RRBS

Materials and Methods

Reduced Representation Bisulfite Sequencing (RRBS) libraries were prepared as described previously (1) with modifications detailed below. Briefly, genomic DNA samples were quantified using a Quant-It dsDNA high sensitivity kit (ThermoFisher, cat# Q33120) and normalized to a concentration of 10ng/ul. A total of 100ng of normalized genomic DNA was digested with *Msp*I in a 20ul reaction containing 1ul *Msp*I (20U/ul) (NEB, cat# R0106L) and 2ul of 10X CutSmart Buffer (NEB, cat# B7204S). *Msp*I digestion reactions were then incubated at 37ºC for 2 hours followed by a 15 min. incubation at 65ºC.

Next, A-tailing reactions were performed by adding 1ul dNTP mix (containing 10mM dATP, 1mM dCTP and 1mM dGTP) (NEB, cat# N0446S), 1ul Klenow 3'-5' exo$^-$ (NEB, cat# M0212L) and 1ul 10X CutSmart Buffer in a total reaction volume of 30ul. A-tailing reactions were then incubated at 30ºC for 20 min., followed by 37ºC for 20 min., followed by 65ºC for 15 min.

Methylated Illumina sequencing adapters (1) were then ligated to the A-tailed material (30ul) by adding 1ul 10X CutSmart Buffer, 5ul 10mM ATP (NEB, cat# P0756S), 1ul T4 DNA Ligase (2,000,000U/ml) (NEB, cat# M0202M) and 2ul methylated adapters in a total reaction volume of 40ul. Adapter ligation reactions were then incubated at 16ºC overnight (16-20 hours) followed by incubation at 65ºC for 15 min. Adapter ligated material was purified using 1.2X volumes of Ampure XP according to the manufacturer's recommended protocol (Beckman Coulter, cat# A63881).

Following adapter ligation, bisulfite conversion and subsequent sample purification was performed using the QIAGEN EpiTect kit according to the manufacturer's recommended protocol designated for DNA extracted from FFPE tissues (QIAGEN, cat# 59104). Two rounds of bisulfite conversion were performed yielding a total of 40ul bisulfite converted DNA.

In order to determine the minimum number of PCR cycles required for final library amplification, 50ul PCR reactions containing 3ul bisulfite converted DNA, 5ul 10X PfuTurbo Cx hotstart DNA polymerase buffer, 0.5ul 100mM dNTP (25mM each dNTP) (Agilent, cat# 200415), 0.5ul Illumina TruSeq PCR primers (25uM each primer) (1) and 1ul PfuTurbo Cx hotstart DNA polymerase (Agilent, cat# 600412) were prepared. Reactions where then split equally into four separate tubes and thermocycled using the following conditions: denature at 95ºC for 2 min. followed by 'X' cycles of 95ºC for 30 sec., 65ºC for 30 sec., 72ºC for 45 sec. (where 'X' number of cycles = 11, 13, 15 and 17), followed by a final extension at 72ºC for 7 min. PCR products were purified using 1.2X volumes of Ampure XP and analyzed on an Agilent Bioanalyzer using a High Sensitivity DNA kit (Agilent, cat# 5067-4626). Once the optimal number of PCR cycles was determined, 200ul PCR reactions were prepared using 24ul bisulfite converted DNA, 20ul 10X PfuTurbo Cx hotstart DNA polymerase buffer, 2ul 100mM dNTPs (25mM each), 2ul Illumina TruSeq PCR primers (25uM each) and 4ul PfuTurbo Cx hotstart DNA polymerase with the thermal cycling conditions listed above. PCR reactions were purified using 1.2X volumes of Ampure XP according to the manufacturer's recommended protocol and analyzed on an Agilent Bioanalyzer using a High Sensitivity DNA kit.

References

1. Gu, H., Smith, Z.D., Bock, C., Boyle, P., Gnirke, A., and Meissner, A. (2011). Preparation of reduced representation bisulfite sequencing libraries for genome-scale DNA methylation profiling. Nat. Protoc. 6, 468-481.