31 Quantitative Methylation Status Assessment in DNA from FFPE Tissues with Bisulfite Modification and Real-Time Quantitative MSP

Ermanno Nardon

Contents

31.1 Introduction and Purpose

Because of its simplicity and requirement of only commonly used equipment, methylation-specific PCR (MSP) is the most widely used assay for detection of methylation. Prior to amplification, the DNA is treated with sodium bisulfite to convert all unmethylated cytosines to uracils. The DNA is then amplified using primers that match one particular methylation status of the DNA, such as that in which DNA is methylated at all CpGs sites. This technique is very sensitive and it was originally estimated to be able to detect one methylated target in the presence of a 1,000-fold excess of unmethylated DNA [1]. Nevertheless, MSP suffers from some drawbacks, such as (1) its proneness to false positives in the presence of unconverted or partially unconverted sequences in the bisulfite-treated DNA [2]; (2) its possible dependence to subjective judgment, since the generated PCR product is visualized on a gel; (3) its low throughput.

Real-Time Quantitative MSP is based on the continuous optical monitoring of a fluorogenic PCR and represents a logical implementation of the MSP strategy, in which a dual-labeled, fluorogenic probe is also included (Methylight technology) [3, 4]. One fluorescent dye serves as a reporter, and its emission spectrum is quenched by a second fluorescent dye. During the extension phase, the 5′ to 3′ exonuclease activity of Taq polymerase cleaves the reporter from the probe, thus releasing it from the quencher, resulting in an increase in fluorescence emission [5, 6]. Three main strategies have been developed for the Methylight assays. In the first one, sequence discrimination occurs at PCR amplification only, with primers specific for either the methylated or the unmethylated sequence and a common probe detecting a portion of the

E. Nardon
Department of Medical, Surgical and Health Sciences,
University of Trieste, Cattinara Hospital,
Strada di Fiume 447, Trieste, Italy

G. Stanta (ed.), *Guidelines for Molecular Analysis in Archive Tissues*,
DOI: 10.1007/978-3-642-17890-0_31, © Springer-Verlag Berlin Heidelberg 2011

amplicon devoid of CpG sites ('Fluorescent MSP'). In the second one, sequence discrimination occurs at probe hybridization level only, with common primers that do not overlap any CpG dinucleotide [7]. In the third approach, primer and probe sets are both designed to detect either fully methylated or fully unmethylated patterns in the targets [3, 4]. This latter is the most widely used strategy and it is the one that assures the highest specificity of methylation detection. In all these assays, data normalization is obtained with an amplification to control for the quantity of input DNA, using primers and probes for a sequence which does not contain any CpG in its unconverted form.

We can take advantage of Methylight technology whenever a quantitative, high-throughput evaluation system is required, e.g. to validate candidate markers arisen from global genome screenings; when a cut-off methylation value needs to be established for a given diagnostic marker, or to stratify a disease according to both the presence and the level of methylation at specific sites [8–11].

Herein is described a Methylight approach to assay for the presence of the CpG island methylator phenotype (CIMP) in human colorectal cancer samples. CIMP affects a significant proportion (15–30%) of sporadic CRC and is characterized by simultaneous aberrant methylation at multiple CpG islands, including several known genes, such as p16, hMLH1, and THBS1 [11, 12]. CIMP-positive CRCs have a distinct clinical, pathologic, and molecular profile, such as association with proximal tumour location, female sex, mucinous and poor differentiated hystology, serrated morphology, microsatellite instability (MSI), and high BRAF and low TP53 mutation rates [10, 13–16]. CIMP+ phenotype association with prognosis is still controversial, but CIMP would be an independent predictor of response to 5-fluorouracil-based treatments [17, 18].

CIMP features can be substantially influenced by CpG islands selected for its detection. In fact, different CIMP panels used in various studies have caused some confusion.

A 6 MINT (Methylated IN Tumor) marker panel was originally developed by Toyota et al. in 1999. CRC tumor population showed a bimodal distribution if stratified according to the number of methylated markers. Tumors testing positive for ≥3 MINTs (CIMP+) displayed a prevalence of KRAS mutations, while tumors with <3 MINTs (CIMP−) showed a prevalence of TP53 mutations [15]. However, subsequent studies failed to observe such a distinct bimodal distribution or to confirm associations with the molecular alterations [14, 19]. These discrepancies may be explained by an overestimation of DNA methylation due to the use of highly sensitive nonquantitative methods such as MSP, by the use of different cutoff values, by the inclusion of other sequences in the panel, or by inadequacy of original panel itself. Recently, an independent genome-wide screening of 195 CpG islands has identified a different set of sequences (CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1) which identifies CIMP status more reliably than the original set [10]. The new panel, developed on a qRT-PCR platform, detects a heavily methylated subset of CRCs that encompasses almost all BRAF mutants and sporadic MSI-H cancers. MSI and BRAF mutations thus would represent a distinctive feature of this type of tumours [16]. This panel has already been successfully validated in independent large sets of colorectal tumours [20, 21].

The procedure herein described is an adaptation of the Weisenberger's protocol (DOI: 10.1038/nprot.2006.152) for quantitatively assessing the methylation status of CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 markers [16] using a TaqMan-based approach. Methylation level at each locus is expressed as a percentage relative to a fully-methylated, M.SssI-treated reference DNA sample (Percentage of Methylated Reference, PMR). A sample is defined as CIMP+ if showing a PMR >10% in at least three of the five markers, while a sample testing positive at two markers or less is considered CIMP negative (CIMP−). The protocol has been divided into four sections:

- DNA extraction from FFPE for methylation analysis
- M.SssI modification
- Bisulfite modification
- Methylight reaction setup and methylation assessment

31.2 DNA Extraction from FFPE tissues for Quantitative Methylation Analysis

Refer to the method described in Sect. 30.2.

31.3 M.SssI Modification

The CpG Methyltransferase M.SssI methylates all cytosine residues within the double-stranded dinucleotide recognition sequence using *S*-adenosyl methionine

(SAM) as a methyl donor. This reaction is performed to create a fully methylated reference sample. Peripheral blood leukocyte DNA (PBL-DNA) is commonly used as a substrate but any genomic DNA can serve the purpose, provided it is of high level purity and bisulfite modification-permissive.

31.3.1 Reagents

- *Human Genomic DNA, 1µg/µl*
- *Nuclease-free water*
- *10X NE Buffer 2* (e.g., New England Biolabs M0226S)
- *32 mM SAM stock* (e.g., New England Biolabs M0226S)
- *M.SssI methyltransferase (4 U/µl)* (e.g., New England Biolabs M0226S)

31.3.2 Equipment

- *Adjustable pipettes*, range: 2–20 µl, 20–200 µl, 100–1,000 µl
- *Nuclease-free aerosol-resistant pipette tips*
- *0.2, 0.5, or 1.5 ml nuclease-free autoclaved tubes*
- *Tabletop refrigerated centrifuge* suitable for centrifugation of 1.5 ml tubes
- *Heating block or Thermal cycler*

31.3.3 Method

- Dilute SAM to 1.6 mM by mixing 2 µl of the 32 mM stock and 38 µl of nuclease-free water. Store unused SAM at −20°C in small aliquots.
- In a 0.5 ml sterile tube add in order[1]:
  - Nuclease-free water ..... 118 µl
  - 10X NEBuffer 2 ..... 16 µl (1X final conc.)
  - Diluted SAM ..... 16 µl (0.16 mM final conc.)
  - Genomic DNA..... 8 µl (0.05 µg/µl final conc.)
  - M.SssI methyltransferase (4 U/µl) ..... 2 µl (0.05 units/µl final conc.)
- Mix, pipette up and down several times.
- Incubate overnight at 37°C in a heating block or in a thermal cycler.
- Add 0.6 µl of M.SssI methylase and 5 µl of 1.6 mM SAM. Mix well.
- Incubate overnight at 37°C in a heating block or in a thermal cycler.
- Stop the reaction by heating at 65°C for 20 min in a heating block or in a thermal cycler.

Modified DNA can now be used for bisulfite conversion. Aliquot unused DNA and store at −20°C.

Since methylation of every CpG site could be incomplete after the described treatment, the whole procedure can be repeated. To verify the completeness of reaction, an aliquot of DNA should be bisulfite-modified and tested with a methylation-specific Methylight reaction (see further on). DNA should be phenol-chloroform extracted before each round of treatment.

31.4 Bisulfite Modification

M.SssI-treated reference and sample DNA are both submitted to Bisulfite Modification (BM) by means of the Zymo EZ DNA methylation kit (Zymo Research, Orange, CA, D5001 or D5002) according to the manufacturer's instructions.[2] For optimal conversion results, the amount of input DNA should be from 200 to 500 ng. DNA amounts from FFPE lower than 200 ng may yield unreliable results in the subsequent Methylight assay.

31.5 Methylight Reaction Setup and Methylation Assessment

The methylation level of each bisulfite-converted sample is detected in real time by a TaqMan chemistry using primers and probes specific for fully methylated sequences at each of the five CIMP-specific loci [16]. Primers and probes are also specific for bisulfite-converted DNA. The amount of fully converted DNA is assessed with a control reaction targeting the repetitive

[1]This reaction can be scaled to accommodate different DNA amounts.

[2]Other bisulfite modification kits can be consistently used, e.g. the Qiagen EpiTect™ Bisulfite Kit and the Invitrogen MethylCode™ Bisulfite Conversion Kit.

element ALU-C4 [16],[3] using primers and probes which are specific for bisulfite-converted DNA and do not have any CpG site in their target sequence. Scalar amounts of the M.SssI–treated, bisulfite-modified reference DNA are real-time amplified to set up six standard curves, one for each gene.

For any sample, methylation amount at a given locus is calculated from the standard curve and input DNA amount is obtained from the ALU-C4 reference curve. Sample methylation percentage with respect to the fully methylated reference (PMR) is then computed as:

$$\text{PMR} = \frac{\text{Methylation amount}}{\text{Input DNA amount}} \times 100 \qquad (31.1)$$

31.5.1 Reagents

- *TaqMan® 1,000 Reactions Gold with Buffer A Pack* (Applied Biosystems, 4304441): includes 10X Buffer, 25 mM MgC_2, and Taq Gold Polymerase
- *Commercial 100 mM stock solution of each dNTP (pH 8)* (e.g., Amersham 27-2035-03). Dilute the dNTP stock solutions to prepare a 10 mM solution of each dNTP in sterile water
- *10× TaqMan Stabilizer solution:* 0.5% gelatin (w/v), 0.1% Tween-20 (v/v)[4]
- *Primers and probes:* lyophilized primers should be dissolved in a small volume of distilled water or 10 mM Tris, pH 8 to make a 300 μM stock solution. Lyophilized probe should be prepared at a final concentration of 100 μM. Dilute the primers and the probe to a working solution of 6 μM (primers) and 2 μM (probe). Make small aliquots of both stock and working solutions and store them at −20°C. In Table 31.1 the specific primer and probe sequences of CACNA1G, IGF2, NEUROG1, RUNX3, and SOCS1 markers are reported.

[3]This multi-copy ALU sequence, which is dispersed throughout the genome, is used for normalization reaction, as it is less prone to fluctuations caused by aneuploidy and copy number changes affecting single-copy gene normalization reactions (e.g. MYOD1, ACTB, and COL2A1). In addition, it also allows for sensitive detection of small amounts of DNA.

[4]Prepare in advance a 20% Tween-20 (Sigma, P-9416) solution in water. Weigh out 0.2 g gelatin (Sigma, G-9391) and add it to a 50 ml conical screw capped tube. Add 20 ml of water. Heat to dissolve, then add 0.2 ml of 20% Tween-20 and bring the final volume to 40 ml with nuclease-free water. Store at −20°C.

31.5.2 Equipment

- *Adjustable pipettes*, range: 2–20 μl, 20–200 μl, 100–1,000 μl
- *Nuclease-free aerosol-resistant pipette tips*
- *0.2 and 1.5 ml nuclease-free microtubes*
- *Sterile laminar flow hood*
- *Tabletop refrigerated centrifuge* suitable for centrifugation of 0.2 ml and 1.5 ml tubes
- *Real-Time thermal cycler for standard 96-well format reactions*
- *PCR microplates/strips*
- *PCR strip caps*

31.5.3 Method

31.5.3.1 Samples and Controls

Sample and M.SssI-treated DNAs previously submitted to bisulfite modification by means of the Zymo EZ DNA methylation kit are usually eluted in 10 μl. We suggest diluting ten times this amount to a 100 μl volume, to allow for pipetting errors in subsequent operations. Since bisulfite modification is highly detrimental to nucleic acid integrity, DNA recovery may vary from sample to sample. Thus, the optimal eluate amount to be used in the assay is hardly predictable. In order to obtain reliable quantifications, the amount of bisulfite-treated sample DNA should yield a Ct value within the linearity range generated by the M.SssI-treated reference DNA. Therefore, we suggest performing a preliminary ALU-C4 control reaction using 2 and 10 μl of the above dilution (testing more than one unknown sample is recommended).[5] Sample and M.SssI-treated DNA amounts herein shown are arbitrary and do not reflect an optimized assay.

To check the specificity of the MethyLight Assay to detect bisulfite-converted DNA only, a preliminary experiment should be conducted using unconverted human genomic DNA, which should not give results to the amplification. All methylation quantification experiments should include an NTC (No Template Control), containing all the components of the reaction except for the template.

[5]The ALU-C4 reaction is highly sensitive and should generate low C_t values. An unknown DNA amount yielding an ALU-C4 C_t value of less than 20 is usually desirable.

Table 31.1 Primer and probe sequences of the five markers used for the assessment of CIMP

Locus name	GeneBank Number	Sequence (5′–3′)	Amplicon size
CACNA1G (Calcium channel, voltage-dependent, alpha 1 G subunit), 17q22	AC021491	F: TTTTTTCGTTTCGCGTTTAGGT R: CTCGAAACGACTTCGCCG F:6FAM-AAATAACGCCGAATCCGACAACCGA-BHQ-1	66 bp
IGF2 (Insulin-like growth factor 2 (somatomedin A)), 11p15.5	AC132217	F: GAGCGGTTTCGGTGTCGTTA R: CCAACTCGATTTAAACCGACG F: 6FAM-CCCTCTACCGTCGCGAACCCGA-BHQ-1	87 bp
NEUROG1 (Neurogenin 1), 5q23-q31	AC005738	F: CGTGTAGCGTTCGGGTATTTGTA R: CGATAATTACGAACACACTCCGAAT F: 6FAM-CGATAACGACCTCCCGCGAACATAAA-BHQ-1	87 bp
RUNX3 (Runt-related transcription factor 3), 1p36	AL023096	F: CGTTCGATGGTGGACGTGT R: GACGAACAACGTCTTATTACAACGC F: 6FAM-CGCACGAACTCGCCTACGTAATCCG-BHQ-1	116 bp
SOCS1 (Suppressor of cytokine signaling 1), 16p13.13	AC009121	F: GCGTCGAGTTCGTGGGTATTT R: CCGAAACCATCTTCACGCTAA F: 6FAM-ACAATTCCGCTAACGACTATCGCGCA-BHQ-1	83 bp
ALU-C4 (Alu-based normalization control reaction)[a]	Consensus sequence [a]	F: GGTTAGGTATAGTGGTTTATATTTGTAATTTTAGTA R: ATTAACTAAACTAATCTTAAACTCCTAACCTCA F: 6FAM-CCTACCTTAACCTCCC-MGBNFQ[b]	98 bp

[a]ALU is a short nucleotide element interspersed across the whole genome; therefore, no gene locus or number is provided.
[b]*MGBNFQ* Minor Groove Binder, nonfluorescent quencher. *F* forward primer, *R* reverse primer

31.5.3.2 Standard Curve Setup

A 5-point standard curve, consisting of 1:10 serial dilutions of an optimized quantity of the M.SssI-treated reference DNA is required for each of the six considered genes (Table 31.2). Each dilution should be run in duplicate. Dilutions are made in 0.2 ml nuclease-free microtubes. Keep tubes in ice.

Table 31.2 Standard curve setup

Curve point	Dilution	Sample	Used for PCR
1	0	M.SssI-treated DNA (1:10 diluted after BM)	10 µl
2	1:10	2.5 µl of 0 dilution + 22.5 µl of sterile H_2O	10 µl
3	1:100	2.5 µl of 1:10 dilution + 22.5 µl of sterile H_2O	10 µl
4	1:1,000	2.5 µl of 1:100 dilution + 22.5 µl of sterile H_2O	10 µl
5	1:10,000	2.5 µl of 1:1,000 dilution + 22.5 µl of sterile H_2O	10 µl

To allow for pipetting errors, it is suggested to prepare each dilution in slight excess, e.g. if 2 µl of M.SssI-treated DNA are theoretically needed for each duplicate, use 2.5 µl instead. The above indicated amounts allow for pipetting errors. For each duplicated standard curve at least 25 µl of M.SssI-treated DNA, 1:10 diluted after BM (bisulfite modification), are needed

31.5.3.3 qReal-Time PCR Setup

Work under a sterile laminar flow hood. We suggest analyzing each unknown sample in duplicate. The herein indicated amount of 1:10-diluted, bisulfite-modified sample does not reflect an optimized assay.

- For each of the six genes and for each duplicate PCR reaction mix the following components in a 0.2 ml nuclease-free microtube:

- 10X Buffer A ..... 6.0 µl (1X final)
- 25 mM $MgCl_2$ ..... 8.4 µl (3.5 mM final)
- 10X stabilizer solution ..... 6.0 µl (1X final)
- 10 mM dNTPs ..... 1.2 µl (200 µM final)
- 6 µM forward primer ..... 3.0 µl (0.3 µM final)
- 6 µM reverse primer ..... 3.0 µl (0.3 µM final)
- 2 µM probe ..... 3.0 µl (0.1 µM final)
- Taq Gold Polymerase ..... 0.2 µl
- Nuclease-free water ..... 9.2 µl
- DNA sample ..... 20.0 µl

- Mix by pipetting, dispense the mixture on the 96-well plate in duplicates of 30 µl each, avoiding bubble formation. Cover microwells with the strips.
- Refer to instrument software instructions for setting up the real-time run.
  - Use the following Thermal Cycler programme:
    - 1×: 95°C for 10′
    - 40–45×: 95°C for 15″/60°C for 1′

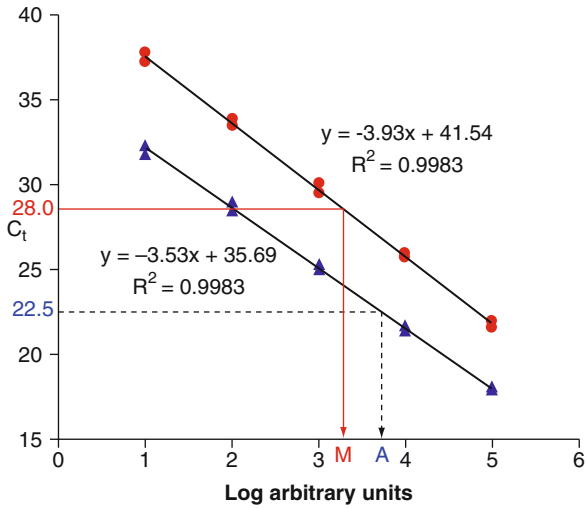

Fig. 31.1 *Example of PMR calculation of an unknown sample - Standard curve method.* Each curve represents the linear regression fitted across C_ts generated by the dilutions of the M. SssI-treated DNA: ▲, Alu C4 reaction; ●, C reaction. Note that since 1,000 copies of the Alu sequence exist per genome, at each control DNA dilution Alu C4 reaction C_ts are considerably lower than the corresponding C_t of the CIMP marker. An unknown sample shows a C_t of 22.5 for the Alu reaction and a C_t of 28.0 for the CIMP marker. From these values sample's log arbitrary amounts *A* and *M* are computed from equations of standard curves as $A = (35.69–22.5)/3.53$ and $M = (41.54–28.0)/3.93$ respectively. PMR is then calculated as: antilog $(M–A) \times 100 = 51.13$

31.5.3.4 Data Analysis and Interpretation

Refer to Sect. 25.6 for the relative quantification using a standard curve. For each control reaction, plot the duplicate C_t value generated from the reference DNA against the corresponding log amount, expressed in arbitrary units (Fig. 31.1). Fit the least squares regression line across tabulated points. Convert the C_t value generated by each unknown sample into arbitrary units, using the standard curve regression equation. To obtain the methylation percentage at a given CIMP marker (PMR), divide the calculated arbitrary units by the arbitrary units calculated from the ALU-C4 standard curve and multiply this quotient by 100.[6]

A sample is scored as CIMP+ if showing a PMR >10% at three or more of the five CIMP markers, while a sample testing positive for ≤2 markers is considered CIMP negative (CIMP–).

31.5.4 Troubleshooting

Troubleshooting suggested in Chap. 25 applies also to the qRT-PCR Methylight assay (Table 31.3).

[6]This model of quantification does not assume that target and control gene have equal amplification efficiencies and it is fully consistent with the efficiency-corrected model of relative quantification. In fact, it can be easily demonstrated that:

$$\mathrm{PMR} = \frac{(E_{\mathrm{target}})^{\Delta C_t,(\mathrm{reference-sample})}}{(E_{\mathrm{control}})^{\Delta C_t,(\mathrm{reference-sample})}} \times 100\,,$$

where E_{target} and E_{control} are the amplification efficiencies of the CIMP marker and of the ALU-C4 reaction, respectively, as calculated from the standard curves $E = 10^{-(1/\mathrm{slope})}$; ΔC_t*,(reference-sample)* is the difference between the C_t value of the fully methylated reference and the C_t value of the unknown sample.

Table 31.3 Troubleshooting

Problem	Possible reason	Solution
The ALU-C4 control reaction yields no amplification signal from unknown samples and/or from reference DNA.	An insufficient amount of bisulfite-modified target DNA is available for amplification. This can be due to:	
	(a) Insufficient DNA submitted to BM: most of the DNA has been degraded during BM.	(a and b): Submit to BM a DNA amount consistent with the instructions provided by the Zymo EZ DNA methylation kit (see Sect. 31.4).
	(b) Too much DNA submitted to BM: poor DNA conversion.	
	(c) Unexhaustive BM due to poor DNA quality.	(c) Repeat DNA extraction. An extensive proteinase K treatment is mandatory to obtain a DNA sample suitable for BM.
CIMP marker(s) reaction yields no amplification signal from the MSss-I -treated reference DNA (the ALU-C4 control reaction is successful).	MSss-I methylation reaction failure. This could be due to:	
	(a) SAM has expired	(a) Use a new batch of SAM. SAM is stable for at least 6 months if stored at −20°C.
	(b) Too much DNA volume in the reaction, which can cause inhibition by changing the pH or salt concentration of the reaction.	(b) Concentrate and desalt the DNA using microcolumns (e.g., Millipore Microcon YM-50)
The C_t value generated by one or more unknown samples is outside the C_t values range of the standard curve.	Nonoptimized assay.	Perform optimization as suggested in Sect. 31.5 of this chapter, using samples whose C_t value is outside the C_t value range of the standard curve.
PMR value of one or more unknown samples is >100%.	Unexhaustive MSss-I methylation treatment of the reference DNA.	MSss-I treatment should be repeated and the DNA Methylight-tested after each round. Refer to Sect. 31.3 of this chapter.

References

1. Herman JG, Graff JR, Myohanen S, Nelkin BD, Baylin SB (1996) Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. Proc Natl Acad Sci USA 93(18):9821–9826
2. Sasaki M, Anast J, Bassett W, Kawakami T, Sakuragi N, Dahiya R (2003) Bisulfite conversion-specific and methylation-specific PCR: a sensitive technique for accurate evaluation of CpG methylation. Biochem Biophys Res Commun 309(2): 305–309
3. Eads CA, Danenberg KD, Kawakami K, Saltz LB, Blake C, Shibata D, Danenberg PV, Laird PW (2000) Methylight: a high-throughput assay to measure DNA methylation. Nucleic Acids Res 28(8):E32
4. Lo YM, Wong IH, Zhang J, Tein MS, Ng MH, Hjelm NM (1999) Quantitative analysis of aberrant p16 methylation using real-time quantitative methylation-specific polymerase chain reaction. Cancer Res 59(16):3899–3903
5. Heid CA, Stevens J, Livak KJ, Williams PM (1996) Real time quantitative PCR. Genome Res 6(10):986–994
6. Holland PM, Abramson RD, Watson R, Gelfand DH (1991) Detection of specific polymerase chain reaction product by utilizing the 5′–3′ exonuclease activity of thermus aquaticus DNA polymerase. Proc Natl Acad Sci USA 88(16): 7276–7280
7. Zeschnigk M, Bohringer S, Price EA, Onadim Z, Masshofer L, Lohmann DR (2004) A novel real-time PCR assay for quantitative analysis of methylated alleles (qama): analysis of the retinoblastoma locus. Nucleic Acids Res 32(16):e125
8. Jeronimo C, Usadel H, Henrique R, Oliveira J, Lopes C, Nelson WG, Sidransky D (2001) Quantitation of GSTP1 methylation in non-neoplastic prostatic tissue and organ-confined prostate adenocarcinoma. J Natl Cancer Inst 93(22): 1747–1752
9. Laird PW (2003) The power and the promise of DNA methylation markers. Nat Rev Cancer 3(4):253–266
10. Ogino S, Cantor M, Kawasaki T, Brahmandam M, Kirkner GJ, Weisenberger DJ, Campan M, Laird PW, Loda M, Fuchs CS (2006) CpG island methylator phenotype (CIMP) of colorectal cancer is best characterised by quantitative DNA methylation analysis and prospective cohort studies. Gut 55(7):1000–1006
11. Toyota M, Ahuja N, Ohe-Toyota M, Herman JG, Baylin SB, Issa JP (1999) CpG island methylator phenotype in colorectal cancer. Proc Natl Acad Sci USA 96(15):8681–8686
12. Issa JP (2004) CpG island methylator phenotype in cancer. Nat Rev Cancer 4(12):988–993
13. Jass JR (2007) Classification of colorectal cancer based on correlation of clinical, morphological and molecular features. Histopathology 50(1):113–130
14. Samowitz WS, Albertsen H, Herrick J, Levin TR, Sweeney C, Murtaugh MA, Wolff RK, Slattery ML (2005) Evaluation of a large, population-based sample supports a CpG island methylator phenotype in colon cancer. Gastroenterology 129(3):837–845
15. Toyota M, Ohe-Toyota M, Ahuja N, Issa JP (2000) Distinct genetic profiles in colorectal tumors with or without the CpG island methylator phenotype. Proc Natl Acad Sci USA 97(2):710–715
16. Weisenberger DJ, Siegmund KD, Campan M, Young J, Long TI, Faasse MA, Kang GH, Widschwendter M, Weener D, Buchanan D, Koh H, Simms L, Barker M, Leggett B, Levine J, Kim M, French AJ, Thibodeau SN, Jass J, Haile R, Laird PW (2006) CpG island methylator phenotype underlies sporadic microsatellite instability and is tightly associated with BRAF mutation in colorectal cancer. Nat Genet 38(7): 787–793
17. Iacopetta B, Kawakami K, Watanabe T (2008) Predicting clinical outcome of 5-fluorouracil-based chemotherapy for colon cancer patients: is the CpG island methylator phenotype the 5-fluorouracil-responsive subgroup? Int J Clin Oncol. Japan Society of Clinical Oncology 13(6):498–503
18. Van Rijnsoever M, Elsaleh H, Joseph D, McCaul K, Iacopetta B (2003) CpG island methylator phenotype is an independent predictor of survival benefit from 5-fluorouracil in stage III colorectal cancer. Clin Cancer Res 9(8):2898–2903
19. Yamashita K, Dai T, Dai Y, Yamamoto F, Perucho M (2003) Genetics supersedes epigenetics in colon cancer phenotype. Cancer Cell 4(2):121–131
20. Nosho K, Irahara N, Shima K, Kure S, Kirkner GJ, Schernhammer ES, Hazra A, Hunter DJ, Quackenbush J, Spiegelman D, Giovannucci EL, Fuchs CS, Ogino S (2008) Comprehensive biostatistical analysis of CpG island methylator phenotype in colorectal cancer using a large population-based sample. PLoS ONE 3(11):e3698
21. Ogino S, Kawasaki T, Kirkner GJ, Kraft P, Loda M, Fuchs CS (2007) Evaluation of markers for CpG island methylator phenotype (CIMP) in colorectal cancer by a large population-based sample. J Mol Diagn 9(3):305–314