**Update 10/11:**

* We’re going to go ahead and use the Zymo buffer, just the easiest way to go
* Stop testing, just go ahead and try the full protocol:
  + Go with 5uL RNAse A and ditch the DTT--though I’m still wondering, is 20uL of buffer enough (why wasn’t it reduced proportionally)?
  + Don’t worry about the hair--just plan to spin down and transfer to a new plate. It only takes a minute and uses one extra plate
  + Go ahead and take Manny’s wing punches through the entire protocol. We’ll split the DNA sample--and validate with Methyl-Seq

**Schedule:**

* Tues 10/11: Overnight digestion
* Wed 10/12: DNA extraction
* Thurs 10/13: Fragmentation reaction?--I need to plan out how we’ll pinpoint the digestion time with titration

**Questions for Manny**

1. What freezer have Cara’s known-age wing tissue samples again?
2. Where is that other sample?--in Sudmant fridge, should throw out everything else...
3. Was the M. thysanodes DNA from wing punches? Just trying to confirm if we’ve done fragmentation tests with anything other than cell lines--it was a cell line
4. How long do you think it will take for methylated adapters to arrive? Probably should pause at the fragmentation testing phase (wait to fragment the full sample) until those arrive? Unless fragmented DNA is stable?
5. What do you think about the above plan and Methyl-Seq validation?--on board

**Questions for Lydia:**

1. Should we try different samples at different reaction sizes? I.e., we do full reactions for sample 1, ½ reactions for sample 2, ¼ reactions for sample 3--then can demonstrate that cutting the reaction doesn’t negatively affect the results?--do a full reaction the first time...
2. We should probably order methylated adapters and bisulfate kit before we jump into this protocol. I don’t think there is anything else to order because you already bought the Kapa HiFi uracil+?

**Old notes:**

Unfortunately, we can’t finalize the protocol until Cara’s samples arrive (scheduled for the end of October, but could be later)--because many details depend on the species and sample type/condition. The goal here is to figure out what we actually can do while we’re waiting.

**Questions for Manny**

1. Our original ratio was originally 200ul buffer--100uL ProK--20uL RNAse A. Now it’s 20-20-20. Why didn’t the RNAse A volume decrease proportionally? Was it because Manny was worried about the interaction with the DTT? Or can we decrease the RNAse A volume to 2-5uL here?
2. Test wing punches--where are they? How many? What species?
3. What freezer have Cara’s known-age wing tissue samples again?
4. Does the experiment plan sound reasonable to you?

**Unknowns**

***DNA Extraction***

* Does the homebrew cell lysis buffer work as well as the Zymo buffer? Does digestion take forever with 0.5% SDS--do we need to increase the concentration?
* Our original ratio was originally 200ul buffer--100uL ProK--20uL RNAse A. Now it’s 20-20-20. Why didn’t the RNAse A volume decrease proportionally? Was it because Manny was worried about the interaction with the DTT? Or can we decrease the RNAse A volume to 2-**5uL** here? MVZ samples
  + Spin down instead. No DTT 5uL RNAse
* Does the hair digest completely? If not, we may need to spin down after digestion and then transfer samples to a new plate. Are the yields roughly the same in quality and quantity if we don’t spin down? We think the DTT might help fully digest the hair. Manny’s wing punches

***Fragmentation reaction***

* Fragmentation reaction time needed for a 150-200bp size range:
  + Does it vary between Pteropus, Eidolon, and Rousettus genera (i.e., will we need different fragmentation times for our different species)? MVZ samples
  + What are the precise fragmentation times needed for Cara’s samples?
* Total volume of the fragmentation reaction? Overloading the reaction with DNA prevents full digestion (we saw two peaks in our fragmentation test #5). We don’t want to have to normalize each individual DNA extraction--but if we find that our DNA extractions often yield more than 500ng in 7uL, then we should increase the reaction size. Cara’s samples
* Bead ratio for size selection after the fragmentation reaction. Do we definitely need a size selection step here? MVZ samples

***KAPA library prep + bisulfite + probe capture***

* We *could* consider taking Manny’s wing punches through the entire protocol and then try to calculate age with the Wilkinson et al. clock. That way we’d confirm whether the protocols and probes actually work, as well as come up with a general workflow. But I wanted everyone’s feedback on whether that would actually be a productive use of time and resources (as opposed to just waiting and doing this with samples we actually need to age)

**Experiment planning:**

***MVZ samples:***

We have 6 organ tissues samples from the MVZ, 2 for each genera: Pteropus, Eidolon, and Rousettus

To test whether the homebrew buffer works and whether we need to increase the SDS concentration--for each pair of genera samples, use the Zymo buffer for one and the homebrew for the other:

* Pteropus: Zymo buffer, 0.5% SDS
* Eidolon: Zymo buffer, 1% SDS
* Rousettus: Zymo buffer, 1.5% SDS

To test how much RNAse we need:

* Split each of the 6 digested samples into two: try 20uL vs 15uL; 20 vs. 10; 20 vs 5; 20 vs 4; 20 vs. 3; 20 vs. 2

Finish DNA extraction method for all 12 samples. Then Quantus to check yield quantity and quality

To test whether fragmentation conditions will need to vary between species:

* Try a fragmentation test, comparing 25, 30, 35, and 40 min within a species or genera (depending on our yield)

Lastly, try the size selection step

***Manny’s wing punch samples*** (not sure yet how many or what species)

To test whether the hair digests sufficiently or whether we need to spin down:

* Split the digested sample, try spinning down vs. not and assess differences in yield quantity and quality