



## Performance characteristics of bisulfite conversion and SYBR green based quantitative PCR for DNA methylation analysis

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### Abstract

Genomic DNA methylation is one of the most important epigenetic modifications in eukaryotes play vital role in development of severe disease like cancer. Many techniques used for assessment of DNA methylation, bisulfite treatment followed by methylation specific polymerase reaction (MSP) are one of them, which introduce conversion of unmethylated cytosine into uracil. The significant level of bisulfite treated DNA degradation results in the failure of methylation detection. Therefore, this step is to be properly controlled to avoid the degradation of DNA. In the present study, an attempt has been made to access the incubation time of DNA with bisulfite treatment at three time points i.e. 2.5, 4 and 16 hrs to get complete conversion of cytosine to uracil. Currently, the experiments were undertaken using oral cancer tissue, with varying incubation time of bisulfite treatment and 2 representative genes viz *MGMT* and *p16* were selected for the quantitative assessment of methylation by real time PCR. Both genes are frequently methylated at promoter region in carcinogenesis. The short term incubation for 4hrs indicated better real time threshold value for *p16* and *MGMT* gene methylation (Ct 25.55, 27.25) and unmethylation (Ct 18.82, 25.84) in tissue whereas it was 28.16, 37.35 and 21.98, 26.19 in blood sample, respectively as compared to other incubation time which shows less degradation of full length DNA.

### Key words

Epigenetic, DNA methylation, Bisulfite conversion, Methylation specific real time PCR

### Introduction

Methylation of cytosine at CpG dinucleotides is a common feature of many higher eukaryotic genomes and it is most likely restricted to CpG dinucleotides. Most of the methylation occurs at the 5' position of the cytosine where methyl group attach covalently by the enzyme DNA methyltransferases. This cytosine modification has become the most intensively epigenetic DNA marker (Shaw, 2006). This modification of genome has important regulatory effects on gene expression, especially when involving CpG-rich areas known as CpG islands, located in the promoter regions of many genes (Bird, 1992). DNA methylation on the other hand, proposed to be a useful marker for cancer diagnosis, screening, surveillance in high-risk individuals, monitoring

of minimal residual disease, and determining optimal therapeutic options (Laird, 2003; Fiegl *et al.*, 2005; Woodson *et al.*, 2005). Therefore, there are increasing demands for reliable assays to measure DNA methylation, particularly for tissue.

A numerous methods to determine DNA methylation status in tumor tissues have been developed among these MSP after sodium bisulfite conversion is widely used (Harman *et al.*, 1996; Sasaki *et al.*, 2003). The sodium bisulfite modification of DNA plays a critical step to analyze the methylation and for mapping at cytosine residue in the promoter region. However, reproducibility of the sodium bisulfite conversion step has not been convincingly investigated and demonstrated. Sodium bisulfite treatment

presents a harsh environment for DNA molecules, and earlier data reported that 84 to 96% of DNA is degraded during 4 hours of bisulfite treatment at 55°C (Grunau *et al.*, 2001). Many attempts have been made to optimize bisulfite treatment by balancing competing goals of maintaining complete cytosine conversion and minimal DNA fragmentation but due to long incubation, high temperature, high molarity, pH rate of bisulfite leads to large degradation of genomic DNA that renders PCR amplification impossible (Tanaka and Okamoto, 2007). Thus random fragmentation of DNA reduces the number of available molecules for PCR amplification, especially with increasing amplicon size. Furthermore, they show large variability when measured repeatedly. Assurance of reproducibility in this critical bisulfite treatment step is essential in applying quantitative methylation assays to clinical practice. Our objectives were to evaluate the precision and performance characteristics of sodium bisulfite treatment and qMSP, keeping both incubation time and temperature factor along with short amplicon size of the selected gene to quantitate methylation.

### Materials and Methods

All the chemicals used were of analytical grade and obtained from Sisco Research Laboratories, Mumbai, India and Sigma Aldrich Co., USA. Trizol reagent and MethylCode Bisulfite conversion kit was purchased by Invitrogen, Carlsbad, CA. SYBR green Premix Ex Taq used for real time PCR was of Takara Bio Inc., USA.

The methylation pattern and analysis was done in Petroleum Toxicology Division of Indian Institute of Toxicology Research (CSIR-IITR), Lucknow, India. Tissue collection and analysis in this study were approved by the King George's Medical College and Saraswati Dental College ethical committee, Lucknow, India. Informed consents from all participants were obtained. Oral cavity tissue and matched blood samples of 10 patients were collected from Out Patient Department and preserved in Trizol reagent and stored at -80°C immediately till further processing for DNA isolation. The present study followed the guidelines of the Helsinki Declaration (Sixth revision, 2008).

**DNA extraction and purification :** Genomic DNA was isolated by phenol/chloroform extraction method followed by ethanol precipitation as described by Sambrook *et al.* (1989). In brief, after removing the upper aqueous phase, collected inter and organic phase and added 300µl of 100% ethanol per 1ml of Trizol followed by gentle vortexing and incubation at 15-30 °C for 2-3min. The mixture was centrifuged at 12,000 rpm for 5min at room temp (25±2 °C). Pellet obtained was washed 2 times in 0.1M sodium citrate solution for 30min at 15-30°C. After washing, DNA pellet was resuspend in 1ml 75% ethanol per ml Trizol for 10-20 min at 15-30°C. After high speed centrifugation (12,000 rpm min<sup>-1</sup>), dry pellet was dissolved in TE buffer. The DNA was

purified by Wizard genomic DNA purification kit (Promega). DNA was quantified by Picodrop spectrophotometer (Picodrop, UK) at 260/280nm wavelength.

**Sodium bisulfite treatment of DNA :** A conventional bisulfite modification reaction was performed as described previously by Epigenome NoE – protocol (Olek, 1996) with slight modifications. Briefly, DNA (500ng-1µg) was denatured by NaOH (3M) with 10min incubation at room temperature and treated with hydroquinone (10mM) and sodium bisulfite (3M) and incubate for 16hrs at 50°C in water bath. DNA was recovered using Wizard DNA cleanup system (Promega) and dissolve in TE buffer. The solution mixed for desulphonating with NaOH (3M) was incubated 15-20min at 37°C. DNA was recovered by adding 17µl ammonium acetate (10M, pH7.0), 1µl glycogen (10mg ml<sup>-1</sup>) and 450µl of cold absolute ethanol and incubated for 20-30 min at -20°C. After precipitation, the DNA was centrifuged at 10,000rpm for 1min, dried at room temperature and dissolved in 20µl autoclaved distilled water. The preparation of bisulfite solution was performed as described by Hayatsu *et al.* (2004). Treatment of DNA by using 3M bisulfite solution was performed as follows: We used 530µl of 3M NaHSO<sub>3</sub> (pre-warmed) was added to alkali denatured DNA solution and incubated accordingly at different time point.

Alternatively, after bisulfite treatment of isolated genomic DNA, 4hr and 2.5hr incubation of 50°C was given and rest protocol was followed as mentioned above for 16hrs. Simultaneously, a bisulfite conversion kit protocol was run parallel as per manufacturer instruction. In brief, DNA (200-500ng) was treated with conversion reagent and incubated in thermal cycler at 64°C for 2.5hrs, followed by purification step and desulphonation and then centrifuge at 12,000rpm for 30sec. The DNA was finally converted by elution buffer and ready for analysis, or storage at -20°C.

**Quantitative methylation specific PCR (qMSP) :** Methylation status of the two genes viz. *p16* and *MGMT* was determined by the method of MSP (Herman, 1996) with the some modifications. We applied two- stage MSP method for promoter methylation analysis. The nucleotide sequences of the primers used for the two-stage MSP for both genes are summarized in Table 1. The real time PCR amplification was carried out in a 20µl reaction mixture containing 10µl of (2X) SYBR premix Ex Taq, 1µl of 10µM of forward and reverse primer for *p16* and *MGMT*, 1µl of template (250ng) and dH<sub>2</sub>O. The reaction was heated at 95°C for 30sec, then amplified for 40cycle [52°C for 30sec (*MGMT*), 72°C for 30sec and 95°C for 20sec, 64°C for 30sec (*p16*)], followed by a final 10min extension at 72°C. PCR products was diluted 2-fold, and 1µl was used for 2<sup>nd</sup> stage real time PCR using the same reagents and conditions as for round 1 PCR excepts the annealing temp for 70°C (*p16*), and 60°C (*MGMT*) for 30sec, and each sample was amplified into two reactions, with one reaction containing primer specific

**Table 1** : Primers used for MS-PCR

Primers	Genes	Position	Sense Primers 5'→3'	Antisense Primers 5'→3'
Primers for stage I PCR	<i>MGMT</i>	E	GGATATGTTGGGATAGTT	CCAAAAACCCCAAACCC
	<i>p16</i>	E	GAAGAAAGAGGAGGGGTTGG	CTACAAACCCCTCTACCCACC
	<i>MGMT</i>	M	TTTCGACGTTTCGTAGGTTTTTCGC	GCACTCTTCCGAAAACGAAACG
Primers for stage II PCR	<i>MGMT</i>	U	TTTGTGTTTTGATGTTTGTAGGTTTTTGT	AACTCCACACTTTCAAAAACAAAACA
	<i>p16</i>	M	TTATTAGAGGGTGGGGCGGATCGC	GACCCCGAACCGCGACCGTAA
	<i>p16</i>	U	TTATTAGAGGGTGGGGTGGATTGT	CAACCCCAAACCACAACCATAA

E, external; M, methylated; U, Unmethylated

for methylated cytosine and the other reaction containing primers specific for unmethylated cytosine. An aliquot of PCR product was separated on a 4% agarose gel electrophoresis (4%AGE). The product size for *MGMT* and *p16* gene was 83 and 150bp for methylation whereas 91 and 151bp for unmethylation. The gel was stained with ethidium bromide and photographed under UV illumination. The reproducibility of the results was confirmed by repeating qMSP for each DNA sample.

### Results and Discussion

This study comprised bisulfite conversion of genomic DNA by different ways which includes incubation time and temp. The bisulfite- qMSP procedures consisted a sodium bisulfite treatment followed by two steps of qPCR using methylation and unmethylation specific primer sets. Efficiency of conversion of cytosine to uracil and degradation of DNA during bisulfite conversion is an additional potential serious problem in the current practice of the bisulfite- MSP. Therefore we examined the expression of certain gene which get methylated in tumor condition with respect to normal subjects by SYBR green based methodology of methylation specific real time PCR after different bisulfite incubation. The threshold cycle (Ct) was determined as the first detectable cycle from the amplification curves.

**qMSP of bisulfite treated DNA:** Table 2 depicts Comparative Ct and Tm value of bisulfite treated DNA after qMSP for all 3 protocols for *p16* gene including kit method. It shows better threshold cycle and melting curve on decreasing the incubation time and temp in blood and tissue of tumor tissue. Better threshold level and melting temp was revealed by kit (22.97Ct/82.83Tm for methylated and 20.40Ct/84.29Tm for unmethylated tissue DNA; 23.25Ct/82.83Tm for methylated and 13.82Ct/83.18Tm for unmethylated blood DNA), 4hrs (25.55Ct/82.33Tm for methylated and 18.82Ct/84.28Tm for unmethylated tissue DNA; 28.16Ct/82.33Tm for methylated and 21.98Ct/84.26Tm for unmethylated blood DNA) incubation time than 16hrs (27.25Ct/82.33Tm for methylated and 21.76Ct/83.68Tm for unmethylated tissue DNA; 33.31Ct/81.81Tm for methylated and 25.75Ct/84.19Tm for unmethylated blood DNA) and 2.5hrs (27.25Ct/82.33Tm for methylated and 21.76Ct/83.68Tm for unmethylated tissue

DNA; 33.31Ct/81.81Tm for methylated and 25.75Ct/84.19Tm for unmethylated blood DNA). The 2.5hrs incubation time at 55°C showed an uncompleted bisulfate conversion, causes high Ct and low Tm (28.33Ct/75.41Tm for methylated and 31.12Ct/72.32Tm for unmethylated tissue DNA; 33.16Ct/75.40Tm for methylated and 29.04Ct/71.92Tm for unmethylated blood DNA).

Similar to *p16* gene, Table 3 shows comparative Ct and Tm value for *MGMT* gene. Here only 4hrs and kit protocol following same patterns whereas 16hrs and 2.5hrs incubation time showed a variation in amplification. (Value of Ct and Tm by qMSP is mentioned in Table 3)

In Fig. 1 and 2 data were validated by 4% AGE for both genes which follows same as Ct value pattern. In Fig. 1, amplified PCR product for *p16* gene of tissue and blood DNA of 4hrs incubation time shows a clear and sharp gel band for the both methylation and unmethylation whereas for 2.5hrs and 16hrs incubation time, the faint or no band is observed. It is clearly visible for 2.5hr incubation that no methylation in tissue and blood DNA whereas a very clear and sharp band for methylated tissue DNA incubated for 4hrs. Simultaneously, in Fig. 2, PCR product of 4hrs incubated methylated and unmethylated DNA shows a clear and sharp band as compared to *MGMT* gene PCR amplified PCR product of 2.5 and 16hrs incubated DNA. This gel pictures depicts that better and less degraded bisulfite converted DNA amplified properly and more reliable for the diagnosis purpose of promoter gene methylation or unmethylation.

Promoter CpG island methylation and transcriptional inactivation of genes are important events during epigenetic pathway of carcinogenesis. DNA methylation protocols play significant role in clinical practice as many genes have been shown to be methylated and functionally silenced in a variety of human neoplasias (Laird, 2005; Issa, 2004) and may be useful marker for predicting prognosis and monitoring efficacy of adjuvant therapy in cancer patients (Fiegl, 2005; Muller *et al.*, 2004). Further may be used for evaluation of tumor recurrence in surveillance of high- or low-risk individuals (Shen *et al.*, 2005; Giovannucci and Oginio, 2005; Woodson *et al.*, 2005). A variety of assays to measure DNA methylation have been developed for

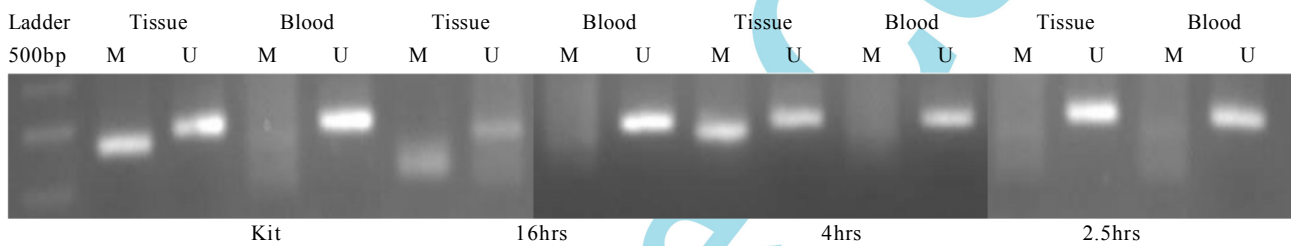
**Table 2 :** Comparative Ct and Tm value of bisulfite treated DNA after qMSP for all 3 protocols for p16 gene including kit method

Bisulfite incubation time	Ct value/Tm of p16 gene in DNA of Oral Cancer Patient			
	Tissue		Blood	
	Methylated	Unmethylated	Methylated	Unmethylated
Kit (64°C)	22.97/82.83	20.40/84.29	23.25/82.83	13.82/83.18
16hrs (50°C)	27.25/82.33	21.76/83.68	33.31/81.81	25.75/84.19
4hrs (55°C)	25.55/82.33	18.82/84.28	28.16/82.33	21.98/84.26
2.5hrs (55°C)	28.33/75.41	31.12/72.32	33.16/75.40	29.04/71.92

**Table 3 :** Comparative Ct and Tm value for MGMT gene

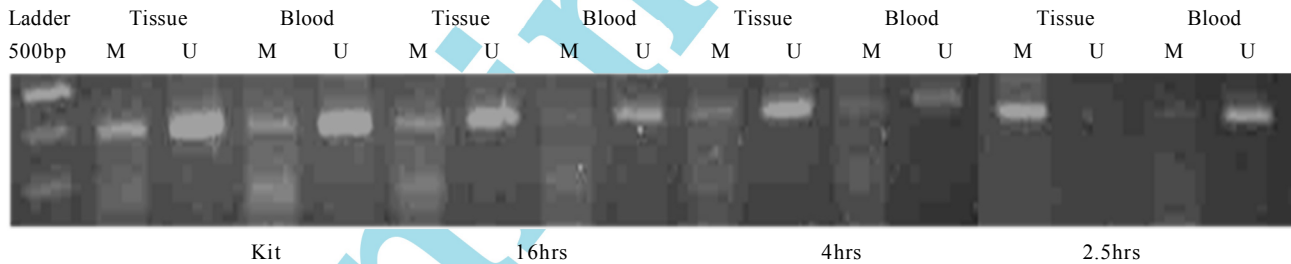
Bisulfite incubation time	Ct value/Tm of MGMT gene in DNA of Oral Cancer Patient			
	Tissue		Blood	
	Methylated	Unmethylated	Methylated	Unmethylated
Kit (64°C)	26.12/83.31	25.29/83.38	37.32/74.23	21.50/83.49
16hrs (50°C)	32.57/73.21	31.31/73.68	37.57/71.24	24.17/82.54
4hrs (55°C)	27.25/83.33	25.84/83.30	37.35/72.23	26.19/83.22
2.5hrs (55°C)	35.37/71.91	26.97/83.33	38.76/69.23	25.56/83.31

**Fig. 1 :** Shows the amplified gel product for both genes (4%AGE) for p16 gene where 4hrs and kit which has less incubation time provides better amplification in comparison to other. (M-150bp and U-151bp amplified product size)



Methylated; M-150bp, Unmethylated; U-151bp

**Fig. 2 :** Represents a methylation pattern of MGMT gene after bisulfite conversion of DNA at different incubation period with varying temp. (M-83bp and U-91bp amplicon size)



Methylated; M-83bp, Unmethylated; U-91bp

paraffin-embedded tissue, and many of these methods rely on sodium bisulfite treatment of genomic DNA from tumor tissue. However, precision and performance characteristics of sodium bisulfite treatment and measurement of methylation have not been comprehensively evaluated (Ogino *et al.*, 2006).

In the present study, we selected two genes viz *p16* and *MGMT* for performance check of our DNA methylation protocol. Both genes contain a CpG island at its promoter region, which is heavily methylated in many cancerous

tumors and unmethylated in normal tissue like lung, oral, gallbladder cancer. In a conventional PCR, bisulfite converted unmethylated DNA shows amplification by the use of primer set of methylation as a false positive result whereas in another technique i.e. COBRA, this conventional MSP product shows double band that indicate contamination of unconverted DNA and overestimation of methylation status. By qMSP, unmethylated samples do not show positive band, while DNA from methylated cancer tissues show positive band by the set of methylation primer without any effects of unmethylated DNA. MSP is a widely

used method to analyze DNA methylation levels at specific genes (Herman *et al.*, 1996; Li and Dahiya, 2002), but it is sometimes incompatible with DNA in insufficient bisulfate modification (Warnecke *et al.*, 2002; Grunau *et al.*, 2001). The present pilot study suggested that 4 hrs are an ideal incubation time to achieve the desired level of bisulfite conversion leading to the accurate results with no false positive or negative effects. We found that by short incubation time and low temperature leads to better sodium bisulfite conversion and qMSP with good threshold level (Ct value) and melting temp, which can be effectively used to quantify DNA methylation and unmethylation in cancerous and normal tissues. In conclusion, sodium bisulfite conversion is reproducible, and subsequent quantitative real-time PCR methylation assays have acceptable precision. Quantitative promoter methylation data are highly correlated with loss of gene expression. Carefully validated quantitative MSP will be useful in both research and clinical molecular diagnostics.

**Conflict of Interest :** None declared

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