TECHNICAL NOTE FOR KARYOSOLVER PRO

Use this technical note for the preparation of hWGS library adapted to Karyosolve analyses.

5. REQUIRED MATERIAL NOT INCLUDED

5.1 Generic Material

- Computer with constantly updated and guaranteed secure internet connection.
- Micropipettes calibrated and periodically verified 0.2-2 µl, 2-20 µl, 20-200 µl or 100-1000 µl and filter tips.
- Vortex.
- Disposable Gloves without powder.
- Thermal cycler calibrated and periodically verified.
- PCR tubes and Caps or 96-wells plate, as needed, DNase and RNase free.
- Dry bath.
- Nuclease-free water.
- 1.5 ml tube magnetic separator or 96-wells plate compatible magnetic separator.
- Fresh 80% ethanol.
- Magnetic beads for the purification of genomic libraries. We recommend to use AMPure XP (Beckman Coulter, cod. A63880) or MAGTIVIO (magSi-NGSPREP Plus, cod. MDKT00010075).
- Nanopore sequencers calibrated and periodically verified.
- 4bases kit hWGS cod: RDC3290.

5.2 Specific Material

The material listed below has been used and validated by 4bases:

- Qubit™ 2.0 Fluorometer or Qubit™ 3.0 Fluorometer or Qubit™ 4.0 Fluorometer calibrated and periodically verified.
- Qubit™ assay tubes.
- Qubit™ dsDNA HS Assay Kit.
- Qubit™ dsDNA BR Assay Kit.

QUALITATIVE ANALYSIS OF DNA (Optional)

- Agilent 2100 Bioanalyzer system with DNA reagent kit calibrated and periodically verified or similar instrument calibrated and periodically verified.

6. IMPORTANT NOTES AND SAFETY INFORMATION

The user is required to apply the following provisions.

- The kit is for professional use, it must be used by trained professionals in molecular biology.
- Do not use if package damaged.
- Biological samples and all reagents should be used in properly equipped rooms, clean and clear of potential contaminants. We suggest cleaning working areas frequently using a solution containing sodium hypochlorite 1-5%.
- Always use safety equipment such as laboratory coat, gloves and safety goggles during all steps described in the protocol.
- Check the risks and safety procedures associated with instruments, electricity, chemicals and other resources applied to the use of the device.
- To avoid contamination of reagents we recommend using DNase/RNase free tubes, filter tips and to pay particular attention to keep all instruments clean and free of contaminants.

- We suggest preparing a unidirectional workflow from the initial phase of DNA isolation following the PCR preparation phase, amplification and post-amplification phases in order to keep working areas separated for the different phases of the procedure using for each phase dedicated laboratory coats, micropipettes, tubes and filter tips.
- Used reagents and biological samples must be wasted according to legal procedures.
- Verify that transportation and storage have been carried out accordingly to the indication of the manufacturer. In case the customer is suspicious that those have not been followed, consider that the results of the tests may not be correct.

➢ **Stopping point**: every time is present a stopping point you can proceed with the following step, or store the samples at 4°C for 24 hours or -20°C for a longer period.

7. LIBRARY PREPARATION

7.1 DNA SAMPLES PREPARATION

Use any commercial kit to obtain DNA from biological tissues. Determine the starting DNA concentration by fluorometric methods for accuracy.

• Use the Thermo Fisher Scientific Qubit dsDNA BR Quantitation Assay kit to accurately quantify the purified gDNA input.

• DNA must be suspended in water for molecular biology, Tris-HCl 10 mM pH 8.0 or EB buffer.

• Accurate DNA input is critical to achieve optimal library fragment yield and length.

• The recommended DNA input is 50 ng, in a final volume of 40 µl

7.2 DNA FRAGMENTATION, END REPAIR AND dA-TAILING

Perform enzymatic fragmentation of gDNA and subsequent dA-tail and end repair to generate dA-tailed DNA fragments.

Reagents required:

- DNA: 50 ng per sample.
- Molecular biology water.
- Qubit dsDNA HS o BR Assay (or equivalent).
- Frag/AT Enzymes (Tube 1).
- Frag/AT Buffer (Tube 2).

Before starting:

Defrost or place on ice:

- Frag/AT Buffer (Tube 2).
- Frag/AT Enzymes (Tube 1).

Make sure the genomic DNA samples are of high quality with an OD 260/280 ratio between 1.8 and 2.0. Use the Qubit system to quantify genomic DNA prior to library preparation.

STEP 1: PREPARE THE THERMOCYCLER, SAMPLES AND REAGENTS

Program the thermal cycler with the following conditions. Set the lid temperature at 105°C. Start the program to pre-cool the thermal cycler as shown below in Table 1.

Note: 5 minutes at 30°C is the condition for samples to obtain an average fragment size between 500 and 600bp.

Stage	Temperature (°C)	Time	Cycles
Stage 1	4	HOLD	1
Stage 2	30	5 min	1
	65	30 min	
Stage 3	4	HOLD	1

Table 1: PCR thermal profile.

WARNING: It is not recommended to measure DNA concentration by absorbance at 260 nm.

Mix the DNA gently. Assay the DNA samples, using the Qubit dsDNA HS or BR Assay kit, to determine their concentration.

Transfer 50 ng of DNA sample in 40 µl of final volume inside a 0.2 ml PCR-strip or inside a well of a 96-well PCR plate.

Spin to make sure all the solution is at the bottom of the tube and place on ice.

STEP 2: FRAGMENTATION, END REPAIR, AND dA-TAILING

Vortex the Frag/AT Buffer tube (Tube 2) for 5 seconds. Spin to make sure all solution is at the bottom of the tube.
Invert the Frag/AT Enzymes tube (Tube 1) a minimum of 5 times to homogenize. Spin to make sure all solution is at the bottom of the tube.
Prepare the Fragmentation Mix in a 1.5-ml microcentrifuge tube kept on ice as reported in Table 3 below.

Mix thoroughly by gently pipetting, avoiding the formation of bubbles.

REAGENT	VOLUME PER REACTION
Frag/AT Buffer (Tube 2)	4 µl
Frag/AT Enzymes (Tube 1)	6 µl
Total	**10 µl**

Table 2: Quantity of reagents for each reaction.

Add 10µl of Fragmentation Mix to each DNA sample. Mix thoroughly by gently pipetting, avoiding the formation of bubbles. Seal the plate or cap the strip/tube and place it on ice.

WARNING: Thorough mixing is critical to achieving desired fragment lengths.

Spin and transfer immediately to the pre-cooled thermal cycler. Start the thermal cycler program from stage 1 to stage 2 as shown in Table 1.

WARNING: While the thermal cycler program is running, prepare the reagents for point 7.3: Ligation of Universal Adapters and Purification **(read before beginning)**.

When the thermal cycler program is complete and the block has reached 4°C, remove the samples and place them on ice.

Proceed immediately to point 7.3: LIGATION OF UNIVERSAL ADAPTERS AND PURIFICATION.

7.3 LIGATION OF UNIVERSAL ADAPTERS AND PURIFICATION

In this phase, the universal adapters are ligated to the DNA fragments with dA tail obtained in point 7.2, once purified they are ready for the introduction of the Indexes by PCR.

Reagents Required

- dA-tailed DNA fragments obtained in point 7.2.
- Ethanol.
- Water for molecular biology.
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution).
- Ligation Master Mix (Tube 3).
- Universal Adapters (Tube 5).
- Magnetic beads.

Before starting:

Defrost or place on ice:

- Universal Adapters (Tube 5).
- Ligation Master Mix (Tube 3).
- Prepare 1 ml of 80% ethanol for each sample.
- Equilibrate the Magnetic Beads at room temperature for at least 30 minutes.
- Program the thermal cycler to incubate samples at 20°C with the lid temperature set to the minimum or turned off. Start the program so that the thermal cycler has reached 20°C at the end of sample preparation.

STEP 1: LIGATION OF UNIVERSAL ADAPTERS

Add 5 µl of Universal Adapters (Tube 5) to each tube or well containing the dA-tailed DNA fragments obtained in point 7.2. Mix thoroughly by gently pipetting and place on ice. Invert the Ligation Master Mix (Tube 3) a minimum of 5 times to homogenize.

WARNING: *Do not vortex the Ligation Master Mix.*

Add 20 µl of Ligation Master Mix (Tube 3) to each sample obtained in point 7.2, mix thoroughly by gently pipetting. Seal the plate or cap the strip and spin to ensure all solution is at the bottom of the tube/well.

REAGENT	VOLUME PER REACTION
Universal adapters (Tube 5)	5 µl
Ligation Master Mix (Tube 3)	20 µl
Total	**25 µl**

Table 3: Quantity of reagents for each reaction.

Incubate the ligation reaction at 20°C for 15 minutes in the thermal cycler, at the end take the samples and proceed with the purification.

Stage	Temperature (°C)	Time	Cycles
Stage 1	20	15min	1

Table 4: Ligation thermal profile.

WARNING: *While the thermal cycler program is running, prepare the reagents for point 7.4: Amplification with UDI primers, purification and quality control.*

STEP 2: PURIFICATION

a) Vortex the pre-equilibrated magnetic beads at room temperature until they are well homogenized.
b) Add 60 µl of homogenized magnetic beads (0.8X) to each sample obtained in the previous step.
c) Incubate the samples for 5 minutes at room temperature.
d) Place the samples on the magnetic support for 1 minute or until the supernatant is clear.
e) The beads form a pellet, leaving a transparent supernatant. Without removing the plate or tubes from the magnetic support, remove and discard the supernatant.
f) Wash the pellet of beads by gently adding 200 µl of fresh 80% ethanol (without disturbing the pellet). Incubate for 1 minute, then remove the ethanol.
g) Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic support. Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the beads pellet.
h) Ensure that all ethanol is removed; put the tube on the thermal bath at 37˚C for 3 minutes, then remove the tube from the thermal bath and incubate 1 or 2 minutes at room temperature, paying attention to not letting crack the beads pellet.
i) Elute the DNA in 17 µl of RNase/DNase free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension with the DNA.
j) Incubate 5 minutes at room temperature.
k) Place tubes on magnetic support and incubate for 2 minutes or until the mixture appears clear; carefully collect 15 µl of the supernatant containing the desired amplicons without touching the pellet.

Proceed with point 7.4: AMPLIFICATION WITH UDI PRIMER, PURIFICATION AND QUALITY CONTROL.

7.4 AMPLIFICATION WITH UDI PRIMERS, PURIFICATION AND QUALITY CONTROL

In this step, the DNA libraries containing the adapters are amplified with the UDI primers, purified and quality control (QC) is performed to complete the protocol.

Reagents Required

- Ligated libraries and containing the adapters from point 7.3.
- Fresh 80% ethanol.
- Magnetic Beads equilibrated.
- Water for molecular biology.
- 10 mM Tris-HCl pH 8 or Buffer EB (optional, for elution).
- Equinox Library Amp Mix (2X) (Tube 4).
- UDI primers.

Before starting

Defrost or place on ice:

- Equinox Library Amp Mix (2X) (Tube 4).
- UDI Primers.

STEP 1: PREPARE THE THERMOCYCLER AND SET UP PCR

Stage	Temperature (°C)	Time	Cycles	Ramping (°C/sec)
Stage 1	98	45 sec	1	4
Stage 2	98	15 sec	8	4
	58	30 sec		1
	72	40 sec		1
Stage 3	72	40 sec	1	4
Stage 4	4	HOLD	1	4

Table 5: PCR thermal profile.

Add 10 µl of UDI Primer contained in the 96-well plate, to each DNA library obtained in point 7.3, mix well by gently pipetting.

WARNING: *To mix, invert the Equinox Library Amp Mix tube (2X) (Tube 4) 5 times before use. Do not vortex.*

Add 25 µl of Equinox Library Amp Mix (2X) (Tube 4) to the DNA library from point 7.3 and mix well by gently pipetting.

Spin the plate or strips and immediately transfer them to the thermal cycler and start the program. At the end of the PCR, remove the samples and proceed with purification.

STEP 2: PURIFICATION

a) Vortex the pre-equilibrated the Magnetic Beads at room temperature until they are well homogenized.
b) Add 30 µl of homogenized Magnetic Beads (0.6X) to each sample obtained in the previous step.
c) Incubate the samples for 5 minutes at room temperature.
d) Place the samples on the magnetic support for 1 minute or until the supernatant is clear.
e) The beads form a pellet, leaving a transparent supernatant. Without removing the plate or tubes from the magnetic support, remove and discard the supernatant.
f) Wash the pellet of beads by gently adding 200 µl of fresh 80% ethanol (without disturbing the pellet). Incubate for 1 minute, then remove the ethanol.
g) Repeat the wash one more time, for a total of two washes, keeping the samples on the magnetic support. Carefully remove all remaining ethanol with a 10 µl pipette, making sure not to disturb the beads pellet.
h) Ensure that all ethanol is removed; put the tube on the thermal bath at 37˚C for 3 minutes, then remove the tube from the thermal bath and incubate 1 or 2 minutes at room temperature, paying attention to not letting crack the beads pellet.
i) Elute the DNA in 27 µl of RNase/DNase free distilled water; pipet up and down 5 times to thoroughly mix the beads suspension with the DNA.
j) Incubate 5 minutes at room temperature.
k) Place tubes on magnetic support and incubate for 2 minutes or until the mixture appears clear; carefully collect 25 µl of the supernatant containing the desired amplicons without touching the pellet.

STEP 3: QUALITY CONTROL

Quantify samples with the Thermo Fisher Scientific Qubit dsDNA HS or BR Assay kit and verify the size of each library on the Agilent DNA 12000 assay chip.

Using 50 ng of good quality gDNA, fragmented for 5 minutes at 30°C and amplified for 8 cycles of PCR should result in a final concentration ≥15 ng/µl. Under these conditions, the average fragment length observed is generally between 500 and 600bp.

➢ **STOPPING POINT**

NOTE: *If not proceeding immediately, store the spiked library sample at –20°C for up to 24 hours.*

NOTE FOR NANOPORE SEQUENCING: Refer to the document IFU_M1050 to continue the protocol for sequence the libraries on Nanopore sequencer.