RRBS Library Prep of Samples for Methylation Comparison

Reduced Representation Bisulfite Sequencing Library Prep with the Zymo Research EZ DNA Methylation RRBS Library Prep Test Kit for Methylation Comparison

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.

MspI Digestion

- Made PCR tubes with 300ng of DNA, 15ng of Spike-in non-mehtylated eColi DNA, and water to 35.5μl

Sample	Volume DNA	Volume spike	Volume ultra pure water
1041	4.81	6	24.69
1471	5.47	6	24.03
1637	3.46	6	26.04
1101	11.07	5	18.43
1548	12.99	6	16.51
1628	17.65	6	11.85

- Made master mix for digestion
  - 10X RRBS buffer 4μl * 6.2 = 24.8μl
  - MspI 0.5μl * 6.2 = 3.1μl
- Added 4.5μl of digestion master mix to each sample and pipetted to mix and spun down
- Placed in thermocycler RRBS digestion program: 4 hours at 37 degrees C and then a 4 degree hold

Adapter Ligation

- Made master mix for adapter ligation
  - 10X RRBS buffer 1μl * 6.2 = 6.2μl
  - rATP 0.5μl * 6.2 = 3.1μl
  - MspI 1μl * 6.2 = 6.2μl
  - T4 DNA ligase 1μl * 6.2 = 6.2μl
  - ultra pure water 6μl * 6.2 = 37.2μl
- Pipetted to mix master mix because ligase enzyme
- Added 9.5μl of master mix to each tube
- added 0.5μl RRBS adapters to each tube (adding separately minimizes adapters ligating to themselves)
- Pipetted to mix
- Put in thermocycler program RRBS adapters overnight:

    - 21 degrees C 3 hours
    - 37 degrees C 1 hour
    - 20 degrees C 1 hour
    - 37 degrees C 1 hour
    - 20 degrees C 1 hour
    - 4 degree hold
- The next morning made gap filling master mix
    - Taq DNA Polymerase 0.5μl * 6.2 = 3.1μl
    - 5-methylcytosine dNTP Mix 1.5μl * 6.2 = 9.3μl
- Added 2μl of gap filling mix to each tube and pipetted to mix
- Placed in theremocyler program 74 degrees C for 30 minutes

Cleanup with DCC

- Made a 1.5mL tube for each sample, added 7:1 ratio DNA binding buffer, so 364μl of DNA binding buffer
- Put elution buffer in thermomixer 56 degrees
- Added DNA sample (52μl) 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 **1 minute and 30 seconds**, discarded flowthrough
- Transferred columns to 1.5mL tubes
- Added 20μl warmed elution buffer to each column directly
- Incubated 1 minute
- Centrifuged 12,000 rcf 30 seconds

Bisulfite Conversion

- Transferred each sample to PCR tubes
- Added 130μl lightning conversion reagent to each sample in at PCR tube
- Put tubes in the thermocycler Pico bisulfite conversion program (the same for this kit)

Cleanup

- Made 6 spin column, one for each sample
- Added 600*µ*l M-binging buffer each to 6 spin columns
- Added 150*µ*l 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 100*µ*l 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 24*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

Index Primer Amplification

- Transferred 24*µ*l of DNA to new PCR tubes
- Added 25*µ*l of Library Amp Master mix to each tube
- Added 0.5*µ*l of each planned primer pairs:

Sample	i5	i7
1041	4	4
1471	5	5
1637	6	6

Sample	i5	i7
1101	13	13
1548	14	14
1628	15	15

- Placed in theremocyler RRBS index amp program for 11 cycles:
  - 94 for 30 sec
  - **94 for 30 sec** (11)
  - **55 for 30 sec** (11)
  - **68 for 1 min** (11)
  - 68 for 5 min
  - 4 hold

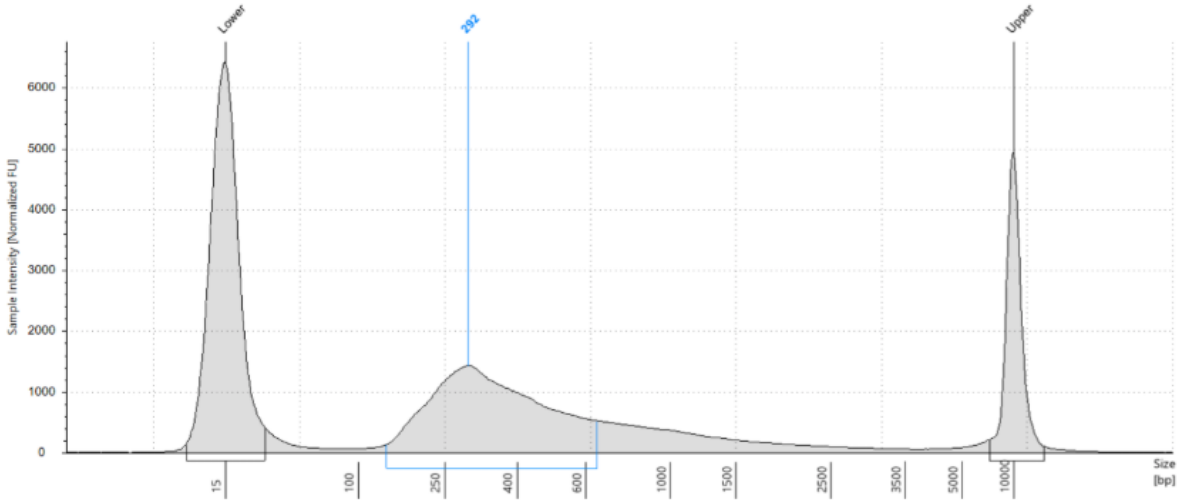
KAPA Pure Bead Cleanup

- Took beads out of fridge beforehand and made fresh 80% EtOH
- Added 50μl (1X) of Kapa Pure beads to each sample
- Performed normal bead cleanup
- Resuspended and eluted samples in 16μl DNA elution buffer

D5000 TapeStation

Full Results

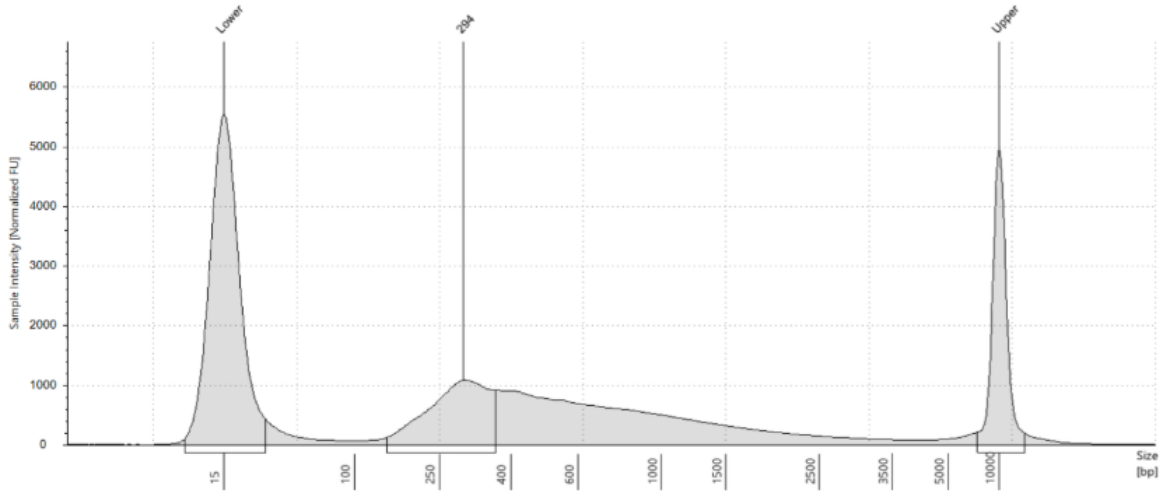
B1: 1041 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
B1	7.78	1041 RRBS		

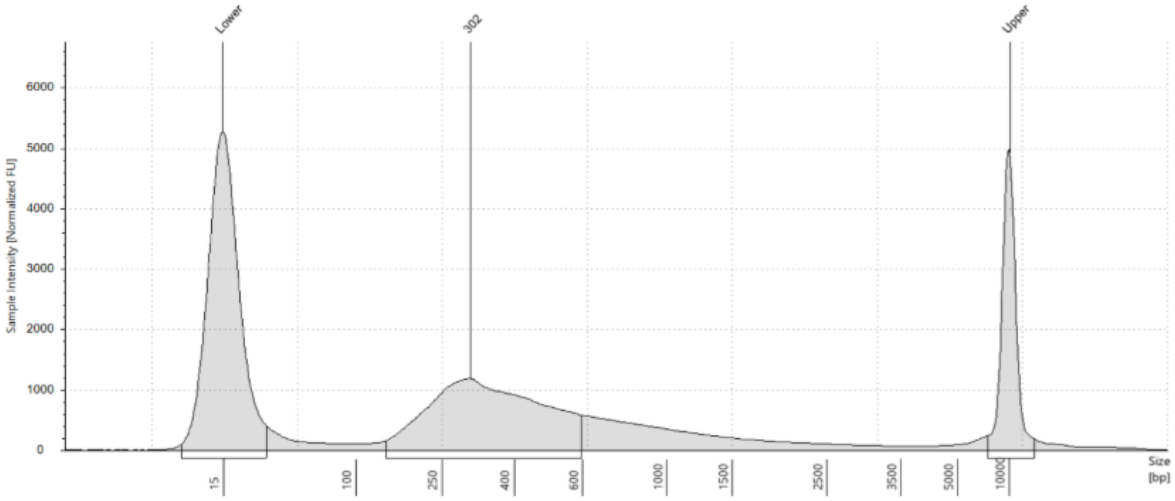
C1: 1471 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
C1	3.57	1471 RRBS		

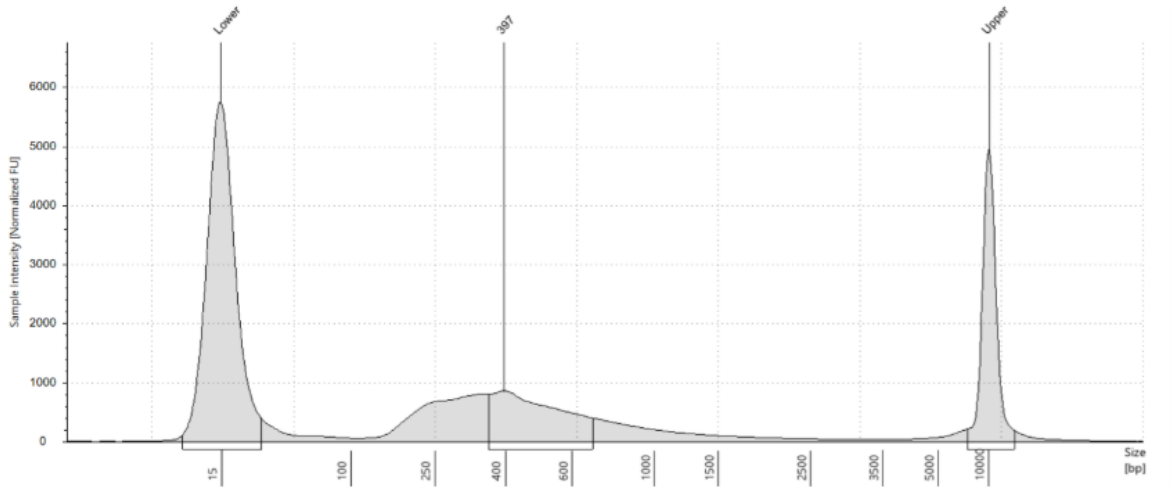
D1: 1637 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
D1	7.35	1637 RRBS		

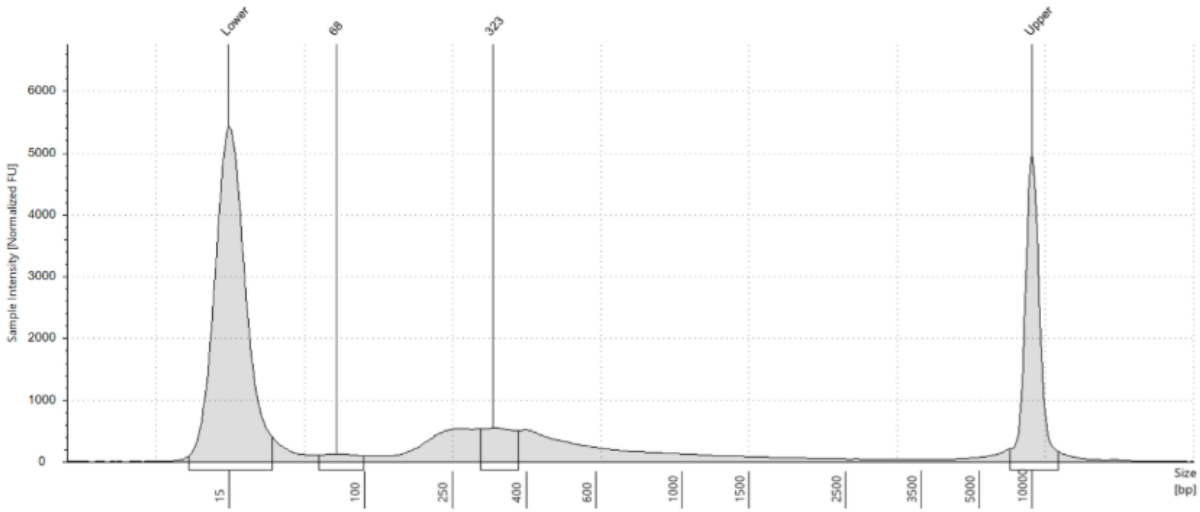
E1: 1101 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
E1	3.14	1101 RRBS		

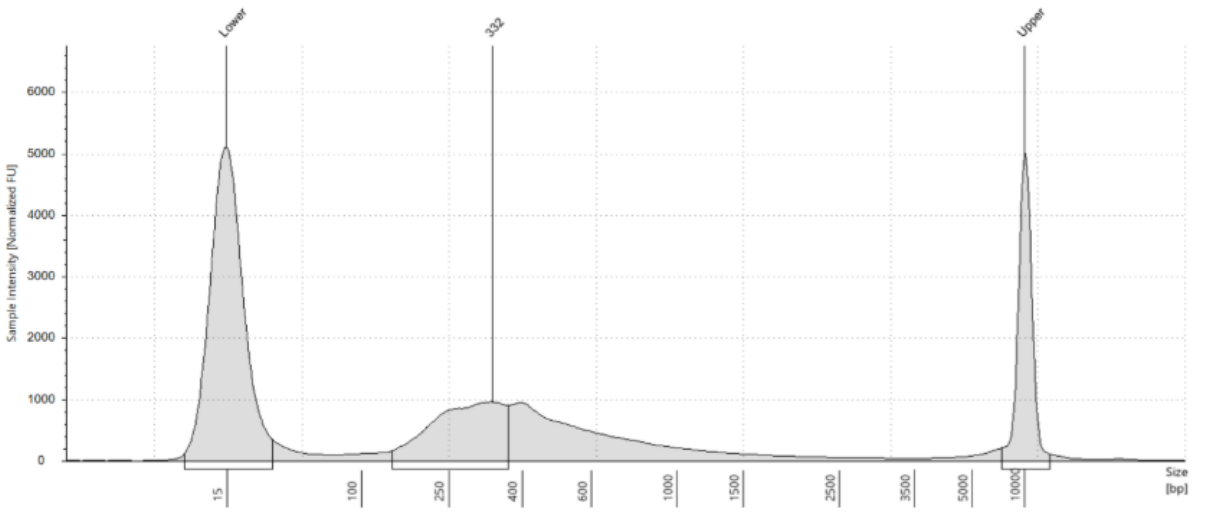
F1: 1548 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
F1	1.17	1548 RRBS		

G1: 1628 RRBS



Sample Table

Well	Conc. [ng/µl]	Sample Description	Alert	Observations
G1	3.73	1628 RRBS		

They have long tails but they all look like they worked!!

Written on October 24, 2019