**Initial notes**

**\*\*\*Before beginning, please read the entire KAPA Hyper Plus Kit manual 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.
* This protocol assumes enough starting material to begin sonication with 1 µg of DNA. However, the Kapa kit is suitable for library construction from 1 ng – 1 μg of double-stranded DNA, so in cases where 1 µg in unrealistic, this same protocol can be used with increased numbers of indexing PCR cycles.
* A refinement of size selection is possible after adapter ligation but it is easiest to get fragments to approximately the correct range before the enzymatic steps of library prep begin.
* The optimal size range of library inserts will vary based on whether data will be de novo assembled, mapped to a reference, or captured. Please consult with the lab manager before making fragmentation decisions.
* SPRI beads and PEG/NaCl solution should only be used at room temperature.
* Fresh 80% ethanol should be diluted each day.
* 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 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.
* 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.

**Fragmentation**

1. Assemble each fragmentation reaction on ice by adding the components in this order:

Double-stranded DNA: 8.75 µL

KAPA Frag Buffer\* (10X): 1.25 µL

KAPA Frag Enzyme\*: 2.5 µL

\*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.

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

Pre-cool block to 4°C

Fragmentation: 37°C for 35 min

Hold at 4°C

1. Transfer reactions to ice, and proceed immediately to End Repair and A-tailing. The ERAT reaction will occur in the same tubes/plate in which the fragmentation was performed.

**End Repair and A-tailing**

1) While the fragmentation reaction is in progress, remove the End Repair and A-Tailing reagents from the Kapa kit. 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) ON ICE: add **2.5 µL of ERAT master mix** to each sample well to make a 15 µL reaction.

4) When adding the ERAT master mix, pipette each well up and down ~1-2 times to mix the fragmented DNA and the ERAT master mix. Pipette gently to avoid introducing excessive bubbles.

5) Seal the tubes, gently vortex\*, and briefly spin down in a centrifuge (< 30 s).

*\*Note: to gently vortex, start with the vortex on the lowest setting. Then gradually increase the speed until the liquid starts to dance around and mix but doesn’t tornado violently or move towards the top of the well.*

Return the reaction plate/tube(s) to ice. Proceed immediately to the next step.

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

65°C: 30 minutes

4°C\*\*: hold

\* Set the lid temperature to 75°C

\*\* If proceeding to the adapter ligation reaction setup without any delay, the reaction may be cooled to 20°C instead of 4°C.

7) Once end repair is proceeding, return the reagents to the Kapa kit and retrieve the Ligation Enzyme, adapter stub, and Ligase Buffer. Leave the enzyme and stub to thaw on ice, and let the buffer thaw at room temperature.

8) After the End-repair and A-tailing reaction is completed and the sample has cooled proceed to Adapter Ligation. The ligation reaction will take place in the same tubes/plate in which the end repair reaction was performed.

**Adapter Ligation**

1) Make a master mix composed of the following and keep it on ice:

Ligation Buffer: 7.5 µL

Ligation Enzyme: 2.5 µL

\*Adapter stub (15 µM) 5.0 µL

*\*Note: do not add the adapter stub to the ligation master mix until immediately before it will be aliquoted. (The ligase will start to make dimer as soon as the adapter stub is introduced into the master mix, even when kept on ice.)*

The master mix can be distributed into a strip tube to facilitate adding to samples with a multichannel pipette

2) ON ICE: add **15 µL of Ligation master mix** to each sample well to make a 30 µL reaction.

3) When adding the ligation master mix, pipette each well up and down ~1-2 times to mix the ERAT reaction and the ligation master mix. Pipette gently to avoid introducing excessive bubbles.

4) Seal the tubes, gently vortex (just so that the liquid starts to move around), and briefly spin down in a centrifuge (< 30 s).

*Note: this is just to mix the eluted DNA and the enzyme. The beads do not need to be resuspended for the ligation reaction. They can remain at the bottom of the tube if they have settled there.*

5) Incubate at 20°C for 15-60 min. or overnight at 4°C.

*Note: The overnight incubation may result in higher ligation efficiency, but it may also result in more adapter dimers. However, the subsequent bead cleanings should do a good job removing most dimer. Overnight ligations are recommended when starting with less sonication input material (< 500 ng), but can also be used when the timing is better to proceed with the Post-Libation Bead Clean-up the next day.*

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

**Post-Ligation Bead Clean-up**

1. 1) Before beginning the bead clean-up, 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) 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

**low-ratio SPRI beads** 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 20 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 **15 minutes** to bind DNA to the beads.

5) Place the tubes on a magnet to capture the beads. Incubate **5 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 residual ethanol has evaporated from the tube but the beads themselves are still damp and shiny. *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 **5-10 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. For larger projects, use the Benchsmart to ensure correct matching of sample well to indexing oligo mix.

*Note: the GSL indexing oligo plates contain a unique P5 and a unique P7 indexing oligo pre-mixed in each well, both at 5µM.*

1) Take the Kapa 2x Ready Mix and indexing oligo plate out to thaw on top of ice (or leave at +4C for ~30 minutes).

2) Label a new plate for indexing PCR for each sample. If you wish to use up all the library product, set up two parallel reactions for each sample.

3) Add 15 µL Kapa 2x Ready Mix to each well of the empty 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 once or twice to mix.

*Note: if you just used the Benchsmart for post-PCR bead cleaning, re-purpose your final set of tips to load 10 µL of adapter-ligated product into the PCR plate and mix.*

5) 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.

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

7) 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 - 10 cycles\*

Extension: 72 °C for 60 seconds /

Final Extension: 72 °C for 5 minutes

Hold at 10 °C

\**6 cycles is sufficient for most samples starting sonication with 500-1000 ng. (Don’t use fewer cycles since 6 is the minimum to ensure that DNA becomes full-length sequence-ready libraries after indexing PCR.) If you start with less input, Table 3 of the Kapa Hyper Prep kit is a useful resource. Generally, about 25% of initial sonication starting material enters the end repair reaction. So, starting with 200 ng of DNA during sonication, we can estimate that there is ~50 ng remaining after size-selection*

**Post-Sonication Double-sided Size Selection**

A ratio of L = 0.75x and R = 0.90x is a good starting point to size-select for libraries with a mode of ~325 bp (insert ~175 bp). But this may need to be adjusted after seeing the initial results.

**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. Here you could add 20 µL water to the 30 µL PCR product for V = 50 µL
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 = 50µL and R = 0.75, add 37.5 µL 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.

1. 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!\*\*\***

1. 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.** To facilitate this it may be best to set the volume to transfer over than the total. (for example, if the total volume is 87.5 µL, possibly move just 85 µL.) The tubes with the beads can then be discarded.
2. 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 0.75x and the left-side ratio (L) is 0.90x, add 0.15 \* 50 µL or 7.5 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.

1. 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.
2. Pipette off the supernatant and discard without removing or disturbing the beads
3. Leave beads on magnet and wash with **200 µL** of freshly prepared **80% ethanol** (make a new ethanol dilution before every SPRI clean-up).

Let stand for **at least 30 seconds** and discard supernatant.

1. Repeat step 9.
2. 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. (For large sets of samples, you may be able to resuspend the first tubes as soon as you are done removing residual ethanol from the final ones.) Avoid overdrying which appears as cracking in the beads
3. Elute as follows:

A) Remove the tubes from the magnetic rack. Add **22 µL** of **EB** or **nuclease-free water** to the wells

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.

1. 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.
2. Place the tubes on a magnet to capture the beads. Incubate for **3 minutes** or until the liquid is clear.
3. Transfer ~**20 μL** of the clear supernatant to a new plate.