Methylation Comparison WGBS and MBDBS With Pico Methyl Seq Library Prep Kit

Using the Zymo Pico Methyl Seq Kit on the MBD enriched 3 *Pocillopora* and 3 *Montipora* samples and non-enriched as whole genome bisulfite sequencing for the purpose of comparing them to RRBS preparations

Samples for methylation comparison are from the Holobiont Integration Project, and were extracted by Emma Strand or myself, see her notebook posts for the extraction specifications: 20190805 and 20180823, 20190718 and 20190903.

Dilution of WGBS samples

Previous trial of WGBS used 100ng input, which didn't work. So our plan is to do 1ng which has previously been shown to work well with this kit for us. However, all the samples for WGBS need to be diluted to avoid pipetting under 1μl. The easiest was to dilute every sample to be 1ng/μl concentration so 1μl could be used as the input for every sample

Sample	volume DNA	volume 10mM Tris HCl
1041	1µl	61.4µl
1471	1µl	53.8µl
1637	1µl	85.8µl
1101	1µl	26.1µl
1548	1µl	22.1µl
1628	1µl	16µl

Dilution of Non-methlated eColi DNA

2.5ng/µl DNA for spike in for bisulfite conversion efficency test is too high a concentration, need it to be .025ng/µl so a 1:100 dilution:

1µl non-methlated DNA
99µl ultra pure water

Sample Prep for PMS

Samples need to all include 1ng of sample, .05ng spike-in, and water up to 20µl

sample + method	tube #	volume DNA	volume diluted spike	volume ultra pure water to 20µl
1041 WGBS	1	1µl	2µl	17µl
1471 WGBS	2	1µl	2µl	17µl
1637 WGBS	3	1µl	2µl	17µl
1101 WGBS	4	1µl	2µl	17µl
1548 WGBS	5	1µl	2µl	17µl

sample + method	tube #	volume DNA	volume diluted spike	volume ultra pure water to 20µl
1628 WGBS	6	1µl	2µl	17µl
1041 Captured MBD	7	1µl	2µl	17µl
1471 Captured MBD	8	1.17µl	2µl	16.8µl
1637 Captured MBD	9	0.5µl	2µl	17.5µl
1101 Captured MBD	10	7.14µl	2µl	10.68µl
1548 Captured MBD	11	5.78µl	2µl	12.22µl
1628 Captured MBD	12	7.63µl	2µl	10.37µl

Bisulfite Conversion

- Added 130µl lightning conversion reagent to each sample in at PCR tube
- Put tubes in the thermocycler Pico bisulfite conversion program

Cleanup

- Made 12 spin column, one for each sample
- Added 600µl M-binging buffer each to 12 spin columns
- Added 150µl of the BS reaction (all) to each individual tube
- Invert columns to mix
- Centrifuged columns at 12,000 rcf for 30 seconds and discarded flowthrough
- Added 100µl M-Wash buffer to each column
- Centrifuged columns at 12,000 rcf for 30 seconds and discarded flowthrough

- Added 200*µ*l L-desulfonation buffer to each column
- Let them sit for 15 minutes
- Centrifuged columns at 12,000 rcf for 30 seconds and discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged columns at 12,000 rcf for 30 seconds and discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged columns at 12,000 rcf for **1 minute and 30 seconds** and discarded flowthrough
- Added 8*µ*l warmed (56C) DNA elution buffer to each column and let sit for 1 minute in new 1.5mL tubes to collect
- Centrifuged columns at 12,000 rcf for 30 seconds

Amplification with PrepAmp Primers

- Made Priming master mix on ice:
  - 2*µ*l 5X PrepAmp buffer * 13 = 26*µ*l
  - 1*µ*l PrepAmp Primers (40*µ*M) * 13 = 13*µ*l
- Made new PCR tubes with 3*µ*l of PrepAmp MM and 7*µ*l of bisulfite treated DNA
- Kept those on ice
- Made PrepAmp Mix on ice:
  - 1*µ*l 5X PrepAmp buffer * 13 = 13*µ*l
  - 3.75*µ*l PrepAmp PreMix * 13 = 48.75*µ*l
  - 0.3*µ*l PrepAmp polymerase * 13 = 3.9*µ*l
- Set thermocylcer program with lid temp restricted to 25 degrees C and place samples inside and run the pico priming program on the thermocycler:
  - 98 for 2 minutes
  - 8 degrees for 1 minute
  - 8 degree hold
  - **During hold vortex, spin tubes down, add 5.05*µ*l PrepAmp Mix to each tube, vortex, spin down, and place back in thermocycler**
  - 8 degrees for 4 minutes
  - 16 degrees for 1 minute with 3% ramp rate
  - 22 degrees for 1 minute with 3% ramp rate

- 28 degrees for 1 minute with 3% ramp rate
- 36 degrees for 1 minute with 3% ramp rate
- 36.5 degrees for 1 minute with 3% ramp rate
- 37 degrees for 8 minutes
- repeat back from the first step one time through again
- **During hold, vortex, spin tubes down, tried to add 0.3µl PrepAmp Polymerase to each tube, it wouldn't really come out of the tip so I ended up adding .5µl, vortex, spin down, and place back into thermocycler**

Cleanup with DNA Clean and Concentrator Columns (DCC)

- Made a 1.5mL tube for each sample, added 7:1 ratio DNA binding buffer, so 107.45µl of DNA binding buffer
- Put elution buffer in thermomixer 56 degrees
- Added DNA sample (15.35µl) to the appropriate 1.5mL tube
- Vortexed, spun down, and added to the column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200µl M-wash buffer to each column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200µl M-wash buffer to each column
- Centrifuged 12,000 rcf for **1 minute and 30 seconds**, discarded flowthrough
- Transferred columns to 1.5mL tubes
- Added 12µl warmed elution buffer to each column directly
- Incubated 1 minute
- Centrifuged 12,000 rcf 30 seconds

First Amplification

- Made 1st Amp master mix:
  - 12.5µl 2X Library Amp Mix * 13 = 162.5µl
  - 1µl Library Amp Primer(10µM) * 13 = 13µl
- Added 13.5µl MM to new PCR tubes
- Added 11.5µl of cleaned and concentrated DNA sample to the appropriate new PCR tube *note here, sample 2 11.5µl in them, could have been carryover of wash buffer in*

the column, even though I increased the spin time 3 fold for the last wash… all of the flow through was added in this step

- Vortexed, spun down, and placed in thermocycler program 1st Pico Methyl Amp program **8 cycles for the 1ng input**

Cleanup with DCC

- Made a 1.5mL tube for each sample, added 7:1 ratio DNA binding buffer, so 175*µ*l of DNA binding buffer
- Put elution buffer in thermomixer 56 degrees
- Added DNA sample (25*µ*l) to the appropriate 1.5mL tube
- Vortexed, spun down, and added to the column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged 12,000 rcf **1 minute and 30 seconds**, discarded flowthrough
- Transferred columns to 1.5mL tubes
- Added 12.5*µ*l warmed elution buffer to each column directly
- Incubated 1 minute
- Centrifuged 12,000 rcf 30 seconds

Amplification with Index Primers

After some consulting of the previous results, we considered that maybe there had not been enough primer in the reaction to get good amplification. So for these libraries, 1*µ*l of each i5 and i7 primer was added. And the 2X LibraryAmp Master Mix was increased in volume to match the total volume (14*µ*l).

- In PCR tubes combined the following:

sample	volume DNA	volume LibAmp MM	volume i5 primer	volume i7 primer
1041 WGBS	12*µ*l	14*µ*l	1*µ*l 1	1*µ*l 1

sample	volume DNA	volume LibAmp MM	volume i5 primer	volume i7 primer
1471 WGBS	12µl	14µl	1µl 2	1µl 2
1637 WGBS	12µl	14µl	1µl 3	1µl 3
1101 WGBS	12µl	14µl	1µl 10	1µl 11
1548 WGBS	12µl	14µl	1µl 11	1µl 10
1628 WGBS	12µl	14µl	1µl 12µl	1µl 12µl
1041 Captured MBD	12µl	14µl	1µl 7	1µl 7
1471 Captured MBD	12µl	14µl	1µl 8	1µl 8
1637 Captured MBD	12µl	14µl	1µl 9	1µl 9
1101 Captured MBD	12µl	14µl	1µl 16	1µl 16
1548 Captured MBD	12µl	14µl	1µl 17	1µl 17
1628 Captured MBD	12µl	14µl	1µl 18	1µl 18

note: samples 1101 WGBS and 1548 WGBS have their i7 indexes swithced

- Vortexed, spun down, and placed in thermocycler program 2nd Pico Methyl Amp program

Cleanup with DCC

- Made a 1.5mL tube for each sample, added 7:1 ratio DNA binding buffer, so 175µl of DNA binding buffer

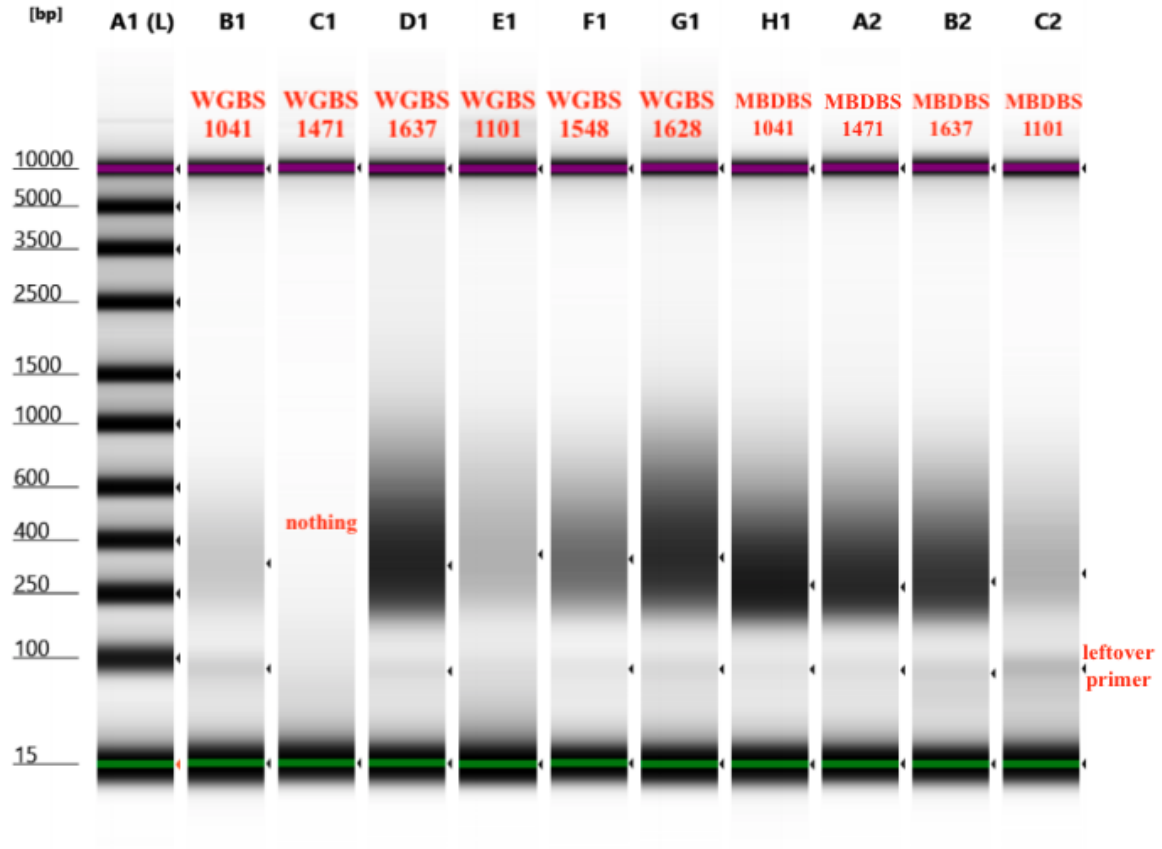
- Put elution buffer in thermomixer 56 degrees
- Added DNA sample (25*µ*l) to the appropriate 1.5mL tube
- Vortexed, spun down, and added to the column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged 12,000 rcf 30 seconds, discarded flowthrough
- Added 200*µ*l M-wash buffer to each column
- Centrifuged 12,000 rcf **1 minute and 30 seconds**, discarded flowthrough
- Transferred columns to 1.5mL tubes
- Added 12*µ*l warmed elution buffer to each column directly
- Incubated 1 minute
- Centrifuged 12,000 rcf 30 seconds

D5000 TapeStation

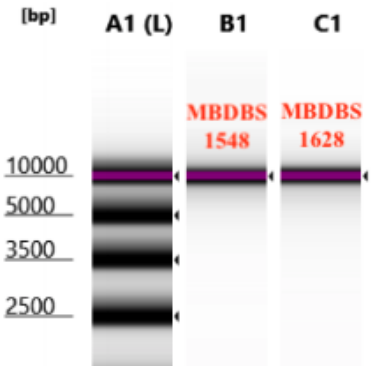
See the full report here here

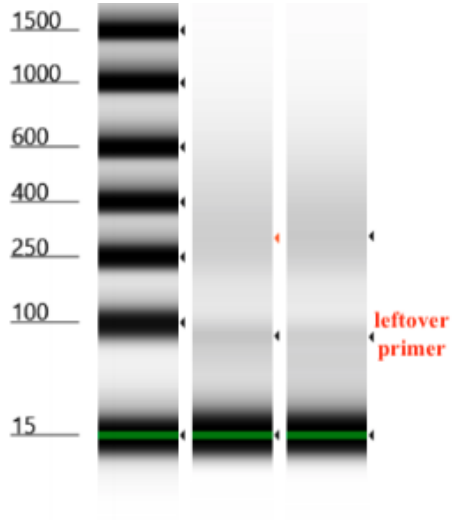
Representative tapes:

Filename: 2019-09-27 - 09.39.40.D5000

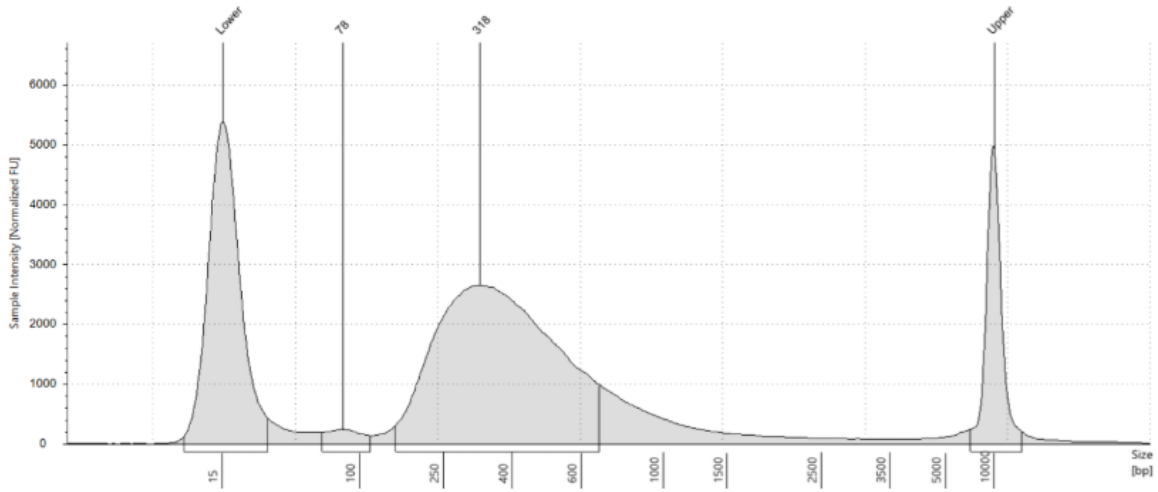


Filename: 2019-09-27 - 10.03.23.D5000

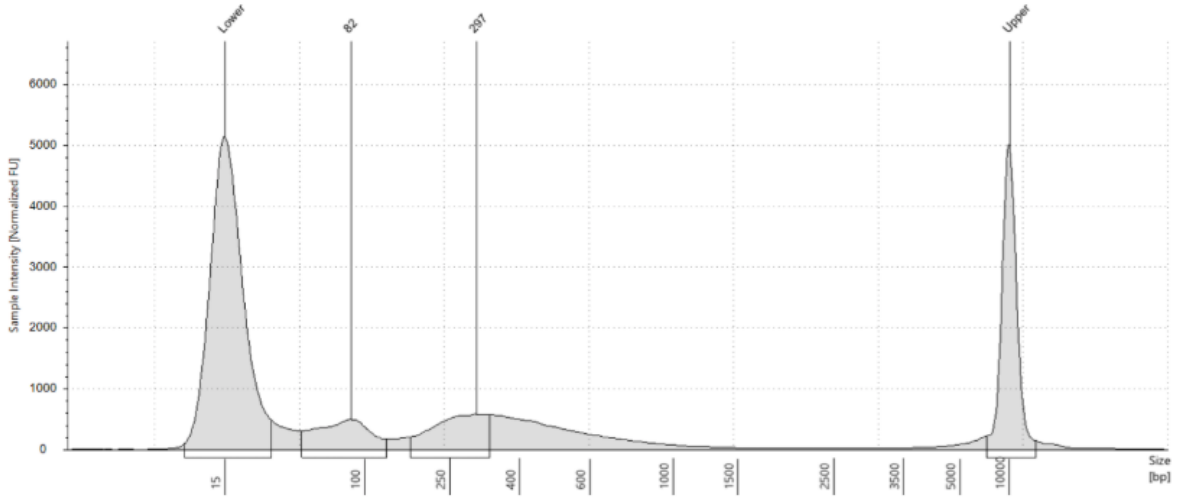



1500
1000
600
400
250
100
15
leftover
primer

D1: 1637 WGBS



C2: 1101 MBDBS



Thoughts: 1471 WGBS didn't work, this is probably because of the extra elution volume following the second cleanup. It will just have to be redone. Although it's weird that there isn't any primer dimer in that trace, like there is in all the others, so I wonder if the tape ran correctly. I might run that one again. For all the others there is primer left over, which makes sense considering how much we increased the volume by, and decreased the

amount of input DNA. For some of the samples, the amplification is not great, which might have been because of an excess of primer inhibiting amplification. Those in theory can be just amplified again, I think all will need to be cleaned up to remove the primer dimer though.

Written on September 26, 2019