Materials and methods for paper writing

*Bisulfite conversion and multiplex amplification*

DNA methylation level was analysis by MethylTargetTM (Genesky Biotechnologies Inc., Shanghai, China), an NGS-based multiple Targeted CpG methylation analysis method. Specifically, the genomic regions of interest were analyzed and transformed to bisulfite-converted sequences by geneCpG software. PCR primer sets were designed with the Methylation Primer software from bisulfate converted DNA.

Genomic DNA (400ng) was subjected to sodium bisulfite treatment using EZ DNA Methylation™-GOLD Kit (Zymo Research) according to manufacturer's protocols. Multiplex PCR was performed with optimized primer sets combination. A 20 µl PCR reaction mixture was prepared for each reaction and included 1x reaction buffer (Takara), 3 mM Mg2+, 0.2 mM dNTP, 0.1 µM of each primer, 1U HotStarTaq polymerase (Takara) and 2 µl template DNA. The cycling program was 95ºC for 2 min; 11 cycles of 94ºC for 20 s, 63ºC for 40s with a decreasing temperature step of 0.5ºC per cycle, 72ºC for 1 min; then followed by 24 cycles of 94ºC for 20 s, 65ºC for 30 s, 72ºC for 1 min; 72ºC for 2 min.

*Index PCR*

PCR amplicons were diluted and amplified using indexed primers. Specifically, a 20 µl mixture was prepared for each reaction and included 1x reaction buffer (NEB Q5TM), 0.3 mM dNTP, 0.3 µM of F primer, 0.3 µM of index primer, 1 U Q5TM DNA polymerase (NEB) and 1 µL diluted template. The cycling program was 98ºC for 30 s; 11 cycles of 98ºC for 10 s, 65ºC for 30 s, 72ºC for 30 s; 72ºC for 5 min. PCR amplicons (170bp-270bp) were separated by agarose electrophoresis and purified using QIAquick Gel Extraction kit (QIAGEN).

*Sequencing*

Libraries from different samples were quantified and pooled together, followed by sequencing on the Illumina MiSeq platform according to manufacturer's protocols. Sequencing was performed with a 2x300bp paired-end mode.

*Data analysis*

Quality control of sequencing reads was performed by FastQC. Filtered reads were mapped to genome by Blast. After reads recalibration with USEARCH, methylation and haplotype were analyzed using Perl script. Statistics were performed by t-test and ANOVA.