SOP of DNA methylation

1. To fragment the genomic DNA, sonicate 5 μg of DNA in 200 μl solution) in a 1.5 ml tube, with Biorupter sonicator (Diagenode) for 4 cycles of 15 min each (30 s "on" and 30 s "off", output H). In between cycles, samples are kept on ice.

2. The DNA fragments are puried with AMpure XP beads (Beckman), and eluted in 25 μl of water (pH=8.0).

3. To repair, blunt, and phosphorylate ends and to add a single "A" base to the 3' end ("Adenylation reaction"), incubate the repaired DNA fragments from previous step with Klenow exo-fragment (3'-5' exo-). These two steps are conbined into one step.

Set up the End-Repair and A-tailing reaction as follows:

Reagent	Volume (μl)
DNA	25
dNTPs (10 mM dATP, 1mM dCTP, 1mM dGTP)	1
NEB buffer 2	3
Klenow Fragment (3'-5′ exo-)	1

4. Incubate 20 min at 30°C followed by incubate 20 min at 37°C.

5. The A-tailed DNA are puried with AMpure XP beads and eluted in 25 μl of water (pH=8.0).

6. To ligate the DNA fragments with the adapters incubate the adenylated fragments from previous step with premethylated, single-end, adapters (Illumina).

Set up the Adapter Ligation reaction as follows:

Reagent	Volume (µl)
DNA	25
10x T4 buffer (with 10 mM ATP)	3.5
Methylated standard adapter: top	2.5
Methylated standard adapter: bottom	2.5
T4 Ligase	1.5

7. Incubate 30 min at 20°C.

8. Purify DNA with AMpure XP beads and eluted in 30 µl of water (pH=8.0).

9. Run DNA from previous step on 2% certied low range ultra agarose gel for 1 h at 100 V. Excise fragments ranging from 150 to 300 bp and extract DNA with QIAquick Gel Extraction Kit. Elute DNA in 40 µL of EB buffer. (May be replaced by the protocl of ★Size Selection Using AMPure XP Beads)

10. At this step, adapter-ligated DNA is ready for bisulfite treatment. Follow the instructions in the EpiTect Bisulfite Kit to prepare buffer BW, buffer BD, and carrier RNA. Follow the instructions in the EpiTect Bisulfite Kit to prepare buffer BL and bisulfite mix on the date of experiment. Follow the instructions in the EpiTect Bisulfite Kit, to prepare the reaction mix on ice.

Set up the Bisulfite Treatment reaction as follows:

Reagent	Volume (µl)
DNA	40
Bisulfite Mix	85

DNA Protection Buffer	15

11. Incubate the tube in the pre-programmed thermal cycler as follows, with the heated lid closed. The complete cycle takes approximately 14.5 hours (Do not hold for more than 5 hours).

Temp (°C)	Time (min)	cycle
95	5	1
60	25	
95	5	1
60	85	
95	5	1
60	175	
95	5	3
60	180	
20	∞	1

12. Follow the instructions in the EpiTect Bisulfite Kit to purify the bisulfite converted DNA (purification protocol for bisulfite conversion of DNA isolated from FFPE Tissue Samples), replacing step 14 as follows: Add 500 buffer BD (desulfonation buffer) to the spin column, and incubate for 20 minutes at room temperature.

13. Purify DNA with AMpure XP beads and eluted in 15 μl of water (pH=8.0).

Enrich Adapters-ligated and Bisulfite-modified DNA fragments by PCR reactions. (May be replaced by the protocol of ★PCR Amplification of Adaptor Ligated DNA)

Set up the Bisulfite Treatment reaction as follows:

Reagent	Volume (μl)
DNA	32
5×buffer	10
dNTPs	5
Illumina PCR primer 1.1 (single-end)	1
Illumina PCR primer 2.1 (single-end)	1
Taq	1

14. Amplify samples by using the following conditions:

Temp (°C)	Time	cycle
95	5 min	1
98	30 sec	1
98	10sec	15~25
65	30 sec	
72	30 sec	
72	5 min	1
4	∞	1

15. Purify DNA with AMpure XP beads and eluted in 15 μl of water (pH=8.0).

★ Purification of DNA with AMpure XP beads

1. Room temperature equilibrium the 1.8× volume beads for 10 min.

2. Thoroughly mix the beads and DNA at least 10 times, then incubate at RT for 10 min.
3. Stand the beads on magnetic plate for 3 min.
4. Aspirate the supenant then wash with 80% fresh EtOH once. Do not take off the tubes from the plate.
5. Dry the beads for 3 min on the plate.
6. Elute the beads with water (pH=8.0) and incubate at RT for 5 min.
7. Stand the beads on magnetic plate for 3 min.
8. Transfer all the supernant to a clean tube.

★ Size Selection Using AMPure XP Beads

1. For AMPure XP Bead-based Size Selection, expect size distributions in the range of 230–270 for 100 bp reads and 310–370 for 200 bp reads.
2. **Table 1: Recommended Conditions for Dual Bead-based Size Selection**

BEAD:DNA RATIO*	INSERT SIZE (bp)	
	100 bp	200 bp
1st Bead Selection	0.9X	0.7X
2nd Bead Selection	0.15X	0.15X

*Bead:DNA ratio is calculated based on the original volume of DNA solution.

3. **AMPure XP Bead-based Dual Bead Size Selection for 100 bp Inserts**

The following size selection protocol is for libraries with 100 bp insert from a 100 µl volume. For libraries with a 200 bp insert please use the bead:DNA ratio listed in the chart above

4. 1st Bead Selection to Remove Large Fragments:

5. This step is used to bind the large, unwanted fragments to the beads. The supernatant will contain the desired fragments.

6. Add 90 μl (0.9X) resuspended AMPure XP beads to 100 μl DNA solution. Mix well on a vortex mixer or by pipetting up and down at least 10 times.

7. Incubate for 5 minutes at room temperature.

8. Place the tube on a magnetic rack to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant to a new tube **(Caution: do not discard the supernatant)** Discard beads that contain the large fragments.

9. 2nd Bead Selection to Remove Small Fragments and to Bind DNA Target:

10. This step will bind the desired fragment sizes (contained in the supernatant from Step 3) to the beads. Unwanted smaller fragment sizes will not bind to the beads.

11. Add 15 μl (0.15X) resuspended AMPure XP beads to the supernatant, mix well and incubate for 5 minutes at room temperature.

12. Put the tube on a magnetic rack to separate beads from supernatant. After the solution is clear (approximately 3

minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets **(Caution: do not discard beads)**.

13. Add 200 μl of 80% freshly prepared ethanol to the tube while in the magnetic stand. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.

14. Repeat Step 6 once.

15. Keeping the tube on the magnetic rack, with the cap open, air dry the beads for 5 minutes.

16. **Caution: Do not overdry the beads. This may result in lower recovery of DNA target.**

17. Remove the tube from the magnet. Elute DNA target from beads into 42 µl sterile 0.1X TE. Mix well on a vortex mixer or by pipetting up and down, incubate for 2 minutes at room temperature.

18. Put the tube in a magnetic rack until the solution is clear, approximately 3 minutes. Transfer approximately 40 µl of the supernatant to a clean tube.

19. Proceed to PCR Amplification.

★ PCR Amplification of Adaptor Ligated DNA (E6270)

1. Mix the following components in a sterile microfuge tube:

For 10 ng – 100 ng

Adaptor Ligated DNA 1-40 µl

Primers 4 µl

Sterile H_2O variable

NEBNext Q5 Hot Start HiFi PCR Master Mix 50 µl

Total volume 100 µl

For 1 *µ*g

Adaptor Ligated DNA 1-40 µl

Primers 10 µl

Sterile H_2O variable

NEBNext Q5 Hot Start HiFi PCR Master Mix 50 µl

Total volume 100 µl

2. **PCR cycling conditions**

STEP	**TEMP**	**TIME**
Initial Denaturation	98°C	30 seconds
4–12 Cycles	98°C	10 seconds
	58°C	30 seconds
	65°C	30 seconds

1 Cycle	65°C	5 minutes
Hold	4°C	∞

3. **Cycling Suggestions:**

DNA	**CYCLES**
10 ng	10–12
100 ng	6–8
1 µg	4–6