**1. The target-specific PCR**

To obtain the target fragments, approximately 50ng of genomic DNA from each sample 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×Taq Master Mix (Vazyme). Here, the primer mixture is formed by mixing specific primers in equal amount. Set up a reaction as follows on ice:

|  |  |
| --- | --- |
| **Components** | **Volume (µL)** |
| Template DNAa | 1µL |
| 2×Taq Master Mix | 5µL |
| iBP\_F mix | 1µL |
| iBP\_R mix | 1µL |
| Total | 10µL |

aThe total 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 | 30sec |
|  |  | 72°C | 10-30secb |
| Stage 3 | Reps: 25 | 95°C | 30sec |
|  |  | 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.

**2. The barcoding PCR**

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

|  |  |
| --- | --- |
|  | **Final volume per 10µL reaction** |
| Template DNAa | 2µL |
| 2×Taq Master Mix | 5µL |
| iBP\_F mix | 1.5µL |
| Barcode primer | 1.5µL |
| Total | 10µL |

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

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

|  |  |  |  |
| --- | --- | --- | --- |
| Stage 1 | Reps: 1 | 95°C | 3min |
| Stage 2 | Reps: 25 | 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.

**3. Next generation sequencing**

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