 20.

Table 20 Pre-hybridization Reaction Conditions

| Temperature | Time  |
|-------------|-------|
| Heated lid  | on    |
| 95°C        | 5 min |
| 65°C        | Hold  |

### 3.11 Hybridization and Capture

- 3.11.1 Prepare the Hybridization Mixture in a new 0.2 mL PCR tube (see Table 21).

Table 21 Hybridization Mixture

| Components   | Volume      |
|--------------|-------------|
| Hyb Buffer 1 | 10 $\mu$ L  |
| Hyb Buffer 2 | 0.4 $\mu$ L |
| Hyb Buffer 3 | 4 $\mu$ L   |
| Hyb Buffer 4 | 5.6 $\mu$ L |
| Total        | 20 $\mu$ L  |

- 3.11.2 Incubate the Hybridization Mixture from step 3.11.1 in the thermocycler at 65°C for at least 5 min.

Before use, ensure there is no precipitation in the mixture.

- 3.11.3 Prepare the Probe mixture on ice (see Table 22) in a new 96-well PCR plate (recommended).

Table 22 Probe Mixture

| Components         | Volume      |
|--------------------|-------------|
| NF water           | 1.5 $\mu$ L |
| Block 5            | 0.5 $\mu$ L |
| MGI Exome V5 Probe | 5 $\mu$ L   |
| Total              | 7 $\mu$ L   |



**Note: The MGI Exome V5 Probe must be thawed on ice and added last to the mixture.**

- 3.11.4 Tightly close the Strip Domed Caps on the 96-well PCR plate from step 3.11.3, then place the Probe Mixture into the thermocycler and run the program in Table 23.

Table 23 Probe Mixture Incubation

| Temperature | Time  |
|-------------|-------|
| Heated lid  | on    |
| 65°C        | 2 min |
| 65°C        | Hold  |

- 3.11.5 Keeping all the mixtures above (pre-hybridization mixture, hybridization mixture, and probe mixture) at 65°C, immediately transfer 13  $\mu$ L of the Hybridization Mixture from step 3.11.2 into the 9  $\mu$ L of Pre-Hybridization Mixture from step 3.10.4 at 65 °C. Pipette up and down to mix thoroughly.



**Note: 100  $\mu$ L Filter Tips are recommended for this step.**

- 3.11.6 Transfer 22  $\mu$ L of the combined pre-hybridization and hybridization mixtures from step 3.11.5 into the 96-well plate with 7  $\mu$ L of the Probe Mixture from step 3.11.4. Pipette up and down to mix thoroughly.



**Note: 100  $\mu$ L Filter Tips are recommended for this step.**

- 3.11.7 Cover the PCR plate with Clear Adhesive Film, press the film firmly against the plate to make sure all wells are completely sealed. Repeat this step to make sure film is completely sealed.
- 3.11.8 Keep the 96-well plate at 65 °C (set the heated lid of thermocycler to 105 °C) for hybridization for **24 hours** according to the program in Table 24.

Table 24 Hybridization Reaction Conditions

| Temperature        | Time |
|--------------------|------|
| Heated lid (105°C) | On   |
| 65°C               | Hold |

### 3.12 Pre-Elution Preparation

- 3.12.1 Turn on the Thermomixer and set at 65 °C at least 30 min before beginning the elution process. For each hybridization reaction, add 1.8 mL of Wash Buffer II to a 2.0 mL tube and place it in the Thermomixer for preheating.
- 3.12.2 Vigorously vortex Dynabeads™ until evenly mixed. For each hybridization reaction, transfer 50 µL of Dynabeads™ to a new 2.0 mL tube.
- 3.12.3 Add 200 µL of Binding Buffer to each tube containing Dynabeads™ and vigorously vortex for 5 seconds to re-suspend the magnetic beads.
- 3.12.4 Centrifuge briefly and place the tube(s) onto a Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.12.5 Repeat step 3.12.3 to 3.12.4 twice more.
- 3.12.6 Finally, add 200 µL of Binding Buffer to each tube containing Dynabeads™ to re-suspend the beads.

### 3.13 Elution

- 3.13.1 Keep the mixtures from step 3.11.8 on the thermocycler following the 24 hours incubation. Slice the adhesive film in-between each row of wells and carefully peel off the film row by row. Quickly use a pipette to estimate the remaining hybridization solutions one by one and transfer each mixture into a separate tube from step 3.12.6 containing prepared Dynabeads™.



**Note:** If the volume of remaining hybridization solution is less than 19 µL, the yield may be low.



**Note:** While handling large number of samples, in order to reduce the vaporization of the hybridization solution, it is recommended to handle the samples in sets of six to eight reactions. In-between sets, the user should re-adhere the film and close the heated lid of the PCR thermal cycler cover for ten seconds before continuing with the next set of reactions.

- 3.13.2 Fix the tubes from step 3.13.1 on a Nutator (or other similar mixer) for mixing by 360° rotations and incubate at room temperature for 30 min.



- 3.13.3 Take the tubes off the mixer.
- 3.13.4 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.13.5 Add 500  $\mu\text{L}$  of Wash Buffer I, close the tube lid, and turn tubes upside down several times to re-suspend the bead mixture, then incubate at room temperature for 15 min.
- 3.13.6 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.13.7 Add 500  $\mu\text{L}$  of pre-heated Wash Buffer II from step 3.12.1. Put the tubes in the Thermomixer and set the mixing speed to 1000 rpm. Press 'short' for 10 s to make sure all the beads are re-suspended, then set speed to 0 rpm. Incubate at 65  $^{\circ}\text{C}$  on the Thermomixer for 10 min.
- 3.13.8 Centrifuge briefly and place the tube onto a Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.13.9 Repeat step 3.13.7 to 3.13.8 twice more.
- 3.13.10 Add 100  $\mu\text{L}$  NF water to each tube to re-suspend the bead mixtures, then transfer all of the solution (including beads) to a new 1.5 mL tube and centrifuge briefly.
- 3.13.11 Place the tube from step 3.13.10 onto a Magnetic Separation Rack for 2 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.13.12 Add 44  $\mu\text{L}$  of NF water to each tube to re-suspend the bead mixtures, then transfer all of the solution (including beads) to a new 0.2 mL PCR tube.

### 3.14 Post-Capture PCR Amplification

- 3.14.1 Take out the MGIEasy Exome Capture Accessory Kit and prepare the Post-capture PCR amplification mixture on ice (see Table 25).

| Components          | Volume           |
|---------------------|------------------|
| Post-PCR Enzyme Mix | 50 $\mu\text{L}$ |
| PCR Primer Mix      | 6 $\mu\text{L}$  |
| Total               | 56 $\mu\text{L}$ |



**Note:** Post-PCR Enzyme Mix and PCR Primer Mix are included in the 'MGIEasy Exome Capture Accessory Kit.'

- 3.14.2 Transfer 56  $\mu\text{L}$  of the Post-capture PCR amplification mixture into each of the PCR tube(s) (including beads) from step 3.13.12. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube.
- 3.14.3 Place the PCR tube(s) from step 3.14.2 into the thermocycler and run the program described in Table 26.

Table 26 Post-capture PCR Amplification Reaction Conditions

| Temperature | Time   | Cycles    |
|-------------|--------|-----------|
| Heated lid  | on     |           |
| 95°C        | 3 min  | 1 cycle   |
| 98°C        | 20 s   |           |
| 60°C        | 15 s   | 13 cycles |
| 72°C        | 30 s   |           |
| 72°C        | 10 min | 1 cycle   |
| 4°C         | Hold   |           |

- 3.14.4 Centrifuge briefly to collect the solution at the bottom of the tube.
- 3.14.5 Place the tube(s) onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer 100  $\mu\text{L}$  supernatant from each tube to a new 1.5 mL tube.

### 3.15 Cleanup of Post-Capture PCR Product and Quantification

- 3.15.1 Take the DNA Clean Beads out of the refrigerator and allow 30 minutes to bring the beads to room temperature. Vortex and mix thoroughly before use.
- 3.15.2 Transfer 100  $\mu\text{L}$  DNA Clean Beads to each tube from step 3.14.5. Pipette up and down at least 10 times to mix thoroughly. Ensure that the liquid and beads are completely expelled from the pipette tip into the tube before proceeding.
- 3.15.3 Incubate at room temperature for 5 minutes.
- 3.15.4 Centrifuge briefly and place the tube(s) onto a Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant with a pipette.
- 3.15.5 Keep the tube(s) on the Magnetic Separation Rack and add 200  $\mu\text{L}$  of freshly prepared 80% Ethanol to each tube to wash the beads and the walls of the tube. Incubate for 30 seconds and carefully remove and discard the supernatant.
- 3.15.6 Repeat step 3.15.5 once, remove all liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, place the tube back on to the

magnet rack, and remove the remaining liquid using a small volume pipette.

- 3.15.7 Keep the tube(s) on the Magnetic Separation Rack with the lid open, and air dry the beads at room temperature until no wetness (reflectiveness) is observed but before the pellet over dries and begins crack.
- 3.15.8 Remove the tube(s) from the Magnetic Separation Rack and add **32  $\mu$ L** TE Buffer to each tube to elute the DNA. Pipette up and down at least 10 times to mix thoroughly.
- 3.15.9 Incubate at room temperature for 5 minutes.
- 3.15.10 Centrifuge briefly, then place the tube(s) back onto the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer **30  $\mu$ L** supernatant from each tube to a different new 1.5 mL tube.
- 3.15.11 Quantify the purified post-capture PCR products with dsDNA Fluorescence Assay Kits such as the Qubit® dsDNA HS Assay Kit or the Quant-iT™ PicoGreen® dsDNA Assay Kit. The required yield for PCR products is  $\geq 1$  pmol. Please refer to Appendix D to calculate the yield. For example, the desired yield for the fragmented DNA with a peak fragment size of 150 bp (Post-hybridization PCR products with a peak fragment size of 234 bp) should be  $\geq 154$  ng.



**Stopping Point:** After cleanup, purified PCR Products can be stored at  $-20^{\circ}\text{C}$ .



**Note:** If the library will be delivered to a service lab for sequencing, please stop here. If the library will be sequenced in your lab, please go to step 3.16 to 3.20.

### 3.16 Denaturation



**Note:** Please read Appendix D carefully before you begin

- 3.16.1 According to the PCR product size and Formula 1 in Appendix D, transfer 1 pmol of each PCR Product to a new 0.2 mL PCR Tube. Add TE Buffer for a total volume of 48  $\mu$ L.
- 3.16.2 Place the PCR tube(s) from step 3.16.1 into the thermocycler and run the program in Table 27.

Table 27 Denaturation Reaction Conditions

| Temperature | Time  |
|-------------|-------|
| Heated lid  | On    |
| 95°C        | 3 min |

- 3.16.3 After the reaction is complete, immediately place the PCR tube(s) on ice for 2 minutes, and centrifuge briefly.

### 3.17 Single Strand Circularization

- 3.17.1 Take out the MGIEasy Circularization Kit and prepare the single strand circularization reaction mixture in a new 0.2 mL PCR tube on ice (see Table 28).

Table 28 Single Strand Circularization Mixture

| Components       | Volume       |
|------------------|--------------|
| Splint Buffer    | 11.6 $\mu$ L |
| DNA Rapid Ligase | 0.5 $\mu$ L  |
| Total            | 12.1 $\mu$ L |

- 3.17.2 Transfer 12.1  $\mu$ L of single strand circularization mixture into each PCR tube from step 3.16.3. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube(s).
- 3.17.3 Place the PCR tube(s) from step 3.17.2 into the thermocycler and run the program in Table 29:

Table 29 Single Strand DNA Circularization Reaction Conditions

| Temperature | Time   |
|-------------|--------|
| Heated lid  | On     |
| 37°C        | 30 min |
| 4°C         | Hold   |

- 3.17.4 After the reaction is complete, immediately place the tube(s) on ice for the next reaction.

### 3.18 Enzymatic Digestion

- 3.18.1 Prepare the following enzymatic digestion mixture (see Table 30) in a new 0.2 mL PCR tube on ice during the reaction in step 3.17.3.

Table 30 Enzymatic Digestion Mixture

| Components       | Volume      |
|------------------|-------------|
| Digestion Buffer | 1.4 $\mu$ L |
| Digestion Enzyme | 2.6 $\mu$ L |
| Total            | 4.0 $\mu$ L |

- 3.18.2 Transfer 4  $\mu$ L of enzymatic digestion mixture into each PCR tube from step 3.17.4. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube(s).
- 3.18.3 Place the PCR tube(s) from step 3.18.2 into the thermocycler and run the following program in Table 31.

Table 31 Enzymatic Digestion Reaction Conditions

| Temperature | Time   |
|-------------|--------|
| Heated lid  | On     |
| 37°C        | 30 min |
| 4°C         | Hold   |

- 3.18.4 After the reaction is complete, centrifuge briefly to collect the solution at the bottom of the tube(s).
- 3.18.5 Immediately add 7.5  $\mu$ L of Digestion Stop Buffer to each PCR tube. Vortex 3 times (3 s each) and centrifuge briefly to collect the solution at the bottom of the tube(s). Transfer all of the solution from each tube into a separate new 1.5 mL tube.

### 3.19 Cleanup of Enzymatic Digestion Product



**Note:** Please read Appendix B carefully before you begin.

- 3.19.1 Remove the DNA Clean Beads from the refrigerator and allow 30 minutes for the solution to come to room temperature. Vortex and mix thoroughly before use.
- 3.19.2 Transfer 170  $\mu$ L of DNA Clean Beads to each tube containing Enzymatic Digestion product from step 3.18.5. Gently pipette at least 10 times to mix thoroughly. Ensure that the solution and beads are fully expelled from the tip into the tube.
- 3.19.3 Incubate at room temperature for 10 minutes.
- 3.19.4 Centrifuge briefly and place each 1.5 mL tube on the Magnetic Separation Rack for 2–5 minutes until the liquid becomes clear. Carefully remove and discard the supernatant using a pipette.
- 3.19.5 Keep the 1.5 mL tube(s) on the Magnetic Separation Rack, add 500  $\mu$ L of freshly prepared 80% Ethanol to each tube without disturbing the beads. Incubate for 30 seconds. Carefully remove and discard the supernatant.
- 3.19.6 Repeat step 3.19.5 once. Remove all of the liquid from the tube without disrupting the beads. You may centrifuge briefly to collect any remaining liquid at the bottom, place the tube(s) back on to the magnetic rack, then remove remaining liquid using a small volume pipette.
- 3.19.7 Keep the 1.5 mL tube(s) on the Magnetic Separation Rack with the lid open, and air-dry beads until no wetness (reflectiveness) is observed but before the pellet begins to over dry and crack.
- 3.19.8 Remove the 1.5 mL tube(s) from the Magnetic Separation Rack and add **22  $\mu$ L** of TE Buffer each tube to elute the DNA. Gently pipette up and down at least 10 times to mix thoroughly.

3.19.9 Incubate at room temperature for 10 minutes.

3.19.10 Centrifuge briefly. Place the 1.5 mL tube(s) back on the Magnetic Separation Rack for 2-5 minutes until the liquid becomes clear. Transfer **20  $\mu$ L** supernatant from each tube to a separate new 1.5 mL tube. Be careful to not disturb the beads.



**Stopping Point: Purified Enzymatic Digestion products can be stored at  $-20^{\circ}\text{C}$  for one month.**

### 3.20 Quality Control of Enzymatic Digestion Product

Quantify the purified Enzymatic Digestion products using the Qubit<sup>®</sup> ssDNA Assay Kit. The final Enzymatic Digestion products (ng of ssDNA)/ input products of PCR (ng of dsDNA) should be  $\geq 7\%$ .

For example, if the input of PCR product used for the circularization has a main band peak around 234 bp by Bioanalyzer (corresponding to the insert fragment peak around 150 bp) is 154 ng total, the final yield after enzymatic digestion should be  $> 10.8$  ng ( $154 \times 0.07 = 10.8$ ).

## Appendix

### Appendix A DNase I Digestion of RNA Sample

If there is DNA contamination in the RNA sample, you will need to perform a DNase I digestion (materials are not provided, see "Equipment and Materials Required but not Prepared"). DNase I digestion can cause some loss of RNA. The input amount of total RNA used in this step should be increased by 20% to 30%. For example, if the required input amount of RNA is 50 ng, the total RNA input should be 60–70 ng for the DNase I digestion. The protocol is shown below:

1. Transfer an appropriate amount of RNA sample to a RNase free 0.2 mL PCR tube and add NF water to the final volume of 42.5  $\mu$ L. Prepare the DNase I digestion reaction mixture on ice (see Table 32):

| Components                 | Volume       |
|----------------------------|--------------|
| Total RNA                  | 42.5 $\mu$ L |
| DNase I                    | 2.5 $\mu$ L  |
| 10 $\times$ DNase I Buffer | 5 $\mu$ L    |
| Total                      | 50 $\mu$ L   |

2. Gently pipette the mixture and place the tube in the Thermocycler. Set up the following reaction conditions as listed in Table 33 below and start the reaction:

| Temperature       | Time     |
|-------------------|----------|
| Heated lid (45°C) | on       |
| 37°C              | 20 min   |
| 4°C               | $\infty$ |

3. When the temperature reaches 4°C, remove and centrifuge the PCR tube briefly, then transfer the mixture to a new 1.5 mL tube, and proceed to the RNA clean up procedure below.



**Note:** Please read Appendix B carefully before you begin.

- 3.1 Remove RNeasy XP beads from 4°C storage and place them at room temperature for 30 min before use. Vortex thoroughly to mix well before use.
- 3.2 Transfer 90  $\mu$ L RNeasy XP beads to the DNase I digestion product in Step 3. Gently pipette at least 10 times to mix thoroughly. Be careful to expel all of the liquid out of the tip and into the tube during the last mix.

- 3.3 Incubate the tube at room temperature for 5 minutes.
- 3.4 Centrifuge briefly. Place the tube on the magnetic rack for 2-5 minutes until the liquid clears. Carefully remove the supernatant with a pipette.
- 3.5 Keep the tube on the magnetic rack and add 200  $\mu$ L freshly prepared 80% Ethanol with NF water to the tube without disturbing the beads. Incubate for 30 seconds and carefully remove and discard the supernatant.
- 3.6 Repeat step 3.5 for a total of two washes, remove all liquid from the tube without disrupting the beads. You may briefly centrifuge the tube to collect any remaining liquid to the bottom, place back on the magnet and then remove any remaining liquid using a small volume pipette tip.
- 3.7 Keep the tube on the magnetic rack with the lid open and allow beads to air dry until no wetness (reflectiveness) is observed. Do not let the beads over-dry or crack.
- 3.8 Remove the tube from the magnetic rack and add appropriate amount (12-13  $\mu$ L) of NF water to elute the RNA. Pipette at least 10 times to mix thoroughly.



**Note: If the purified RNA sample needs to be quantified, elute the RNA by adding 13  $\mu$ L of NF water, transfer 11  $\mu$ L of supernatant to a new RNase free PCR tube and take 1  $\mu$ L of product for quantification using "Qubit™ RNA HS Assay Kit" (See "Equipment and Materials Required but not Provided"). If the quantification step is not performed, elute the RNA by adding 12  $\mu$ L of NF water and transfer 10  $\mu$ L of supernatant to a new RNase free PCR tube for RNA Fragmentation.**

- 3.9 Incubate the tube at room temperature for 5 minutes.
- 3.10 Briefly centrifuge. Place the tube on the magnetic rack for 2-5 minutes until liquid is clear. Carefully transfer the supernatant to a new RNase free PCR tube and proceed to the RNA Fragmentation.



## Appendix B Magnetic Beads and Cleanup Procedures

For magnetic bead-based purification, we recommend using DNA Clean Beads supplied by MGI (MGI, Cat. No. 1000005278) or AMPure® XP (Agencourt, Cat. No. A63882). If you choose Magnetic Beads from other manufacturers, please optimize the cleanup conditions before getting started.

### Before You Use

- To ensure capture efficiency of the Magnetic Beads, remove the beads from 4°C refrigerator storage, and equilibrate to room temperature for 30 minutes before use. Vortex and mix thoroughly immediately before use.
- Vortex or pipette up and down to ensure that the beads are thoroughly mixed before each use.
- The volume of the beads (ratio beads: sample volume) 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.
- In the magnetic separation step, please allow the solution to become completely clear before removing the supernatant. This process should take 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. If the supernatant is not completely clear, beads and product will be removed, and therefore lead to a reduction in sample eluted.
- Avoid contacting the beads with pipette tips when pipetting. 2-3 µL of fluids 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. Keep the tube on the Magnetic Separation Rack when washing with Ethanol. Do not shake or disturb the beads in any way.
- After the second bead wash with 80% Ethanol, try to remove all of the remaining Ethanol from within the tube. Centrifuge briefly to collect any remaining liquid at the bottom. Separate the beads magnetically and remove any remaining liquid by using a small volume pipette.
- After washing twice with 80% Ethanol, air-dry the beads at room temperature. Drying takes approximately 2-5 minutes depending on your laboratory 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 tip when removing the supernatant. Contamination of the sample with remaining beads may affect subsequent purification steps. Therefore, the total volume of TE buffer used for elution should be 2  $\mu\text{L}$  more than the volume that is going to be eluted off. The remaining 2  $\mu\text{L}$  should ensure no beads are removed with the purified sample.
- Pay attention when opening/ closing the tube lids 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.

## Appendix C Combination Barcode Adapters Strategies

- This set includes one MGIEasy DNA Adapters-16 (Tube) Kit. This kit was developed to meet the requirements for multiplex sequencing. We selected the best Adapter combinations based on the principle of balanced base composition. However, the number of Barcode Adapters are not always continuous. For optimal performance, please carefully read instructions in Appendix C-1.
- MGI Adapters are double stranded. Please do not incubate above room temperature to avoid structural changes such as denaturation, which may affect performance.
- Before use, centrifuge to collect the Adapter liquid at the bottom of tube. Gently remove the cap to prevent spills and cross-contamination. Mix Adapters with a pipette before you use. Remember to close the cap immediately after use.
- Adapters from other MGI Library Prep Kits (number 501-596) are designed differently and are incompatible for mixed use. Mixed use will cause errors in barcode de-multiplexing during the data analysis procedures.
- Adapter quality as well as quantity directly affects the efficiency and quality of the final library. Please follow Table 34 to dilute the Adapter according to the input amounts of RNA sample used during processing.

Table 34 Recommended Adapter Input according to the Amount of total RNA

| total RNA ( ng ) | MGI Adapter    | MGI Adapter               |
|------------------|----------------|---------------------------|
|                  | Dilution Ratio | Input after Dilution (μL) |
| 51-100           | 5              | 5                         |
| 10-50            | 10             | 5                         |

- For other amounts of RNA sample input, please adjust the Adapter input appropriately.

### C-1 MGIEasy DNA Adapters-16 (Tube) Kit Instructions

Based on the principles of balanced base composition, Adapters must be used in specific groups. Please follow the instructions below to use Adapters in proper combination:

2 sets of 4 Adapters: (01-04) and (13-16)

1 set of 8 Adapters: (97-104)

If the sequencing data output requirement is the same for all samples in one lane, please refer to Table 35 below to choose the appropriate barcode Adapter combinations.

Table 35 MGI Easy DNA Adapters-16 (Tube) Kit Instruction

| Sample(s)<br>/lane | Instructions (Example)  |
|--------------------|---|
| 1                  | <p>Requires at least 1 set of Adapters:</p> <p>1. Take a set of 4 Adapters (e.g. 01-04), mix 4 Adapters with equal volumes, then add the mixture to the sample.</p> <p>Or 2. Take a set of 8 Adapters (e.g. 97-104), mix 8 Adapters with equal volumes, then add the mixture to the sample.</p>   |
| 2                  | <p>Requires at least 1 set of Adapters:</p> <p>1. Take a set of 4 Adapters (e.g. 01-04), mix Adapters with equal volumes in pairs to obtain 2 mixtures of equal volumes. Add 1 mixture to one sample. (e.g. Mix 01 &amp; 02, then add to sample 1; Mix 03 &amp; 04, then add to sample 2)</p> <p>Or 2. Take a set of 8 Adapters (97-104), mix Adapters with equal volumes in groups of 4 to obtain 2 mixtures of equal volumes. Add 1 mixture to one sample. (e.g. Mix 97-100, then add to sample 1; Mix 101-104, then add to sample 2)</p> |
| 3                  | <p>Requires at least 2 sets of Adapters:</p> <p>For sample 1&amp;2, use the method for (2 samples/lane) above. For sample 3, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for samples 1-2 and for sample 3.</p>  |
| 4                  | <p>Requires at least 1 set of Adapters:</p> <p>1. Take a set of 4 Adapters (e.g. 01-04), add 1 Adapter to each sample in an equal volume. (e.g. Add Adapters 01, 02, 03, 04 to samples 1, 2, 3, 4, respectively.)</p> <p>Or 2. Take a set of 8 Adapters (97-104), mix Adapters with an equal volume in pairs to obtain 4 mixtures of equal volumes. Add 1 mixture to each sample. (e.g. Mix 97-98, 99-100, 101-102, 103-104, then add respectively to samples 1, 2, 3, 4.)</p>  |
| 5                  | <p>Requires at least 2 sets of Adapters:</p> <p>For samples 1-4, use the method for (4 samples/lane) above. For sample 5, use the method for (1 sample/lane) above. Note that you should use different Adapter sets for samples 1-4 and for sample 5.</p>   |
| 6                  | <p>Requires at least 2 sets of Adapters:</p> <p>For samples 1-4, use the method for (4 samples/lane) above. For sample 5-6, use the method for (2 sample/lane) above. Note that you should use different Adapter sets for samples 1-4 and for samples 5-6.</p>  |

|   |   |
|---|---|
| 7 | <p>Requires all 3 Adapter sets and follow these 3 steps:</p> <ol style="list-style-type: none"> <li>1) For samples 1-4, use the method for (4 samples/lane) above (Use 1st Adapter set).</li> <li>2) For samples 5-6, use the method for (2 samples/lane) above (Use 2nd Adapter set).</li> <li>3) For sample 7, use the method for (1 sample/lane) above (Use 3rd Adapter set). You can add a single Adapter within the Adapter set. Or add the Adapter mix which is mixed from all Adapters within the Adapter set with an equal volume.</li> </ol> <p>Note that you should use different Adapter sets for samples 1-4, for samples 5-6 and for sample 7.</p> |
| 8 | <p>Requires at least 1 set of Adapters:</p> <ol style="list-style-type: none"> <li>1. Take a set of 8 Adapters (97-104), respectively add 1 Adapter to each sample in an equal volume.</li> <li>Or 2. Take 2 sets of 4 Adapters (01-04 and 13-16), add 1 Adapter to each sample in an equal volume.</li> </ol>  |

For situations in which the sequencing data output requirements are different between samples, any sample with a data output of more than 20% for each lane must use a separate set of Adapters.

For example, 9 samples are pooled into 1 lane, one of which requires 30% of the total data output. In this case, the other 8 samples may use Adapters (97-104), whereas the final sample must use a full Adapter set instead of using only a single Adapter (e.g. Adapter set (01-04) or (13-16)).

## Appendix D Conversion between DNA Molecular Mass and Number of Moles

The formula below shows the calculation of the mass in (ng) that corresponds to 1 pmol of dsDNA sample with varying fragment sizes.

$$\text{The mass (ng) corresponding to 1 pmol PCR Products} = \frac{\text{DNA Fragment Size (bp)}}{1000 \text{ bp}} \times 660 \text{ ng}$$

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