MethylC-seq library preparation protocol (Ecker lab)

Citation: Lister *et al. Human DNA methylomes at base resolution show widespread epigenomic differences*. **Nature**. 2009 Nov 19;462(7271):315-22.

Sonication of gDNA:

- Start with 5.3 µg gDNA
- Prepare Promega unmethylated control Lambda DNA at 25.4 ng/µl: [stock lambda DNA] = 813 ng/µl. Add 2 µl of Lambda DNA to 62 µl of Qiagen buffer AE or EB to give working conc. of 25.4 ng/µl.
- Add unmethylated lambda control DNA to 5.3 µg of sample gDNA at a final concentration of 0.5% (w/w). Make DNA solution up to 300 µl total in Qiagen buffer AE or EB. The final [gDNA] = 17.7 ng/µl for each sample.
- Sonicate (Bioruptor) gDNA for 30 sec intervals, rest for 2 min, do 5 cycles (12.5 minutes total).
- Remove tubes mix by flicking, centrifuge briefly, repeat above cycle a total of 4 times (4 x 5 cycles)
- Spin down a final time, remove 300 ng of gDNA to run on a 1% agarose gel (17 µl gDNA + 4.5µl of 5x loading dye)
- Run all samples on a gel using the 2 log DNA ladder to check sonication.

- PCR cleanup of sonicated gDNA samples:
    - Follow **MinElute** PCR Purification Kit protocol
    - Elute twice, each time in **17 µl of EB**, for a total of ~32 µl elution

Perform End Repair using the End-It Kit (Epicentre):

- Add the following to the elution (Prepare a master mix of Kit contents)
    - DNA sample 32 µl
    - Water 2 µl
    - 10x End-it Buffer 5 µl
    - 10mM dNTP mix 5 µl
    - 10mM ATP 5 µl
    - End-it Enzyme Mix 1 µl

- Mix tube gently, spin briefly, incubate at **RT** for **45 min**
- PCR cleanup using **MinElute** PCR Purification Kit, Elute in **2x 16 µl of EB.**

Add “A” bases to 3’ End:

- Prepare the following reaction mix in each PCR tube
    - DNA sample 32 µl
    - Klenow buffer 5 µl
    - 1mM dATP mix 10 µl
    - Klenow exo - 3’ to 5’ exo minus 3 µl

- Mix tube gently, spin briefly, put in thermal cycler for 30 min at 37°C
- PCR cleanup using **MinElute** PCR Purification Kit, elute in **10 µl of EB**

Prepare a 2% Agarose Gel for size selection of ligation products:

- 1.1 g of Low Range ultra agarose (Biorad)
- 55 ml of 1xTAE (no ethidium bromide)
- Boil to dissolve
- Add 2.2 µl of ethidium bromide (10 mg/ml)

Ligate Adapters to Fragments:

- Prepare the following reaction mix in each tube (from elution above)
  - DNA sample 10 µl
  - 2x DNA ligase buffer 25 µl
  - 15 µM single end methylated adapters 10 µl
  - DNA ligase 5 µl

- Mix tube gently, spin briefly, incubate for 15 min at RT
- Purify using **MinElute** PCR Purification Kit, elute in **2x 10 µl of EB (20 µl total)**
- Add 8 µl of 4x Loading buffer to each sample.
- Prepare Low Molecular Weight ladder; 8 µl ladder + 3 µl of 4x Loading buffer
- Load gel to give max amount of space (keep libraries far apart)
- Weigh 1 empty tube for each library (for Agarose Plug), and label 175-225
- Remove tray, slide top of gel first onto blue light box.
- Use one razor blade to cut vertical (use new one for each library); cut directly beside the lane and position the top of the razor blade at same spot on the gel to minimize contamination between fragment sizes.
- Get new razor blade, cut at 175 bp (b/w 150 & 200 marker), cut at 225 bp (b/w 200 & 250 marker) - use same razor blade
- Use a needle to remove the slice of gel and put the 175-225bp slice in the tube.
- Repeat for each library.
- Weigh the tubes containing the 175-225 slice to calculate the weight of the agarose plug
- Use the **minElute Gel Extraction Kit** according to the Qiagen protocol and elute: 10 µl EB, wait 5 min, spin, 10 µl EB, wait 5 min, spin (**2x 10µl EB**)
- Can freeze sample overnight at -20˚C.

Bisulfite Conversion of gDNA:

- Thaw the 20 µl gDNA sample on ice
- Prepare 3 N NaOH: 1g NaOH pellets + H_2O to 8.3 ml
- Prepared Solution 1+2 at 80°C (contains Sodium Bisulfite; keep away from the light with foil).

- Perform **HGS Bisulfite treatment** (Human Genetic Signatures – MethylEasy Xceed ME002) exactly as the protocol describes.
- Warm up the Solution #5 at ~70°C before the elution
- Elute in **2x 15µl** Solution #5 for a total elution of 30µl (Remember to heat sample to 95°C to finish conversion

- Whatever sample is NOT used in PCR, freeze and store at -80°C

Low Amplification (4 cycle PCR) of BS Converted gDNA:

- PCR mix:

Components:	1x
BS Converted DNA	10 µl
10x Pfu Cx buffer	5 µl
F gDNA Primer (25uM)	1 µl
R gDNA Primer (25uM)	1 µl
dNTPs (12.5uM)	1.25 µl
H_2O	30.75 µl
Pfu Turbo Cx Polymerase	1 µl
Total:	50µl

- Add 40 µl of MM to each 10µl of BS Converted DNA and amplify as follows:

PCR Setup
1. 2 min. @ 95
2. 30 sec. @ 98
3. 15 sec. @ 98
4. 30 sec. @ 60 4 cycles (of steps 3-5)
5. 4 min. @ 72
6. 10 min. @ 72
7. Hold at 4

- Minelute PCR purify, elute samples in 2x10µl of EB, add 8µl of 4x Sample Buffer, run a 2% Agarose Gel (large gel) with a DNA ladder at 50V 1hr.

2% Gel:	
Agarose	1.1g
1xTAE	55 ml
Ethidium bromide	2.2 µl

- Cut out bands as before. Minelute Gel Purify the sample. Elute in **2x10µl of EB.**
- Library is ready to sequence. Quantitate using Qubit dsDNA HS kit.