Ontario Institute for Cancer Research

WGS Library Preparation - KAPA

Purpose

To define the Whole Genome Sequencing (WGS) library preparation procedure, using the KAPA Hyper Prep Kit.

Scope

Following extraction and initial Quality Control (QC), samples in the WGS workflow must undergo library preparation. A library consists of DNA fragments with ligated adapters and indices that is suitable for sequencing. This Standard Operating Protocol (SOP) outlines the procedure for creation of WGS libraries from extracted gDNA, using the KAPA Hyper Prep kit, and applies to all staff who perform WGS library preparation.

Responsibilities

Management:
Review and update procedure as required

QA Manager:
Monitor quality output from this procedure

Laboratory Staff:
Follow this procedure and report any non-conformances that may occur

Reagents and Consumables

Item Description	Vendor	Catalogue #
NA12878 DNA from LCL (gDNA positive control); GLCS_0002	Coriell	NA12878
KAPA Hyper Prep Kit	Roche	7962363001
High Sensitivity D1000 ScreenTape	Agilent	5067-5584
High Sensitivity D1000 Reagents	Agilent	5067-5585
NucleoMag® NGS Clean-up and Size Select beads	Macherey-Nagel	744970

This SOP has been approved for use by the OICR Genomics Medical Director.

or AMPure XP Beads	Beckman/Cedarlane	A36881 (60 ml)
100% Ethanol	Sigma-Aldrich	E7023
1X Low TE Buffer (10 mM Tris-HCl (pH 8.0), 0.1 mM EDTA	Life Technologies	12090-015
Ambion Nuclease-free Water	ThermoFisher	AM9937
Qubit dsDNA HS Assay	Life Technologies	Q32854
IDT xGen Stubby Adapters 15uM stock	Integrated DNA Technologies	10005924
IDT Dual Index (IDT unique indexing primer pool 20uM)	Integrated DNA Technologies	custom with standard desalting
Pipette Tips	Any Vendor	Various
Strip Tubes	Any Vendor	Various
96-Well Plate	Any Vendor	Various
Microcentrifuge Tubes (1.5mL)	Any Vendor	Various
High Sensitivity NGS Fragment Analysis Kit	Agilent	DNF-474
Standard Sensitivity NGS Fragment Analysis Kit	Agilent	DNF-473
Covaris 96 microTube plate	Covaris (via D-Mark)	520078

Note: Plastic consumables may be substituted for an approved equivalent product

Equipment

Item Description	Vendor	Catalogue #
Qubit 4.0 Fluorometer	Life Technologies	Q33226
TapeStation 2200	Agilent	G2964AA
TapeStation 4200	Agilent	G2991AA
Centrifuge	Eppendorf	Various

Fragment Analyzer Automated CE System 48/96 Cap	Agilent	FSv2-CE10F
c1000 Thermocycler	Bio-Rad	1841100
Mini-centrifuge	VWR/Fisher	C1413-VWR230, 05-090-100
Pipettes (mechanical)	Eppendorf, Gilson, Rainin	Various
Vortex	Fisher	02215365
Covaris E220	Covaris (via D-Mark)	COV-500239
Covaris M220	Covaris (via D-Mark)	COV-500295

Variables and Observations to Record

Record
Quantity of input DNA (ng)
Qubit quantification (ng/µL) (final library)
% Adapter contaminant (final library)
Library size (bp) (Tapestation or Fragment Analyzer)
KAPA kit reagent lots
Index and other reagent lots

Batch Controls

A no template control (NTC) and positive DNA control must be included in each library synthesis batch. DNA positive controls are purchased from the Coriell Institute (NA12878, MISO alias GLCS_0002). Control libraries are not sequenced but are included in **each** library synthesis batch and recorded in a MISO batch within the respective MISO CAP project(s). Control libraries must be synthesized at the same time as a production library batch. The following library information is recorded and logged in MISO for **all** libraries.

Sample Name	IDT Dual Indices	Insert Size (bp)	Qubit (ng/ul)
1) NTC negative control			

2) gDNA positive control (GLCS_0002)			

Important Considerations

Ensure that the following are used

- IDT xGen Stubby Adapters
- IDT Dual Index (custom order, no cat#)
- Number of Library Amplification cycles **(12)**
- Use freshly-prepared 80% ethanol

Reagent Lots and Expiration

- Prior to starting an assay, inspect reagents to ensure that they have not expired.
- Lot numbers for critical reagents must be recorded in MISO.

Procedure

1. DNA Shearing

Shearing can be performed using either the Covaris M220 (individual samples) or E220 (larger batches) at the discretion of the operator and/or Production Manager. Brief instructions for each instrument are provided below, with detailed instructions appearing in the TM. Covaris M220_E220 Use and Maintenance SOP.
Note: E220 degassing takes approximately 2 hours, so plan experiments accordingly.

An input amount of 100 ng for FFPE tumor or buffy coat gDNA or 25ng of fresh frozen tumor gDNA is used for shearing in a total volume of 50 ul Low TE. Remember to include both the gDNA positive (GLCS_0002) and NTC negative controls.

Instructions for Covaris M220

1. Transfer DNA solution to a Covaris microTUBE-50 AFA Fiber Screw-Cap (PN520166).
   a. Pipette slowly and carefully to avoid creating bubbles in the tube.
2. Load microTUBE into the M220 insert, lower tube lever, and close the chamber door.
3. For Kapa Hyper Prep WGS, use the following parameters:
   a. M220 Shearing Parameters(WGS)
   b. Shearing Program Name: **DNA_0550_bp_microtube-50_CAP**
   c. Target Shearing Size: **~550bp**
   d. Temperature Range:
      Min: 18C/ **Set Point**:20.0C / Max: 22.0C

Running Time	Peak Power	Duty Factor	Cycles/Burst
40 Seconds	75.0	10.0	200

4. After verifying parameters, select "**Run**"
5. Once run is complete, remove microTUBE and dry with Kim wipes

   SAFE STOP: The Covaris microTUBE-50 or E220 96 microtube plates are **not** designed for long term storage. Samples must be transferred as soon as possible to a new PCR plate or strip tube, which may be stored at -20°C for a maximum of 5 days.

Instructions for Covaris E220

1. Transfer the total volume into individual wells of the Covaris 96 microTube plate (no.520078).
2. Briefly centrifuge shearing plate once all samples have been added.
3. Ensure the correct E220 shearing protocol is used: **CAP - KAPA Hyper Prep WG Library Prep 60s**
4. E220 shearing parameters:
   - Peak Power 140W
   - Duty Factor: 10
   - Cycles/burst 200
   - Time: 60 seconds
   - Temperature Range: 4-7℃
5. Briefly centrifuge and transfer sheared DNA from Covaris microTUBE-50 or E220 96 microtube plate to PCR tubes for End Repair and A-tailing (ER &AT).

   SAFE STOP: The Covaris microTUBE-50 or E220 96 microtube plates are **not** designed for long term storage. Samples must be transferred as soon as possible to a new PCR plate or strip tube, which may be stored at -20°C for a maximum of 5 days.

2. End Repair and A-tailing (ER & AT):

Plan Ahead

- Thaw IDT xGen Stubby Adapter 15 uM on ice during ER/AT incubation, in preparation for **Step 3, Adapter Ligation.**
- Thaw IDT index primer pool 20uM (unique dual index) on ice during ER/AT incubation, in preparation for **Step 5, Library Amplification.**
- Warm Nucleomag (or AMPure) beads to room temperature for at least 30 min, in preparation for **Step 4, SPRI Purification 1**.

1. **Prepare "ER & AT Mix" master mix, pipette to mix and keep on ice**

ER & AT Mix	**1x**
End Repair & A-Tailing Buffer	7 uL
End Repair & A-Tailing Enzyme	3 uL
Total ER & AT Mix	**10 uL**

2. Add **10uL** of **ER & A-T Mix** to 50 uL of sheared DNA from step 1.5; 60ul total rxn volume.
3. Pipette to mix and centrifuge briefly.
4. Incubate in a thermal cycler using program "CAP WG ER AT". Proceed **immediately** to Ligation once "CAP WG ER AT" program completes.

Thermocycler Conditions:
- Program Name: CAP WG ER AT
- Reaction Volume: 60 uL
- Cover Temperature: 85 °C

Step	**Temperature (⁰C)**	**Time**	**Cycle1**
1	20	30 min	1
2	65	30 min	1
3	4	Hold	1

3. Adapter Ligation

Plan Ahead
- Thaw 2X KAPA HiFi HotStart ReadyMix for use in **Step 5, Library Amplification.**

1. **Prepare the "Adapter Ligation Mix", pipette to mix, and keep on ice**

Adapter Ligation Mix	**1x**
Ligation Buffer	30 uL
DNA Ligase	10 uL
PCR-grade water	5 uL
IDT xGen Stubby Adapter 15 uM	5uL
Total Adapter Ligation Mix	**50 uL**

2. Add **50ul** of prepared '**Adapter Ligation Mix**' to the "ER & AT" Product (60ul) for each sample and pipette to mix.

ER & AT Product	60 uL
Adapter Ligation Mix	50 uL
Total Mix	**110 uL**

3. Centrifuge briefly and incubate at **20^oC** for 15 min, using thermal cycler program: CAP WG Ligation

   Thermocycler Conditions:
   - Program Name: CAP WG Ligation
   - Reaction Volume: 110 uL
   - Cover Temperature: Off (not heated)

Step	Temperature (^oC)	Time	Cycle1
1	20	15 min	1
2	4	Hold	1

4. Proceed **immediately** to next step.

4. SPRI Purification 1

1. Add **66uL** of NucleoMag (may substitute with AMPure) beads to each 110 uL ligation reaction product for a 0.6X SPRI clean-up.
2. Pipette to mix and incubate at room temperature for 7 min.
3. Place on the magnet and allow the solution to clear (3-5min).
4. Remove supernatant without disturbing beads.
5. Add 200 uL of fresh 80% EtOH while still on magnet and let sit for 1min, then remove EtOH.
   a. Repeat for a total of 2 washes.
6. Dry beads at room temperature for 3-5 min or until all residual ethanol evaporates*
   a. ***Over drying beads may reduce yield.**
7. Add **21uL** of 1X Low TE to each sample, remove from magnet, and resuspend beads. Incubate at room temperature for 4min.
8. Place tubes/plate back on magnet until liquid is clear and beads bound to magnet (2-3 min). Transfer the supernatant (20uL) to a new PCR well. Do not transfer beads. If residual beads are transferred, bind to magnet and transfer again.

5. Library Amplification

1. Record index well location for each library for entry in MISO; each IDT index primer pool (20uM) contains dual indexes.
2. Add **25 uL** of KAPA HiFi HotStart to each sample.
3. Add **5 uL** of IDT Index to each sample.

2X KAPA HiFi HotStart ReadyMix	25 uL
IDT index primer pool 20uM (unique dual index)	5.0 uL
Purified Adapter Ligated Library	20 uL
TOTAL REACTION	**50 uL**

4. Pipette to mix, centrifuge briefly, and run the following program:

Thermocycler Conditions:
- Program Name: CAP WG PCR Amplification
- Reaction Volume: 50 uL
- Cover Temperature: 105°C

Step	Temperature (C)	Time	Cycles
1	98	45 seconds	1
2	98	15 seconds	12
3	60	30 seconds	
4	72	30 seconds	
5	72	60 seconds	1
6	4	Hold	1

Proceed directly to post-amplification clean-up (**Spri Purification 2)**.

Plan Ahead
- Thaw Qubit standards and warm TapeStation or Fragment Analyzer Reagents to room temperature for use in **Step 7, Library Quantity and Quality Assessment.**

6. SPRI Purification 2

1. Add **40uL** of NucleoMag (may substitute with AMPure) beads to each 50 uL PCR reaction product for a 0.8X SPRI clean-up.
2. Pipette to mix and incubate at room temperature for 7 min.
3. Place on the magnet and allow the solution to clear (3-5 min).
    a. Remove supernatant without disturbing beads.
4. Add 200uL 80% EtOH while still on magnet and let sit for 1 min, then remove EtOH.
5. Repeat for a total of 2 washes
6. Dry beads at room temperature for 3-5 min or until all residual ethanol evaporates*
    a. ***Over drying beads may reduce yield.**
7. Add **21uL** 1X Low TE to each sample well while on magnet. Remove plate/tube from magnet and resuspend beads. Incubate at room temperature for 4 min.
8. Place the tube/ plate back on the magnet until clear (3-5 min), then transfer the supernatant (20uL) to a new well/tube. Do not transfer beads. If residual beads are transferred, bind to magnet and transfer again.
9. Store at -20°C or proceed to library **Quantity and Quality Assessment.**

7. Library Quantity and Quality Assessment

1. Assess the library quantity by running **1 uL** of each library on Qubit dsDNA HS Assay.
    a. Refer to the TM. Genomics Qubit Fluorometric Quantitation SOP, located on the Genomics Quality SharePoint.
2. Library Quality may be assessed using either the TapeStation or Fragment Analyzer.
    a. Assess library quality using the TapeStation (High Sensitivity Tape).
        i. Refer to the TM. High Sensitivity TapeStation Assay SOP, located on the Genomics Quality SharePoint.
    b. Assess library quality using the Fragment Analyzer.
        i. Refer to the TM. Fragment Analyzer Assays SOP, located on the Genomics Quality SharePoint.
3. Store at -20°C.

8. LIMS Entries

1. For general MISO instruction and training, see TM. LIMS Usage - MISO.
2. Propagate one or more MISO Libraries from their respective gDNA Aliquots. If using the MISO bulk edit function, ensure that the order of samples has not been changed in MISO when propagating samples. Enter the following information for each Library:
    - Matrix Barcode: Use barcode scanner while column is selected
    - Box Alias and Position: Use Box Search column to find correct Box Alias
    - Creation Date: Defaults to current date, but set to date of library creation
    - SOP: Set to WG Library Prep – KAPA Hyperprep v.X (see version at the footer of SOP)
    - Thermal Cycler

- Design: Set to WG
    - Design tells analysis pipeline to do alignment against a whole genome
- Platform: Set to Illumina
- Type: Set to Paired End
- Index Kit: Set to IDT Dual Index UDI
- Index 1: Set to the index used for the library. Will autofill Index 2
- Kit: Set to KAPA Hyper Prep
- Kit Lot: The lot number of the KAPA Hyper Prep Kit
- QC Passed: See Library Quality Assessment for criteria
- Size (bp): Determined during Library Quality Assessment
- Volume: Set to 18 uL, unless elution volume was less than expected
    - Elution volume is 20 uL, but 2 uL are used for QC
- Concentration Determined during Library Quality Assessment
- GroupID: Do not enter a groupID unless you are certain that it is required (e.g. for re-validation). Group ID is inherited has been set during sample accessioning.
    - GroupIDs are used to determine which entries are top-ups: https://wiki.oicr.on.ca/x/6ARdBw
    - WGS and WT GroupIDs must match for analysis to proceed.

3. Propagate Library Aliquot. Prepare a 15 uL aliquot at 5 ng/ul of the stock library and propagate a matching MISO Library Aliquot from the MISO Library:
    a. Matrix Barcode: Use barcode scanner while column is selected
    b. Box Alias and Position: Use Box Search column to find correct Box Alias
    c. QC Passed: Set to True
    d. Conc. 5 ng/uL
    e. Volume: 15 uL
    f. Parent ng Used: Record the amount of stock library used to create the dilution
    g. Parent Vol. Used: Record the volume (uL) of stock library used to create the dilution
    h. GroupID: Do not enter a groupID unless you are certain it is required (e.g. for re-validation). Group ID is inherited and has been set during sample accessioning.
        i. GroupID is used by the analysis pipeline to determine which samples/libraries belong together, e.g. to determine which entries are top-ups: https://wiki.oicr.on.ca/x/6ARdBw
        ii. WGS and WT GroupIDs must match for analysis to proceed
    i. Creation Date: Defaults to current date, but set to date of dilution/aliquot creation
    j. Prior to saving Libraries, check that all information is correct and that the MISO entry matches the LIMs tracking sheet
4. Add Library QC to all stock libraries that were prepared during the same library synthesis batch:
    a. Select all stock libraries prepared together in the same batch
    b. Select Add QCs: In the pop up window, enter 2 for QCs per Library (Qubit, TapeStation or Fragment Analyzer) and 2 Controls per QC (Positive control, No Template Control)

    c. Fill in the two QC areas per Library, ensuring each individual Library has both QC values entered
    d. Control 1: Assign as positive control with the lot number of the control used and indicate if the control passes
    e. Control 2: Assign as negative control, enter the lot number of nuclease-free water used and indicate if control passes
    f. When all QC metrics have been entered, review to ensure all QC values are paired to the correct Library, and then Save
5. Attach batch QC files to all stock libraries that were prepared during the same library synthesis batch:
    a. Select all stock libraries prepared together in the same batch
    b. Select Attach Files: Upload New Files
    c. Category:
        i. LIMS tracking sheet for scanned batch worksheet
        ii. Misc for Qubit csv file
        iii. TapeStation for run report
    d. Choose files: Attach the appropriate file corresponding to the category previously selected. Only one file can be uploaded at a time
    e. Upload
    f. Repeat previous steps until all three Batch QC files have been uploaded.

6. After all QC has been entered and the appropriate files have been uploaded Library aliquots can be passed to GRP as follows for sequencing:
    a. Move the Library aliquot tubes into the GRP “CAP Miseq Inbox” BOX1580 and update the locations in MISO
    b. Add the LDI for every sample passed on for sequencing into the Workset- GRP MiSeq QC Queue (For CAP-stream NovaSeq 2x151) in MISO.
    c. Send an email to GenomicsLibrarySubmissions@oicr.on.ca with the LDI’s that were added to the Workset to alert the sequencing team that there are CAP libraries pending sequencing.