

Development and Performance Evaluation of TaqMan Real-time Fluorescence Quantitative Methylation Specific PCR for Detecting Methylation Level of PER2

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Abstract

Background: PER2 gene methylation is closely related to the occurrence and progress of some cancers, but there is no method to quantitatively detect PER2 methylation in conventional laboratories. So, we established a TaqMan real-time fluorescence quantitative methylation specific PCR (TaqMan real-time FQ-MSP) assay and use it for quantitative detection of PER2 methylation in leukemia patients.

Methods: According to the PER2 sequence searched by GenBank, a CpG sequence enrichment region of the PER2 gene promoter was selected, and the methylated and unmethylated target sequences were designed according to the law of bisulfite conversion of DNA to construct PER2 methylation positive and negative reference materials. Specific primers and probe were designed. The reference materials were continuously diluted into gradient samples by 10-fold ratio to evaluate the analytical sensitivity, specificity, accuracy and reproducibility of the method, and the analytical sensitivity of TaqMan real-time FQ-MSP assay was compared with that of the conventional MSP assay. At the same time, the new-established TaqMan real-time FQ-MSP assay and the conventional MSP assay were used to detect the PER2 methylation level of 81 patients with leukemia, and the samples with inconsistent detection results of the two assays were sent to pyromethylation sequencing to evaluate the clinical detection performance .

Results: The minimum detection limit of TaqMan real-time FQ-MSP assay for detecting PER2 methylation level established in this study was 6 copies/uL, and the coefficient of variation(CV) of intra-assay and inter-assay was less than 3%. Compared with the conventional MSP assay, it has higher analytical sensitivity. For the samples with inconsistent detection results, the results of pyrosequencing and TaqMan real-time FQ-MSP assay are consistent.

Conclusion: TaqMan real-time FQ-MSP assay of PER2 methylation established in this study has high detection performance and can be used for the detection of clinical samples.

1. Introduction

The PER2 gene is a member of the PER gene family composed of PER1, PER2 and PER3, and is one of the core genes that control the circadian rhythm[1]. PER2 is mainly expressed in the central nervous system (CNS) including the suprachiasmatic nucleus (SCN) and the peripheral nervous system[2]. A large number of studies have shown that the abnormal expression of PER2 is closely related to the occurrence and development of a variety of tumors, such as breast cancer[3], ovarian cancer[4], pancreatic cancer[5], oral squamous cell carcinoma[6], liver cancer[7], large intestine cancer et al.[8]. Importantly, the abnormal expression of PER2 can be detected in a variety of hematological malignancies[9]. As a common diseases of hematological malignancy, leukemia usually leads to high mortality and poor prognosis, posing a huge threat to human health[10]. Compared with mononuclear cells of normal human bone marrow and tonsil, the PER2 level of patients with acute myeloid leukemia (AML) is significantly down-regulated[11]. Especially in the case of chronic myeloid leukemia (CML), not only the expression of PER2

is reduced, and the CpG site in its promoter region has a hypermethylated state, which is related to the clinical stage of the clinical patient[12, 13]. Epigenetic changes such as DNA methylation can lead to silencing of tumor suppressor gene, which can be seen in the early stages of many malignant tumors[14, 15]. As the tumor progresses, the degree of abnormal gene methylation also increases[16]. Studies have shown that many tumor suppressor genes are methylated in patients with leukemia, such as SOCS1, GPX3, p15, p16, etc. [17, 18]. In recent years, the role of methylation of tumor suppressor gene in tumorigenesis and progression has received widespread attention. In human hematological malignancies, abnormal methylation of the CpG island in the gene promoter region is related to the transcription inactivation of tumor suppressor genes [19, 20]. In leukemia, PER2 methylation may cause down-regulation of PER2 expression, which can disrupt and cause abnormal circadian rhythms of leukemia cells[13]. Therefore, PER2 methylation may be an important epigenetic marker associated with leukemia.

Commonly used methods for methylation detection include methylation-specific PCR (MSP)[21], bisulfite sequencing (BSP)[22], methylation-sensitive high-resolution melting (MS-HRM), etc.[23]. These methods have certain shortcomings, such as low sensitivity, long time-consuming or high cost. In particular, traditional DNA methylation detection method is mostly qualitative methods, which cannot accurately and quantitatively detect the level of gene methylation, nor can it dynamically observe change of methylation level[24, 25]. Therefore, the correlation between methylation level and clinical characteristics and the role of methylation level changes in the occurrence and development of diseases cannot be better studied.

Currently, there are several methods reported for quantitative detection of PER2 methylation, such as BSP, MethyLight, Sequenom's Massarray, but these methods are not only expensive, but also usually need to be sent to independent laboratories for testing [26-28]. Up to now, there is no specific assay for quantitative detection of PER2 methylation that has been confirmed by detection performance and can be performed in common laboratories. Therefore, we have established a reliable and stable TaqMan real-time FQ-MSP assay to detect the methylation level of PER2 gene promoter and assessed the detection performance of the method. We also verified the clinical detection performance of the new-established method by testing the bone marrow samples of 81 patients with AML and CML, using pyrosequencing as the gold standard.

2. Materials And Methods

2.1. Reagents

Premix Ex Taq™, puc57 vector, Top10 competent cells and a small amount of plasmid extraction kit were purchased from Takara, Japan. Genomic DNA extraction kit and gel recovery kit were purchased from Beijing Tiangen Biochemical Technology Company. EZ DNA Methylation-Gold™ Kit was purchased from ZYMO Research, USA.

2.2. Patients and samples

This study collected the bone marrow samples of 81 patients with AML and CML who were admitted to the outpatient and inpatient departments of Yantai Yuhuangding Hospital affiliated to Qingdao University from September 2020 to January 2021. Among these 81 patients, 41 patients were diagnosed as AML and 40 patients were diagnosed as CML (Supplementary Table 1). All patients were diagnosed by cytomorphology, cytogenetics and molecular genetic analysis of bone marrow samples, and were classified according to the revised 2008 World Health Organization (WHO) standards. This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital affiliated to Qingdao University. All participants provided signed informed consent.

2.3. Construction of reference materials

According to the PER2 sequence retrieved by GenBank, a CpG-rich region in the promoter region of the PER2 gene was selected as the original template sequence. According to the rule of DNA modification by bisulfite, the methylated and unmethylated bisulfite modified DNA sequences were designed as the positive (MSP-5) and negative (MSP-6) reference materials. The two sequences were linked with pUC57 vector respectively, and then transformed into Escherichia coli Top10 competent cells. After oscillating culture at 37 °C for 1 h, they were coated on AMP + AGAR plate to screen positive clones and expand the culture. The bacteria liquid PCR identification of MSP-5 and MSP-6 was performed with methylated primers and non-methylated primers respectively. Primer sequences were presented in Supplementary Table 2[29]. The positive recombinant plasmid was extracted and sent to Shanghai ShengGong Biological Company for sequencing, and the sequence was compared and analyzed by BLAST on NCBI.

2.4. Conversion of plasmid copy number concentration

The recombinant plasmid obtained in the above operation was first used to measure the OD260/OD280 value and the concentration by Nanodrop Lite spectrophotometer. The concentration of plasmid DNA is 20 ng/uL. The calculation formula for converting plasmid concentration into copy number is: concentration of copy number (copies/ μ L) = [plasmid concentration(ng/ μ L) \times 10⁻⁹] \times (6.02 \times 10²³) / (base number \times 660). According to this formula, the concentration of plasmid copy number was 6 \times 10⁹ copies/uL.

2.5. Primers and probe design

The specific primers and probe were designed based on the CpG distribution characteristics of MSP-5 and MSP-6, the distance between the upstream and downstream primers and the Tm value. The sequences of the primers and probes are shown in the Table 1. The 5' end of the probe is labeled with

FAM fluorophore and the 3' end is labeled with BHQ1 fluorophore. All primers and probes are synthesized by Invitrogen.

Table 1

Primers and probe used for TaqMan real-time FQ-MSP

Primers and probe	Sequence (5'-3')	Length (bp)	Tm	GC	Product length (bp)
MF5	TGCGGTTTCGTTGCGGTTTAC	21	62.56	52%	140
MR5	GCCGACGCCGTTTCAAACCG	20	65.37	65%	
Probe	AAACCCGCGCGCCGACGACGA	21			

2.6. TaqMan real-time FQ-MSP assay and standard curve

MSP-5 was continuously diluted by 10-fold ratio to different concentrations as a template for TaqMan real-time FQ-MSP. In order to determine the best reaction system and reaction conditions of the TaqMan real-time FQ-MSP assay, different concentrations of primers and probe were used in the experiment (primers concentration: 100nM~400nM, probe concentration: 200nM~500nM) (Supplementary Fig. 1, Supplementary Fig. 2), and annealing temperature is gradually increased from 55°C to 65°C to screen out the best primers and probe concentration and the best annealing temperature, so that it has better amplification efficiency. In the experiment, each concentration was measured 3 times. Then, according to the established optimal reaction system and reaction conditions, perform TaqMan real-time FQ-MSP reaction on the gradiently diluted MSP-5. Finally, we analyze the data results and draw a standard curve. The Y-axis of standard curve represents the number of cycles experienced by the sample's fluorescence signal reaching the set threshold (Ct value), and the X axis represents the logarithm of the DNA copy number.

2.7. Analytical specificity, analytical sensitivity, accuracy, reproducibility

Analytical specificity

The optimized system and reaction conditions were used to perform TaqMan real-time FQ-MSP on MSP-5 and MSP-6, to see if there was specific amplification, so as to evaluate the specificity of TaqMan real-time FQ-MSP assay.

Analytical sensitivity

According to the optimal reaction system and reaction conditions, perform 20 times of TaqMan real-time FQ-MSP on the 10-fold dilution of MSP-5. According to the MIQE guidelines, We consider the lowest concentration that can be detected in at least 95% of the samples in the test results as the minimum detection limit[30]. At the same time, conventional MSP assay and real-time fluorescence quantitative PCR(SYBR GREEN) assay(qPCR) were used to amplify the same template. MSP-6 was set as a negative control in each experiment. Finally, the minimum detection limit of the three methods was determined, and then the detection results of the various methods were compared to evaluate the sensitivity of the methods.

Accuracy

Choose plasmids with a concentration of 6 to 6×10^7 copies/uL higher than the minimum detection limit, perform 10 detections on each concentration sample with the method established in this study. According to the number of positive results detected, the accuracy of the method is evaluated.

Reproducibility

Four positive standards in different concentrations (6×10^7 copies/uL, 6×10^5 copies/uL, 6×10^3 copies/uL, and 6×10 copies/uL) were used as templates for TaqMan real-time FQ-MSP assay. The intra-batch reproducibility experiment is to do 10 repetitions of the template in one TaqMan real-time FQ-MSP assay. The inter-batch reproducibility experiment is to measure the same template 10 times in 10 days. Finally, the obtained test results are analyzed to obtain SD and CV, so as to evaluate the reproducibility of the method.

2.8. DNA extraction, bisulfite modification

Genomic DNA extraction kit was used to extract genomic DNA from the collected bone marrow samples, and the absorbance value was detected with Nanodrop Lite spectrophotometer to determine the DNA concentration and purity. Subsequently, Zymo Research's EZ DNA Methylation-Gold™ Kit was used for bisulfite conversion of DNA and stored DNA at -20°C .

2.9. TaqMan real-time FQ-MSP assay and conventional MSP assay

In order to assess and compare the detection effect of TaqMan real-time FQ-MSP assay and conventional MSP assay, these two assays were used to detect the PER2 methylation level of 81 leukemia patients from the Yantai Yuhuangding Hospital affiliated to Qingdao University. MSP-6 was set up in each experiment as a negative control, and a blank control is also set up. After that, the clinical samples with

inconsistent detection results between the TaqMan real-time FQ-MSP assay and conventional MSP assay were sent to Shanghai Shengong Biological Company for pyrophosphate methylation sequencing to verify the accuracy of the assay.

3. Results

3.1. Identification of PCR products and recombinant plasmid sequencing

The product length of MSP-5 is 140 bp, and the product length of MSP-6 is 160 bp. The PCR amplified product was identified by agarose gel electrophoresis, and the result showed that it was consistent with the size of the fragment shown on the electrophoresis graph, which was in line with the expected result (Fig.1). After sequencing, the sequence of the recombinant plasmid MSP-5 and MSP-6 completely matched the reference sequence. The sequencing chromatograms of MSP-5 and MSP-6 are shown in Supplementary Fig.3.

3.2. Optimized TaqMan real-time FQ-MSP assay

After continuously optimizing the experimental conditions, the optimal reaction system and reaction conditions were determined. The optimal concentration of primers is 200 nmol/L, and the optimal concentration of probe is 400 nmol/L. In a PCR reaction system, the optimized reaction mixture consisted of 10 μ L Premix Ex Taq, 2.0 μ L template DNA, 0.8 μ L TaqMan probe, 0.4 μ L forward primer and 0.4 μ L reverse primer. RNase-free water was added to a total volume of 20.0 μ L. The PCR amplification procedure was as follows: 95 °C for 30 s; 40 cycles of 95 °C for 5 s and 60 °C for 20s.

3.3. Standard curve

The Ct value amplified by Taqman real-time FQ-MSP assay and the logarithm value of DNA copy number concentration were used as a standard curve (Fig. 2). According to the standard curve, it can be seen that the DNA copy number has a wide linear relationship at 6×10^4 to 6×10^7 copies/ μ L, with a linear correlation (R^2) of 0.9987 and efficiency of 103%. The slope is -3.229. The intercept is 39.89. The 95% CI of slope is -3.362 to -3.096 and the 95% CI of intercept is 38.20 to 40.58. The standard error of slope is 0.051 and the standard error of intercept is 0.268. The slope is statistically significant ($P < 0.0001$). The linear regressive equation of standard curve is $y = -3.229x + 39.89$.

3.4. Analytical specificity of TaqMan real-time FQ-MSP assay

Two different concentrations (2×10^{-4} ng/uL and 2×10^{-5} ng/uL) of MSP-5 and MSP-6 were used as templates for TaqMan real-time FQ-MSP assay (For the qPCR detection of PER2 gene, the Ct values of the results are in the range of 25-35, so we chose these two concentrations of plasmids). MSP-5 showed an amplification curve, while the control group MSP-6 did not show a specific amplification curve (Fig. 3). According to the results, this assay has good specificity.

3.5. Analytical sensitivity of TaqMan real-time FQ-MSP assay

TaqMan real-time FQ-MSP, conventional MSP and qPCR were used to detect the MSP-5 after gradient dilution. The results showed that the minimum detection limit of conventional MSP assay is 60 copies/uL, the minimum detection limit of qPCR is 60 copies/uL, and the minimum detection limit of TaqMan real-time FQ-MSP assay established in this study is 6 copies/uL (Fig. 4). The analytical sensitivity of TaqMan real-time FQ-MSP is 10 times higher than that of conventional MSP and qPCR, which suggested a high degree of analytical sensitivity of this assay.

3.6 Accuracy

For plasmids with a concentration of 6 to 6×10^7 copies/uL above the minimum detection limit, the results showed that each concentration of plasmids had a positive amplification result, and the positive rate was 100%, indicating that this assay has good accuracy.

3.7. Reproducibility of TaqMan real-time FQ-MSP assay

The results of intra-assay reproducibility experiments showed that the intra-assay CVs were 0.85%, 1.06%, 0.90%, 0.41% ($P < 0.0001$), and the inter-assay reproducibility experiments showed that the inter-assay CVs were 2.69%, 2.90%, 1.61%, 0.86% ($P < 0.0001$, Table 2), which were all less than 3%, indicating a high degree of reproducibility of this assay.

Table 2

Intra and inter-assay reproducibility results of TaqMan real-time FQ-MSP assay

Concentration of plasmid copy number(copies/uL)	Intra-assay reproducibility			Inter-assay reproducibility		
	Mean Ct	SD	CV(%)	Mean Ct	SD	CV(%)
6×10 ⁷	13.98	0.1195	0.85	14.31	0.3859	2.69
6×10 ⁵	20.26	0.2149	1.06	21.33	0.6196	2.90
6×10 ³	27.83	0.2500	0.9	28.06	0.4517	1.61
6×10	32.69	0.1351	0.41	32.91	0.2843	0.86

3.8. Comparison of concordance between TaqMan real-time FQ-MSP assay and conventional MSP assay

The test results of 81 leukemia clinical samples are shown in Table 6. 37 positive samples were detected by TaqMan real-time FQ-MSP assay with a positive rate of 45.7%, while 17 positive samples were detected by conventional MSP assay with a positive rate of 20.9% (Table 3). 20 samples with inconsistent results from the two assays were sent for pyrophosphomethylation sequencing. The sequencing results showed that 20 samples had different degrees of PER2 methylation (Supplementary Fig. 4), which verified the accuracy of the new-established TaqMan real-time FQ-MSP assay of this research. The PER2 methylation levels of different stages of leukemia are shown in the Table 4.

Table 3

Comparison of TaqMan real-time FQ-MSP assay and conventional MSP assay

	TaqMan real-time FQ-MSP+	TaqMan real-time FQ-MSP-	Total
Conventional MSP+	17	0	17
Conventional MSP-	20	44	64
Total	37	44	81

Table 4

Results of PER2 methylation in different stages of leukemia

Clinical response	Methylation detection rate	Quantitative level of methylation(copies/uL) M(Q _L ~Q _U)
Initial treatment of AML	6/11 (54.5%)	59.04 (30.69, 234.59)
Remission of AML	8/25 (32%)	25.84 (21.84, 102.83)
Relapsed/Refractory of AML	5/5 (100%)	518.01 (102.81, 1066.48)
Chronic phase of CML	14/30 (46.7%)	77.95 (33.27, 105.99)
Accelerated phase of CML	1/3 (33.3%)	32.20
Blast crisis of CML	3/7 (42.9%)	110.61 (81.15, 289.09)

4. Discussion

DNA methylation is observed as an early event in tumorigenesis[16, 31]. In previous studies, hypermethylation of PER2 gene has been reported in various malignant tumors, such as breast cancer, endometrial cancer, lung cancer, myeloid leukemia, etc.[16, 32-34]. At the same time, as the demethylation treatment of hematological malignancies is widely used today, quantitative data of methylation may provide another observation index for efficacy evaluation[35]. The current methylation detection methods reported in articles are mainly MSP, BSP, but MSP is less sensitive and can only be qualitatively detected, and BSP is expensive and time-consuming[36]. In clinical applications, a more sensitive and convenient method that can accurately quantify and dynamically monitor changes in PER2 methylation level is needed to understand the relationship between the patient's methylation level and the development of the disease. It provides important significance for the diagnosis, staging, treatment and prognosis of the disease. Therefore, we have developed and verified a TaqMan real-time FQ-MSP assay that can detect PER2 methylation level, which effectively solves the problems of MSP and BSP.

In this study, we used the conventional MSP assay and qPCR assay to detect PER2 methylation positive standards, and the minimum detection limit of both methods is 60 copies/ul. However, in the detection of leukemia clinical samples later, we found that the concentration of copy number of PER2 methylation in many clinical samples was less than 60 copies/ul, so these two methods were difficult to meet the detection requirements of PER2 trace methylation in leukemia. Interestingly, when the same primers were used to detect PER2 methylation levels by qPCR assay and conventional MSP assay, while the amplification curve appeared at a concentration of copy number of 60 copies/ul, non-specific bands appeared on agarose gel electrophoresis of the amplified product at that concentration. Therefore, these two methods have low specificity trace methylation of PER2. When the template concentration is low, the improper operation of these methods is extremely likely to cause non-specific amplification and affect the test results. TaqMan real-time FQ-MSP assay established in this study has a minimum detection limit of 6 copies/ul for PER2 methylation detection, which greatly improves the detection sensitivity. And TaqMan

real-time FQ-MSP assay has higher specificity due to the addition of the probe recognition step[37]. Through intra-batch and inter-batch reproducibility tests of different concentrations of plasmid standards, it was shown that the CV of Ct value of the amplification reaction was less than 3%. In order to detect the level of PER2 methylation in leukemia patients, we evaluated and tested 81 clinical samples. The positive rate of PER2 methylation detected by TaqMan real-time FQ-MSP assay was 45.7%, while the positive rate of PER2 methylation detected by conventional MSP assay was 20.9%. After that, the gold standard of DNA methylation, bisulfite pyrosequencing, was used to detect the methylation status of samples that were inconsistent with the two methods, which also confirmed that TaqMan real-time FQ-MSP assay is more accurate[38]. Moreover, the level of PER2 methylation in each stage of AML and CML was statistically analyzed in order to facilitate the later analysis of the relationship between PER2 methylation and the occurrence and development of leukemia. In short, compared with conventional MSP assay, we can quantitatively detect PER2 methylation level and dynamically detect the changes in the PER2 methylation level by designing and synthesizing specific primers and TaqMan probe, and constructing quantitative standard products. TaqMan real-time FQ-MSP assay established in our study has high specificity, high sensitivity, good reproducibility, low cost, simple operation, and no need for further gel electrophoresis separation, which also reduces the chance of sample contamination and errors, and greatly reduces the labor involved in DNA methylation analysis.

In this study, for the first time, the methylation reference material was constructed innovatively by directly synthesizing the corresponding sequence as a template according to the law of DNA transformation by bisulfite. In the past, for the construction of methylation reference materials, some researchers used cell lines to extract DNA and modified with bisulfite, and some researchers extracted DNA from cord blood and then treated with methyltransferase and modified with bisulfite to construct reference materials[39, 40]. However, these methods can affect the reliability and accuracy of the established reference materials due to the degradation and transformation efficiency of DNA in the early process of DNA extraction or chemical modification. Therefore, accurate and quantitative reference materials cannot be constructed. In our study, the methylation reference material was constructed by directly synthesizing sequences of 100% methylated target gene and 100% non-methylated target gene after modified with bisulfites, so the standards with different copy concentrations could be constructed accurately and quantitatively. The advantage is that it can avoid the problems of cell calculation errors, partial loss in the DNA extraction process, methyltransferase catalyzed methylation efficiency and bisulfite conversion efficiency that affect the evaluation of the final detection performance. Therefore, the scope of the detection performance evaluation in this study does not include the cell line count ratio and the evaluation of bisulfite conversion efficiency, but directly evaluates the performance of the detection system composed of primers, probe designed by template sequence and corresponding reagents. Importantly, this study provides an idea for the quantitative detection of methylation of other genes, and provides a reference and basis for the construction of similar methylation reference materials.

TaqMan real-time FQ-MSP assay established in this study provides a basis for the detection of clinical PER2 methylation, but its practical application detection efficiency still needs more clinical samples for verification and evaluation. Although we used leukemia samples for clinical verification in this study, the

occurrence of PER2 methylation is not unique to leukemia. This method can also be used to quantitatively detect other diseases with abnormal methylation of PER2 gene, which provide researchers with the most cost-effective detection method.

5. Conclusion

In summary, this study established a TaqMan real-time FQ-MSP assay, which has a wide detection range, high sensitivity and specificity, and can be used for accurate detection and dynamic monitoring of PER2 methylation levels in clinical practice. It is of great significance to understand the relationship between the degree of DNA methylation and the tumor burden, the course of the disease and even the prognosis of tumorous diseases. Furthermore, this study provides useful data for future studies of PER2 methylation and a better strategy for understanding the epidemiology of PER2 methylation in myeloid leukemia. PER2 methylation has the potential to become a leukemia biomarker. The characteristics and its clinical value need to be supported by further basic research evidence.

Abbreviations

MSP: methylation specific PCR; TaqMan real-time FQ-MSP: TaqMan real-time fluorescence quantitative MSP; PER2: Period 2; CNS: central nervous system; SCN: suprachiasmatic nucleus; AML: Acute myeloid leukemia; CML: Chronic myeloid leukemia; BSP: bisulfite sequencing; MS-HRM: methylation-sensitive high-resolution melting

Declarations

Author contributions

Material preparation, data collection and analysis were performed by Huihui Jiang, Xin Yang, Miaomiao Mi, Xiaonan Wei, Hongyuan Wu, Yu Xin, Liping Jiao and Shengjun Sun. The first draft of the manuscript was written by Huihui Jiang and Xin Yang. Chengming Sun critically revised the work. All authors have read and approved the manuscript, and all authors commented on previous versions of the manuscript.

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Conflict of interest

The authors declare that they have no conflict of interest.

Ethical statement

This study was approved by the Ethics Committee of Yantai Yuhuangding Hospital affiliated to Qingdao University with the protocol code of 2017210.

Consent for participate

All participants provided signed informed consent.

Consent for publication

Not applicable

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Figures

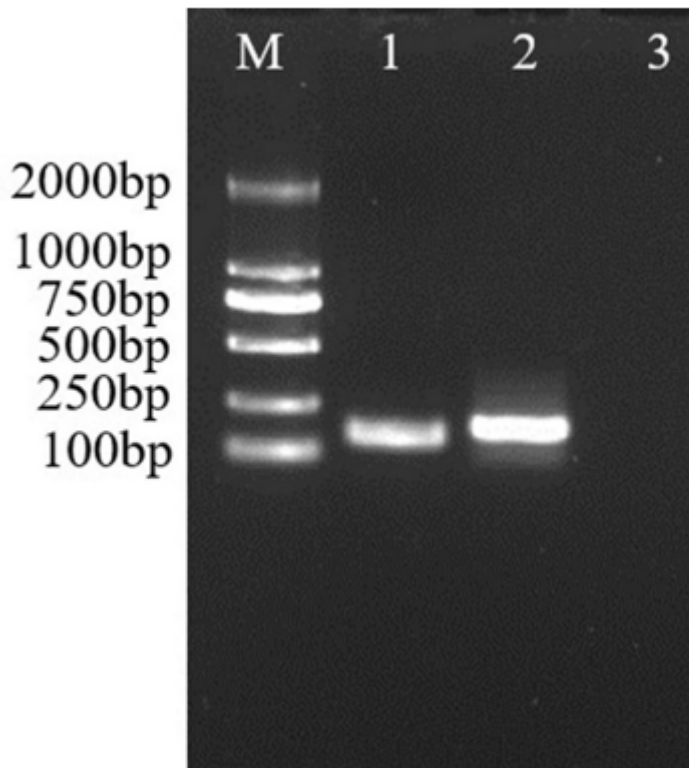


Figure 1

PCR amplification of target gene M DNA marker 1 MSP-5 2 MSP-6 3 Negative control

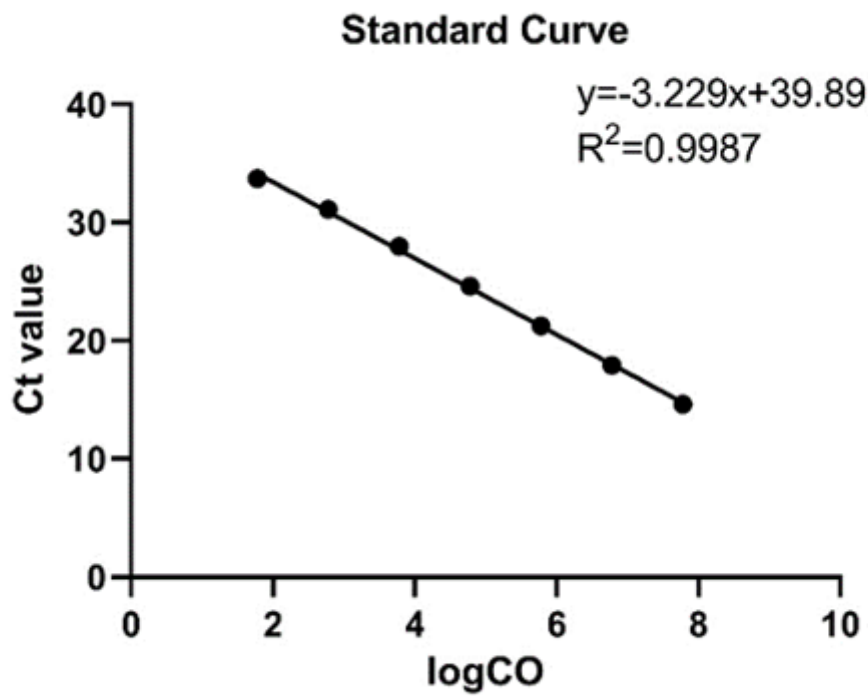


Figure 2

Standard curve of TaqMan real-time FQ-MSP

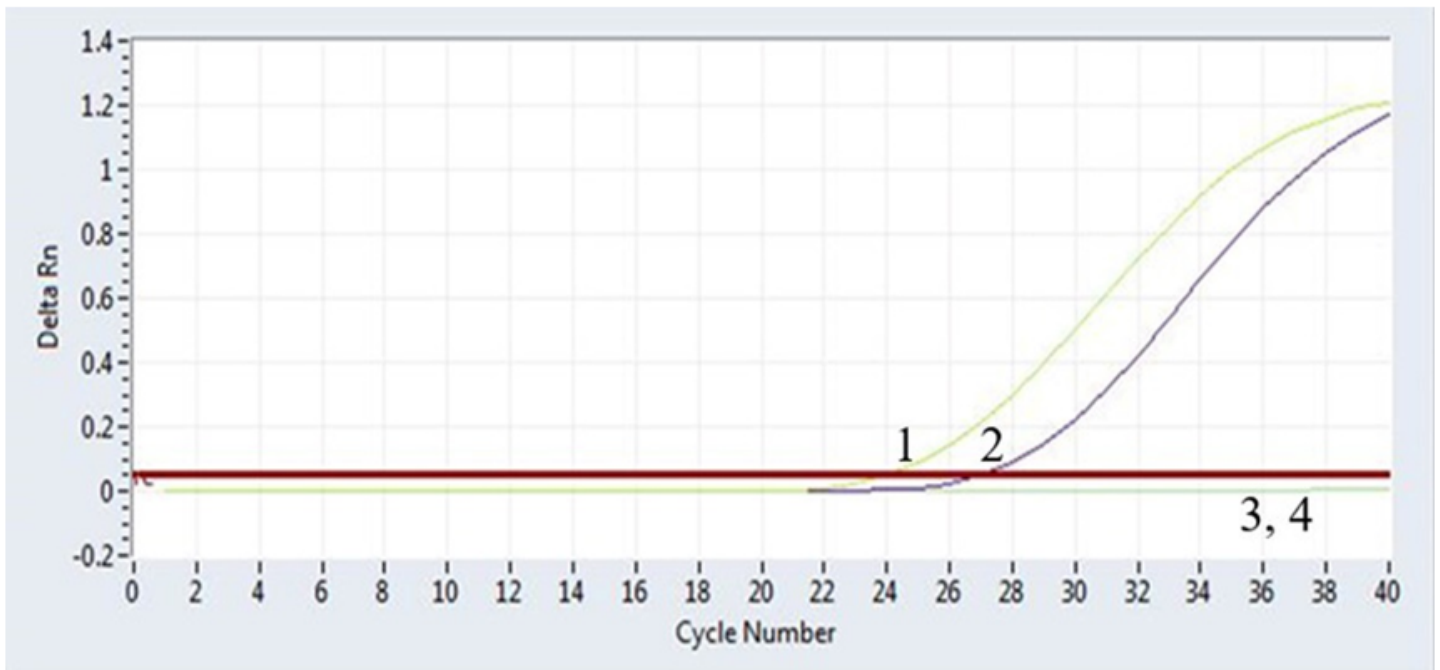


Figure 3

Analytical specificity of TaqMan real-time FQ-MSP assay 1-2×MSP-5, 3-4×MSP-6

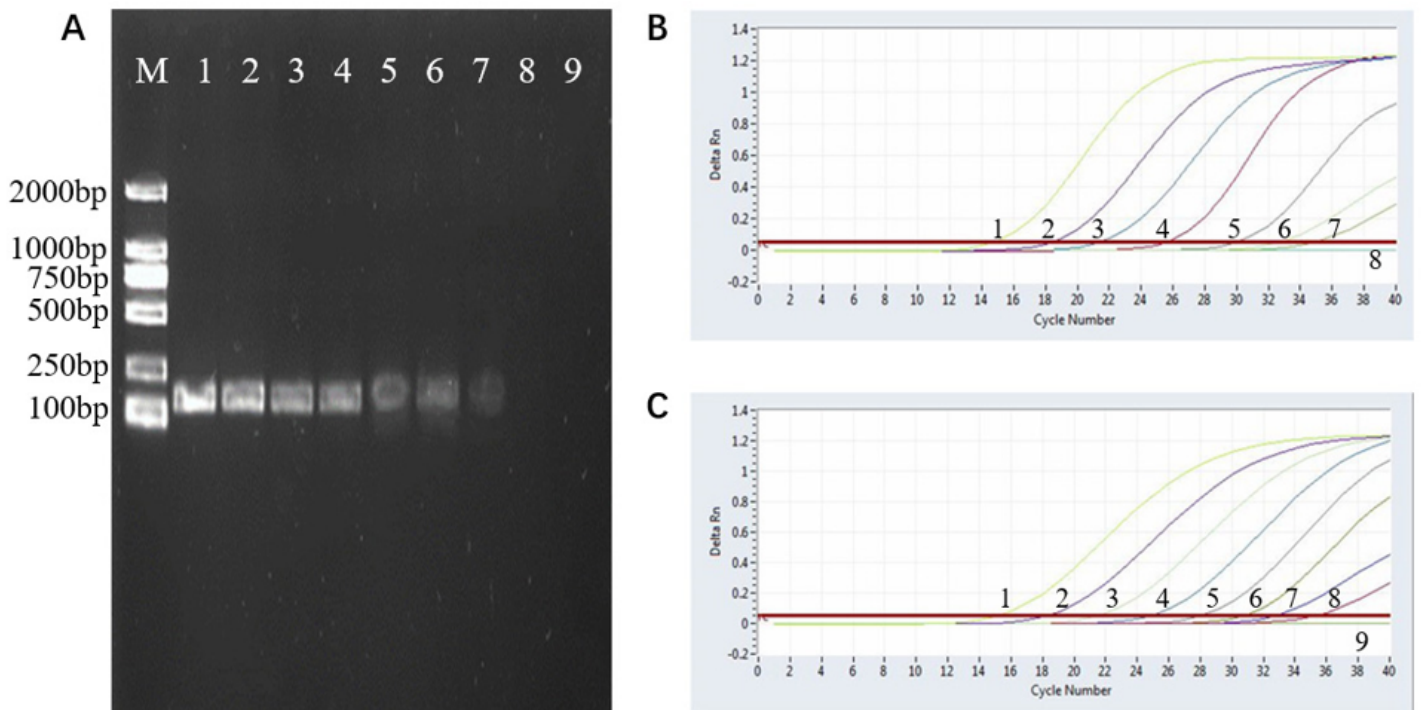


Figure 4

Analytical sensitivity of the conventional MSP (Fig. 4A), qPCR (SYBR GREEN) (Fig.4B), TaqMan real-time FQ-MSP (Fig.4C) assays M: DNA Marker. Labels 1-9: 6×10^7 copies/uL, 6×10^6 copies/uL, 6×10^5 copies/uL, 6×10^4 copies/uL, 6×10^3 copies/uL, 6×10^2 copies/uL, 6×10^1 copies/uL, 6 copies/uL, MSP-6.

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