**Update**

1. MVZ samples
   1. Learn general library prep
   2. Get a sense of size selection bead ratios
2. Manny’s wing punches
   1. Validate with Methyl Seq

**Objective** = Design a high throughput, affordable DNA methylation assay for bats in line with the epigenetic clock developed by Wilkinson et al. (2020)

**Steps:**

1. Design an in-solution probe assay from Wilkinson et al. microarray—DONE
2. Design and optimize a library prep protocol
3. Validate assay with **both** Steve’s and Cara’s aged samples (and probably Cara’s colleagues’ ~100 samples from Singapore)

**To do:**

1. Get our DNA extraction and fragmentation protocols (drafts [here](https://docs.google.com/document/d/1uJ0sBPjzmAg-3goP_nDvrr0McJHrlB3QuDqMM2kOT_Y/edit?usp=sharing)) running on the Benchsmart 96 automated pipetting system. We’re waiting on Pteropus spp., Eidolon spp., and Rousettus spp. tissue samples from the EGL--they’re really busy so these samples will not be available for pick up until late September. The advantage of using the EGL samples is that it will be more similar to our Mada samples with respect to species, sample type, and DNA yield.
2. Keep mapping out subsequent library prep steps in more detail. Full protocol [here](https://docs.google.com/document/d/1EmrXPH9B905kXVXGotFnXyCEpKPkMVLR/edit?usp=sharing&ouid=106307316144479120356&rtpof=true&sd=true)

**...once Mada samples arrive (Sept 30th)**

1. Finalize fragmentation reaction conditions
   1. What is the DNA yield from the Mada wing punches? → will determine total reaction volume
   2. What is the precise fragmentation time between 30-45 minutes that will give us 150-200bp?
2. High throughput DNA extractions

**...and once we have a protocol running:**

1. Follow up with Jerry about using his samples to validate our assay

**Summary of protocol optimization progress**

**Fragmentation tests:**

1st test: Lydia digested varying amounts of falcon, pigeon, and guanaco DNA (all extracted the same way) while varying the total volume of the reaction, but kept the total digestion time constant (20 minutes). She found that each DNA sample had remarkably consistent fragmentation shapes regardless of 1) the total amount of DNA added and 2) total volume of the reaction BUT there were dramatic differences between the falcon, pigeon, and guanaco DNA samples.

2nd test: Lydia and I digested varying amounts of Myotis lucifugus (cell line) and Myotis thysanodes (wing tissue) DNA (which Manny and I extracted using the Qiagen kit) in a full reaction volume, but varying the digestion time: 15, 20, or 30 minutes. There was again, differences between species; but overall, it looked like we needed a slightly longer digestion time—at 30 minutes the peak fragment size was around 275bp and we’re shooting for 150-200bp.

3rd test: We wanted to keep playing around with the digestion time while replicating conditions we’ll have for Cara’s Mada samples. I extracted DNA from Manny’s Pteropus, Rousettus, and Eidolon cell lines (same genera as the Mada samples) using Stacy’s bead-based DNA extraction method that can be high throughput. I then digested a total of 200ng in a 1/5th reaction for 20, 30, 40, and 50 minutes. This time, nothing digested. Lydia’s first test suggested that changing the amount of DNA to 200ng and total reaction size to 1/5th shouldn’t have an impact. So we thought I had probably done something wrong

4th test: Replicated the 3rd test with Lydia to try to figure out what I was doing wrong. We got the same results as before--nothing digested. Then we realized I had accidentally been using 1M Tris instead of 10mM Tris as a DNA buffer. I did a buffer swap, replacing the 1M Tris with nuclease-free water.

5th test: Swapping out the buffer for water solved the problem! Replicated the 3rd and 4th test and the DNA digested. Except this time, I didn’t normalize the input DNA amount—though I did do a 1:2 dilution to save DNA, which gave me a total input amount of 539ng in column 1, 259 ng in column 2 and 567 ng in column 3. Results were a bit unclear because some of the samples in the 30 minute and 40 minute treatments had two peaks. This is likely because the reaction was overloaded with DNA (KAPA recommends <500ng on [page 7](https://rochesequencingstore.com/wp-content/uploads/2018/01/KAPA-Frag-Kit.pdf)). And in our test 5 results, it looks like the higher the input, the larger the second peak—samples 2, 5, 8, and 11 had an input amount of 259ng and there was no second peak; whereas the other two sample sets had an input amount >500ng and had second peaks (and samples 3, 6, 9, and 12 had the highest input amount and also the largest second peaks). Overall, it looks like we want to shoot for a time between 30 and 45 minutes.

***What we’ve learned so far about the fragmentation reaction:***

1. The relationship between the fragmentation time and fragment size distribution is species-specific--DNA from different species, digested for the same amount of time, had different fragment size distributions
2. Total input DNA and reaction volume do not affect the fragment size distribution
3. That being said, there does seem to be a risk of overloading the reaction with DNA--so if we notice we’re getting highly concentrated DNA from the wing punch samples (>500ng in 7uL), we should probably increase the total reaction volume
4. Make sure you’re using 10mM Tris not 1M…

***Next steps:*** We still don’t know what fragmentation time we’ll need to get ~150-200bp. Based on our tests with bat DNA, we know it will be roughly between 30 and 50 minutes. But it’s probably worth waiting until the Mada samples arrive to fine tune fragmentation conditions--because the fragmentation time is species-specific and the total reaction volume will depend on the DNA yield from the Mada wing punches. In the meantime, it would be worthwhile to modify our protocol for the EGL Benchsmart 96 automated pipetting system. We were hoping to use the MVZ samples to test out that protocol--but the MVZ is really busy and those samples won’t be available until the last week of September