This protocol was derived from: Wendt et al. (2018) “Targeted bisulfite sequencing using the SeqCap Epi Enrichment System”, replacing the SeqCap Epi system with the Arbor probe protocol. Eventually, we will also cut the KAPA kit to save money.

***Initial notes***

* 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
* 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

**PROTOCOL**

**Fragmentation**

1. Enzymatic fragmentation: 150-200bp size range
2. Following fragmentation, transfer 50 μL of the fragmented DNA to a 0.2 mL PCR tube and construct the sample library following the procedure described in the KAPA Library Preparation Kit Technical Data Sheet, beginning at End Repair and A-tailing (step 2)

*End product:* 50μL of fragmented DNA

*Materials?*

**KAPA library prep**

**End Repair and A-tailing**

1. In the same tube(s)/plate in which enzymatic fragmentation was performed, assemble each end repair and A-tailing reaction as follows:

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1. Vortex gently and spin down briefly. Return the reaction tube(s)/plate to ice. Proceed immediately to the next step
2. Incubate in a thermocycler programmed as outlined below. A heated lid is required for this step. If possible, set the temperature of the heated lid to ~80°C (instead of the usual 105°C)

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1. Proceed immediately to the next step.

**Adapter Ligation**

1. Dilute adapter stocks to the appropriate concentration, as outlined in Table 3 below

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1. In the same plate/tube(s) in which end repair and A-tailing was performed, assemble each adapter ligation reaction as follows:

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1. Mix thoroughly and centrifuge briefly
2. Incubate at 20°C for 15 minutes
   1. Note: to achieve higher conversion rates and library yields, particularly for low-input samples, consider increasing the ligation time, to a maximum of 4 hours at 20°C, or overnight at 4°C. Please note that longer ligation times may lead to increased levels of adapter-dimer. Adapter concentrations may have to be optimized if ligation times are extended significantly
3. Proceed immediately to the next step

*End product:* 110μL with adapters ligated

*Materials*

* KAPA HyperPrep gDNA kit
* Methylated P5 and P7 adapters

**Post-ligation Cleanup**

1. SPRI bead cleanup—EGL protocol
2. Resuspend beads in 55 μL of PCR-grade water

*End product:* 55μL

*Materials:*

* SPRI beads (EGL)

**Dual-SPRI Size Selection**

*Need to decide on ideal fragment length to develop protocol*

*Materials*

* SPRI beads (EGL)

**Bisulfite treatment with the Zymo EZ-DNA Methylation Lightning Kit:** <1.5 hours for test run, <3 hours for high throughput

* Converts unmethylated cytosines to uracils
* DNA input: Samples containing between 100 pg to 2 μg. For optimal results, the amount of input DNA should be from 200-500ng

**Reagent preparation**

* Add 24 ml of 100% ethanol to the 6ml M-Wash Buffer concentrate (D5030) or 96 ml of 100% ethanol to the 24 ml M-Wash Buffer concentration (D5031) before use
* M-DNA Wash Buffer included with D5030S & D5030T is supplied ready-to-use and does not require the addition of ethanol prior to use

**Protocol**

1. Add 130 μl of **Lightning Conversion Reagent** to 20 μl of a DNA sample in a PCR tube. Mix, then centrifuge briefly to ensure there are no droplets in the cap or sides of the tube.

***Note:*** *if the volume of DNA is less than 20 μl, compensate with water.*

1. Place the PCR tube in a thermal cycler and perform the following steps:

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1. Add 600 μl of **M-Binding Buffer** to a **Zymo-Spin IC Column** and place the column into a provided **Collection Tube**
2. Load the sample (from Step 2) into the **Zymo-Spin IC Column** containing the **M-Binding Buffer**. Close the cap and mix by inverting the column several times
3. Centrifuge at full speed (>=10,000 x g) for 30 seconds. Discard the flow-through
4. Add 100 μl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds
5. Add 200 μl of **L-Desulphonation Buffer** to the column and let stand at room temperature (20-30°C) for 15-20 minutes. After the incubation, centrifuge at full speed for 30 seconds
6. Add 200 μl of **M-Wash Buffer** to the column. Centrifuge at full speed for 30 seconds. Repeat this wash step.
7. Place the column in a 1.5 ml microcentrifuge tube and add 15 μl of **M-Elution Buffer** directly to the column matrix. Centrifuge for 30 seconds at full speed to elute the DNA

* **PAUSE POINT** *The DNA is ready for immediate analysis or can be stored at or below -20* °*C for later use. For long term storage, store at or below -70*°*C. Zymo recommends using 1-4 ul of eluted DNA for each PCR, however, up to 10 ul can be used if necessary. The elution volume can be > 10 ul depending on the requirements of your experiments, but small elution volumes will yield higher DNA concentrations*

*End product:* 15 μl of bisulfite-treated sample

*Materials*

* Zymo EZ-DNA Methylation Lightning Kit

**Indexing PCR**

*Materials*

* Kapa HiFi uracil+ (Peqlab)
* P5 and P7 primers
* Dual-indexing oligo plates
* Internal barcodes (same as unique molecular identifies, UMIs?)

**Probe capture with Arbor Custom Methyl Seq-manual**

*Arbor recommends pooling 8 libraries, 200 ng each (for a total of 1.6 ug), concentrated to* ***7 ul****, and suspended in a nuclease-free buffer or water. So for our test run, we can stick to this exact protocol. But for our actual samples, if we pool 48/reaction, we’ll 1600/48ng for each sample in 7 ul?*

**Part 1: Hybridization setup—**sample libraries are mixed with various adapter-blocking nucleic acids, denatured, and then combined with other hybridization reagents (including baits). The hybridization reaction incubates for several hours

**1.1 Prepare materials**

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**1.2 Hybridization Mix setup**

1. Once the Hyb reagents have thawed, vortex them to homogenize and then briefly centrifuge



1. Assemble the Hybridization Mix in a microcentrifuge tube, briefly vortex and briefly centrifuge to collect the sample. **The following volumes are already adjusted for pipetting error:**

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1. Incubate Hybridization Mix at **60°C** for **10 minutes** in the heat block. **Vortex occasionally** to collect condensate. Remove from the heat block and **let sit for 5 minutes** before proceeding
2. For each capture reaction, aliquot 18.5μl of Hybridization Mix to a 0.2ml well/tube (now referred to as HYBs)

**1.3 Blockers Mix setup**

1. Assemble the Blockers mix in an appropriately-sized tube and mix by pipetting. **The following volumes are already adjusted for pipetting error:**

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1. For each capture reaction, aliquot 5 μl of Blockers Mix to a 0.2 ml well/tube
2. Add 7 μl of pooled libraries to each Blockers Mix aliquot and mix by pipetting (now referred to as LIBs)

**1.4 Reaction assembly**

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1. Put the LIBs (the 0.2 ml tubes with the pooled libraries + blocker mix) in the thermal cycler, close the lid, and start the thermal program

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1. Once the cycler reaches 63°C during step 2, pause the program, put the HYBs (the 0.2 ml tubes with the 18.5μl of Hybridization Mix aliquots) in the thermal cycler, close the lid, and resume the program

Diagram

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1. After step 2 of the program is complete, leaving all the tubes in the thermal cycler, pipette 18 μl of each HYB to each LIB. Use a multichannel pipettor for easier execution. Gently homogenize by pipetting up and down 5 times.

Diagram

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1. Dispose of the HYB tubes. Briefly spin down the LIBs, return to the thermal cycler, close the lid, and allow the reactions to incubate overnight (16 to 24 hours)

**Part 2: Bind and Wash (“Cleanup”)—**the hybridized baits + targets are bound to streptavidin-coated magnetic beads, and then most non-target DNA is removed with several rounds of washing with a warm buffer. This is typically performed the day following completion of Part 1.

**2.1 Prepare materials**



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**2.2 Wash Buffer X preparation**

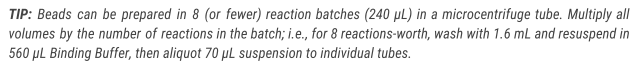
This step generates enough Wash Buffer X for 44 reactions in microcentrifuge tube cleanup format, and 68 reactions in 0.2ml cleanup format; scale up or down if needed

1. Thaw and thoroughly homogenize Wash Buffer and Hyb S prior to aliquoting in order to dissolve any visible precipitate; warm slightly if necessary
2. Combine 400 μl Hyb S, 39.6 ml nuclease-free water and 10 ml Wash Buffer in a 50 ml tube. Vortex thoroughly, label “Wash Buffer X.” *Wash Buffer X can be stored at 4°C for 1 month*
3. Heat the Wash Buffer X to 63°C in the water bath or oven for at least 30 minutes before use

**2.3 Bead preparation**

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1. For each capture reaction, aliquot 30 μl beads to a microcentrifuge tube
2. Pellet the beads in the magnetic particle collector until the suspension is clear (1-2 minutes). Leaving the tubes on the magnet, remove and discard the supernatant
3. Add 200 μl Binding Buffer to each bead aliquot. Vortex to resuspend the beads the centrifuge briefly. Pellet in the magnetic particle collector, remove and discard the supernatant
4. Repeat Step 3 above twice for a total of three washes
5. Resuspend each washed bead aliquot in 70 μl Binding Buffer. If proceeding to washing in 0.2 ml format, transfer the aliquots to PCR strips



**2.5 Binding beads and hybrids**

1. Heat the bead aliquots to 63°C for at least 2 minutes
2. Transfer each capture reaction to the heated bead aliquots. Mix by pipetting.
3. Incubate the libraries + beads on the heat block or thermal cycler for 5 minutes. Agitate at the 2.5 minute mark by flicking or inverting the tubes to keep the beads suspended, followed by briefly centrifuging to collect

**2.6 Bead washing**

1. Pellet the beads with the magnetic particle collector until the solution is clear. Remove and discard the supernatant.
2. Add 375 ul (MC tube format) or 180ul (0.2 mL format) warmed Wash Buffer X to the beads, remove from the magnetic particle collector, place on the heat block for 15 seconds, and briefly vortex or mix by pipetting. Briefly centrifuge to collect.
3. Incubate for 5 minutes at 63°C in the heat block or thermal cycler. Agitate at the 2.5 minute mark via gentle vortexing and briefly centrifuge
4. Repeat steps 1 through 3 two times for MC tube format (three washes total), or three times for 0.2 mL format (four washes total). After the last wash and pelleting, remove as much fluid as possible without touching the bead pellet

**Part 3: Library Resuspension and Amplification—**bead-bound enriched library is resuspended in Buffer E and amplified

**3.1 Prepare materials**

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**3.2 Enriched library recovery**

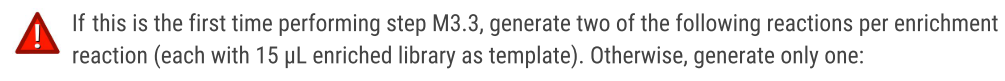
Add 30 μl buffer E to the washed beads and thoroughly resuspend by pipetting. Then, depending on your library amplification system, choose workflow A or B:

**Table

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**3.3 Library amplification**

This is an example post-capture amplification using KAPA HiFi HotStart ReadyMix and Illumina libraries



1. Assemble the following PCR master mix:

Table

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1. Cycle the reactions with the following thermal program:

Table

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1. After amplification:

* If the beads were included in the amplification reaction and you intend to use silica columns for purification, pellet the beads first and purify only the supernatant
* Otherwise, purify the reaction using your preferred PCR cleanup (e.g., silica columns or SPRI beads)

**3.4 Perform a second round of enrichment**

1. Combine both purified amplification reactions generated above and concentrate to 7 μl
2. Repeat steps M1.1 through M3.3 using this once-enriched template as input

**If this is the end of your second time through step 3.3 (two of two)**, the enriched libraries are now ready for quantification, quality-assessment, and sequencing

*Materials*

**Need to order:**

* Arbor custom MyBaits 48 reaction kit: we’re planning to multiple 48 libraries per reaction
* 0.2 mL PCR strips with attached lids (2 per 8 reactions) (e.g., VWR Cat# 93001-118)
* PCR primers to amplify sequencing libraries after capture, e.g.:
* PCR reagents for post-capture amplification (1 per reaction, e.g., Roche Cat#07958927001)
* PCR purification system—SPRI bead clean up materials (1 per reaction)

**Available in lab:**

* 50 mL tubes (1 per 44 reactions)
* Microcentrifuge tubes (1.5, 1.7, or 1.8mL) (2 per 8 reactions)
* Pipettors and tips for 0.5-500 μl
* Thermal cycler with heated lid compatible with 0.2 ml strips
* Magnetic particle collector for microcentrifuge tubes (e.g., ThermoFisher Cat#12321D)
* Magnetic particle collector for 0.2 ml strips (e.g., Permagen Cat# S500)
* Vortex mixer and mini-centrifuge for tubes and strips
* Water bath or incubation oven at 63°C
* Heat block for microcentrifuge tubes at 60°C
* Nuclease-free H20

**

**Pool libraries for sequencing**

*With whoever is sequencing and would be willing to take on a few extra samples*

**What other steps need to happen before sequencing? Bioanalyzer/gel?**

**Sequencing**

*Depends on the project we’re adding our samples to*