**Background**

Wilkinson et al. (2020) developed an epigenetic clock for bats, identifying a set of ~2K (1,994) CpG sites that are conserved and correlated with chronological age across bat genomes—from a custom mammalian microarray that assays 37.5K sites, comprised of 35.5K 50bp CpG-terminal sequences that are conserved across 62 mammals (including 5 bat species) + 2K human biomarker probes. While not all 1,994 probes map to all bat species genomes, a more-than-sufficient subset map across species to allow for robust age analyses.

Our goal was to adapt the Wilkinson et al. (2020) probe set to in solution hybridization capture, which required extending their probe sequences to 80bp. First, in order to identify the 1,994 Wilkinson et al. (2020) age-correlated CpG sites and flanking sequences in bat genomes, we mapped their 50bp probe sequences to 17 bat species genomes. We extended those 50bp sequences by 30bp up- and downstream and ran a multiple sequence alignment of the extended sequences across all 17 species. We defined our 80bp probe sequences by identifying the most conserved 80bp frame within the 110bp multiple sequence alignment (i.e., the frame that had the highest number of bp matches and lowest number of gaps summed across species) and recording the most common nucleotide at each base pair within that frame. To design our probe set for post-bisulfite-conversion capture, we made 9 variants for each 1,994 probe sequence—4 bisulfite converted sequences for both strands and 1 unconverted sequence for a bisulfite conversion control—for a total of 17,946 probes. The majority of our probes include multiple CpG sites, but only 1-2 of those sites (coordinates recorded on our annotation file) are identified as age-correlated by Wilkinson et al. (2020).

We will first apply this protocol to three Madagascar bat species (*Pteropus rufus*, *Eidolon dupreanum*, and *Rousettus madagascariensis*) to obtain age data that we will then combine with serological datasets in order to construct age-seroprevalence models of potentially zoonotic viruses circulating in these populations. However, by building off the Wilkinson et al. (2020) microarray probes that target conserved CpG sites, we have maintained the flexibility of their assay and thus, our protocol can flexibly be applied to obtain age data for any bat species of interest.

**Protocol sources**

* Wang et al. 2013. Tagmentation-based whole-genome bisulfite sequencing. Nature Protocols 8(10): 2022-2032—8 reactions, 101bp paired-end sequencing on HiSeq2000—our only major change = targeted approach (probe capture) instead of whole genome
* Zymo EZ DNA Methylation Lightning Kit for test run (Zymo EZ-96 DNA Lightning Kit for high throughput on actual samples)
* Arbor Custom Methyl-Seq protocol

**Overview**

**Day 1**

1. Pre-annealing of stub adaptors (1 hour)
2. Assembly of transposome (1 hour)

--potential pause point

1. Tagmentation (15 min)
2. Bio-analyzer/gel size check?
3. SPRI bead clean up (30 min)

--potential pause point

1. Oligonucleotide replacement and gap repair
2. SPRI bead clean up (30 min)

--potential pause point

1. Bisulfite treatment (3 hours)

--potential pause point

1. Indexing PCR
2. Double-sided bead purification for size selection?

--potential pause point??

**Day 2**

1. Pool for probe capture
2. Probe capture (round 1)
   1. Part 1: Hybridization

--incubates overnight (16-24 hours)

**Day 3**

* 1. Part 2: Bind and wash (“cleanup”)
  2. Part 3: Library resuspension and amplification

1. Probe capture (round 2)
   1. Part 1: Hybridization

--incubates overnight (16-24 hours)

**Day 4**

* 1. Part 2: Bind and wash (“cleanup”)
  2. Part 3: Library resuspension and amplification

1. Pool libraries for sequencing
2. Any other steps that need to happen before sequencing? Bioanalyzer/gel?

**Nextera-style library prep protocol** (for 8 DNA samples—from Manny’s bat cell lines)

**Pre-annealing of stub adaptors:** anneals methylated top adapter **Tn5mC-Apt1** (methylated sequencing primer 1 and transposon end recognition sequence) and bottom adapter **Tn5mC1.1-A1block**, *1 hour (Wang et al. 2013)*

1. In a 200-μl PCR tube, mix 10 μl each of oligonucleotides **Tn5mC-Apt1** and **Tn5mC1.1-A1block** (100 μM each) and 80 μl of H20.
2. Set the thermocycler conditions for adaptor assembly as follows:

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*End product:* 100μl of Tn5mC-Adapt

*Materials:*

**Need to order:**

* Tn5mC-Apt1—**methylated** top adaptor stub, *Wang et al.* (methylated sequencing primer 1 + transposon end recognition sequence)—modified version of [Illumina Nextera Transposase Adapter, Read 1](https://dnatech.genomecenter.ucdavis.edu/wp-content/uploads/2013/06/illumina-adapter-sequences_1000000002694-00.pdf):

5’ TcGTcGGcAGcGTcAGATG**TGTATAAGAGAcAG** 3’

* Tn5mC1.1-A1block—**phosphorylated and dideoxycytidylated** bottom adaptor stub (reverse complement to end 15 bp of Tn5mC-Apt1 top adaptor stub, part of the read 2 adapter, which will be completed with the replacement oligo):

p**CTGTCTCTTATACA**ddC

**Available in lab:**

* H20
* Thermocycler
* 200-μl PCR tube (1)

**Assembly of the transposome:** attaches adapter stubs to Tn5,*1 hour (Wang et al. 2013)*

1. Heat 100 μl glycerol (≥99.5%) to 90 °C in a thermocycler. Hot glycerol can be readily and exactly pipetted
2. Transfer 50 μl of hot glycerol to a 1.5 ml reaction tube and cool it down to RT by incubation on ice for 3 min
3. Add 50 μl of adaptor **Tn5mC-Adapt** (10 μM) from Step 2 and mix it by repeated pipetting
4. Transfer 10 μl of the glycerol-adaptor mixture (stable at -20 °C for at least 6 months) to a new 1.5 ml reaction tube, add 10 μl of **Tn5 transposase**; mix by repeated pipetting
5. Maintain the adaptor-transposase mixture, the transposome, at RT for 30 min, and then place it on ice. The 20 μl mixture is sufficient for eight tagmentation reactions

* **PAUSE POINT** The **transposome** can be stored at -20 °C for at least 1 month

*End product:* 20 μl assembled transposome

*Leftovers:*

* 50 μl pre-annealed Tn5mC-Adapt
* 40 μl glycerol-adaptor mix

*Materials*

**Need to order:** nothing

**Available in lab:**

* Glycerol (≥99.5%)
* Tn5 transposase
* Thermocycler
* 1.5 ml reaction tubes
* Dry ice

**Tagmentation of genomic DNA:** fragments + adapter stub attachment,*15 min (Wang et al. 2013)*

1. Qubit samples to obtain concentrations
2. Dilute a portion of each sample to desired concentration
3. Remove the 2x TD buffer and transposome solutions from the freezer and thaw on ice. After thawing, gently vortex to mix (briefly spin down for ~1 second if splashing occurs)
4. Set up 8 tagmentation reactions, one in each well of an eight-well PCR strip. Per well, mix on ice **10 μl of 2x TD** and **7.5 μl of aqueous DNA solution** containing **20 ng of genomic** DNA and 10 pg of phage-λ DNA.
5. Add **2.5 μl of the assembled transposome** (from Step 7) to each well and mix by repeated pipetting
6. Set the thermocycler conditions for tagmentation as follows

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*End product:* Eight-well PCR strip with 20 μl tagmentation reaction

*Materials*

**Need to order:**

* 2x TD buffer (or another tagmentation buffer) = 20 mM Tris(hydroxymethyl)aminomethane; 10 mM MgCl2; 20% (vol/vol) dimethylformamide. Before the addition of dimethylformamide, adjust the pH to 7.6 with 100% acetic acid
* Phage-λ DNA, **unmethylated** (Promega, order no. D1521): spiked in to calculate bisulfite conversion efficiency

**Available in lab:**

* Qubit + buffer, dye, and standards
* Dry ice
* Vortex
* Eight-well PCR strip

**Post tagmentation SPRI bead purification:** *30 min estimated for eight reactions (Wang et al. 2013)*

1. To each well, add **15 μl of 5 M guanidinium thiocyanate** (total volume of DNA solution 35-μl), 10μl of AMPure beads and 36 μl of AMPure buffer (total volume of bead solution 46 μl) and mix homogeneity by repeated pipetting
2. Keep reactions at RT for 10 min
3. Transfer the 8-well strip to a magnetic separator, wait for 1 min and remove the supernatant thoroughly by pipetting without disturbing the bead pellet in each well; discard the supernatant

* **CRITICAL STEP** To avoid bead carryover or loss of beads, removal of the supernatant in several small volume steps rather than in a single large volume may be preferred

1. While the strip is still on the magnetic separator, wash the beads in each well with 50 μl of 80% ethanol by repeated (10x) pipetting without disturbing the pellets. Remove the supernatant in each well completely by pipetting without disturbing the bead pellet; discard the supernatant. Keep the eight-well strip open on the magnetic separator for 10 min.

* **CRITICAL STEP** Keep the washing step as short as possible. Complete removal of any liquid and droplets is essential; the beads must be completely dry before the next step.

1. Remove the eight-well strip from the magnetic separator and thoroughly resuspend the magnetic beads in 12 μl of H2O per well
2. Transfer the plate to the magnetic separator, wait for 1 min and transfer the supernatant (the eluate containing the tagmented DNA) from each well into a new well of a new eight-well strip, without disturbing the bead pellets. Transfer 1 μl of the eluate from each well to a DNA LoBind reaction tube for troubleshooting by monitoring the fragment size using the Quant-iT dsDNA HS assay kit and the Bioanalyzer 2100

* **PAUSE POINT** The **purified tagmented DNA** can be stored at -20 °C for at least 1 month

*End product:* 12 μl (1 μl of gel size check) of purified tagmented DNA in each well

*Materials:* Substitute in bead cleanup protocol used in the lab where you end up doing this protocol

***Add a bioanalyzer step? Or gel*** *(not sure what is available)?*Test a small aliquot to optimize the tagmentation step and even after optimization, make sure the step worked?

**Oligonucleotide replacement and gap repair:** the unmethylated bottom adapter stub is replaced with a methylated adapter stub and a gap of 9 base pairs is repaired by the combined action of DNA polymerase and DNA ligase,*1 hour (Wang et al. 2013)*

1. To the 11 μl of eluate in each well, add 2 μl of dNTP mix (2.5 mM each, 10 mM), 2 μl of 10x Ampligase buffer and 2 μl of **replacement oligo Tn5mC-Repl01** (10 μM); mix by repeated pipetting
2. Set the thermocycler conditions for replacement and annealing as follows:

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1. While the eight-well strip remains in the thermocycler, add 1 μl of **T4 DNA polymerase** and 2.5 μl of Ampligase per well; mix by repeated pipetting
2. Continue the reaction in the thermocycler with the following conditions for gap repair:

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*End product:* 20.5 μl in each well

*Materials*

**Need to order:**

* dNTP mix (2.5 mM each, 10 mM)
* 10x Ampligase buffer
* Replacement oligo Tn5mC-Repl01—**phosphorylated, methylated, dideoxycytidylated, and inverted** (just the modified version of the Tn5mC1.1-A1block sequence = bottom adapter sequence + the methylated sequencing primer 2), *Wang et al.*—modified version of the **reverse complement** of the [Illumina Nextera Transposase Adapter, Read 2](https://dnatech.genomecenter.ucdavis.edu/wp-content/uploads/2013/06/illumina-adapter-sequences_1000000002694-00.pdf)

p**cTGTcTcTTATAcA**cATcTccGAGccCAcGAGAcinvT

* **T4 DNA polymerase**

**Available in lab:**

* Thermocycler

**SPRI bead purification after oligonucleotide replacement:** *30 min estimated for 8 reactions (Wang et al. 2013)*

1. To the samples (20.5 μl per well) in the eight-well strip from Step 23, add 10 μl of AMPure beads and 26 μl of AMPure buffer (total volume of bead solution 36 μl) per well; mix to homogeneity by repeated pipetting
2. Repeat steps 15-18, resuspending the beads in 25 μl of H20 per well
3. Transfer the eight-well strip to the magnetic separator. Wait for 1 min and transfer the supernatant (the eluate containing the tagmented and gap-repaired DNA) from each well into a new well of a new eight-well strip, without disturbing the bead pellets. Transfer 5 μl of the eluate from each well to a LoBind reaction tube for troubleshooting by real-time PCR or for preparation of a sequencing library for genome analysis without bisulfite treatment

* **PAUSE POINT** The **purified gap-repaired DNA** can be stored at -20 °C for at least 1 month

*End product:* 25 μl per well, 5 μl for gel size check

***Gel size check:*** Test 5 μl to optimize the tagmentation step and after optimization, to make sure the step worked

**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*

**Need to order:**

* Zymo EZ-DNA Methylation Lightning Kit

**Available in lab:**

* 100% Ethanol

**Indexing PCR**

*End product:*

* Following PCR, the unmethylated cytosines that had been converted to uracils are now thymines

*Materials:*

**Need to order:**

* Kapa HiFi uracil+ (Peqlab)
* Dual-indexing plate (i5/i7 indexes)
* Primers
  + PCR primer Tn5mCp1—no modifications, *Wang et al.—*[Illumina Nextera Index Kit, Index Read 2](https://dnatech.genomecenter.ucdavis.edu/wp-content/uploads/2013/06/illumina-adapter-sequences_1000000002694-00.pdf) 384 UDIs

AATGATACGGCGACCACCGAGATCTACAC [i5 index]TCGTCGGCAGCGTC

* + PCR barcode primer Tn5mCBar—no modifications, *Wang et al.*—[Illumina Nextera Index Kit, Index Read 1](https://dnatech.genomecenter.ucdavis.edu/wp-content/uploads/2013/06/illumina-adapter-sequences_1000000002694-00.pdf) 384 UDIs

CAAGCAGAAGACGGCATACGAGAT[i7 index]GTCTCGTGGGCTCGG

**Double-sided bead purification?**

**Pool for capture**

**Pre-treating Nextera-style libraries (Arbor workaround)**

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1. **Prepare materials**

**Text, letter

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1. **Amplification of indexed Nextera libraries**

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1. **Prepare streptavidin magnetic beads**

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1. **Deplete the residual streptavidin-affinity molecules**

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**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**

Table

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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**

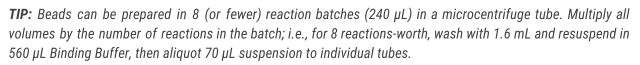
This step generates enough Wash Buffer X for 44 reactions in microcentrifuge tube cleanup format, and 68 reactions in 0.2ml cleanup format; scale up or down if needed

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**

**Graphical user interface, text, application, email

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**3.2 Enriched library recovery**

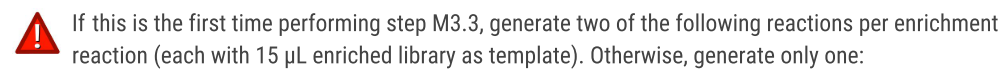
Add 30 μl buffer E to the washed beads and thoroughly resuspend by pipetting. Then, depending on your library amplification system, choose workflow A or B:

**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:

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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 or 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*