**Supplementary Materials**

**Ⅰ. Equipment**

CFX96 Real-Time PCR Detection System(Bio-Rad, USA)

Multiskan GO spectrophotometer(Thermo Scientific™, USA)

0.5-10μL, 10-100μL, 100-1000μL pipettes(Eppendorf Research®, Germany)

Centrifuge(Thermo Scientific™, USA)

**Ⅱ. Reagents and materials**

Nasopharynx swab(COPAN, Cat. No. 502CS01, Italia)

digene® Specimen Transport Medium(QIAGEN, Cat. No. 5128-1220, Germany)

QIAamp® DNA extraction mini kit(QIAGEN, Cat. No. 51106, Germany)

EZ DNA Methylation-Gold™ conversion Kit( Zymo, Cat. No. D5006, USA)

qPCR pre-mix(Tsingke, Cat. No.1FD21A01, 1FD21B01, China)

Double distilled water(Tsingke, Cat. No.TSP002, China)

Tris-EDTA buffer solution(Leagene, Cat. No.ND0092, China)

Primers(Tsingke, China)

Hydrolysis probes(Sangon Biotech, China)

**Ⅲ. Sequence of calibrator**

gtgTGtTGag tgTtatTttt ggaaTagtag aaaattgaaT TttgttggTG ggagaaggaa

taaTGTTtta tTtgggagga gTGaTGgatt atagTTaata agagagTtTa agaTGTaggg

taaCGTTtta tTtgggagga gCGaCGgatt atagTTaata agagagTtTa agaCGTaggg

TtTGTaaagt atagtggTTT TGtgggaTTt tagaggtgga gTaaTGtTta aagtggtaat

Note: yellow mark represent the target sequence of E-CpMQ primers, red mark represents target sequence of unmethylation hydrolysis probe, green mark represents target sequence of methylation hydrolysis probe.

**Ⅳ. Sequence of synthetic methylation bisulfite-converted standard.**

GTGCGTCGAGTGTTATTTTTGGAATAGTAGAAAATTGAATTTTGTTGGCGGGAGAAGGAATAACGTTTTATTTGGGAGGAGCGACGGATTATAGTTAATAAGAGAGTTTAAGACGTAGGGTTCGTAAAGTATAGTGGTTTCGTGGGATTTTAGAGGTGGAGTAACGTTTAAAGTGGTAATAATATTAGGCGGGGTTGGGTAAAGGGGTTTTACGGGCGGGATTAATTACGTTTTGTTTACGTAAGTTTAGTTAATTCGTTTACGATTTGAAAAATGTAGTTTTTAATTAATTGGCGGTTT

**Ⅴ. Sequence of synthetic unmethylation bisulfite-converted standard.**

GTGTGTTGAGTGTTATTTTTGGAATAGTAGAAAATTGAATTTTGTTGGTGGGAGAAGGAATAATGTTTTATTTGGGAGGAGTGATGGATTATAGTTAATAAGAGAGTTTAAGATGTAGGGTTTGTAAAGTATAGTGGTTTTGTGGGATTTTAGAGGTGGAGTAATGTTTAAAGTGGTAATAATATTAGGTGGGGTTGGGTAAAGGGGTTTTATGGGTGGGATTAATTATGTTTTGTTTATGTAAGTTTAGTTAATTTGTTTATGATTTGAAAAATGTAGTTTTTAATTAATTGGTGGTTT

**Ⅵ. Principle diagram of E-CpMQ assay**

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**Basic principle of E-CpMQ assay:**

In the E-CpMQ reaction system, the threshold line needs to be set in the exponential phase of qPCR. To prevent the threshold from falling outside the exponential phase, it is necessary to adjust the threshold line manually. However, for preventing the result deviation caused by manual adjustment of a single threshold line, threshold lines of two channels need to be adjusted simultaneously. Therefore, to provide an adjustment reference for the two channels, a calibrator was invented to calibrate the raw data of qPCR.

A pair of universal primers can be used to amplify the methylated Cp sequence, unmethylated Cp sequence, calibrator. Methylated probe and unmethylated probe are capable to recognize the corresponding methylated sequence or unmethylated sequence. Therefore, if methylated and unmethylated sequences containing in one reaction system, both sequences will be amplified by universal primers unchanging its original proportions. After one round of amplification, a methylated sequence releases 1 FAM fluorescence and an unmethylated sequence releases 1 TexasRed fluorescence, while a calibrator releases 1 FAM fluorescence plus 1 TexasRed fluorescence. With the same amplification efficiency, the ratio of fluorescence released by methylated and unmethylated sequences can be used to determine the ratio of the original sequence. For the calibrator, the two fluorescence released by the calibrator are consistent in each round of amplification, this also means the Cq values from two channels of the calibrator are invariable. When the qPCR is done, it is only necessary to adjust the threshold of the FAM channel of the calibrator within the exponential phase of all other sample wells, and then adjust the Cq of the TexasRed channel of the calibrator to be consistent with the FAM channel(calibrator well: Cq-FAM = Cq-TexasRed). Then Cp methylation ratio can be calculated with the calibrated Cq values using following formula: Methylation ratio = methylation/ (methylation + unmethylation) = 2ΔCq/(2ΔCq +1), the ΔCq = CqFAM - CqTexasRed.

**Ⅶ. Interpretation rules of the E-CpMQ assay result**

|  |  |  |
| --- | --- | --- |
| **FAM channel** | **TexasRed channel** | **Methylation ratio** |
| Cq < 37 | Cq < 37 | # |
| Cq ≥ 37 or negative | 100% |
| Cq ≥ 37 or negative | Cq < 37 | 0% |
| Cq ≥ 37 or negative | 0% |

**#** Methylation ratio = 2ΔCq/(2ΔCq +1), the ΔCq = CqFAM - CqTexasRed