**1. Bisulfite Conversion**

The single sample and pooled DNA samples were treated respectively using the EZ DNA Methylation-Lightning Kit (Zymo, D5031) kit following the instruction manual.

**2. The target-specific PCR**

Approximately 50ng of the bisulfite-converted DNA was used as the template for a multiplex PCR reaction using a site-specific forward primer mixture (iBP\_F mix), a site-specific reverse primer mixture with bridge sequence (iBP\_R mix), and 2×KAPA HiFi HotStart Uracil+ReadyMix (KK2801). Set up a reaction as follows on ice:

|  |  |
| --- | --- |
| **Components** | **Volume (µL)** |
| Template DNAa | 2µL |
| 2×KAPA HiFi HotStart Uracil+ReadyMix (KK2801) | 5µL |
| iBP\_F mix | 1.5µL |
| iBP\_R mix | 1.5µL |

aThe template DNA means the bisulfite-converted DNA, and the total the amount of template added to the reaction will need to be determined empirically by the user. Usually, 50ng is enough.

A standard thermocycler with touchdown PCR program was used to run the reaction using the following parameters:

|  |  |  |  |
| --- | --- | --- | --- |
| Stage 1 | Reps: 1 | 95°C | 3min |
| Stage 2 | Reps: 10-15a | 95°C | 30sec |
|  |  | 65°C-55°C | 20sec |
|  |  | 72°C | 10-30secb |
| Stage 3 | Reps: 25 | 95°C | 30s |
|  |  | 60°Cc | 30sec |
|  |  | 72°C | 10-30secb |
| Stage 4 | Reps: 1 | 72°C | 5min |

aThe means the decrease the annealing temperature of the reaction 1 °C per cycle.

bThe extension time is determined by the size of the PCR product.

cAnnealing temperature is dependent on the *T*m values of mixed primers.

The first round PCR products were purified by AMPure XP Reagent (A63882).

**3. The barcoding PCR**

The cleaned first-round PCR products were further barcoded during the second-round PCR using synthetic barcode primer. Set up a reaction as follows on ice:

|  |  |
| --- | --- |
| **Components** | **Volume (µL)** |
| Template DNAa | 1µL |
| 2×Taq Master Mix | 10µL |
| iBP\_F mix | 2µL |
| Barcode primer | 2µL |
| Total | 20µL |

aThetemplate DNA means the products of the cleaned first-round PCR.

A standard PCR program was used to run the reaction using the following parameters:

|  |  |  |  |
| --- | --- | --- | --- |
| Stage 1 | Reps: 1 | 95°C | 5min |
| Stage 2 | Reps: 34 | 95°C | 30s |
|  |  | 60°Ca | 30sec |
|  |  | 72°C | 10-30secb |
| Stage 3 | Reps: 1 | 72°C | 5min |

aAnnealing temperature is dependent on the *T*m values of mixed primers and barcode primer.

bThe extension time is determined by the size of the PCR product.

**4. Next generation sequencing**

The products were pooled in equal amount, purified and sent for next generation sequencing.