Meyer & Kircher Library Preparation Consumables

Check the following list of consumables before beginning the Meyer & Kircher protocol. All reagent PDFs on Dropbox.

1. AMPure XP/SPRIselect and homemade SPRI beads – ≤3 months old, see last bullet in "Things to Consider…"
2. Fragment DNA
   – microTUBE AFA Fibre Pre-Slit Snap-Cap 6x16mm
3. Blunt-End Repair
   – Buffer Tango (10X)
   – dNTPs (25 mM each)* [we use in-house dNTPs from ancient lab (2.5mM each), volume change noted]
   – ATP (100 mM)
   – T4 polynucleotide kinase (10 U/µL)
   – T4 DNA polymerase (5 U/µL) [we might have 3 U/µL, volume change noted]
4. Adapter Ligation
   – T4 DNA ligase buffer (10X) #
   – PEG-4000 (50%) [comes with T4 DNA ligase buffer]
   – T4 DNA ligase (5 U/µL) [comes with T4 DNA ligase buffer]
   – p5p7 adapter mix
5. Adapter Fill-In
   – ThermoPol reaction buffer (10X)
   – dNTPs (25 mM each)* [see above]
   – *Bst* polymerase, large fragment (8 U/µL)
6. Indexing PCR
   – KAPA HiFi Hotstart ReadyMix
   – Primer IS4 (10 µM)
   – Reverse indexing primers (10 µM)

Things to Consider Before Starting Library Prep

- Blue text = alterations from original protocol, orange text = aDNA lab procedures by SM. **Steps 12-14 are optional.**
- All master mix calculations should include **extra 0.5 reactions**. Ex: if you have 3 samples, calculate reagent volume needed for 3.5 samples.
- Barcodes = "indexes"
  - Perry Lab utilizes Primer IS4 as a universal forward primer.
  - Indexing primers are reverse primers (set ordered by Logan Kistler).
- *Up to 94 samples can be processed in parallel on a 96-well reaction plate; two wells should be reserved for a blank and a positive control. Seal each reaction plate with ~~strip caps~~ sealing tape and centrifuge to 2000g in a plate centrifuge after setting up each reaction in order to collect the liquid in the bottom of the wells. This prevents cross-contamination while removing the ~~caps~~ tape.*
  - *…strongly recommend carrying a positive and a blank control* throughout protocol. *Positive control could be 200-500 ng of purified PCR product with discrete size of 200-300 bp.*
  - When instructed to "briefly spin down" the 96-well plate or strip tube, set large centrifuge near walk-in fridge to 2000 g and spin plate/strip tube for 5 seconds. It will come up to about 300 g in this amount of time, which is more than enough.
- *Carboxyl-coated magnetic beads (Solid Phase Reversible Immobilization [SPRI] beads) are ideally suited for reaction purification in a 96-well plate setup, [but] does not retain molecules shorter than 100-150 bp…If retention of short molecules is desired, adjust size cutoff by varying the volume of SPRI bead/buffer suspension added to the sample.*
  - We make our own SPRI beads in the Ancient Lab, and bring them over to the Modern Lab as needed (stored in foil-wrapped falcon tubes in walk-in refrigerator).
  - Use AMPure XP or SPRIselect beads for size selection step after Blunt End Repair – based on SPRI Based Size Selection protocol by Beckman Coulter.
  - M&K suggest drying the beads for 20:00 minutes during bead clean up steps. This amount of time could over-dry your sample while it's bound to the SPRI beads. Check every couple of minutes for "cracked" beads.

M&K Library Prep (0): Preparation of Adapter Mix

This step produces sufficient adapter mix for 200 reactions. The adapter mix can be used repeatedly and stored at -20°C before and after usage. We make the adapter mix in the Ancient Lab, then bring aliquots to the Modern Lab as needed. All aliquots are currently stored in the -20°C freezer – white box, orange tape, made by S. Marciniak.

M&K Library Prep (1): Fragmentation of Sample DNA

This process describes how the Perry lab optimally fragments DNA to a specific insert size (<600 bp for sequencing). This step is not always required as fragmentation may not be necessary for highly degraded DNA, aDNA, etc.

1. Schedule time on the Covaris M220 ultrasonicator [Millennium Science Complex, Life Sciences Building].
2. Make dilutions:
    a. Need 1 µg (1000 ng) DNA in a 100-130 µL total solution of qH_2O to make 1 library – speed vac down to 50 µL after shearing. [Can do 2 µg DNA in a 100-130 µL solution that will result in two libraries if need be.]
    b. Record the following table and calculations:

Sample ID	Sample Concentration (ng/µL)	1000ng/ [Sample Conc. (ng/µL)]	2000ng/ [Sample Conc. (ng/µL)]	qH_2O to 1___ µL
X	#	1000ng/#	2000ng/#	

3. Pack up and take with you the following equipment:
    - ☐ DNA samples
    - ☐ Kim wipes
    - ☐ qH_2O aliquot
    - ☐ Marker
    - ☐ Gloves
    - ☐ P200 micropipette and tips
    - ☐ 1.5-mL microtubes
    - ☐ [Tough tags]
    - ☐ Microtube AFA Fibre screw caps
    - ☐ Mini centrifuge
    - ☐ APS/CAH PSU ID
4. Set up Covaris:
    a. Open laptop and turn on machine (back left). Open Covaris program.
    b. Load pedestal and add water until level with the pedestal stage.
    c. Select/edit appropriate program* and wait for temperature to equilibrate.
    d. Label microtube AFA fibre screw caps [sample name] & new 1.5-mL tubes [sample name, _ bp sheared, date].
5. Load sample into AFA fibre microtubes.
6. Place AFA fibre microtube with sample into the hole in the stage of the pedestal, lower hammer, and close door. If all of the three green check marks are present, click RUN. Click COMPLETE when the shearing program ends.
7. Transfer sheared DNA into new, labeled 1.5-mL tube. Discard AFA fibre microtbue in allotted waste jar.
8. When finished, clean and put away the pedestal, and suck up water with syringe or Kim wipe. Turn off Covaris machine, close program, and leave laptop as you found it.
9. If you diluted 1 µg DNA in 100 µL qH_2O, can either bead clean up (elute in 50 µL) to reduce volume and concentrate sample OR speed vac down to 50 µL (speed vac is preferred for retaining the same mass, but beware of salts).

*Shearing protocol:

Target bp	Peak Incident Power	Duty Factor (%)	Cycles Per Burst	Treatment Time (sec)	Temperature (°C)	Volume (µL)
	50	20	200		20	100-130

According to Illumina TruSeq Nano DNA library prep, for really long, well-preserved DNA, shear for 65 seconds for a target size of 350 bp and 45 seconds for 550 bp.

Safe stopping point – store sheared DNA extracts @ -20°C

M&K Library Prep (2): Blunt-End Repair

Covaris shearing generates DNA fragments with 3' or 5' overhangs. *During BER, these overhanging ends are filled in or removed by T4 DNA polymerase. 5'-phosphates are attached using T4 polynucleotide kinase (PNK).*

1. Place 25-50 µL of each sheared DNA sample into separate strip tube/plate wells. Add blank control (25-50 µL qH_2O) and positive control to two empty wells.
2. Make master mix as follows. Flick to mix, then briefly spin down. **Avoid vortexing after addition of enzymes*.

Reagent (Kept in -20°C)	Volume (µL) per Sample		Volume (µL) per Sample [25 uL template input]	Final Concentration	Vol. (µL) per .5 Samples
qH_2O	7.12 (5 U/µL)	3.70	12.83		
Buffer Tango (10X) Lot # [record]	7		NEB Buffer 2 – 5.00 BSA – 0.25	1X	
dNTPs (25 mM each) Lot # [record]	0.28	2.8 (2.5 mM each)	2.00 (2.5 mM each)	100 µM each	
ATP (100 mM) Lot # [record]	0.7		0.5	1 mM	
*T4 PNK** Lot # [record]	3.5		2.50	0.5 U/µL	
*T4 DNA Poly** Lot # [record]	1.4 (5 U/µL)	2.3 (3 U/µL)	1.67 (3 U/µL)	0.1 U/µL	
Total Volume (µL)	20		25	-	

3. Add 20 µL of master mix to 50 µL of sample. Mix by pipetting up and down.
4. Seal (lid/tape), spin down briefly, and incubate in thermal cycler – 25°C for 15:00 minutes, 12°C for 5:00 minutes, 4°C for forever. ["LIB" folder → "BLUNT", 50 µL total reaction volume]
   a. Take AMPure XP/SPRIselect beads out of fridge to bring up to room temperature.
   b. After incubation, briefly spin down in case any condensation has accumulated on the lid.
5. Place plate on ice or immediately proceed to next step.

After Blunt-end Repair, do a Size-Selection Bead Cleanup (3)

See "Things to Consider Before Library Prep" for more info about beads. For ancient samples, a regular bead cleanup with homemade SPRI beads is sufficient for this step (see Step 5). 25 µL sample input + 20 µL BER (2) = 45 µL per sample. Add 45 * 1.8 = **81 µL beads**.

1. Resuspend AMPure XP/SPRIselect beads by vortexing.
2. BER (2) = 70 µL per sample. Add 70 * 0.5x = **35 µL** of beads to each sample.
3. Put on a new seal* (caps/tape) and vortex for several seconds. Ensure beads are properly suspended. *Use new seal whenever possible.
4. Let samples rest at room temperature for 5:00 minutes, then briefly spin down residual sample/beads.
5. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
6. Aspirate off supernatant and transfer to new well; fragments of DNA that are too large will be bound to beads.
7. Resuspend AMPure XP/SPRIselect beads by vortexing, then add 70 * (1.8x – 0.5x) = **91 µL** of beads to each sample.
8. Seal and vortex (see Step 3), let samples rest at room temperature for 5:00 minutes, then briefly spin down.
9. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
10. Aspirate off supernatant and discard; target insert DNA will be bound to beads.
11. Leave samples on magnet, wash with 150 µL of freshly made (<1 week old) 70% EtOH, and let sit 1:00 minute.
12. Aspirate off EtOH and discard.
13. Repeat steps 11 and 12 for second wash.
14. Remove any residual EtOH, then let dry for 5-20:00 minutes undisturbed at room temperature unsealed.
15. Take samples off magnet and elute with 22 µL TEt by directly aiming stream at beads stuck to well wall.
16. Seal wells and vortex (see Step 3) to dislodge beads from wall. Let sit 1:00 minute undisturbed, then briefly spin down.
17. Magnetize in ring stand and let sit undisturbed 2:00 minutes, then transfer 20 µL eluate into new well. This eluate now contains cleaned DNA of your target length.

Safe stopping point – seal and store DNA samples @ -20°C

M&K Library Prep (4): Adapter Ligation

1. Make master mix as follows, flick to mix, briefly spin down, and set aside. **Avoid vortexing after addition of enzyme*.
    a. IMPORTANT: The ligase works at room temperature. Be sure to add ligase to master mix last, and immediately distribute master mix among samples *after* adding the p5p7 adapter mix to each sample.
    b. T4 Ligase Buffer may have precipitate. Can warm to 37°C on heat block or in incubator, with periodic vortexing, to resuspend.
    c. Since PEG is highly viscous, be sure to vortex the Master Mix well before adding T4 DNA ligase, then mix gently thereafter.

Reagent (Kept in -20°C)	Volume (µL) per 1 Sample		Volume (µL) per Sample [25 uL template input]	Final Concentration	Vol. (µL) per .5 Samples
qH_2O	10	8	10.2		
PEG (see **1c**) Lot #	4 (4000)	6 (6000)	4 (4000)	5%	
T4 Ligase Buffer (see **1b**) Lot #	4		4	1X	
*T4 DNA Ligase** (see **1a**) Lot #	1		0.84 (5.97 U/uL)	0.125 U/µL	
p5p7 adapter mix [Aliquot from aLab]	*1*		*1*	*2.5 µM each*	
Total Volume (µL)	19		19	-	

2. Add 1 µL p5p7 adapter mix to each sample.
3. Add 19 µL Master mix to the 20 µL DNA. Pipette to mix, then seal, and briefly spin down.
4. Incubate in thermal cycler – 22°C for 30:00 minutes. ["LIB" folder → "LIG", 40 µL total reaction volume]
    a. Take SPRI beads out of fridge to bring up to room temperature.
    b. After incubation, briefly spin down in case any condensation has accumulated in the lid.

After Adapter Ligation, do a Bead Cleanup (5)

1. Resuspend SPRI beads by vortexing.
2. AL (4) = 40 µL per sample. Add 1.8 volumes of beads to each sample → 40 µL x 1.8 volumes = **72 µL beads**.
3. Switch vortex to "On" instead of "Touch", then adjust speed to about 1/3 maximum. Put on a new seal* (caps/tape) and vortex for several seconds. Ensure beads are properly suspended. *Use new seal whenever possible.
4. Let samples rest at room temperature for 5:00 minutes, then briefly spin down residual sample/beads.
5. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
6. Aspirate off supernatant and discard; DNA will be bound to beads.
7. Leave samples on magnet, wash with 150 µL of freshly made (<1 week old) 70% EtOH, and let sit 1:00 minute.
8. Aspirate off EtOH and discard.
9. Repeat steps 7 and 8 for second wash.
10. Remove any residual EtOH, then let dry for 5-20:00 minutes undisturbed at room temperature unsealed.
11. Make master mix for Adapter Fill-in while samples are drying.
12. Take samples off magnet and elute with 22 µL TEt by directly aiming stream at beads stuck to well wall.
13. Seal wells and vortex (see Step 3) to dislodge beads from wall. Let sit 1:00 minute undisturbed, then briefly spin down.
14. Magnetize in ring stand and let sit undisturbed 2:00 minutes, then transfer 20 µL eluate into new well. This eluate now contains cleaned DNA of your target length.

M&K Library Prep (6): Adapter Fill-in

1. Make master mix as follows, flick to mix, and briefly spin down. **Avoid vortexing after addition of enzyme*.

Reagent (Kept in -20°C)	Volume (µL) per 1 Sample		Volume (µL) per Sample [25 uL template input]	Final Concentration	Vol. (µL) per .5 Samples
qH₂O	14.1	10.5	10		
ThermolPol Buffer Lot #	4		4	1X	
dNTPs (10mM) Lot #	0.4	4.0 (2.5 mM each)	4	250 µM each	
*Bst DNA Polymerase** Lot #	1.5		2	0.3 U/µL	
Total Volume (µL)	20		20	-	

2. Add 20 µL Master mix to each sample. Pipette to mix slightly, then seal, and briefly spin down.
3. Incubate in thermal cycler – 37°C for 20:00 minutes. ["LIB" folder → "FILL", 40 µL total reaction volume]
4. After incubation, briefly spin down in case any condensation has accumulated in the lid.

After Adapter Fill-in, do a Bead Cleanup (7)

1. Resuspend SPRI beads by vortexing.
2. AF (6) = 40 µL per sample. Add 1.8 volumes of beads to each sample → 40 µL x 1.8 volumes = **72 µL beads**.
3. on a new seal* (caps/tape) and vortex for several seconds. Ensure beads are properly suspended. *Use new seal whenever possible.
4. Let samples rest at room temperature for 5:00 minutes, then briefly spin down residual sample/beads.
5. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
6. Aspirate off supernatant and discard; DNA will be bound to beads.
7. Leave samples on magnet, wash with 150 µL of freshly made (<1 week old) 70% EtOH, and let sit 1:00 minute.
8. Aspirate off EtOH and discard.
9. Repeat steps 7 and 8 for second wash.
10. Remove any residual EtOH, then let dry for 5-20:00 minutes undisturbed at room temperature unsealed.
11. Make master mix for Indexing PCR while samples are drying.
12. Take samples off magnet and elute with 22 µL TEt by directly aiming stream at beads stuck to well wall.
13. Seal wells and vortex (see Step 3) to dislodge beads from wall. Let sit 1:00 minute undisturbed, then briefly spin down.
14. Magnetize in ring stand and let sit undisturbed 2:00 minutes, then transfer 20 µL eluate into new well. This eluate now contains cleaned DNA of your target length.

Safe stopping point – seal and store DNA samples @ -20°C

M&K Library Prep (8): Indexing PCR

1. Make master mix as follows, flick to mix, and briefly spin down.

Reagent (Kept in -20°C)	Volume (µL) per 1 Sample	Volume (µL) per Sample [25 uL template input]	Final Concentration	Vol. (µL) per ___.5 Samples
qH₂O	3	3		
KAPA HiFi Hotstart Ready Mix [aLab aliquot]	25	25	1X	
Primer IS4 [universal forward]	1	1	200 nM	
Barcode (1-80) [unique reverse]	*1*	*1*	*200 nM*	
Total Volume (µL)	29	29	-	

2. Add 1 µL of the respective barcode to each sample, then add 29 µL Master Mix to each sample.
3. PCR – load following reaction parameters [50 µL total reaction volume]

98°C	98°C	60°C	72°C	Repeat # times	72°C	8°C
2:00 minutes	0:20 seconds	0:30 seconds	0:30 seconds		5:00 minutes	Forever

   - If you went into library prep with 1 µg DNA, 8 cycles will be plenty.
   - If you start with 0.75 µg DNA, do 10 cycles; if you start with 0.5 µg DNA, do 12 cycles.

After Indexing PCR, do a Bead Cleanup (9)

1. Resuspend SPRI beads by vortexing.
2. PCR (8) = 50 µL per sample. Add 1.8 volumes of beads to each sample → 50 µL x 1.8 volumes = <u>**90 µL beads**</u>.
3. Vortex briefly, let samples rest at room temperature for 5:00 minutes, then briefly spin down residual sample/beads.
4. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
5. Aspirate off supernatant and discard liquid; DNA will be bound to beads.
6. Leave samples on the magnet, and wash with 150 µL of freshly made (<1 week old) 70% EtOH, and let sit 1:00 minute. Aspirate off EtOH and discard.
7. Repeat step 6 for second wash.
8. Remove any residual EtOH, then let dry for 5-20:00 minutes undisturbed at room temperature unsealed.
9. Take samples off magnet, and elute with 22 µL TEt by directly aiming buffer stream at beads stuck to well wall.
10. Put on a new seal, vortex briefly, let sit 1:00 minute undisturbed, then briefly spin down.
11. Magnetize in ring stand and let sit undisturbed 2:00 minutes, then transfer 20 µL eluate into new 1.5-mL tubes. This eluate now contains your cleaned DNA library.

M&K Library Prep (10): 2% Check Gel

1. Make 2% agarose gel:
   - If using a 30-mL gel rack, use 30-mL 1X TAE Running Buffer + 0.6 g Agarose. + 0.75 µL Ethidium Bromide.
   - If using a 60-mL gel rack, use 60-mL 1X TAE Running Buffer + 1.2 g Agarose + 2 µL Ethidium Bromide.
   - If using a 120-mL gel rack, use 120-mL 1X TAE Running Buffer + 2.4 g Agarose + 5 µL Ethidium Bromide.
2. Load 6 µL 50 bp GeneRuler ladder.
3. Load 1 µL DNA + 5 µL load dye (mix prior to loading) to respective wells.
4. Run out at ~100 volts. Make sure you do not let the samples run off the gel.
5. Visualize on the UV block.

M&K Library Prep (11): Check DNA Library Concentration

Qubit – follow Qubit instructions, use 1 uL of sample.

Nanodrop

1. Turn on computer and load Nanodrop program. Select DNA.
2. Initialize laser with water (1.5 µL), then blank machine with TEt (1.5 µL).
3. Load 1.5 µL each sample respectively.

OPTIONAL – M&K Library Prep (12): Gel Visualization and Size Selection

1. Make a 1.8% low-melt agarose gel.
   – Be sure to make it pretty deep so that the entire sample fits in the well of the gel*.
   – Combs with medium teeth are better, as they allow more DNA to fit in each well without being wider than the gel-cutting tips*.

   *PJ's 160-200-mL gel rack works best (I use 180 mL for this). If using the 160-mL rack, use 180 mL 1X TAE buffer and 3.24 g low-melt agarose. Use 5-10 µL Ethidium Bromide (I usually use 7 or 8 µL). If you use GelRed 10,000, use 1 µL stain per 10 µL buffer (in a 40-mL gel, use 4 µL GelRed 10K).
2. Add 10 µL load dye to each sample.
3. Load gel with 6 µL 50 bp GeneRuler ladder and then, leaving a space between each sample**, load all of sample/dye mixture. **You will need to cut out the appropriate section later.
4. Run gel at ~100 volts. Make sure you do not let the samples run off the gel.
5. Visualize gel on UV block to make sure it worked and take photos. If the gel seems mushy, let it sit for a few minutes to cool, or wrap it up in plastic wrap (twice) and foil and put in fridge for a few minutes.
6. Transfer gel to blue visualization block (not UV).
7. Mark the appropriate location of the band you want with pipette tips.
8. Size selection:
   a. Take a gel punch from each sample at the appropriate band length, as well as one longer and one shorter.
   b. Store these in separate, labeled, clean tubes.

Target gel punch ___________bp Second gel punch ___________bp Third gel punch ___________bp

OPTIONAL – M&K Library Prep (13): Gel Extraction of Size-selected Bands (OMEGA)

***Can use Qiagen gel extraction kit (follow protocol in box). Elute with 35 µL then 35 µL again.**

1. Weigh tubes with gel punches and note specifically the heaviest weight (grams).
2. Add 1.5 – 2.0 volumes (compared to heaviest gel punch weight) Binding Buffer XP2 to sample.
   – Heaviest weight of gel punch ___________bp
   – Volume XP2 to add to each sample ___________µL
3. Incubate at 60°C for 7:00 minutes with a slight agitation or shake every 2-3 minutes. Quick spin.
   – At this point, pH should be 7.0-8.0. If the pH is too high (too basic), add 8.3 µL NaOAL (pH 5.2)***.

   ***I typically add the NaOAL every time as a precaution and to standardize the samples.
4. Add to spin column and centrifuge at 10,000g for 1:00 minute. Discard filtrate; reuse collection tube.
5. Add 300 µL Binding Buffer XP2, centrifuge at Maximum Speed for 1:00 minute. Discard filtrate; reuse collection tube.
6. Add 700 µL SPW wash buffer, centrifuge at Maximum Speed for 1:00 minute. Discard filtrate; reuse collection tube.
7. Repeat step 6 for second wash with SPW buffer.
8. Spin at Maximum Speed for an additional 2:00 minutes to dry tube.
9. Transfer spin column to new, labeled collection tube (with lid).
10. Add 35 µL extraction buffer and let rest 5:00 minutes @ room temperature.
11. Spin at Maximum Speed 1:00 minute.
12. Repeat steps 10 and 11 for second elution.

OPTIONAL – Pick: Bead Cleanup, Qiagen PCR Purification*, or Omega Column Cleanup* (14)

***Column-based kit: Follow protocol in kit box if doing a spin column kit**

1. Resuspend SPRI beads by vortexing.
2. Gel Extraction (13) = 70 µL sample. Add 1.8 volumes of beads to each sample → 70 µL x 1.8 volumes = <u>126 µL beads</u>.
3. Spin down samples, then add 1.8 volumes beads to samples.
4. Switch vortex to "On" instead of "Touch", then adjust speed to about 1/3 maximum. Put on a new seal* (caps/tape) and vortex for several seconds. Ensure beads are properly suspended. *Use new seal whenever possible.
5. Let samples rest at room temperature for 5:00 minutes, then briefly spin down residual sample/beads.
6. Magnetize on ring stand. Let sit at room temperature for 5:00 minutes to separate the beads from the solution.
7. Aspirate off supernatant and discard; DNA will be bound to beads.

8. Leave samples on magnet, wash with 150 µL of freshly made (<1 week old) 70% EtOH, and let sit 1:00 minute.
9. Aspirate off EtOH and discard.
10. Repeat steps 8 and 9 for second wash.
11. Remove any residual EtOH, then let dry for 5-20:00 minutes undisturbed at room temperature unsealed.
    – Make master mix for Indexing PCR while samples are drying.

1. Take samples off magnet and elute with 20 µL TEt by directly aiming stream at beads stuck to well wall.
2. Seal wells and vortex (see Step 3) to dislodge beads from wall. Let sit 1:00 minute undisturbed, then briefly spin down.
3. Magnetize in ring stand and let sit undisturbed 1:00-2:00 minutes, then transfer eluate into new well. This eluate now contains cleaned DNA of your target length.