# gDNA Kapa Quarter Protocol with Indexing PCR

## Initial notes

## ***Before beginning, please read the entire KAPA Hyper Prep Kit protocol that this document is based on***

- What is true for all library preparation methods is just as true here: the vast majority of researcher time and effort should go into obtaining the best quality DNA fragmented to the optimal range. Take your time to get it right here and it will pay off with easier library preparation and better quality data.

- SPRI beads and PEG/NaCl solution should only be used at room temperature.

- Fresh 80% ethanol should be diluted each day.

- Safe stopping points: The protocol can be paused after sonication, post-sonication size selection, and post-ligation bead clean-up. Do not stop after end repair.

- Always ensure that KAPA Hyper Prep Kit components have been fully thawed and thoroughly mixed before use. The KAPA Hyper Prep End Repair & A-tailing Buffer and Ligation Buffer may contain precipitates when thawed at 4°C. These buffers must be thawed at room temperature and vortexed thoroughly before use.

- Reaction master mixes prepared from the enzymes and buffers for end repair and A-tailing, as well as for ligation, are very viscous and require special attention during pipetting. Keep all enzyme components and master mixes on ice as long as possible during handling and preparation.

- Always thaw the adapter stub on ice. Do not warm in hands or leave at room temperature for long periods.

# Sonication

*Before using the qSonica instrument for the first time, consult with the lab manager for proper training.*

1) In a qSonica 0.2 mL tube, dilute a 100µL aliquot of each sample to 10 ng/µL (by qubit) or 15 ng/µL (by nanodrop) using 1x LTE (10mM Tris, 0.1 mM EDTA), 1x TE or EB(T) (10mM Tris with or without 0.05% tween). Make sure tubes are completely closed. Spin down samples to keep all liquid at the bottom. Keep samples very cold before sonicating. (It is okay to freeze them; then they can be thawed just before beginning sonication.)

[Note: this protocol can use less starting material if 1 µg is not available. It is best to keep the mass of material within each 18 sample batch as similar as possible. The sonication tube volume should always be identical within each batch)

2) Ensure that all tubing is properly connected. Add ~1.5 L of cold deionized water to the qSonica clear bath. Turn on the water cooler and wait 5-10 to allow the system to cool to 4°C. (If the bath is filled with room temperature water, it will take 15-20 minutes to cool.)

3) Turn the water adjustment dial to "-" to add water to the reservoir in back. Fill this to ~50% full. The water level inside the bath should be ~2 cm above the titanium horn. If not, add more cold water to achieve this level.

4) Turn on the power supply ("|" icon on the top right). Ensure that the cabinet door is latched closed. Select the "degas" program which will run for 10 minutes.

5) While degassing, load your samples into the blue 18-place tube holder. (If you have < 18 tubes to sonicate, fill empty spaces with blank tubes containing water.) Cover with the white donut and screw on the top section. Leave the assembly on ice or at +4C until ready for use.

6) Sonication times will vary by DNA size, genome size, tube volume, and extraction method. The following is just an example protocol. Before you begin, please consult with the lab manager for the best trial conditions to select based on other recent results. Additional user validated protocols can be found here:
https://www.sonicator.com/pages/publications-and-protocols-chromatin-dna-shearing
(Note: in all cases qSonica and users report the "Total Sonication On Time". A protocol using a pulse such as 15s on/15s off will take twice as long to complete)

7) After degrassing, use the setting on the power supply to set the sonication conditions:
Example:
Timer = 3:00 (total sonication on time)
Pulse = 15s ON / 15s OFF
Amplitude = 25%

8) Attach the sample rack to the lid of the sonicator bath. Make sure the lid is plugged in.

9) Assess the bath water level by eye and adjust using the water adjustment dial if it is

too high or too low. The water level in the bath should match that of the sample tubes as closely as possible. However, if they are not a perfect match it is better if the water level of the bath is slightly below the water level of the sample tubes. (Otherwise excessive splashing may occur.)

10) Close the cabinet door and use the red start/stop button to start the sonication run. Check that you see only minor splashing during the first ON cycle. You can adjust the water level while sonication is proceeding if small adjustments need to be made.

11) Optional but recommended: pause the sonication process using the blue button midway through in order to spin down any splashing in your tubes. This will result in more even sonication and very little residual HMW DNA.

12) After sonication is complete, open the cabinet and remove the sample rack. Spin down tubes and either proceed directly to bead cleaning or store frozen until ready to proceed.

13) When optimizing, after sonicating, take an aliquot (5-10 µL) of sample to run on an agarose gel to assess the sizing pattern. This is not necessary once the sonication parameters have been worked out for your project.

14) Continue with the next set of tubes to sonicate. When you are done for the day, turn off the chiller pump and use the “0” button on the power supply to turn it off. Consult with the lab manager about when and how to empty the water from the batch.

# Post-Sonication Double-sided Size Selection

The following protocol was used successfully for a double-sided selection centered around 350bp, using 0.5x for right-side selection and 0.65x for left-side. However, the best bead ratios will be dependent on the shearing profile of your samples. Initial testing of size selection ratios on non-essential samples is recommended before beginning.

**This protocol follows the notation of an Rx/Lx double-sided clean-up, where R is the right-side ratio and L is the left-side ratio.**

1) Resuspend your aliquot of **low-ratio formula** SPRI (Sera-Mag) bead suspension by inverting or vortexing. Take the bead tube out to warm up to room temperature (~30 min)

2) Standardize all reactions/extractions to the same volume (V) by adding water to your samples if necessary

3) Add SPRI bead suspension to samples as follows reaction to start the right-side clean-up:
   A) Add an **R-fold volume** of SPRI bead suspension to each (ex: if V=100µL and R=0.5, add 50µL low-ratio formula SPRI beads to each sample).
   B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.
   C) Let the tubes incubate for **15 minutes** at room temperature.
   D) While the samples are incubating, label a new set of 0.2mL strip tubes for the supernatant in step 5.

4) Briefly (0.5s) spin down the contents of the tubes. Place them in a magnetic plate or stand and let sit for **3 minutes** (or until the supernatant is clear) to separate the beads from solution.

*****Reminder: when you remove the supernatant from the beads in step 5, keep the liquid. Do not discard it!*****

5) Carefully pipette the supernatant without removing or disturbing the beads and **move it to the new, empty strip tubes. Be very careful not to carry over any beads at this step.** (The tubes with the beads can then be discarded.)

6) Add **low-ratio formula** SPRI (Sera-Mag) bead suspension to the sample in the new tube as follows to start the left-side clean-up:
   A) If the second ratio is L, add an **L - R -fold volume** of low-ratio formula SPRI bead suspension to each reaction based on the original volume
   (ex: if the right-side ratio (R) is 0.5x and the left-side ratio (L) is 0.65x, add 0.15 * 100 µL or 15 µL).
   B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.
   C) Let the tubes incubate for **15 minutes** at room temperature.

7) Briefly (0.5s) spin down the contents of the tubes. Place them in a magnetic plate or stand and let sit for **3 minutes** (or until the supernatant is clear) to separate the beads from solution.

8) Pipette off the supernatant and discard without removing or disturbing the beads

9) Leave beads on magnet and wash with **200 µL** of freshly prepared **80% ethanol** (need to make a new dilution before every SPRI clean-up).
Let stand for **at least 30 seconds** and discard supernatant.

10) Repeat step 9.

11) Then use a small volume pipette (such as Rainin 20µL LTS pipette) to remove as much residual ethanol as possible without disturbing the beads. A toothpick can be used to soak up alcohol spots. Let the beads air-dry for **3-5 minutes** at room temperature without caps. Avoid overdrying which appears as cracking.

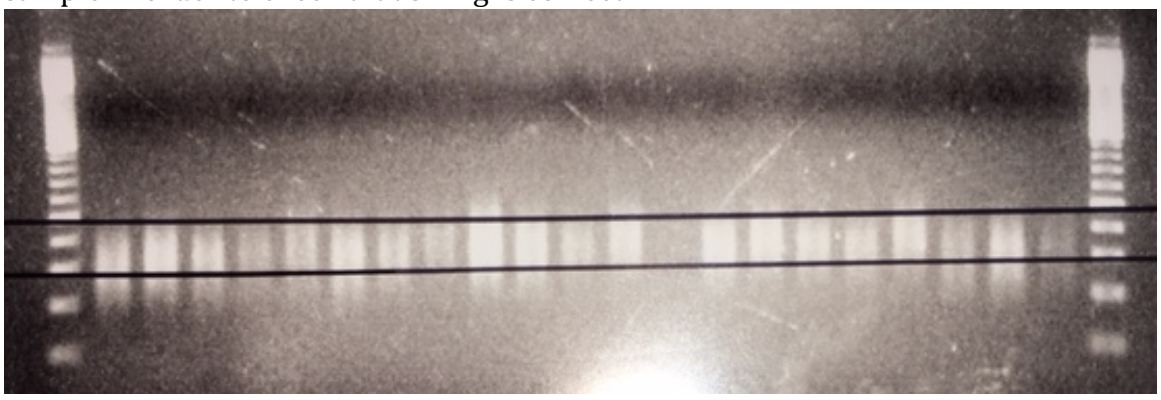
12) Elute as follows:
A) Remove the tubes from the magnetic rack. Add **12.5 µL** of **EB** to the wells and seal the tubes with caps.
B) Pipette up and down 10 times to mix well and/or seal the tubes and vortex gently. Ensure that the beads are resuspended and homogenized in the liquid.
C) Let the tubes incubate for **10 minutes** at room temperature.

13) Briefly (0.5s) spin down the contents of the tubes. Samples can stay in the bead solution if you do not need to assess them before continuing to end repair.

14) If assessment is required, place the plate back on the magnetic rack, let stand for **3 minutes** (or until the supernatant is clear) to separate the beads from solution.

15) Optional: remove 1 µL of clear DNA solution to qubit these samples to know how much DNA is available as input material for End Repair and A-Tailing

16) Optional: if sufficient material is available, run an agarose gel with ~1-2 µL of clear sample in order to check that sizing is correct



17) If required, add water to return the volume of the sample/bead solution to 12.5 µL

# End Repair and A-tailing

1) Remove the End Repair and A-Tailing reagents from the yellow Kapa kit on the top shelf of the 4122 freezer. Keep the enzyme on ice, and let the buffer thaw at room temperature.

2) Make a master mix composed of:

| | |
|---|---|
| End Repair & A-Tailing Buffer: | 1.75 µL |
| End Repair & A-Tailing Enzyme: | 0.75 µL |

Gently vortex and briefly spin before aliquotting. The master mix can be distributed into a strip tube in order to facilitate adding to samples with a multichannel pipette.

3) Add **2.5 µL of ERAT master mix** to each sample well to make a 15 µL reaction.

4) Then use a 20 µL multichannel to pipette each well up and down ~10 times to thoroughly mix the eluted bead solution and the ERAT master mix. Pipette gently to avoid introducing excessive bubbles. (Alternatively, seal tubes and gently vortex.)

5) Seal the tubes and briefly spin down in a centrifuge.

6) Incubate* in a thermocycler programmed as outlined below:

20°C: 30 minutes

65°C: 30 minutes

4°C: hold

*A heated lid is required for this incubation. If possible, set the temperature of the lid at tracking 10 °C above the block (PTC-200)

7) Return end repair enzymes to the yellow Kapa gDNA box and retrieve the Ligation Enzyme and Ligase Buffer. Leave the enzyme on ice, and let the buffer thaw at room temperature.

8) Leave the adapter stub to thaw on ice.

9) After the End-repair and A-tailing reaction is completed and the sample has cooled to 4°C, proceed to Adapter Ligation

# Adapter Ligation

1) Make a master mix composed of:

| | |
|---|---|
| Ligation Buffer: | 7.5 µL |
| Ligation Enzyme: | 2.5 µL |
| Adapter stub (50 µM) | 1.5 µL |
| Nuclease-free water | 3.5 µL |

2) Add **15 µL of Ligation master mix** to each sample well to make a 30 µL reaction.

3) Use a 200 µL multichannel to pipette each well up and down ~10 times to thoroughly mix the ERAT bead solution and the Ligation master mix. Pipette gently to avoid introducing excessive bubbles. (Alternatively, seal tubes and gently vortex.)

4) Seal the tubes and briefly spin down in a centrifuge.

5) Incubate at 20°C for 15 min. or overnight at 4°C. (The overnight incubation may result in higher ligation efficiency, but it may also result in more adapter dimers.)

6) Proceed to the next step. (Post-Ligation Bead Clean-up)

# Post-Ligation Bead Clean-up

1) Before beginning the bead clean up, warm a tube of PEG/NaCl solution to room temperature. [Note that for short-term use, the PEG/NaCl Solution may be stored at 2°C to 8°C (protected from light) for ≤2 months.]

2) In the same tubes as the ligation reaction, perform a 0.8X bead-based cleanup by combining the following:

Adapter ligation reaction product: 30 µL

**PEG/NaCl solution** at room temperature: **24 µL**

Note: if you removed the beads from your samples after double-sided cleaning, you will need to add beads again at this step. In place of PEG/NaCl solution, use 24 µL of room-temperature **low-ratio SPRI beads**)

3) Mix thoroughly by gently vortexing and/or pipetting up and down multiple times.

4) Incubate the tube at room temperature for **10 minutes** to bind DNA to the beads.

5) Place the tubes on a magnet to capture the beads. Incubate **3 minutes** or until the liquid is clear.

6) Carefully remove and discard the supernatant.

7) Keeping the tubes on the magnet, add **200 µL** of freshly prepared **80% ethanol**.

8) Incubate the tubes on the magnet at room temperature for **30 seconds**

9) Carefully remove and discard the ethanol.

10) Keeping the tubes on the magnet, add **200 µL** of freshly prepared **80% ethanol**.

11) Incubate the tubes on the magnet at room temperature for **30 seconds**

12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads. To accomplish this, use a small volume (10µL) tip to remove ethanol remaining at the bottom of the tube. Then use wooden toothpicks to blot up any dots of ethanol on the sides of the tubes.

13) Dry the beads at room temperature for **3 minutes or less**, until all of the ethanol has evaporated. *Caution: over-drying the beads may result in reduced yield.*

14) Remove the tubes from the magnet. Then thoroughly resuspend the beads: in **22 µL** of elution buffer (10 mM Tris-HCl, pH 8.0 – 8.5) a.k.a. Buffer EB or EBT

15) Incubate the tubes at room temperature for **3 minutes** to elute DNA off the beads.

16) Place the tubes on a magnet to capture the beads. Incubate for **3 minutes** or until the liquid is clear.

17) Transfer **20** µL of the clear supernatant to a new set of tubes and seal.

SAFE STOPPING POINT: samples can be stored here until ready to proceed to indexing PCR

## Indexing PCR

Before starting indexing PCR, make a detailed plan of which sample will be matched with which well of the indexing oligo plate and which color plate. Then make a very clear map that you can bring with you into the lab to be certain that the correct library will be matched with the chosen index well. Obviously working in sets of 8 and using the multichannel makes it far less likely that mistakes will occur.

Note: the GSL indexing oligo plates contain a unique P5 and a unique P7 indexing oligo pre-mixed in each well, both at 5µM. A new plate contains 10 µL of oligo mix, so you can set up two reactions total.

1) Take the Kapa 2x Ready Mix and GSL indexing oligo plate out to thaw on ice.

2) Label a new set of tubes for Indexing PCR for each sample. If you wish to use up all the library product in order to increase diversity, set up two parallel reactions for each sample to run simultaneously.

3) Add 15 µL Kapa 2x Ready Mix to each well of the indexing PCR plate.

4) Then add 10 µL of adapter-ligated product to each reaction well. Ideally, add 8 at a time using the LTS 20 µL multichannel. Gently pipette up and down to mix. Be sure to indicate which tubes have had product added by covering them or moving the position after the library has been added.

6) Finally, add 5 µL of the corresponding indexing oligo plate. Ideally add 8 at a time using the LTS 20 µL multichannel. Gently pipette up and down to mix. Then seal tubes well.

7) Briefly spin down the tubes and place in a cycler **with a heating lid.**

8) Amplify using the following cycling protocol:

Initial Denaturation: 98 °C for 45 seconds
Denaturation: 98 °C for 15 seconds \
Annealing: 60 °C for 30 seconds | x 6 - 9 cycles
Extension: 72 °C for 60 seconds /
Final Extension: 72 °C for 1 minute
Hold at 10 °C

# Post-PCR Bead Clean-up

1) Before beginning the bead clean up, warm a tube of **low-ratio SPRI bead solution (SeraMag)** to room temperature.

2) In the same tubes as the PCR reaction, perform a 0.8X bead-based cleanup by combining the following:

Indexing PCR product: 30 µL

**SPRI bead solution** at room temperature: **24 µL**

(Note: if you set up two indexing PCR reactions, they can be combined before bead cleaning. In that case, add 48 µL beads to preserve the 0.8X ratio)

3) Mix thoroughly by gently vortexing and/or pipetting up and down multiple times.

4) Incubate the tube at room temperature for **10 minutes** to bind DNA to the beads.

5) Place the tubes on a magnet to capture the beads. Incubate **3 minutes** or until the liquid is clear.

6) Carefully remove and discard the supernatant.

7) Keeping the tubes on the magnet, add **200 µL** of freshly prepared **80% ethanol**.

8) Incubate the tubes on the magnet at room temperature for **30 seconds**

9) Carefully remove and discard the ethanol.

10) Keeping the tubes on the magnet, add **200 µL** of freshly prepared **80% ethanol**.

11) Incubate the tubes on the magnet at room temperature for **30 seconds**

12) Carefully remove and discard the ethanol. Try to remove all residual ethanol without disturbing the beads. To accomplish this, use a small volume (10µL) tip to remove ethanol remaining at the bottom of the tube. Then use wooden toothpicks to blot up any dots of ethanol on the sides of the tubes.

13) Dry the beads at room temperature for **3 minutes or less**, until all of the ethanol has evaporated. *Caution: over-drying the beads may result in reduced yield.*

14) Remove the tubes from the magnet. Then thoroughly resuspend the beads: in **22 µL** of **nuclease-free water**

15) Incubate the tubes at room temperature for **3 minutes** to elute DNA off the beads.

16) Place the tubes on a magnet to capture the beads. Incubate for **3 minutes** or until the liquid is clear.

17) Transfer **20** µL of the clear supernatant to a new set of **low bind/siliconized** tubes and seal. This will be where your libraries will be stored until pooling for capture. So

label well.

## Quality control and pooling

1) Qubit all samples using 1 µL of final library product. If any sample values are too low, they can be re-amplified using IS5 and IS6 oligos.

2) Run a subset of samples on the bioanalyzer using a DNA 1000 chip to obtain library sizing information for sequencing and to verify that no adapter dimer is present.

3) If all looks well, prepare samples to be pooled for capture by:

a) group into sets of 8-12 individuals

b) determine how much library DNA to pool per sample (ex: 1000 ng / 10 samples = 100 ng each)

c) for each sample, determine how much volume to pool by dividing the target ng by the concentration (ex. 100ng / 20 ng/µL = 5 µL to pool)

## What to sign out in the EGL (reagents)

For this protocol, please keep track of the following in addition to any plastic consumables. Everything can be signed out on the freezer in 4122:

1. mL of SPRI bead solution (SeraMag) used

2. number of Kapa DNA prep equivalents. If you do 96 quarter preps sign this out as either “24 Kapa DNA preps”, or “96 * 0.25 Kapa DNA preps”. The ERAT enzyme & buffer, the Ligation enzyme & buffer, and the PEG/NaCl solution are considered a part of the Kapa DNA kit and do not need to be signed out separately.

3. amount of adapter stub used

4. amount of 2x ReadyMix used for indexing PCR

You do not need to sign out EB/EBT and indexing oligos.