Application Note

WES of FFPE samples on MGI sequencing platforms

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The KAPA HyperCap Workflow enables high-performance whole exome sequencing of FFPE DNA on MGI sequencing platforms

MGI sequencing platforms, based on innovative DNBSEQ™ technology, offer high-accuracy, fast, and flexible solutions for high-throughput Whole Exome Sequencing (WES). The KAPA HyperCap Workflow combines KAPA HyperPlus Kits and KAPA HyperExome Probes to overcome challenges associated with FFPE sample preparation, and enable high-performance WES of FFPE DNA on MGI systems.

Introduction

Solid tumor biopsies are routinely stored in formalin-fixed, paraffin-embedded (FFPE) tissue blocks. Next-generation whole exome sequencing (WES) of such samples is now routinely performed in basic, translational and clinical research settings, to comprehensively survey the somatic mutations (point mutations, short indels, copy number variants, and gene fusions) in the coding regions of a cancer genome. However, WES from FFPE DNA samples remains challenging, due to limited sample availability and the molecular damage caused by sample archiving and DNA extraction methods.

MGI sequencing platforms, based on DNBSEQ technology, offer flexibility, ease-of-use, fast turnaround times, and competitive per-base sequencing cost for high-throughput WES. High sequencing accuracy and lower duplication rates in WES are achieved through innovative single-stranded circularization (ssCir) and DNA Nanoball (DNB) template preparation strategies and combinatorial probe anchor synthesis (cPAS) sequencing chemistry.[1]

Library construction methods for Illumina® sequencing are easily adaptable for MGI systems. The KAPA portfolio of high-performance library preparation and KAPA HyperCap target enrichment reagents from Roche provide industry-leading sample preparation solutions, particularly for challenging sample types such as FFPE and cell-free DNA.[2] We have previously demonstrated the utility of Roche sample preparation solutions for human whole genome and whole exome sequencing from high-quality samples on MGI platforms.[3,4] In this Application Note, we show that the KAPA HyperCap Workflow, with KAPA HyperPlus Kits and KAPA HyperExome Probes, supports high-quality WES from FFPE samples in MGI sequencing pipelines. Results generated with a commercial FFPE sample (100 ng input, 3-plex exome capture) demonstrate that a modified hybridization reaction setup, combined with more stringent hybridization and washing conditions, improve all key sequencing metrics for FFPE libraries. A step-by-step optimized protocol for FFPE samples is included.

Materials and methods

Experimental design

Whole exome libraries were prepared from a commercial FFPE reference standard using the KAPA HyperPlus Kit, the KAPA HyperCap Workflow v3.1[5] and KAPA HyperExome Probes.[6] The standard KAPA HyperCap Workflow was modified to obtain MGI-compatible libraries and achieve improved sequencing metrics with FFPE samples. A workflow overview, as well as a step-by-step protocol, are included in the Appendix. Single-stranded circularization (ssCir), DNA Nanoball (DNB) preparation, and DNBSEQ™ using a DNBSEQ-G400 sequencer* was performed by MGI Tech Co., Ltd (Shenzhen) using standard protocols.[7,8] Data analysis was performed with open source bioinformatics tools.

Library construction and sequencing

Input DNA and Enzymatic Fragmentation: A commercial FFPE reference standard (HD300)[9] was obtained from Horizon Discovery. Three 100 ng aliquots of HD300 DNA were fragmented for 25 min at 37°C using KAPA Frag Enzyme and Buffer. Fragmentation reaction products were used directly in the End Repair and A-Tailing reaction.

Library construction: The KAPA HyperPlus library construction workflow outlined in the KAPA HyperCap Workflow v3.1 User Guide,[5] is similar to the protocol used for library construction with the MGI Easy Universal Library Prep Kit.[10] The standard protocol was modified as follows to obtain MGI-compatible libraries:

- *Adapter Ligation:* 5 µL of 10 µM MGIEasy DNA Adapter (MGI Tech Co., Ltd) was used (instead of KAPA Universal Adapter) in each ligation reaction. A unique adapter (barcode) was chosen for each library (according to manufacturer's instructions)[10] to support multiplexed sequencing. Ligation was performed for 30 min at 20°C.
- *Pre-capture PCR:* Pre-capture PCR was performed with KAPA HiFi HotStart ReadyMix and 5 µL of MGI Primer Mix[11] (containing 10 µM of each primer per reaction; provided by MGI Tech Co., Ltd). Six cycles of pre-capture amplification were performed.

Pre-capture Library QC: The fragment size distribution of amplified, bead-purified Sample Libraries was determined with an Agilent® Bioanalyzer 2100 instrument and DNA 1000 Kit (Agilent Technologies). Libraries were quantified using a Qubit® Fluorometer and dsDNA HS Assay Kit (Thermo Fisher Scientific).

Target enrichment (exome capture): Two identical Multiplex DNA Sample Library Pools were constructed (as outlined in Table 1 in the next section), by pooling 350 ng of each of the three uniquely indexed KAPA HyperPlus libraries, for a combined mass of 1.05 µg per 3-plex pool. Pools were hybridized to KAPA HyperExome Probes, using (i) the standard KAPA HyperCap Workflow v3.1 for panels with a capture target size ≥40 Mb[5] (with modifications for the MGI platform), or (ii) an optimized protocol for FFPE samples (detailed in the Appendix). To ensure compatibility with the MGI sequencing platform, MGI-specific reagents were employed in both versions of the workflow: the hybridization sample was prepared with MGI Blocking Oligos (instead of KAPA Universal Enhancing Oligos), and post-capture library amplification was performed with the same MGI Primer Mix used in the Pre-capture PCR. To achieve optimal results with FFPE samples, the hybridization reaction setup was modified, and all high-temperature incubations during pre- and post-hybridization processing, as well as the hybridization incubation itself, were performed at 65°C (instead of the 55°C used for the standard protocol).

Post-capture PCR: Recovery of the captured Multiplex DNA Sample Pools was performed as per the standard KAPA HyperCap Workflow. Post-capture PCR was performed with KAPA HiFi HotStart ReadyMix and 5 µL of MGI Primer Mix[11] (10 µM of each primer per reaction; provided by MGI Tech Co., Ltd). Eight cycles of post-capture amplification were performed for both pools.

Post-capture Library QC: The fragment size distribution of the two enriched, amplified Multiplex DNA Sample Libraries was determined with an Agilent Bioanalyzer 2100 instrument and DNA 1000 Kit (Agilent Technologies). Libraries were quantified using a Qubit Fluorometer and dsDNA HS Assay Kit (Thermo Fisher Scientific).

DNA Nanoball (DNB) Preparation: Library denaturation, ssCir, Exo digestion and cleanup, and DNB generation were performed at MGI Tech Co., Ltd (Shenzhen), using standard protocols. The recommended input amount (1 pmol per sequencing-ready library) was used for ssCir with the MGIEasy Circularization Kit (MGI Tech Co., Ltd).[7] Of each purified ssCir library, 40 fmol was converted to DNBs using the DNBSEQ-G400RS High-throughput Sequencing Set (MGI Tech Co., Ltd).[8]

Sequencing: Paired-end Sequencing (2 x150 bp) was performed on a DNBSEQ-G400 instrument (MGI Tech), using standard combinatorial probe anchored synthesis (cPAS) technology. Sequencing parameters were tailored to obtain >100 million read pairs per library.

Alignment: Read quality was assessed using FASTQC (v0.10.1). Adapter and quality trimmed sequencing reads (obtained with SOAPnuke, v2.1.2) were aligned to the human reference genome (build GRCh37/hg19) using BWA-MEM (v0.7.12) with default parameters. Duplicates were marked and removed with Picard (v1.134).

Quality control analysis: Quality control metrics (library insert size, alignment, GC bias, and genome coverage metrics) were calculated with Picard. Picard AlignmentSummaryMetrics was used to summarize the alignment metrics.

*In parts of Asia-Pacific, e.g., China, the DNBSEQ-G400/G400 Fast is known as MGISEQ-2000/2000 Fast and the DNBSEQ-G50 as the MGISEQ-200. All references to the DNBSEQ-G400 also apply to the MGISEQ-2000.

Results and discussion

Library construction metrics

Key library construction parameters and QC metrics are summarized in Table 1. Pre-capture library yields obtained with the KAPA HyperPlus Kit ranged between 924 and 984 ng. These yields were slightly lower than expected from the standard KAPA HyperCap Workflow, and were attributed to a lower than usual primer concentration in the pre-capture PCR (when using MGI-compatible reagents, as compared to using the standard HyperCap reagents for Illumina® sequencing). Nevertheless, the amount of each amplified Sample Library was sufficient for pre-capture QC, target enrichment, and archiving.

A smear analysis performed with the Bioanalyzer software (across the region of 150 – 1,000 bp) yielded an average fragment size of approximately 300 bp for all three pre-capture libraries (Figure 1A). Both the standard and optimized capture workflows produced post-capture libraries with an average fragment size of approximately 280 bp; Figure 1B). Pre- and post-capture fragment sizes were slightly smaller than expected for libraries prepared with the same protocol from high-quality DNA (~320 bp). This is not unusual, as molecular damage incurred during tissue fixation and deparaffinazation typically result in less efficient amplification for longer FFPE DNA molecules.

Sequencing metrics

Important sequencing metrics are shown in Figure 2 and Table 2 on the next page.

On-target rates: On-target rates achieved with the standard protocol were high (average of 73.4% reads on primary target; average of 79.9% bases in the padded capture target), and comparable to results obtained with high-quality genomic DNA.[4] A significant (>10%) improvement in on-target rates (average of 83.9% reads on primary target, and average of 89.1% bases in padded capture target) was achieved with the optimized FFPE protocol.

Duplication rates: Duplication rates were in the range of 10 – 11% for both protocols, with the standard protocol performing only marginally better (resulting in fewer duplicates). These rates were regarded as acceptable for the sequencing depth.

Fold-80 base penalty: This metric is defined as the fold of additional sequencing required to ensure that the mean coverage is achieved for 80% of the target bases, and is an important indication of coverage uniformity. Fold-80 values were low (<2.0) for both

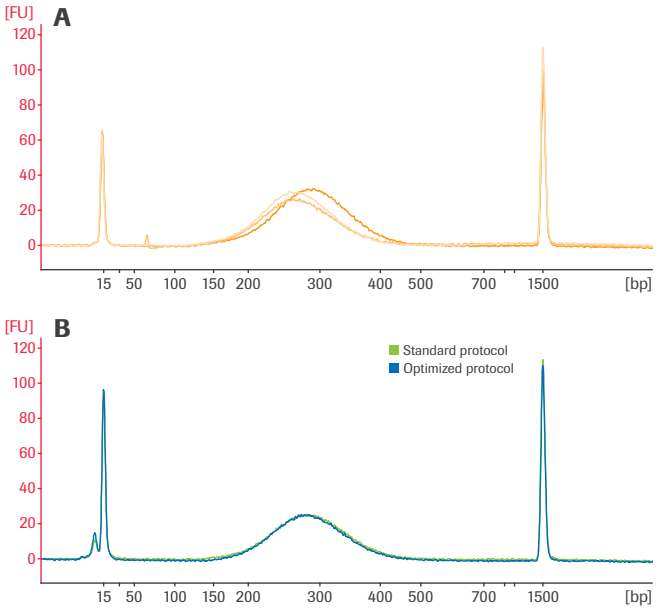

Figure 1. Fragment size distributions of (pre-capture) Sample Libraries (A) and enriched, amplified (post-capture) Multiplex DNA Sample Library Pools (B). Libraries were prepared as described in Materials and Methods. Library fragment size distributions were determined with an Agilent® 2100 Bioanalyzer instrument and DNA 1000 Kit.

protocols. The optimized FFPE protocol resulted in a significant improvement in fold-80 base penalty over the standard protocol (averages of 1.69 and 2.07, respectively), and approached values obtained for exome sequencing of high-quality genomic DNA on the MGI platform.[4]

GC bias: GC bias plots (which illustrate the dependence of normalized coverage on the GC content of capture targets) provide another means for assessing coverage uniformity. Figure 3 on p. 5 clearly demonstrates the improvements in coverage uniformity achieved with the more stringent hybridization and wash conditions used in the optimized FFPE protocol.

Coverage depth: An average of 101 million high-quality reads were obtained for each of the three libraries, translating to an average coverage depth of 239X and 278X with the standard and optimized protocols, respectively. Below 50X coverage, the benefits of the optimized protocol were not significant (97.1% of bases covered, vs. 96.6% with the standard protocol; not shown). However, at 100X, the impact was evident (94.1% of bases covered with the optimized protocol, vs. 85.6% with the standard protocol). This typically translates to improved variant calling sensitivity, which is critical for tumor samples.

Table 1. Library construction parameters and QC metrics

Input	Library Prep Kit	Fragmentation parameters	Replicate numbers	Adapter IDs	Pre-capture amplification cycles	Average pre-capture library yield	Capture protocol	Post-capture amplification cycles	Post-capture library yield
HD300 FFPE reference DNA (100 ng)	KAPA HyperPlus	25 min at 37ºC	1, 2, 3	15, 16, 104	6	944 ng ±35 ng	Standard (55ºC)	8	618 ng
							Optimized (65ºC)	8	468 ng

The three uniquely indexed KAPA HyperPlus libraries were mixed in equal proportions (350 ng each) to create two Multiplex (3-plex) DNA Sample Library Pools, each comprising a total of 1.05 µg pre-capture library DNA. Pool 1 was processed with the standard KAPA HyperCap v3.1 Workflow (hybridization and wash steps at 55ºC), whereas Pool 2 was processed with the workflow optimized for FFPE samples (hybridization and wash steps at 65ºC).

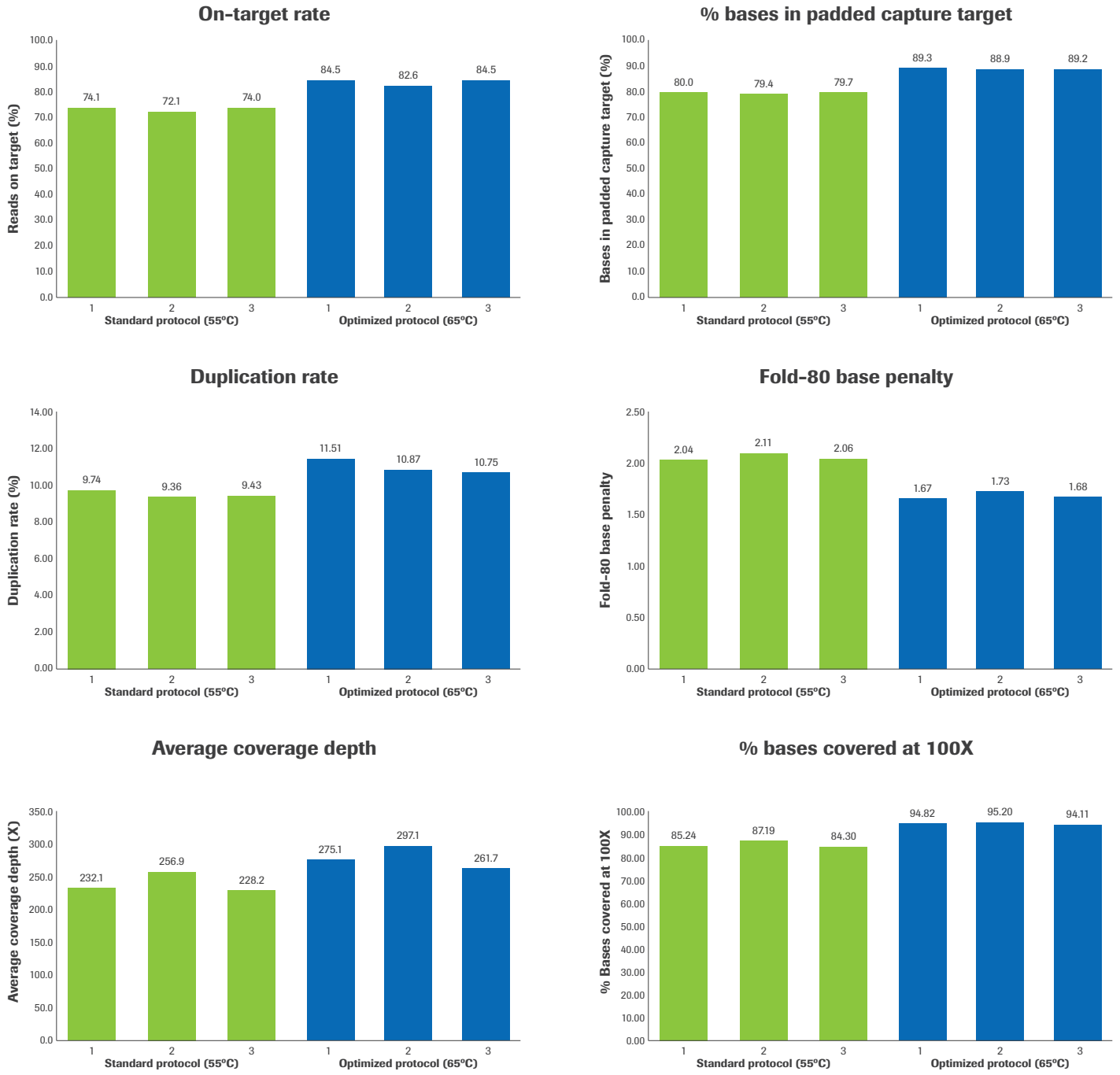

Figure 2. Key sequencing metrics for whole exome FFPE libraries prepared with the KAPA HyperCap Workflow and sequenced on an DNBSEQ™-G400 instrument. Each bar represents a uniquely indexed KAPA HyperPlus library in a 3-plex Multiplex DNA Sample Library Pool. Green bars represent results generated with the standard protocol (hybridization and washes at 55°C), whereas blue bars represent results generated with the workflow optimized for FFPE samples (modified hybridization reaction setup, hybridization and washes at 65°C). Libraries were prepared and sequenced, and data were analyzed as described in Materials and Methods.

Table 2. Additional sequencing metrics

Metric	Standard protocol (55°C)			Optimized protocol (65°C)		
	Library 1	Library 2	Library 3	Library 1	Library 2	Library 3
Mean insert size (bp)	199	211	198	199	211	198
Mapping rate	99.84	99.86	99.84	99.84	99.84	99.83
Number of reads after R1/R2 trimming	108,847,113	118,204,407	106,903,990	111,959,864	117,875,240	105,586,712
Percentage high-quality reads (≥Q30)	89.48	92.88	88.61	89.89	93.15	89.07
% bases covered with at least 1 read	98.21	98.27	98.22	98.07	98.15	98.06

Data on file.

Conclusions

The aim of this study was to demonstrate the utility of the KAPA HyperCap Workflow from Roche—with KAPA HyperPlus Kits and KAPA HyperExome Probes—for high-quality human whole exome sequencing of FFPE samples on the MGI sequencing platform.

Libraries prepared from 100 ng inputs of a commercial FFPE reference sample (HD300) and enriched in a 3-plex exome capture, yielded high-quality sequencing data. On-target rates, coverage uniformity, and overall coverage was significantly improved by modifying the hybridization reaction setup and increasing the temperature (stringency) of hybridization and post-hybridization washes from the 55°C used in the standard protocol to 65°C. In particular, this resulted in a more balanced (uniform) normalized coverage across the entire range of GC content. Since less sequencing reads were associated with AT-rich regions, more reads were available to improve coverage of GC-rich regions. Despite a small (1.5%) increase in the average PCR duplication rate, improvements in coverage uniformity (fold-80 base penalty; -0.38 points or an 18.2% improvement) and specificity (% reads on-target; +10.4%) were achieved with the optimized FFPE protocol. This translated to broader and deeper coverage of the whole exome target. Mean coverage improved by 39X and the percentage of bases covered at 100X improved by 9.1%. Overall, this demonstrates the robustness and flexibility of the KAPA HyperCap Workflow and the KAPA HyperExome Probes, and the ability to accommodate different sample types across different sequencing platforms.

Only a few minor modifications to the standard KAPA HyperCap Workflow are required to generate MGI-compatible libraries that can be used directly in standard ssCir, DNB generation, and sequencing protocols. Mainly, standard barcoded adapters, library amplification primers, and blocking oligos (designed for Illumina® sequencing) have to be replaced with MGI-compatible reagents. Care must be taken to maintain the recommended working concentrations of all these reagents to ensure optimal library conversion rates, capture efficiency, and library quality.

The detailed protocol included in the Appendix provides all the instructions needed to unlock the industry-leading performance of KAPA Sample Prep reagents when performing whole exome sequencing of FFPE samples on the MGI platform.

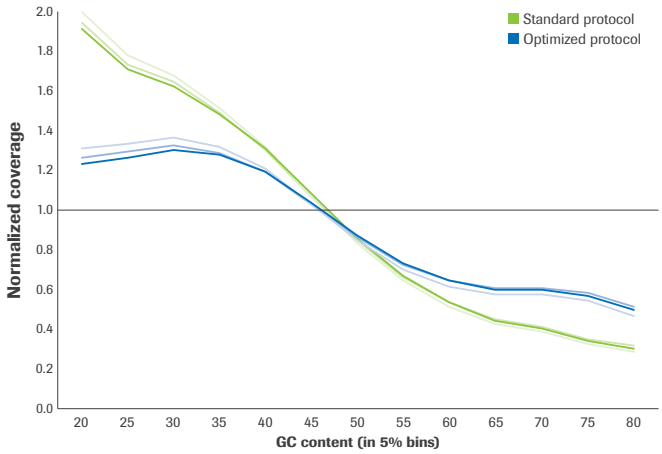

Figure 3. Normalized coverage across %GC bins. Green lines represent the results for the three libraries generated with the standard protocol (hybridization and washes at 55°C), and blue lines the results obtained with the optimized protocol (modified hybridization reaction setup, hybridization and washes at 65°C). The black line designates the theoretical optimum normalized coverage of 1. Libraries were prepared and sequenced, and data analyzed as described in Materials and Methods.

Appendix

Detailed sample preparation workflow

1. Required reagents

Reagents supplied by Roche:

- KAPA HyperPlus Kit
  - Roche PN: 07962401001 (24 libraries)
  - Roche PN: 07962428001 (96 libraries)
- KAPA HyperPure Beads
  - Roche PN: 08963835001 (5 mL)
  - Roche PN: 08963843001 (30 mL)
  - Roche PN: 08963851001 (60 mL)
  - Roche PN: 08963878001 (4 x 60 mL)
  - Roche PN: 08963860001 (450 mL)
- KAPA HyperCapture Reagent Kit
  - Roche PN: 9075810001 (24 reactions)
  - Roche PN: 9075828001 (96 reactions)
- KAPA HyperCapture Bead Kit
  - Roche PN: 9075780001 (24 reactions)
  - Roche PN: 9075798001 (96 reactions)
- KAPA HyperExome Probes
  - Roche PN: 9062548001 (12 reactions)
  - Roche PN: 9062556001 (24 reactions)
  - Roche PN: 9062564001 (48 reactions)
  - Roche PN: 9062572001 (96 reactions)
  - For larger pack sizes, please contact your Roche sales representative
- KAPA HyperChoice Probes
  - Different custom probe options are available (see https://sequencing.roche.com/en/products-solutions/products/sample-preparation/dna-reagents/target-enrichment/hybridization/custom-design.html).
  - HyperDesign Tool: free online custom design portal (www.hyperdesign.com)
- KAPA Probes Resuspension Buffer
  - Roche PN: 9075879001 (1 mL)
  - Roche PN: 9075887001 (5 mL)

The following reagents are not supplied by Roche, and must be sourced from general laboratory stocks or third-party suppliers:

- MGIEasy DNA Adapters Kit V1.0
  - MGI PN: 1000005284 (16 rxn, 16 barcodes)
  - MGI PN: 1000005282 (96 rxn, 96 barcodes)
- MGI-compatible library amplification primer mixes for pre- and post-capture PCR*
- Compatible blocking oligos for MGIEasy DNA Adapters*
- Freshly prepared 80% ethanol
- Elution Buffer (10 mM Tris-HCl, pH 8.0 at 25ºC)
- Nuclease-free or PCR-grade water

The following equipment and plasticware are required for this workflow:

- Pipettes and suitable, sterile barrier/filter tips
- PCR tubes/96-well plates (0.2 mL)
- Low DNA-binding microtubes (1.5 mL)
- Magnetic block e.g., DynaMag™-96 Side Magnet (Thermo Fisher Scientific Cat. No. 12331D)
- Themocyclers (for standard PCR)
- Agilent® Bioanalyzer 2100 or similar instrument and appropriate reagent kits
- Qubit® Fluorometer and appropriate assays (Thermo Fisher Scientific)

*Primer and blocking oligo sequences may be found on the MGI website, in the document entitled *Oligos and primers for BGISEQ&DNBSEQ NGS system*.[11] Order primers as ready-to-use solutions, or lyophilized and re-suspend according to supplier's recommendations. For both pre- and post-capture amplification, prepare an amplification primer mix consisting of 10 µM of each primer in 10 mM Tris-HCl, pH 8.0 at 25ºC.

Both Block 3 and 4 are prepared with nuclease-free water to a final concentration of 300 µM. Block 4 is prepared as in the following example: resuspend each barcoded blocking oligo "Ad153_newBC_indexblock_xx" (depending on the barcoded adapters used for the libraries that are pooled in the hybridization) to a working concentration of 300 µM with nuclease-free water. Prepare a mix of the respective blocking oligos by using equal volume per oligo. This mix is the "Block 4". Use 10 µL of Block 4 per hybridization.

2. Workflow overview

Figure A1. KAPA HyperCap Workflow for the preparation of MGI-compatible whole exome FFPE libraries. Triplicate aliquots of HD300 FFPE DNA (100 ng) were subjected to enzymatic fragmentation for 25 min at 37°C to obtain an average fragment size of approximately 200 bp (as described in **Section 4.1**). Pre-capture libraries were prepared with the KAPA HyperPlus Kit as described in **Sections 4.3 – 4.6**. After library QC (**Section 4.7**), two 3-plex capture pools were prepared and exome capture was performed as described in **Sections 4.8 – 4.11**. Target enrichment steps that were specifically optimized for FFPE samples are highlighted in red. To obtain libraries for MGI sequencing, Illumina®-specific adapters, library amplification primers, and blocking oligos used in the standard KAPA HyperCap Workflow were exchanged for reagents compatible with the MGI platform. Standard protocols and MGI reagents were used for ssCir and DNB generation. The total workflow time (excluding hybridization, library QC, ssCir, and DNB generation) is approximately 5 hours.

3. Quality control of input DNA

Quality control of input DNA is strongly recommended. In this study, DNA was quantified using an Qubit® Fluorometer and Qubit dsDNA HS (High Sensitivity) Assay Kit (Thermo Fisher Scientific). These assays are designed to provide accurate quantification of double-stranded DNA molecules present in the sample.

4. Library construction protocol

This protocol describes construction of individual *Sample Libraries* using the KAPA HyperPlus Kit and is based on the protocol outlined in the **KAPA HyperCap Workflow v3.1 User Guide**.[5]

4.1 Enzymatic Fragmentation

4.1.1 Prepare 100 ng of DNA in Elution Buffer for Enzymatic Fragmentation. Assemble each Fragmentation reaction on ice, as outlined below:

Component	Volume
Input DNA (100 ng)	35 µL
KAPA Frag Buffer (10X)[1]	5 µL
KAPA Frag Enzyme[1]	10 µL
Total volume	**50 µL**

[1]The KAPA Frag Buffer and Enzyme may be pre-mixed and kept on ice prior to reaction setup, and dispensed as a single solution. Please note the volume of buffer is less than the volume of enzyme in this reaction.

4.1.2 Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.

4.1.3 Incubate the plate/tube(s) in a thermocycler, pre-cooled to 4°C and programmed as outlined below. A heated lid is not required for this step. If used, set the temperature of the heated lid to ≤50°C.

Step	Temp	Time
Pre-cool block	4°C	N/A
Fragmentation	37°C	25 min
HOLD	4°C	∞

4.1.4 Quickly transfer the reaction to ice and proceed immediately to **End Repair and A-Tailing**.

NOTE: Enzymatic fragmentation parameters are expected to yield DNA with a median fragment size of approximately 200 bp. Optimal fragmentation conditions for FFPE samples of variable quality may have to be determined empirically. In addition, fragmentation time may be increased to obtain shorter inserts (to improve coverage metrics), or decreased to obtain longer inserts (to obtain more information about intronic content).

4.2 End Repair and A-Tailing

4.2.1 Assemble each End Repair and A-Tailing reaction on ice in a tube or well of a PCR plate, as outlined below:

Component	Volume
Fragmented dsDNA	50 µL
KAPA End Repair & A-Tailing Buffer[1]	7 µL
KAPA HyperPlus End Repair & A-Tailing Enzyme Mix[1]	3 µL
Total volume	**60 µL**

[1]The buffer and enzyme mix should preferably be premixed and added in a single pipetting step. Premixes are stable for <24 hr at room temperature, for ≤3 days at 4ºC, or for ≤4 weeks at -20ºC.

4.2.2 Vortex gently and spin down briefly. Return the plate/tube(s) to ice. Proceed immediately to the next step.

4.2.3 Incubate in a thermocycler programmed as outlined below:

Step	Temp	Time
End Repair and A-Tailing	65ºC[1]	30 min
HOLD	4ºC	∞

[1]A heated lid is required for this incubation. If possible, set the lid temperature to 85ºC, instead of the default ~105ºC.

4.2.4 Centrifuge the plate/tubes(s) briefly and proceed immediately to **Adapter Ligation**.

4.3 Adapter Ligation

4.3.1 In the same plate/tubes(s) in which End Repair and A-Tailing was performed, assemble each Adapter Ligation reaction as follows:

Component	Volume
End Repair and A-Tailing Reaction Product	60 µL
MGIEasy DNA Adapter (10 µM)[1]	5 µL
Ligation Buffer[2]	30 µL
DNA Ligase[2]	10 µL
Nuclease-free Water[2]	5 µL
Total volume	**110 µL**

[1]Libraries that will be pooled for sequencing must each receive a unique Adapter ID or index.

[2]The water, buffer and ligase enzyme should preferably be premixed and added in a single pipetting step. Premixes are stable for ≤24 hr at room temperature, or for ≤3 days at 4ºC.

4.3.2 Mix thoroughly and centrifuge briefly.

4.3.3 Incubate in a thermocycler programmed as outlined below:

Step	Temp	Time
Set heated lid	50ºC	On
Adapter Ligation	20ºC	30 min
HOLD	4ºC	∞

4.3.4 Centrifuge plate/tube(s) briefly and proceed immediately to **Post-ligation Cleanup**.

4.4 Post-ligation Cleanup

4.4.1 Make sure that the KAPA HyperPure Beads have been equilibrated to room temperature and thoroughly resuspended to obtain a homogeneous mixture.

4.4.2 In the same plate/tube(s) from step 4.3.4, perform a 0.8X bead-based cleanup by combining the following:

Component	Volume
Adapter Ligation Reaction Product	110 µL
KAPA HyperPure Beads	88 µL
Total volume	**198 µL**

4.4.3 Mix thoroughly by vortexing and/or pipetting up and down multiple times.

4.4.4 Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.

4.4.5 Place the plate/tube(s) on a magnetic stand to capture the beads. Incubate until the liquid is clear.

4.4.6 Carefully remove and discard the supernatant.

4.4.7 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of freshly prepared 80% ethanol.

4.4.8 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.4.9 Carefully remove and discard the ethanol.

4.4.10 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of 80% ethanol.

4.4.11 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.4.12 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4.4.13 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. Be careful not to over dry the beads as this may result in reduced yield.

4.4.14 Remove the plate/tube(s) from the magnetic stand.

4.4.15 Thoroughly resuspend the beads in 22 µL of Elution Buffer (10 mM Tris-HCl, pH 8.0).

4.4.16 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

4.4.17 Place the plate/tube(s) on a magnetic stand to capture the beads. Incubate until the liquid is clear.

4.4.18 Transfer 20 µL of each eluate to a fresh well/tube and proceed with **Pre-capture Library Amplification**.

4.5 Pre-capture Library Amplification

4.5.1 Assemble the pre-capture library amplification reaction as follows:

Component	Volume
Purified, Adapter-ligated DNA	20 µL
KAPA HiFi HotStart ReadyMix (2X)[1]	25 µL
MGI-compatible Pre-capture Library Amplification Primer Mix[1,2]	5 µL
Total volume	**50 µL**

[1]KAPA HiFi HotStart ReadyMix and primers should be premixed and added in a single pipetting step.

[2]Refer to Section 1 (materials not supplied by Roche).

4.5.2 Mix well by pipetting up and down several times.

4.5.3 Amplify the library using the following thermocycling profile:

Step	Temp	Time	Cycles[1]
Initial Denaturation	98°C	45 sec	1
Denaturation	98°C	15 sec	6
Annealing	60°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	1 min	1
HOLD	4°C	∞	1

[1]The number of PCR cycles may require optimization depending on the input amount.

4.5.4 Proceed immediately to **Library Amplification Cleanup**.

4.6 Library Amplification Cleanup

4.6.1 In the same plate/tube(s), perform 1.4X bead-based cleanup by combining the following:

Component	Volume
Amplified Pre-capture Library DNA	50 µL
KAPA HyperPure Beads	70 µL
Total volume	**120 µL**

4.6.2 Thoroughly resuspend the beads by pipetting up and down multiple times.

4.6.3 Incubate the plate/tube(s) at room temperature for 5 min to bind DNA to the beads.

4.6.4 Place the plate/tube(s) on a magnetic stand to capture the beads. Incubate until the liquid is clear.

4.6.5 Carefully remove and discard the supernatant.

4.6.6 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of 80% ethanol.

4.6.7 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.6.8 Carefully remove and discard the ethanol.

4.6.9 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of 80% ethanol.

4.6.10 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.6.11 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4.6.12 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. Be careful not to over dry the beads as this may result in reduced yield.

4.6.13 Remove the plate/tube(s) from the magnetic stand.

4.6.14 Thoroughly resuspend the beads in 32 µL of Elution Buffer (10 mM Tris-HCl, pH 8.0) or nuclease-free water.

4.6.15 Incubate the plate/tube(s) at room temperature for 2 min to elute DNA off the beads.

4.6.16 Place the plate/tube(s) on a magnetic stand to capture the beads. Incubate until the liquid is clear.

4.6.17 Transfer 30 µL of each eluate to a new well/tube and proceed with **Pre-capture Library QC**.

NOTE: At this point, the amplified, purified pre-capture libraries may be stored at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C (long term storage).

4.7 Pre-capture Library QC

Evaluate the success of library construction by determining the library fragment size distribution with an electrophoretic method (e.g. with an Agilent® Bioanalyzer 2100 instrument and DNA 1000 or DNA High Sensitivity Kit). Pre-capture libraries should have a fragment size distribution of 150 – 1,500 bp.

A Qubit® Fluorometer and dsDNA HS Assay Kit (Thermo Fisher Scientific) are recommended for the quantification of pre-capture libraries.

If capturing a single sample, utilize 1000 ng of a uniquely indexed amplified DNA Sample Library per capture. For multiplexed capture, mix equal amounts (by mass) of the appropriate number of uniquely indexed DNA Sample Libraries to obtain a combined DNA mass of 1.0 – 1.5 µg. At least 350 ng of each individual library is required for 3-plex exome capture of FFPE samples.

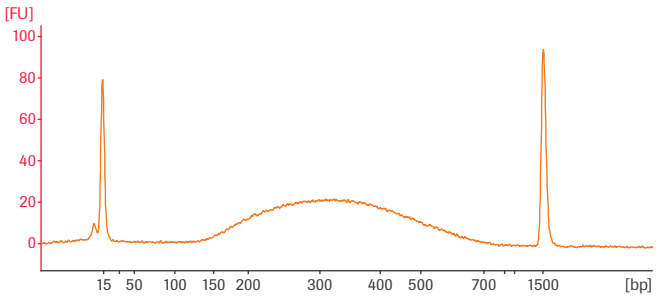

Figure A2. Expected size distribution of pre-capture libraries. Adapter-ligated libraries generated with the KAPA HyperPlus Kit were amplified for six cycles using KAPA HiFi HotStart ReadyMix and MGI-compatible Library Amplification Primers. The size distribution was assessed using an Agilent® 2100 Bioanalyzer instrument and DNA 1000 Kit.

4.8 Preparing for hybridization

4.8.1 Thaw the appropriate number of 4 µL KAPA Target Enrichment Probes aliquots (aliquot per capture pool or hybridization reaction).

4.8.2 Prepare capture pools by combining equal amounts (by mass) of uniquely indexed pre-capture libraries, to obtain a combined mass of 1.0 – 1.5 µg. For exome capture of FFPE samples with KAPA HyperExome Probes, 3-plex capture pools (3 x ≥350 ng pre-capture library) are recommended. This mixture will subsequently be referred to as the *Multiplex DNA Sample Library Pool.*

4.8.3 Add nuclease-free water to adjust the final volume of each pool to 45 µL.

NOTE: If the volume of the Multiplex DNA Sample Library Pool exceeds 45 µL, please refer to the Troubleshooting section of the KAPA HyperCap Workflow v3.1 User Guide.

4.8.4 Prepare the hybridization sample as follows:

- Add 20 µL of COT Human DNA to the Multiplex DNA Sample Library Pool, to bring the total volume up to 65 µL.

NOTE: When working with non-human DNA, the KAPA Hybrid Enhancer Reagent should be used instead of COT Human DNA. Some optimization of the protocol may be required for non-human species.

- Add 130 µL of KAPA HyperPure Beads to each well or tube containing Multiplex DNA Sample Library/ COT Human DNA mixture.
- Mix thoroughly by vortexing for 10 sec and spin down briefly.
- Incubate at room temperature for 10 min to allow the Multiplex DNA Sample Library and COT Human DNA to bind to the beads.
- Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant.
- Keeping the plate/tube(s) on the magnetic stand, add 200 µL of freshly prepared 80% ethanol.
- Incubate the plate/tube(s) on the magnetic stand at room temperature for at least 30 sec.
- Carefully remove and discard the ethanol. Try to remove any residual ethanol without disrupting the beads.
- Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. Be careful not to over dry the beads as this may result in reduced yield.
- Prepare the MGI Blocking Oligos as follows:

Component	Volume
Block 3	1.0 µL
Block 4 (see bottom of p. 6)	10.0 µL
Nuclease-free Water	2.4 µL
Total volume	**13.4 µL**

- Remove the plate/tube(s) from the magnetic stand and add 13.4 µL of MGI Blocking Oligos to the bead-bound DNA sample in each well/tube.
- Mix thoroughly by vortexing to ensure a homogeneous mixture.

4.8.5 Prepare the Hybridization Master Mix according to the capture target size as shown in the tables below:

- *For KAPA Target Enrichment Designs <40 Mb in capture target size:*

Component	Volume
Hybridization Buffer	28 µL
Hybridization Component H	12 µL
Nuclease-free Water	3 µL
Total volume	**43 µL**

- *For KAPA Target Enrichment Designs >40 Mb in capture target size:*

Component	Volume
Hybridization Buffer	28 µL
Hybridization Component H	9 µL
Nuclease-free Water	6 µL
Total volume	**43 µL**

4.8.6 Add 20 µL of the Hybridization Master Mix to the mixture of Multiplex DNA Sample Library Pool, COT DNA and Blocking Oligos (from Step 4.8.4).

4.8.7 Mix thoroughly and spin down briefly. Incubate at room temperature for 2 min.

4.8.8 Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.8.9 Transfer the full volume of each eluate (33.4 µL) to a fresh well/tube.

4.8.10 Incubate the eluate in a thermocycler programmed as outlined below (with the lid temperature set to 105ºC). Leave the tubes in the thermocycler.

Step	Temp	Time
Denaturation	95ºC	5 min
Incubation	65ºC	HOLD

4.8.11 Prepare the KAPA Target Enrichment Probes as described below:

- To each aliquot of 4 µL KAPA Target Enrichment Probes (from Step 4.8.1), add the remaining 23 µL of Hybridization Master Mix (from Step 4.8.5). Mix well and incubate the mix (27 µL) in a thermocycler (with lid temperature set to 105ºC) at 95ºC for 2 min. Transfer to ice immediately, and incubate on ice for 5 min.
- Add the full volume of the KAPA Target Enrichment Probes + Hybridization Master Mix (27 µL) directly into the 33.4 µL of eluate from Step 4.8.10, while the tubes containing the eluate are still in the thermocycler.

4.8.12 Mix well by pipetting up and down, and continue the 65ºC incubation for 16 – 20 hours to hybridize the KAPA Target Enrichment Probes to the Multiplex DNA Sample Library Pool before proceeding to **Wash and Recover the Captured Multiplex DNA Sample Library Pools**. The sample must be kept at 65ºC until it is transferred to the Capture Beads in Step 4.9.5.

4.9 Wash and Recover the Captured Multiplex DNA Sample Library Pools

4.9.1 Before completion of the hybridization incubation (step 4.8.12), thaw the Hybridization Wash Buffers. Mix well before using the buffers.

4.9.2 Dilute the 10X Wash Buffers (I, II, III and Stringent) and the 2.5X Bead Wash Buffer from the KAPA HyperCapture Reagent Kit, to create 1X working solutions. Volumes listed in the table on the next page are sufficient for **one capture**. These should be scaled up when performing multiple captures in parallel.

Buffer	Vol. of conc. buffer	Vol. of nuclease-free water	Total volume of 1X buffer
Stringent Wash Buffer (10X)	40 µL	360 µL	400 µL[1]
Wash Buffer I (10X)	10 µL	90 µL	100 µL[1]
	20 µL	180 µL	200 µL[2]
Wash Buffer II (10X)	20 µL	180 µL	200 µL[2]
Wash Buffer III (10X)	20 µL	180 µL	200 µL[2]
Bead Wash Buffer (2.5X)	120 µL	180 µL	300 µL[2]

[1]Place the 1X Stringent Wash Buffer and the smaller (100 µL) aliquot of 1X Wash Buffer I at 65ºC for Step 4.9.8.

[2]1X working solutions of these buffers may be stored at room temperature (15ºC to 25ºC) for up to 2 weeks.

4.9.3 Pre-warm the 1X Stringent Wash Buffer and 100 µL aliquot of 1X Wash Buffer I at 65ºC for at least 15 min.

4.9.4 Prepare the Capture Beads as follows:

- Allow the Capture Beads to equilibrate to room temperature before use.
- Vortex Capture Beads for 15 sec to ensure a homogeneous mixture before use.
- Aliquot the appropriate amount of beads per capture into a new well/tube, as outlined below:

Panel size	Volume of beads per capture
<40 Mb Capture Target Size	50 µL
>40 Mb Capture Target Size	100 µL

- Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant without disturbing the beads.
- Keeping the plate/tube(s) on the magnetic stand, add two times the initial volume of beads of 1X Bead Wash Buffer.
- Remove the plate/tube(s) from the magnetic stand and mix thoroughly by vortexing. Spin down briefly.
- Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant, without disturbing the beads.
- Keeping the plate/tube(s) on the magnetic stand, perform a second wash by adding two times the initial volume of beads of 1X Bead Wash Buffer.
- Remove the plate/tube(s) from the magnetic stand and mix thoroughly by vortexing. Spin down briefly.
- Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant, without disturbing the beads.
- Add the initial volume of beads of 1X Bead Wash Buffer.
- Remove plate/tube(s) from the magnetic stand and mix thoroughly by vortexing. Spin down briefly.
- Aliquot the appropriate amount of resuspended beads per capture as shown in the table below in a new well/tube:

Panel size	Volume of resuspended beads per capture
<40 Mb Capture Target Size	50 µL
>40 Mb Capture Target Size	100 µL

- Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.
- Carefully remove and discard the supernatant, without disturbing the beads. The Capture Beads are now ready to bind to the hybridized DNA. Proceed immediately to the next step.

4.9.5 Transfer each hybridization sample (from step 4.8.12) into a single well/tube with the prepared Capture Beads.

4.9.6 Mix thoroughly by vortexing for 10 sec and spin down briefly.

4.9.7 Incubate the capture reaction in the thermocycler at 65ºC for 15 min (set lid temperature to 105ºC).

4.9.8 Add 100 µL of pre-warmed 1X Wash Buffer I to each capture reaction.

4.9.9 Mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly.

4.9.10 Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.9.11 Carefully remove and discard the supernatant, without disturbing the beads.

4.9.12 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of pre-warmed 1X Stringent Wash Buffer.

4.9.13 Remove the plate/tube(s) from the magnetic stand and mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly.

4.9.14 Incubate the mixture in a thermocycler pre-heated to 65ºC for 5 min (set lid temp to 105ºC).

4.9.15 After incubation, remove the plate/tube(s) from the thermocycler and place on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.9.16 Carefully remove and discard the supernatant, without disturbing the beads.

4.9.17 Repeat steps 4.9.12 through 4.9.16 once, for a total of two washes with the pre-warmed 1X Stringent Wash Buffer.

4.9.18 To each well/tube, add 200 µL of room temp 1X Wash Buffer I.

4.9.19 Mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly.

4.9.20 Incubate the plate/tube(s) at room temperature for 1 min.

4.9.21 Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.9.22 Carefully remove and discard the supernatant, without disturbing the beads.

4.9.23 To each well/tube, add 200 µL of room temp 1X Wash Buffer II.

4.9.24 Mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly and transfer the contents to a new tube.

4.9.25 Incubate the plate/tube(s) at room temperature for 1 min.

4.9.26 Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.9.27 Carefully remove and discard the supernatant, without disturbing the beads.

4.9.28 To each well/tube, add 200 µL of room temp 1X Wash Buffer III.

4.9.29 Mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly.

4.9.30 Incubate the plate/tube(s) at room temperature for 1 min.

4.9.31 Place the plate/tube(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.9.32 Carefully remove and discard the supernatant, without disturbing the beads.

4.9.33 Remove the plate/tube(s) from the magnet.

4.9.34 To each well/tube, add 20 µL of nuclease-free water and mix thoroughly. Spin down briefly.

4.9.35 Proceed to **Amplify Enriched Multiplex DNA Sample**.

4.10 Amplify Enriched Multiplex DNA Sample

4.10.1 Refer to Section 1 (reagents not supplied by Roche) for guidelines on preparing the post-capture amplification primer mix.

4.10.2 In fresh well(s)/tube(s), prepare one post-capture PCR master mix for each Enriched Multiplex DNA Sample by thoroughly mixing the components listed in the table below:

Component	Volume
KAPA HiFi HotStart ReadyMix (2X)	25 µL
MGI-compatible Post-capture Library Amplification Primer Mix	5 µL
Total volume	**30 µL**

4.10.3 Retrieve the bead-bound DNA from Step 4.9.34, and mix thoroughly by vortexing for 10 sec. Spin down briefly.

4.10.4 Transfer 20 µL of the bead-bound DNA (PCR template) to each well/tube containing 30 µL Post-Capture PCR Master Mix. Mix well.

4.10.5 Amplify the enriched Multiplex DNA Samples using the thermocycling profile given in the next table. The total number of Post-Capture PCR cycles is dependent on the Capture Target Size.

Step	Temp	Time	Cycles[1]
Initial Denaturation	98ºC	45 sec	1
Denaturation	98ºC	15 sec	Variable (see table below)[1]
Annealing	60ºC	30 sec	
Extension	72ºC	30 sec	
Final Extension	72ºC	1 min	1
HOLD	4ºC	∞	1

Capture Target Size	Number of Post-capture PCR Cycles[1]
<100 kb	18
100 kb - 2 Mb	16
>2 Mb - 40 Mb	10
>40 Mb	8

[1]Numbers of post-capture PCR cycles are recommendations and may have to be optimized depending on individual experimental needs.

4.10.6 Once post-capture amplification has been completed, proceed immediately to **Purification of Amplified, Enriched Multiplex DNA Sample**.

4.11 Purification of Amplified, Enriched Multiplex DNA Sample

4.11.1 Equilibrate the KAPA HyperPure Beads to room temperature before use. Mix well to obtain a homogeneous solution before use.

4.11.2 Place the plate/tube(s) containing the amplified, enriched Multiplex DNA Sample(s) on the magnetic stand to capture the beads. Incubate until the liquid is clear. Transfer each supernatant (50 µL) to a new well or tube.

4.11.3 Add 70 µL of KAPA HyperPure Beads to each amplified, enriched Multiplex DNA Sample.

4.11.4 Mix thoroughly by vortexing for 10 sec to ensure that the mixture is homogeneous. Spin down briefly. Incubate at room temp for 5 min to allow the Multiplex DNA Sample to bind to the beads.

4.11.5 Place the plate/tube(s) containing the bead-bound DNA on the magnetic stand to capture the beads. Incubate until the liquid is clear.

4.11.6 Carefully remove and discard the supernatant without disturbing the beads.

4.11.7 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of 80% ethanol.

4.11.8 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.11.9 Carefully remove and discard the ethanol.

4.11.10 Keeping the plate/tube(s) on the magnetic stand, add 200 µL of 80% ethanol.

4.11.11 Incubate the plate/tube(s) on the magnetic stand at room temperature for ≥30 sec.

4.11.12 Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads.

4.11.13 Dry the beads at room temperature for 3 – 5 min, or until all of the ethanol has evaporated. Be careful not to over dry the beads as this may result in reduced yield.

4.11.14 Remove the plate/tube(s) from the magnetic stand.

4.11.15 Thoroughly resuspend the beads in 22 µL of Elution Buffer (10 mM Tris-HCl, pH 8.0) or nuclease-free water.

4.11.16 Vortex for at least 10 sec to ensure that all of the beads are resuspended. Briefly spin down.

4.11.17 Incubate the plate/tube(s) at room temperature for 2 min to elute the DNA off the beads.

4.11.18 Place the plate/tube(s) on a magnetic stand to capture the beads. Incubate until the liquid is clear.

4.11.19 Transfer 20 µL of each eluate to a new well/tube and proceed with **Post-capture Library QC**.

NOTE: At this point, the amplified, purified, post-capture libraries may be stored at 2°C to 8°C for 1 – 2 weeks, or at -15°C to -25°C (long term storage).

4.12 Post-capture Library QC

Evaluate post-capture libraries by determining the library fragment size distribution and post-capture library yield. For these assays, a 10-fold dilution of each final library (2 µL diluted to 20 µL in nuclease-free water) is recommended.

To determine the library fragment size distribution, analyze 1 µL of each diluted, post-capture library with an Agilent® Bioanalyzer 2100 instrument and DNA 1000 or DNA High Sensitivity Kit. Post-capture libraries are expected to have a fragment size distribution in the range of 150 – 1,000 bp, with an average fragment size in the range of 250 – 320 bp, as shown in Figure A3.

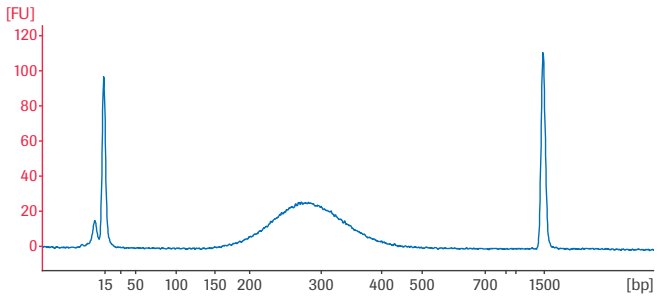

Figure A3. Expected size distribution of post-capture libraries. Sequencing-ready amplified, enriched Multiplex DNA Samples were prepared as described. The size distribution was assessed using an Agilent 2100 Bioanalyzer instrument and DNA 1000 Kit.

Use 5 µL of the 10-fold diluted library to determine the library concentration with a Qubit® Fluorometer and dsDNA HS Assay Kit (Thermo Fisher Scientific). Convert the concentration to mass. The final yield of each Multiplex DNA Sample library should be >100 ng.

DNA Sample libraries that meet all of the above criteria may be used for sequencing on MGI Sequencing Platforms. Refer to MGI protocols for ssCir and DNB generation.

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