DNA methylation: Bisulphite modification and analysis

Susan J Clark[1], Aaron Statham[1], Clare Stirzaker[1], Peter L Molloy[2] & Marianne Frommer[3]

[1]Cancer Program, Garvan Institute of Medical Research, 384 Victoria St, Darlinghurst, Sydney, NSW 2010, Australia. [2]CSIRO Molecular and Health Technologies, Preventative Health National Flagship, PO Box 184, North Ryde, NSW 1670, Australia. [3]School of Biological Sciences, University of Sydney, NSW 2006, Australia. Correspondence should be addressed to S.J.C. (s.clark@garvan.org.au).

Published online 21 December 2006; doi:10.1038/nprot.2006.324

DNA methylation is an important epigenetic modification of DNA in mammalian genomes. DNA methylation patterns are established early in development, modulated during tissue-specific differentiation and disrupted in many disease states, including cancer. To understand further the biological functions of these changes, accurate and reproducible methods are required to fully analyze the DNA methylation sequence. Here, we describe the 'gold-standard' bisulphite conversion protocol that can be used to re-sequence DNA from mammalian cells in order to determine and quantify the methylation state of a gene or genomic region at single-nucleotide resolution. The process of bisulphite treatment exploits the different sensitivities of cytosine and 5-methylcytosine (5-MeC) to deamination by bisulphite under acidic conditions—in which cytosine undergoes conversion to uracil, whereas 5-MeC remains unreactive. Bisulphite conversion of DNA, in either single tubes or in a 96-well format, can be performed in a minimum of 8 h and a maximum of 18 h, depending on the amount and quality of starting DNA.

INTRODUCTION

Epigenetics is the term used to describe heritable changes in gene function that occur without a change in the DNA sequence. The predominant epigenetic modification of DNA in mammalian genomes is the methylation of cytosine (5-MeC), which does not alter the function of the gene product, but provides information as to where and when the gene should be expressed. The primary target sequence of methylation in the DNA of mammals is at 5′-CpG-3′ dinucleotides. However, not all CpG dinucleotides in normal mammalian cells are methylated; in fact, CpG sites in CpG island promoter regions are protected from DNA methylation, whereas CpG sites in gene-coding or non-coding regions are commonly methylated. Genome-wide DNA methylation patterns are established in early development and are then essentially maintained during differentiation, with only some modulation in DNA methylation patterns spanning tissue-specific promoters[1]. However, genomic DNA methylation patterns can be disrupted in disease, in particular in cancer cells, in which aberrant methylation of CpG island promoters is associated with gene silencing and demethylation is associated with gene activation[2]. To understand further the biology that promotes methylation changes in normal development, and that is influenced by diet, environment and heredity to modify DNA methylation patterns in disease, requires accurate and reproducible methods to analyze and quantify the DNA methylation sequence in detail.

A number of methods for determining the distribution of 5-MeC in DNA have been described (reviewed in refs. 3–5). These can be divided into three broad groups: differential enzymatic cleavage of DNA; selective chemical cleavage of DNA; and differential sensitivity to chemical conversion. The detection of 5-MeC using sodium bisulphite to chemically convert the DNA prior to DNA sequencing was first reported by Frommer *et al.* in 1992 (ref. 6) and was optimised by Clark *et al.* in 1994 (ref. 7). Since that time, bisulphite genomic sequencing has become the standard for DNA methylation analysis, as this method defines the methylation state of every cytosine residue in the target sequence, at single-molecule resolution. The key to the bisulphite protocol for determining DNA methylation is based on the selective chemical reaction of sodium bisulphite with cytosine versus 5-MeC residues. The reaction is highly single-strand specific and cannot be performed on double-stranded DNA. A summary of the bisulphite genomic conversion and PCR amplification protocol is outlined in **Figure 1**. The deamination of cytosine by sodium bisulphite and subsequent PCR involves five critical steps: denaturation of the DNA into single strands; reaction of bisulphite with the 5–6 double bond of cytosine to give a cytosine sulphonate derivative; hydrolytic deamination of the resulting cytosine–bisulphite derivative to give a uracil sulphonate derivative; removal of the sulphonate group by a subsequent alkali treatment to give uracil; and PCR amplification. Because the conversion of cytosines to uracils creates non-complementary strands (i.e., uracils opposite guanines), DNA must be amplified with separate pairs of primers that are specific for either the top or bottom DNA strands. Following PCR amplification, the uracils are amplified as thymines, whereas 5-MeC residues are amplified as cytosines. To determine methylation at single-nucleotide resolution, the PCR amplicon can either be sequenced directly or cloned and sequenced (**Fig. 2**). DNA methylation in the PCR target region is then read by scoring the remaining cytosine resides in the sequence.

The majority of new data on DNA methylation is now based on prior treatment of the DNA with bisulphite, followed by DNA amplification with target specific primers. However, the method of analysis of the amplified PCR fragments can vary considerably depending on the degree of specificity and detail of methylation required. Some of the different analysis options that have been described for post-PCR analyses after bisulphite treatment are summarized in **Tables 1** and **2** . The various post-bisulphite analysis options can be divided into either 'Non-selective Detection' for quantitative methylation analysis (**Table 1**) or 'Selective Detection' for analysis of only methylated DNA or only unmethylated DNA (**Table 2**). Bisulphite genomic clonal sequencing analysis is still the

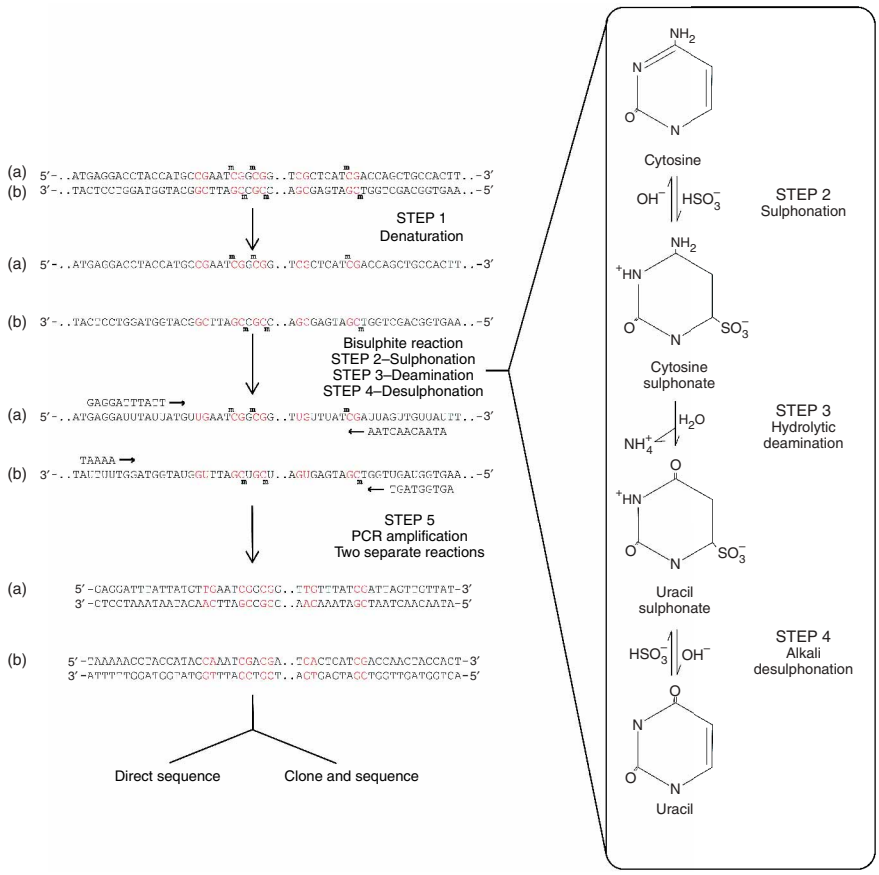

Figure 1 | Schematic diagram of the five critical steps in the bisulphite conversion and PCR amplification reaction. An example DNA sequence, 5′ to 3′ orientation, with the complementary top (a) and bottom (b) DNA strands is shown. The CpG sites are colored red and methylation of a CpG site is indicated by ^{m}CpG. After denaturation, the DNA is single stranded and each strand, a and b, can be amplified independently with strand-specific bisulphite-specific primers to determine the methylation state of each strand. Example strand-specific and bisulphite-specific PCR primers are indicated above and below the DNA strands (in reality, primers are longer; see text). In the forward primers, the cytosine residues are replaced by thymine residues and, in the reverse primers, the guanines (opposite cytosine residues) are replaced by adenine residues. Detailed design parameters of the bisulphite-specific PCR primers are given in the text. After PCR amplification, methylation of the CpG sties in the target sequence can be determined by either direct PCR sequencing of the product or cloning and sequencing (for example results, see **Fig. 4**).

only method that can give resolution at every cytosine site in the target sequence at the single-molecule level; other analytical procedures are less detailed, but can give either a semi-quantitative or an average estimate of the methylation state of the amplified target sequence.

It is now clear, with the completion of the human genome sequence, that re-sequencing the genome for DNA methylation is necessary to fully understand the complexity and function of DNA methylation in different normal and diseased cell types. The need for such detailed analysis is gaining momentum and is enabled by bisulphite methylation sequencing in a large project termed the Human Epigenome Project[8–14]. Here, we present the 'gold-standard' bisulphite genomic conversion protocol that can be used for sequencing every cytosine residue in a target

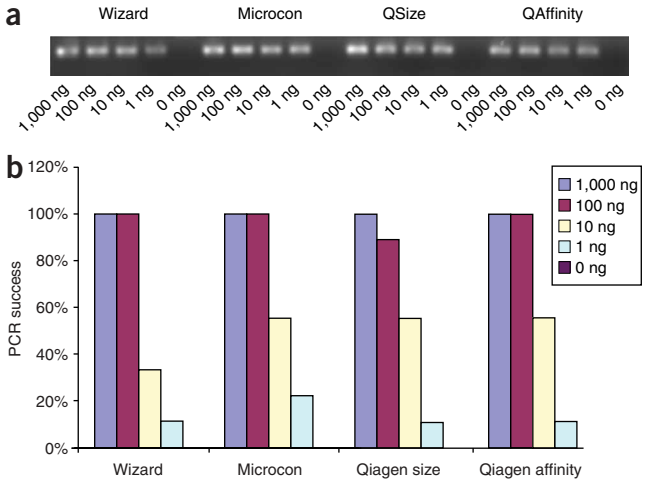

Figure 2 | Comparison of efficiency of bisulphite PCR amplification after different desalting and desulphonation procedures. (**a**) Human genomic cell line DNA samples (1,000 ng, 100 ng, 10 ng, 1 ng and 0 ng (negative control)) were bisulphite-treated in triplicate, according to either the single tube or 96-well plate procedures, and the bisulphite-treated DNA was resuspended in 50 µl after each reaction. A total of 2 µl from each 50 µl bisulphite-treated DNA was used in a 20 µl single-round PCR, also in triplicate. This converts to an equivalent of 40 ng, 4 ng, 400 pg, 40 pg or 0 pg starting genomic DNA in each of nine replicate PCRs (3 bisulphites × 3 PCR amplifications) for each of the different clean-up procedures, respectively. (**b**) The graph plots the percentage of successful PCR amplifications from the nine replicates. Essentially, all protocols provided efficient bisulphite conversion and recovery for 1,000 ng and 100 ng of starting DNA and were able to robustly amplify DNA after the different clean-up and desulphonation steps, for both single tube and 96-well reactions. However, when the starting DNA is limiting (10 ng) — that is, when only the equivalent of 400 pg of bisulphite-treated DNA is used for PCR — the number of successful PCRs is reduced by about 50%. The PCR becomes even more stochastic when only 1 ng DNA is treated, and 40 pg is used in the reaction with only 1–2 PCRs out of 9 giving a product. It is clear that having more DNA (4–40 ng) in the PCR reaction results in robust PCR amplification; however, if DNA is limiting, multiple PCR reactions are necessary to ensure successful amplification and representative sampling[18]. In summary, in the single tube reactions, the Microcon performs slightly better with a greater yield of DNA for a more robust PCR amplification. DNAs purified by Qiagen size and Qiagen Affinity are essentially equivalent in PCR amplification efficiency after bisulphite treatment, and both procedures also compare favorably to single tube reactions.

TABLE 1 | Summary of different methylation analysis options of PCR-amplified bisulphite-treated DNA.

Technique	Applications	Comments	References
Bisulphite genomic sequencing: clonal analysis	Determination of cytosine methylation at each CpG site in individual molecules	Informative at single-molecule and single-base resolution Detects DNA methylation heterogeneity Potential for allele discrimination Time-consuming and laborious, as a high number of clones need to be sequenced	Frommer *et al.* (1992)[6] Clark *et al.* (1994)[7] Warnecke *et al.* (2002)[18] Grunau *et al.* (2001)[19]
Bisulphite genomic sequencing: direct PCR analysis	Determination of average level of methylation at each CpG site in amplicon	Semi-quantitative methylation profile at single-base resolution New algorithms improve quantification Not very sensitive ($>$10–20% methylation) GC-tag primers improve background and read length	Paul *et al.* (1996)[20] Boyd & Zon (2004)[21] Lewin *et al.* (2004)[22] Han *et al.* [23]
Bisulphite genomic sequencing: direct pyrosequencing PCR analysis	Quantification of methylation of individual CpG sites in amplicon	Quantitative down to a level of about 10% methylation High-throughput method Limited to sites within up to 100 bp	Tost *et al.* (2003)[24] Collella *et al.* (2003)[25]
Bisulphite genomic sequencing: hairpin-bisulphite-PCR	Determination of methylation on complementary strands of same molecule	Detects CpG methylation on both DNA strands Most informative for DNA hemi-methylation analysis Difficult to design primers	Laird *et al.* (2004)[26]
RNA transcript fragmentation/mass spectrometry (e.g., Sequenom)	Quantifying methylation at multiple CpG sites using fragmentation of T7 RNA polymerase transcripts	Very high throughput Quantitative data for multiple CpG sites Fragmentation-specific Combined CpG sites Multiple steps and sophisticated equipment	Schatz *et al.* (2004)[27] Ehrich *et al.* (2005)[28]
Fluorescence melting-curve analysis	Provides average methylation of amplicon (from PCR melting profile)	Simple method to give overall estimate of methylation Low sensitivity	Worm *et al.* (2001)[29] Guldberg *et al.* (2002)[30]
Amplicon cytosine content	Provides average methylation level across amplicon	Can provide rapid, high-throughput estimation of methylation levels Direct quantification of cytosine or measure of incorporation	Yang *et al.* (2006) [31] Yamamoto *et al.* (2004)[32]
MS-SSCA[(i)]	Discriminates methylated and unmethylated molecules based on secondary structure	Alleles can be separated and further analysed Not all methylation differences resolved	Maekawa *et al.* (1999)[33] Bianco *et al.* (1999)[34]
MS-DHPLC[(ii)]	Adaptation of MS-SSCA to HPLC	High throughput versus gel-based method	Baumer *et al.* (2001)[35]
COBRA[(iii)]	Determination of methylation at specific restriction enzyme sites within amplicon	Quantitative and rapid Analysis limited to restriction sites Analysis can be affected by incomplete restriction enzyme cutting	Sadri & Hornsby (1996)[36] Xiong & Laird (1997)[37] Ead *et al.* (2002)[38]
MS-SNuPE[(iv)]	Assess methylation at single CpG sites	Quantitative and rapid Only single specific CpG sites analyzed Each assay needs to be individually optimized Recently adapted for higher-throughput multiplexing	Gonzalgo & Jones (2002)[39] Kaminsky *et al.* (2005)[40]
SNuPE- IP RP HPLC[(v)]	Application of ion-pair reverse-pair HPLC to analysis of SNuPE products	Higher throughput than gel-based analysis	El-Maarri *et al.* (2002)[41]
SNuPE/Mass spec	MS-SNuPE combined with mass-spectrometry of products	Quantitative and high throughput Allows a level of multiplexing	Tost *et al.* (2003)[24]

TABLE 1 | (Continued).

Technique	Applications	Comments	References
Methylation Microarray	Quantification of methylation at multiple CpG sites	Not all sites work well; limited dynamic range	Gitan *et al.* (2002)[42] Adorjan *et al.* (2002)[43]
MethylQuant	Quantify methylation level in range down to 0.1% of a single CpG site through real-time PCR	Quantification with SyberGreen and no probes Primer design and PCR optimization are critical Requires second amplification round	Thomassin *et al.* (2004)[44]
Multiplex ligation/bead array (Illumina)	Quantifies methylation at multiple individual sites	High throughput Needs pairs of oligonucleotides for each site	Bibikova (2006)[45]

(i) MS-SSCA (methylation-sensitive – single-strand confirmation analysis). (ii) MS-DHPLC (methylation-sensitive – denaturing high-performance liquid chromatography). (iii) COBRA (combined bisulphite restriction analysis). (iv) Ms-SNUPE (methylation-sensitive – single nucleotide primer extension). (v) SNuPE-IP RP HPLC (sensitive single nucleotide primer extension ion paired reverse phase high-performance liquid chromatography).

sequence, in either a single tube reaction or a 96-well format for high-throughput analyses. The protocol provides information on primer design and conditions to enable representative amplification of molecules with varying methylation states, and compares the sensitivities of procedures for clean-up, amplification and analysis.

MATERIALS

REAGENTS

- Genomic DNA isolated from cell lines, tissues, or clinical samples, prepared as described in REAGENT SETUP. ! CAUTION Clinical materials need to be regarded as potentially carrying infectious agents, and appropriate safety and disposal measures should be followed. Preferably, handle in a biosafety cabinet.
- Needle: 26 G × 0.5 in (0.45 × 13 mm) (Terumo, cat. no. NN 2613R)
- NaOH pellets (Ajax Finechem, cat. no. A482) ! CAUTION Toxic; avoid skin contact.
- tRNA (10 mg ml^{-1}) (Sigma, cat. no. R-4251)
- Sodium metabisulphite (di-sodium disulphite, Na_2O_5 S_2) (BDH Chemicals, cat. no. 103564D) ! CAUTION Toxic; avoid inhalation and skin contact; weigh in a safety cabinet.
- Quinol (1,4 dihydroxybenzene) (BDH Chemicals, cat. no.103122E) ! CAUTION Toxic; avoid inhalation and skin contact; weigh in a safety cabinet.
- DNA clean-up resin (Wizard DNA Clean-up System; Promega, cat. no. A7280)
- Microcon YM-100 desalting columns (Millipore, cat. no. 42414)
- QIA quick 96 PCR Purification Kit (Qiagen, cat. no. 28181)
- Qiagen MinElute 96 UF PCR Purification Kit (Qiagen, cat. no. 28051)
- Isopropanol (BDH Chemicals, cat. no. 10224.6L)
- Ammonium acetate (APS, cat. no. 27-500G)
- 100% Ethanol (BDH Chemicals, cat. no. 1017)
- Restriction enzymes; for example, *Hind*III (New England BioLabs, cat. no. RO104S) or *Eco*R1 (New England BioLabs, cat. no. RO101S) ▲ CRITICAL The enzyme used to digest the DNA must not have a site within the target sequence.
- Agarose gel (Omnigel-sieve Agarose; Edwards Instruments Co., cat. no. 3025)
- Gel buffer (1 × TAE) (Sigma, cat. no. T-1503)
- Acetic acid (BDH Chemicals, cat. no. 10001)
- EDTA (BDH Chemicals, cat. no. 100935V)
- Ethidium bromide, 0.1% wt/vol (Sigma, cat. no. E-8751) ! CAUTION Carcinogen.
- Guanidinium hydrochloride (Sigma, cat. no. G-3272)
- Tris/HCl, pH 6.4 (Sigma, cat. no. T-1503)
- Triton-X-100 (Sigma, cat. no. T-8787)
- Proteinase K (Amresco, cat. no. 0706)
- tRNA (Sigma, cat. no. R-4251)
- SDS (BDH Chemicals, cat. no. 442444H)
- Phenol:Chloroform:Isoamyl alcohol, 25:24:1 (Fluka Bichemika, cat. no. 77617), saturated with 100 mM Tris at pH 8.0
- TRIzol reagent (Invitrogen, cat. no. 15596-018)
- Mineral oil (Sigma, cat. no. M-5904)
- H_2O, water for irrigation (Baxter, cat. no. AHF114)
- PCR film (Eppendorf, cat. no. 00030127.480)
- Parafilm (Pechiney, cat. no. PM-996)
- 96-well PCR plates (ABgene, cat. no. AB-0900)
- Methylated DNA (Serological DNA:CpGenome Universal methylated DNA; Chemicon International, cat. no. S7821)
- Unmethylated DNA (Human Genomic DNA; Roche, cat. no. 11691 112 001)
- 2 × PCR Master Mix (Promega, cat. no. M7502)
- SYBRGreen 1 nucleic acid gel stain (10 000 × conc.) (Molecular Probes, cat. no. S-7563)
- Power SybrGreen PCR Master Mix (Applied Biosystems, cat. no. P/N 4367659 and L/N 0606025)
- Oligonucleotide primers designed against target sequence (100 ng ul^{-1})

EQUIPMENT

- 90 °C heating block or thermal cycler or water bath
- Thermal cycler or closed-lid waterbath at 50 °C
- PCR System (7900 model, Applied Biosystems) or any other thermal cycler
- Electrophoresis power source
- Electrophoresis chamber
- UV transilluminator or any gel-imaging system
- Vacuum regulator (Qiagen, cat. no. 19530)
- Multi-channel pippetteman (1–20 µl and 20–200 µl)
- Heated-lid thermocycler (Gene Amp 9600 model; Perkin Elmer)
- Plate-spinning centrifuge (58014R model; Eppendorf)
- QIAvac Multiwell Vacuum manifold (Qiagen, cat. no. 9014579)
- 96 well twin tec PCR plate (Eppendorf, cat. no. 0030128.672)
- QIAvac 96 Vacuum manifold (cat. no. 19504)

REAGENT SETUP

50× TAE 24.2% Tris, 5.71% acetic acid, 5% EDTA.

Lysis buffer 1 (1×) 7 M Guanidinium hydrochloride, 5 mM EDTA, 100 mM Tris/HCl, pH 6.4, 1% Triton-X-100, 50 µg ml^{-1} Proteinase K, 100 µg ml^{-1} tRNA.

Lysis buffer 2 (10×) 2 µl of 10 mg ml^{-1} tRNA, 140 µl of 20 mg ml^{-1} Proteinase K, 500 µl of 20% SDS, 358 µl H_2O. Dilute to 1× before use.

DNA buffer (1×) 10 mM Tris at pH 8.0/0.1 mM EDTA

DNA isolation kit A range of methods and kits can be used to isolate DNA from a variety of tissue and cells, as isolation of high-molecular-weight DNA is not required for bisulphite conversion. ▲ CRITICAL It is important when choosing a method of isolation that the DNA is free of proteins, as this ensures efficient denaturation and, therefore, complete bisulphite conversion. Note that the bisulphite deamination reaction only occurs on the single-stranded DNA, and does not work on double-stranded DNA. DNA isolation protocols that are suitable for bisulphite conversion from a variety of mammalian tissue and cell sources are summarized in **Box 1**. The amount and quality of DNA isolated can be estimated using a variety of standard methods, including traditional spectrophotometry, NanoDrop Spectrophotometry (NanoDrop Technologies), gel electrophoresis and PCR amplification with a reference gene (such as ribosomal RNA). Gel electrophoresis using size markers will give an estimation

TABLE 2 | Methods for selective amplification of methylated or unmethylated sequences from bisulphite-treated DNA.

Technique	Applications	Comments	References
Methylation-specific PCR (MSP)	Sensitive detection of methylated (or unmethylated) sequences present as a minority sequence Applicable to clinical samples	Rapid qualitative method with high sensitivity and specificity for methylation detection False positives can arise due to inadequate conversion and mispriming Primer design is critical	Herman *et al.* (1996)[46]
Quantitative Real-Time Methylation specific PCR (MethylLight Conlight, QAMA(i))	Quantitative version of MSP with probes for real-time detection	High throughput and readily quantifiable Can use control probes for methylated, unmethylated DNA and for unconverted DNA	Eads *et al.* (2000)[47] Rand *et al.* (2002)[48] Zeschnigk *et al.* (2004)[49]
HeavyMethyl PCR	Quantitative real-time alternative to MSP	High sensitivity, quantitative Real-time detection with fluorogenic probe Requires more components and optimization than conventional MSPCR	Cottrell *et al.* (2004)[50]
Headloop PCR	Quantitative real-time alternative to MSP	Sensitive; can detect $1/10^4$ methylated molecules Less liable to false-positives than MSP More complex primer design and optimization than MSP	Rand *et al.* (2005)[51]
MEP (Methylation enrichment pyrosequencing)	MSP with pyrosequencing validation of amplicon	High sensitivity and specificity Detects mispriming and non-conversion Requires additional pyrosequencing step and primer	Shaw *et al.* (2006)[52]
Bisulphite differential denaturation PCR	Selective amplification of hypomethylated DNA	Simple adaptation of standard bisulphite-PCR and able to be quantified	Rand *et al.* (2006)[53]

(i) QAMA (quantitative analysis of methylated alleles).

of DNA degradation and PCR will provide an estimate of amplifiable DNA prior to the bisulphite reaction.

Pretreatment of DNA To efficiently denature DNA, it can be advantageous to reduce the size of high-molecular-weight DNA. The fragment size of the DNA that is to be bisulphite-converted needs to be no larger than 2 kb to aid denaturation to single DNA strands. DNA isolated from fixed tissue samples is normally of low molecular weight after isolation and therefore can be added directly into the bisulphite reaction. DNA that is of high molecular weight can be fragmented prior to use by either digestion with a six-base restriction enzyme, such as *Eco*RI or *Hind*III, or sonication in DNA buffer at 50–75% power on ice for 2 min, or shearing the DNA, in DNA buffer, by passing the solution five times through a 26 G needle. Sonication or shearing the DNA is less precise but allows the amplification of all target sequences, whereas restriction enzyme digestion is predictive but limits the amplification to known PCR targets.

PROCEDURE

Bisulphite Conversion of DNA

1| Denaturation: For single tube reactions follow option A; for high-throughput methylation analysis in a 96 well plate follow option B. First prepare a fresh 3M NaOH solution by dissolving 6 g of NaOH pellets in 50 ml of sterile H_2O.

(A) Single tube reactions

(i) Denature the genomic DNA (that has previously been resuspended in H_2O or DNA buffer) in a 1.5 ml tube by adding 2 µl of 3 M NaOH to 18 µl DNA to make a final volume of 20 µl.

(ii) Incubate samples at 37 °C for 15 min, followed by incubation at 90 °C for 2 min and then immediately place the tube on ice for 5 min, to ensure the DNA remains denatured.

(iii) Centrifuge the tubes at 4 °C for 10 sec at 10,000*g*, to ensure the DNA is at the bottom of the tube.

(B) 96 well reaction plate for high throughput methylation analysis

(i) For 96 well reactions, add 9 µl of DNA to each of the wells in the PCR plate.

(ii) Denature the genomic DNA by adding 1 µl of freshly prepared 3 M NaOH to make a final volume of 10 µl.

(iii) Seal the plate with a plate sealer, vortex and centrifuge briefly at 10,000*g* for 30 s.

(iv) Incubate the plate at 37 °C for 15 min, in a hot-lid thermocycler followed by incubation at 90 °C for 2 min.

(v) Place the plate on ice for 5 min and centrifuge briefly at 10,000*g* for 30 s at 4 °C.

▲ **CRITICAL STEP** Ensure that DNA is of sufficiently low molecular weight prior to denaturation and bisulphite treatment; see REAGENT SETUP (Pretreatment of DNA).

BOX 1 | EXAMPLES OF DNA ISOLATION PROTOCOLS

● Tissue samples and cell lines: Freeze cells (1×10^6) or tissue ($\sim$100 mg) in liquid N_2 and store at –70 °C or in liquid N_2. Prior to use, grind the tissue under liquid N_2 with a mortar and pestle and add 400 µl of Lysis buffer 1 to the ground tissue, transfer the mixture to a 1.5 ml tube and incubate for 16 h at 55 °C. For cell lines, add 400 µl of 1× Lysis buffer 2 to the frozen cells and incubate for 16 h at 55 °C. Vortex both samples after the lysis reaction and centrifuge at top speed for 2 min to pellet the cell debris. Remove the supernatant into a clean 1.5 ml tube and dilute with 200 µl of H_2O to reduce the salt concentration. Extract the supernatant twice with 400 µl of phenol:chloroform:isoamylalcohol mix and collect the upper aqueous phase. Add 900 µl of ice-cold ethanol to the aqueous phase and leave for 1 h or more at –20 °C. Centrifuge the solution at top speed for 10 min at 4 °C, then remove the supernatant and resuspend the pellet in 40 µl of H_2O. After the DNA is dissolved, the concentration can be determined and the DNA should be frozen at –20 °C.

■ **PAUSE POINT** The DNA can be stored for many years at –20 °C until required for bisulphite treatment.

● DNA extraction from paraffin block samples: Trim the excess wax from cored tissue (5 mm punches) from the paraffin block. Place the core in a 1.5 ml tube and add 400 µl of 1× Lysis buffer 2. Thoroughly homogenize the samples with disposable 1.5 ml pestles and incubate in 1.5 ml tubes for 48 h at 55 °C. After incubation, subject the samples to five freeze–thaw cycles in dry ice for 5 min, followed by 95 °C for 5 min. Vortex the samples and centrifuge at top speed for 2 min to pellet the cell debris. Remove the supernatant into a clean 1.5 ml tube and dilute with 200 µl of H_2O to reduce the salt concentration. Extract the supernatant twice with 400 µl of phenol:chloroform mix. Add 900 µl of ice-cold ethanol and leave for 1 h or more at –20 °C. Centrifuge the solution at top speed for 10 min at 4 °C, then remove the supernatant and resuspend the pellet in 18 µl of H_2O. After the DNA is dissolved, it can be frozen at –20 °C or used directly in the bisulphite reaction.

■ **PAUSE POINT** The DNA can be stored for at least one year at –20 °C until required for bisulphite treatment.

● DNA extraction from slide samples: Scrape into clean 1.5 ml tubes, approximately 2 cm × 1 cm sections of the cell monolayer from a slide. Add 400 µl of 1× Lysis buffer 2. Thoroughly homogenize the samples with disposable 1.5 ml pestles and incubate for 48 h at 55 °C. After incubation, subject the samples to five freeze–thaw cycles of dry ice for 5 min, followed by 95 °C for 5 min. Vortex the samples and centrifuge at top speed for 2 min to pellet the cell debris. Remove the supernatant into clean tubes and dilute with 200 µl of H_2O to reduce the salt concentration. Extract the supernatant twice with 400 µl of phenol:chloroform:isoamylalcohol mix. Add 900 µl of ice-cold ethanol and leave for 1 h or more at –20 °C. Centrifuge the solution at top speed for 10 min at 4 °C, then remove the supernatant and resuspend the pellet in 18 µl of H_2O. After the DNA is dissolved, it can be frozen at –20 °C or used directly in the bisulphite reaction.

■ **PAUSE POINT** The DNA can be stored for at least one year at –20 °C until required for bisulphite treatment.

● DNA isolation after isolation of RNA using TRIzol Reagent (Invitrogen) for small numbers of cultured cells ($1 \times 10^{3-5}$): Add 1 ml TRIzol reagent to cultured cells in a 24- or 6-well plate. Add 0.2 ml chloroform per 1 ml TRIzol, shake, centrifuge at 12,000 *g* for 15 min at 2–8 °C. Remove the aqueous phase from the organic phase. To the organic phase, add 300 µl of 100% ethanol. Mix by inversion and store at 15–30 °C for 2–3 min. Centrifuge at 14 000*g* for 15 min at 4 °C. Discard the supernatant and resuspend the DNA pellet in 18 µl lysis buffer 2. Incubate the samples at 55 °C overnight. Either freeze at –20 °C or use directly in the bisulphite reaction.

■ **PAUSE POINT** The DNA can be stored for at least one year at –20 °C until required for bisulphite treatment.

2| Sulphonation & Hydrolytic Deamination: For single tube reactions follow option A; for high-throughput methylation analysis in a 96 well plate follow option B.

(A) Single tube reactions

(i) Add the following to the denatured DNA sample immediately after the centrifugation.

Denatured DNA (100 ng–2 µg)	20 µl
Saturated sodium metabisulphite at pH 5.0	208 µl
10 mM Quinol	12 µl
Mineral oil	200 µl

Gently mix all reagents, centrifuge for 10 sec and incubate at 55 °C in a water bath with a lid, for 4–16 h.

▲ **CRITICAL STEP** It is important to prepare the Saturated Sodium Metabisulfite at pH 5.0 freshly, by dissolving 7.6 g $Na_2O_5S_2$ in 15 ml sterile H_2O, and adding 464 µl of 10 M NaOH. 10 M NaOH is also prepared freshly, by dissolving 20 g NaOH in 50 ml H_2O. The solution of saturated sodium bisulphite is achieved by gently inverting the reagent/H_2O mixture, with minimum mixing and aeration, and may require pH adjustment with 10 M NaOH before all is nearly dissolved. As it is saturated some small lumps may still remain undissolved. Use the freshly made solution either immediately or on the same day. If not used immediately store the solution in the dark or in foil after preparation.

▲ **CRITICAL STEP** It is important that the bisulphite conversion reaction takes place in the dark to avoid oxidation, so if this is not possible, wrap the tubes in foil prior to incubation. After incubation, centrifuge briefly for 10 sec and remove and discard the mineral oil that is on the top phase. The mineral oil can be easily removed by freezing the tubes and since the oil does not freeze it can be pipetted from the bisulphite reaction. The mineral oil is required to prevent evaporation, however, it is not required if the reaction is performed in a PCR thermocycler with a heated lid.

▲ **CRITICAL STEP** The two variables to consider in deciding on the length of bisulphite treatment are the quantity and quality of DNA to be converted. Recommended incubation times for limited amounts or poor quality, degraded DNA, such as that isolated from paraffin embedded tissue, is 4 h. DNA that is of high molecular weight, such as isolated from tissue culture cells, can be incubated for 4 h, if the concentration is less than 100 ng DNA; 6 h for 100 to 500 ng DNA; and 6-16 h for 500 ng to 2 µg DNA.

(B) 96 well reaction plate

(i) Add the following to the denatured DNA sample in the 96 well plate immediately after the centrifugation.

Denatured DNA (100 ng–2 μg)	10 μl
Saturated sodium metabisulfite at pH 5.0	104 μl
10 mM Quinol	6 μl

Seal plate again, vortex and spin down briefly, and incubate at 55 °C for 6 h.

▲ **CRITICAL STEP** As for the single tube reaction it is important to prepare the Saturated Sodium Metabisulfite at pH 5.0, freshly by dissolving 7.6 g $Na_2O_5S_2$ in 15 ml sterile H_2O, and adding 464 μl of 10 M NaOH. 10 M NaOH is also prepared freshly, by dissolving 20 g NaOH in 50 ml H_2O. The solution of saturated sodium bisulphite is achieved by gently inverting the reagent/H_2O mixture, with minimum mixing and aeration, and may require pH adjustment before all is nearly dissolved. As it is saturated some small lumps may still remain undissolved. Use the freshly made solution on the same day and store in the dark or in foil after preparation.

? TROUBLESHOOTING

3| Desalting and Alkali Desulphonation: For single tube reactions there are two alternative procedures (A and B) listed below, that can be followed. For 96-well-based reactions there are two alternative procedures (C and D) listed below, that can be followed. The desalting step is to remove the bisulphite ions, the alkali step is required to successfully complete the bisulphite conversion.

(A) Wizard Clean-up: Single tube reaction

(i) Add 1 ml of Promega Wizard DNA Clean-up resin.
(ii) Mix gently by inversion to bind the DNA to the silica gel slurry.
(iii) Pipette into a 3-ml syringe barrel attached to a Wizard minicolumn.
(iv) Insert plunger and slowly push through the resin mix.
(v) Detach the syringe from the minicolumn and remove the plunger.
(vi) Re-attach the syringe barrel to the minicolumn and wash the minicolumn with 2 ml of freshly prepared 80% isopropanol.
(vii) Remove the syringe barrel and transfer the minicolumn to a microfuge tube.
(viii) Spin in a microfuge for 2 min to dry the resin.
(ix) Transfer the minicolumn to a new microfuge tube. Add 50 μl of H_2O to the minicolumn.
(x) Leave at room temperature for 5 min.
(xi) Spin for 20 s to collect the eluant. Discard the minicolumns.
(xii) Desulphonate by adding 5.5 μl of 3 M NaOH.
(xiii) Incubate at 37 °C for 15 min.
(xiv) Centrifuge briefly and add 1 μl tRNA (10 mg ml^{-1}).
(xv) Add 33.3 μl of 5 M ammonium acetate at pH 7.0.
(xvi) Add 330 μl of ice cold (–20 °C) 100% ethanol.
(xvii) Mix well by inversion and leave at –20 °C for at least 1 h or overnight to precipitate the DNA.
(xviii) Centrifuge at 14,000*g* for 15 min at 4 °C.
(xix) Remove all traces of supernatant and air dry for ~20 min.
(xx) Resuspend the DNA pellet in 50 μl DNA buffer or H_2O at room temperature for ~2 h with occasional vortexing to ensure that the DNA is dissolved, and then either use in the PCR amplification or store at –20 °C. See **Figure 2** for further information.

■ **PAUSE POINT** The bisulphite-treated DNA can be stored at –20 °C for 1–10 years, depending on the quality and quantity of the starting genomic DNA.

▲ **CRITICAL STEP** Ensure that all traces of ethanol are removed as this can affect the PCR reaction. We recommend this clean-up protocol if the DNA is resuspended in lysis buffer prior to the bisulphite reaction, as an ethanol precipitation step is required to remove the SDS and proteinase K solution. Desalting by Wizard columns is simple and takes a minimum of 2 h to complete after the bisulphite reaction for a maximum of 24 tubes (24-well microfuge).

(B) Microcon Clean-up: Single tube reaction

(i) Add 150 μl of H_2O to the bisulphite reaction.
(ii) Transfer to a Microcon YM-100 column.
(iii) Spin at 500*g* for 25 min at room temperature.
(iv) Discard the filtrate and add 350 μl H_2O to the column to wash, and spin at 500*g* for 25 min.
(v) Repeat the wash step and add 350 μl 0.1 M NaOH to the column to desulphonate, and spin at 500*g* for 25 min.
(vi) Discard the filtrate and add 350 μl H_2O to the column to wash, and spin at 500*g* for 25 min.
(vii) Place the column in a new 1.5 ml tube. Add 50 μl DNA buffer or H_2O and pipette up and down to release the DNA from the column.
(viii) Invert the column and spin to transfer into a microfuge tube. Either use in the PCR amplification or store at –20 °C. See **Figure 2** for further information.

■ PAUSE POINT After the desalting and desulphonation steps, the bisulphite-treated DNA can be stored for 1–10 years at −20 °C, depending on the quality of the starting DNA.

▲ CRITICAL STEP This procedure does not require an ethanol precipitation step, but is more labor-intensive and takes a minimum of 3 h to complete after the bisulphite reaction for 24 tubes. The length of time to ensure complete washing at each spin varies according to the centrifuge. We recommend this procedure if the DNA is resuspended in H_2O or DNA buffer prior to the bisulphite reaction and/or if $<$500 ng of DNA is used in the bisulphite reaction, as there is less loss when no ethanol precipitation is required. However, if the bisulphite reaction contains lysis buffer, the size-exclusion clean-up protocol will concentrate factors that are inhibitory to PCR. Therefore, in this case, a subsequent ethanol precipitation step is required after elution.

(C) Qiagen size-exclusion clean-up: 96 well reactions

(i) Seal unused wells of MinElute plate from the QIAGEN MinElute 96 UF Kit (if all the wells are not in use) with parafilm and tape, and transfer the bisulphite reaction to the MinElute plate using a multi-channel pippetteman.

(ii) Apply vacuum for 10 min.

(iii) Add 150 µl H_2O, and apply vacuum for a further 10 min.

(iv) Repeat with another 150 µl H_2O wash and 10 min vacuum.

(v) Add 150 µl 0.1 M NaOH and incubate at room temperature for 10 min to complete the desulphonation reaction and apply vacuum for 10 min.

(vi) Add 150 µl H_2O and apply vacuum again for 10 min.

(vii) Finally, add 50 µl H_2O and vortex briefly in a plate vortex or pipette up and down 20× and transfer to a new 96-well plate (elution). See **Figure 2** for further information.

■ PAUSE POINT The plate can be sealed and the bisulphite-treated DNA can be stored at −20 °C until use or for 1–10 years.

▲ CRITICAL STEP This procedure is simple, takes 2 h to perform and can be used on DNA that has been resuspended in lysis buffer.

(D) Qiagen Affinity Clean-up: 96 well reactions

(i) Follow the manufacturers' instructions for clean-up using the QIAquick 96 Kit.

(ii) Seal unused wells of QIAquick plate with parafilm and tape, and add three volumes of buffer PM (i.e., 360 µl) to QIAquick plate.

(iii) Transfer the bisulphite reaction using a multi-channel pipette and mix by pipetting up and down.

(iv) Apply vacuum for 2 min, add 900 µl of PE buffer and apply vacuum for a further 2 min to wash.

(v) Add another 900 µl of PE buffer, and apply vacuum for 2 min (wash). Apply vacuum for 10 min to dry the residual ethanol, and blot the excess liquid from the bottom of the plate on paper towels.

(vi) Add 70 µl of supplied RNAse-free H_2O and incubate at room temperature for 1 min.

(vii) Apply vacuum to elute into a new 96-well plate (∼50 µl volume).

(viii) Add 5.5 µl 3 M NaOH, vortex, spin briefly, seal the plate and incubate at 37 °C for 15 min (desulphonation).

(ix) Add 1 ul tRNA (10 mg ml^{-1}) and 33.3 µl 5 M ammonum acetate, then add 300 µl ice-cold EtOH, seal and store at −20 °C overnight.

(x) Spin down at 2250*g* at 4 °C for 30 min, remove seal, invert plate onto paper towels and spin at 180 *g* at 4 °C for 1 min.

(xi) Allow to air-dry at room temperature for 15 min and resuspend in 40 µl H_2O. Seal and store at −20 °C until use. See **Figure 2** for further information.

■ PAUSE POINT The bisulphite-treated DNA can be stored, sealed, at −20 °C for 1–10 years.

▲ CRITICAL STEP This procedure is more costly and labor-intensive and requires an ethanol precipitation step.

Bisulphite PCR amplification

4| To perform methylation bisulphite sequencing on a given target sequence, the bisulphite-treated DNA is amplified using PCR oligonucleotide primers that can amplify both methylated (M) and unmethylated (U) DNA in proportion.

? TROUBLESHOOTING

5| Reaction conditions need to be optimized for each amplicon and primer set.

? TROUBLESHOOTING

6| To initially optimize PCR conditions, use the following conditions: Mix 50% methylated bisulphite-converted DNA (50 M) and 50% bisulphite-converted unmethylated DNA (50 U) to test for proportional PCR amplification with bisulphite conversion-specific primers and PCR master mix.

DNA (10 ng) (50 M:50 U)	2 µl
Bisulphite conversion-specific primers (10 ng each)	2 µl
2 × Promega Master Mix	12.5 µl
H_2O	8.5 µl

In a temperature-gradient thermocycler, set the run reaction in a gradient + and – 3 °C from the predicted Tm of the primer across 10 tubes.

Example of PCR cycling conditions:

95 °C	4 min	× 1
95 °C	45 s	
60 °C (predicted Tm)	1 min 30 s	× 5
72 °C	2 min	
95 °C	45 s	
60 °C (predicted Tm)	1 min 30 s	× 25
72 °C	1 min 30 s	
72 °C	4 min	× 1
Hold 4 °C		

? TROUBLESHOOTING

7| To test that the methylated and unmethylated DNA have amplified in proportion, the amplicon can be digested with an informative restriction enzyme, such as *Taq*1 (TCGA), that will digest methylated DNA but will not digest unmethylated DNA when the restriction enzyme site is altered after bisulphite conversion to TTGA. The products can then be visualized for the extent of digestion after electrophoresis of the products on an agarose gel (**Fig. 3a**). Alternatively, SYBRGreen (0.2 µl) (1:1,000 dilution) can be added to the PCR reaction and the extent of methylation can be assessed by performing heat-dissociation plots in a real-time PCR thermocycler (**Fig. 3b**).

? TROUBLESHOOTING

Conversion-specific primers

8| The design of PCR bisulphite conversion-specific primers is crucial for ensuring that the efficient amplification of completely converted DNA occurs. The list of criteria given in **Box 2** can be used to aid design.

● **TIMING**

Sample and solution set-up: 30–45 min
Step 1: DNA denaturation: 25 min
Step 2: Sulphonation and hydrolytic deamination (bisulphite reaction): 4–16 h
Step 3: Desalting and desulphonation: Wizard, 2 h for 24 tubes; Microcon, 3 h for 24 tubes; Qiagen size-exclusion, 2 h for 96 wells; Qiagen Affinity, 4 h for 96 wells
Step 4: PCR, 2.5 h

BOX 2 | CRITERIA TO AID DESIGN OF CONVERSION-SPECIFIC PRIMERS

1. 24–30 base pairs in length to ensure specificity, as the template contains only 3 bases because the cytosine residues are lost after bisulphite conversion.
2. Each primer should be designed with a similar predicted Tm above 50 °C.
3. Contains multiple (at least 25%) C ⇒ T bases, to ensure conversion specificity.
4. Last base at the 3′ end of the primer should be a C ⇒ T base to ensure amplification of converted DNA.
5. CpG dinucleotides should not be present in the primers (especially at the 3′ end), to avoid potential bias for either methylated or unmethylated templates or unconverted DNA. This may not be possible in dense CpG islands, so alternately, an inosine or degenerate base should be substituted (Y for C position for forward primer, R for G position for reverse primer).
6. Amplicon length should be no more than ~450 bp to ensure maximum yield, but smaller amplicons (100–200 bp) are advised from clinical samples, as the DNA is commonly more degraded from paraffin-embedded tissue.
7. Semi-nested PCR may also be needed to obtain sufficient quantities of PCR product, and to ensure specificity.
8. A macro for Microsoft Word that converts a given genomic DNA sequence to its bisulphite modified form has been published by Singal and Grimes[15].
9. Free online software for alignment of bisulphite-treated sequences and to aid in the design of primers for bisulphite-converted DNA is available at http://biq-analyzer.bioinf.mpi-inf.mpg.de, http://www.urogene.org/methprimer/index1.html[16], http://bisearch.enzim.hu[17] and Methyl Primer Express (http://www.appliedbiosystems.com). However, these programs do not necessarily follow these guidelines and, in particular, are not stringent for conversion-specific amplification. We therefore advise that PCR fragments that are amplified using primers designed using these programs be stringently validated for bisulphite conversion and sequence specificity by DNA sequencing.

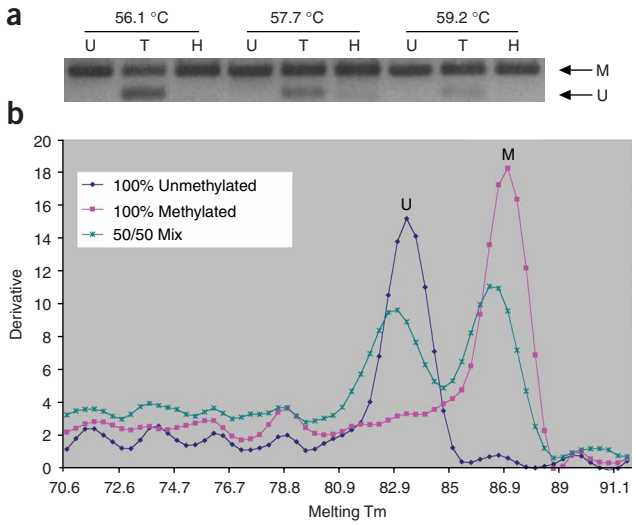

Figure 3 | Examples of bisulphite PCR amplification evaluation. (**a**) An agarose gel showing an example of a temperature-gradient bisulphite PCR amplification profile from a 50% mixture of methylated (M) and unmethylated (U) DNA. Proportional amplification can be tested by digestion of the PCR product with an informative enzyme, such as *Taq*I (T), that will digest methylated DNA after bisulphite conversion (T^{m}CGA to TCGA), as the restriction enzyme site is conserved. In contrast, unmethylated DNA will remain uncut (U), as the restriction enzyme site is changed after bisulphite treatment (TCGA to TTGA). Therefore, an equal mixture of cut and uncut products are expected if the PCR has amplified the 50% mixture of methylated and unmethylated DNA in proportion. In this example, the PCR amplifies methylated and unmethylated DNA in proportion at 56.1 °C, but at 59.2 °C the unmethylated DNA is amplified preferentially, as determined by the reduced digestion of the PCR product. To test for complete bisulphite conversion, the DNA can be digested with a cytosine site-specific enzyme, such as *Hpa*II (H). *Hpa*II will only digest if the bisulphite conversion reaction has failed, as the restriction enzyme site is maintained (CCGG), but will not digest if the bisulphite reaction is successful as the site is converted to TCGG or TTGG depending on the methylation state of the DNA. (**b**) A real-time heat-dissociation plot of a bisulphite PCR amplification from a 50% mixture of methylated (M) and unmethylated (U) DNA. In this example, the PCR has amplified the methylated and unmethylated DNA in proportion compared with the control amplification from fully methylated and unmethylated DNA that dissociate at 82.9 °C and 86.9 °C, respectively.

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Solution
2	Incomplete bisulphite conversion (from sequence or restriction analysis of products)	Re-purify DNA – treat with Proteinase K/SDS and phenol/chloroform extraction Restriction digest or shear DNA to facilitate denaturation Add high temperature pulses during bisulphite treatment – e.g. 30 sec at 90 °C once per hour Prepare new reagents For more details on incomplete conversion see Warnecke *et al.*[18]
4–6	No PCR product	Test primers on control bisulphite-converted DNAs Use a control reaction to test for bisulphite conversion of DNA or loss of DNA using an established set of primers and PCR conditions Evaluate a matrix of PCR conditions – annealing temperature, primer and Mg^{2+} concentrations
4–6	More than one PCR product (from gel or melting curve analysis)	Optimize PCR conditions – try higher annealing temperatures, matrix of primer concentrations and shorter extension times Alternatively, perform second round nested or semi-nested PCR
4–6	Primer dimers in PCR product	Evaluate variation in Mg^{2+} concentration, a matrix of lower primer concentrations and higher annealing temperatures to minimise level Increase input DNA levels if possible. If necessary, purify PCR product from contaminating primer dimers using a size exclusion column such as a Wizard column prior to sequence or further analysis
4	Contamination of PCR product	Always prepare and keep genomic DNA, and perform bisulphite reactions in a clean room separate from where PCR amplifications are performed or analysed Always include a no DNA control when setting up the bisulphite reaction so that any PCR contamination can be monitored Always include a no DNA control in the PCR reaction so that contamination of the PCR solutions and primer dilutions can also be monitored If PCR contamination is detected make fresh solutions and start again
7	Biased amplification of methylated and unmethylated amplicons	Vary temperature and/or Mg^{2+} concentration and/or lengthen extension time in PCR Evaluate primers directed to other strand or move primer location For more details on PCR bias see ref. 54

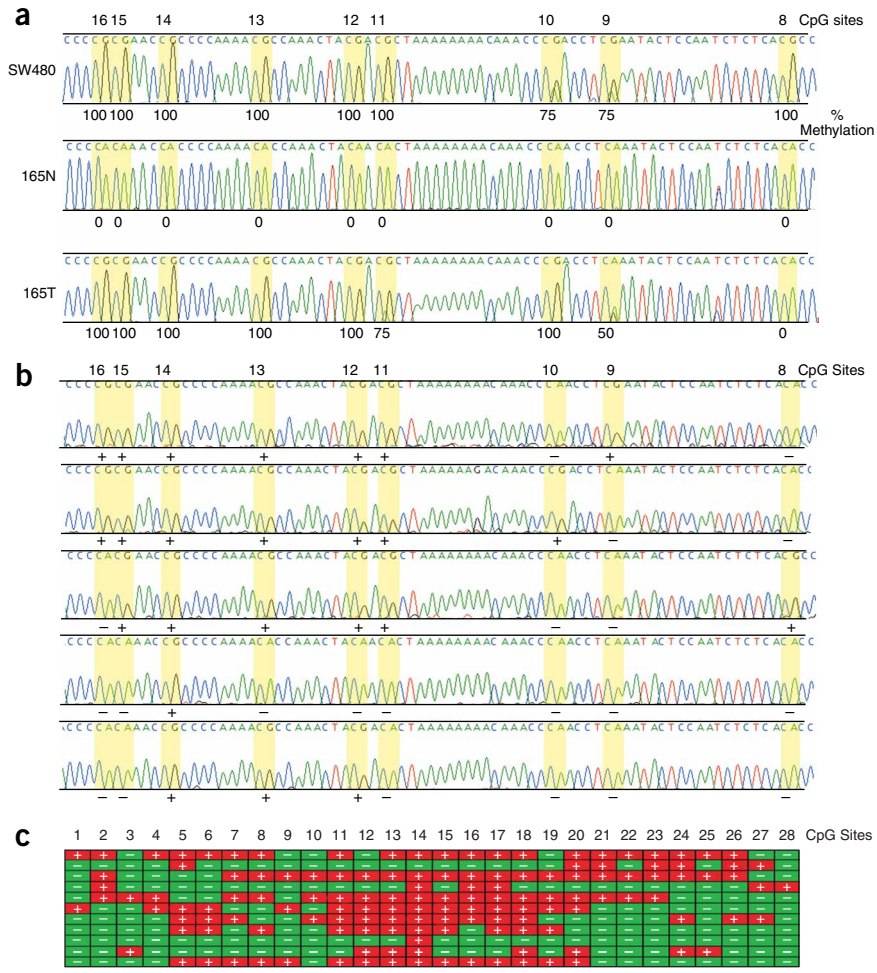

Figure 4 | Typical examples of direct and clonal PCR sequencing analysis after bisulphite conversion. Genomic DNAs from a human colorectal cancer cell line (SW480), colorectal tumor (165T) and matched normal sample (165N) were bisulphite converted and amplified in triplicate with bisulphite-specific primers to the target region of interest. The pooled PCR products were purified using the Wizard PCR DNA purification system to remove any primer dimers before sequencing. (**a**) The PCR products were directly sequenced using the reverse primer of the PCR amplification in the Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase, and the automated 3730 DNA analyser with KB^{TM} basecaller in Sequence analysis v5.1 (Applied Biosystems). Direct DNA sequencing through CpG sites 8–16 within the PCR fragment is shown. The degree of methylation at each CpG site from the direct sequencing profile was estimated by measuring the relative peak height of the cytosine (C) versus thymine (T) profile. As this can only be regarded as semi-quantitative, the degree of methylation was expressed as either 0%, 25%, 50%, 75% or 100%. (**b**) The pooled PCR products from the tumor sample were cloned into the pGEM^{R}-T-Easy Vector (Promega) using the Rapid Ligation Buffer System (Promega). Eleven individual clones were sequenced from the PCR reactions using the Dye Terminator cycle sequencing kit with AmpliTaq DNA polymerase, FS (Applied Biosystems) and the automated 373A NA Sequencer (Applied Biosystems). Clonal DNA sequencing examples from five independent colonies, spanning CpG sites 8–16 within the PCR fragment, are shown. The '+' below the sequence indicates a methylated CpG and a '–' indicates an unmethylated CpG. (**c**) Summary of the DNA methylation data from tumour samples, based on clonal analysis of 11 clones from the pooled PCR fragments. Bisulphite sequencing of individual clones validated the semi-quantitative methylation levels obtained from direct PCR sequencing analysis, but the level of detail obtained from clonal analysis highlights the degree of methylation heterogeneity that is often observed in clinical samples and this detail is often lost using direct PCR sequencing.

ANTICIPATED RESULTS

After bisulphite treatment, the DNA is amplified with strand-specific and bisulphite-specific primers in either a single or semi-nested PCR reaction. The resulting PCR fragments can be visualized by agarose-gel electrophoresis, as shown in **Figure 2a** and sequenced either directly (**Fig. 4a**) or by cloning and sequencing (**Fig. 4b**). After cloning and sequencing, the methylation state of the individual molecules can be tabulated to visualize the heterogeneity of methylation (**Fig. 4c**). Cloning and sequencing is still the only available method that can give single-nucleotide resolution for methylation across the DNA molecule.

ACKNOWLEDGMENTS The authors thank Rebecca Hinshelwood for help with the figures. The work was partly supported by grants from NH and MRC to S.J.C. (ID9293811, ID293810, ID325622).

COMPETING INTERESTS STATEMENT The authors declare that they have no competing financial interests.

Published online at http://www.natureprotocols.com
Rights and permissions information is available online at http://npg.nature.com/reprintsandpermissions

1. Warnecke, P.M. & Clark, S.J. DNA methylation profile of the mouse skeletal alpha-actin promoter during development and differentiation. *Mol. Cell. Biol.* **19**, 164–172 (1999).
2. Jones, P.A. Overview of cancer epigenetics. *Semin. Hematol.* **42**, S3–S8 (2005).
3. Grigg, G. & Clark, S. Sequencing 5-methylcytosine residues in genomic DNA. *Bioessays* **16**, 431–436 (1994).
4. Clark, S.J. & Frommer, M. in *DNA and Nucleoprotein Structure In Vivo Springer-Verlag* (eds. Saluz, H.P. & Wiebauer, K.) 123–132 (R.G. Landes Company, Austin, Texas, 1995).
5. Clark, S.J. & Frommer, M. in *Laboratory Methods for the Detection of Mutations and Polymorphisms in DNA* (ed. Taylor, G.) 151–162 (CRC Press, New York, 1997).
6. Frommer, M. *et al.* A genomic sequencing protocol that yields a positive display of 5-methylcytosine residues in individual DNA strands. *Proc. Natl. Acad. Sci. USA* **89**, 1827–1831 (1992).
7. Clark, S.J., Harrison, J., Paul, C.L. & Frommer, M. High sensitivity mapping of methylated cytosines. *Nucleic Acids Res.* **22**, 2990–2997 (1994).
8. Rakyan, V.K. *et al.* DNA methylation profiling of the human major histocompatibility complex: a pilot study for the human epigenome project. *PLoS Biol.* **2**, e405 (2004).

9. Rauscher, F.J. It is time for a Human Epigenome Project. *Cancer Res.* **65**, 11229 (2005).
10. Murrell, A., Rakyan, V.K. & Beck, S. From genome to epigenome. *Hum. Mol. Genet.* **14** (Spec No. 1): R3–R10 (2005).
11. Jones, P.A. & Martienssen, R. A blueprint for a Human Epigenome Project: the AACR Human Epigenome Workshop. *Cancer Res.* **65**, 11241–11246 (2005).
12. Callinan, P.A. & Feinberg, A.P. The emerging science of epigenomics. *Hum. Mol. Genet.* **15** (Spec No. 1): R95–R101 (2006).
13. Garber, K. Momentum building for human epigenome project. *J. Natl. Cancer Inst.* **98**, 84–86 (2006).
14. Esteller, M. The necessity of a human epigenome project. *Carcinogenesis* **27**, 1121–1125 (2006).
15. Singal, R. & Grimes, S.R. Microsoft Word macro for analysis of cytosine methylation by the bisulfite deamination reaction. *Biotechniques* **30**, 116–120 (2001).
16. Li, L.C. & Dahiya, R. MethPrimer: designing primers for methylation PCRs. *Bioinformatics* **18**, 1427–1431 (2002).
17. Tusnady, G.E., Simon, I., Varadi, A. & Aranyi, T. BiSearch: primer-design and search tool for PCR on bisulfite-treated genomes. *Nucleic Acids Res.* **33**, e9 (2005).
18. Warnecke, P.M. *et al.* Identification and resolution of artifacts in bisulfite sequencing. *Methods* **27**, 101–107 (2002).
19. Grunau, C., Clark, S.J. & Rosenthal, A. Bisulfite genomic sequencing: systematic investigation of critical experimental parameters. *Nucleic Acids Res.* **29**, E65–5 (2001).
20. Paul, C.L. & Clark, S.J. Cytosine methylation: quantitation by automated genomic sequencing and GENESCAN analysis. *Biotechniques* **21**, 126–133 (1996).
21. Boyd, V.L. & Zon, G. Bisulfite conversion of genomic DNA for methylation analysis: protocol simplification with higher recovery applicable to limited samples and increased throughput. *Anal. Biochem.* **326**, 278–280 (2004).
22. Lewin, J., Schmitt, A.O., Adorjan, P., Hildmann, T. & Piepenbrock, C. Quantitative DNA methylation analysis based on four-dye trace data from direct sequencing of PCR amplificates. *Bioinformatics* **20**, 3005–3012 (2004).
23. Han, W., Cauchi, S., Herman, J.G. & Spivack, S.D. DNA methylation mapping by tag-modified bisulfite genomic sequencing. *Anal. Biochem.* **355**, 50–61 (2006).
24. Tost, J., Dunker, J. & Gut, I.G. Analysis and quantification of multiple methylation variable positions in CpG islands by Pyrosequencing. *Biotechniques* **35**, 152–156 (2003).
25. Colella, S., Shen, L., Baggerly, K.A., Issa, J.P. & Krahe, R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. *Biotechniques* **35**, 146–150 (2003).
26. Laird, C.D. *et al.* Hairpin-bisulfite PCR: assessing epigenetic methylation patterns on complementary strands of individual DNA molecules. *Proc. Natl. Acad. Sci. USA* **101**, 204–209 (2004).
27. Schatz, P., Dietrich, D. & Schuster, M. Rapid analysis of CpG methylation patterns using RNase T1 cleavage and MALDI-TOF. *Nucleic Acids Res.* **32**, e167 (2004).
28. Ehrich, M. *et al.* Quantitative high-throughput analysis of DNA methylation patterns by base-specific cleavage and mass spectrometry. *Proc. Natl. Acad. Sci. USA* **102**, 15785–15790 (2005).
29. Worm, J., Aggerholm, A. & Guldberg, P. In-tube DNA methylation profiling by fluorescence melting curve analysis. *Clin. Chem.* **47**, 1183–1189 (2001).
30. Guldberg, P., Worm, J. & Gronbaek, K. Profiling DNA methylation by melting analysis. *Methods* **27**, 121–127 (2002).
31. Yang, I. *et al.* Rapid quantification of DNA methylation through dNMP analysis following bisulfite-PCR. *Nucleic Acids Res.* **34**, e61 (2006).
32. Yamamoto, T. *et al.* Methylation assay by nucleotide incorporation: a quantitative assay for regional CpG methylation density. *Biotechniques* **36**, 846–50 852, 854 (2004).
33. Maekawa, M. *et al.* DNA methylation analysis using bisulfite treatment and PCR-single-strand conformation polymorphism in colorectal cancer showing microsatellite instability. *Biochem. Biophys. Res. Commun.* **262**, 671–676 (1999).
34. Bianco, T., Hussey, D. & Dobrovic, A. Methylation-sensitive, single-strand conformation analysis (MS-SSCA): A rapid method to screen for and analyze methylation. *Hum. Mutat.* **14**, 289–293 (1999).
35. Baumer, A., Wiedemann, U., Hergersberg, M. & Schinzel, A. A novel MSP/DHPLC method for the investigation of the methylation status of imprinted genes enables the molecular detection of low cell mosaicisms. *Hum. Mutat.* **17**, 423–430 (2001).
36. Sadri, R. & Hornsby, P.J. Rapid analysis of DNA methylation using new restriction enzyme sites created by bisulfite modification. *Nucleic Acids Res.* **24**, 5058–5059 (1996).
37. Xiong, Z. & Laird, P.W. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* **25**, 2532–2534 (1997).
38. Eads, C.A. & Laird, P.W. Combined bisulfite restriction analysis (COBRA). *Methods Mol. Biol.* **200**, 71–85 (2002).
39. Gonzalgo, M.L. & Jones, P.A. Quantitative methylation analysis using methylation-sensitive single-nucleotide primer extension (Ms-SNuPE). *Methods* **27**, 128–133 (2002).
40. Kaminsky, Z.A., Assadzadeh, A., Flanagan, J. & Petronis, A. Single nucleotide extension technology for quantitative site-specific evaluation of metC/C in GC-rich regions. *Nucleic Acids Res.* **33**, e95 (2005).
41. El-Maarri, O., Herbiniaux, U., Walter, J. & Oldenburg, J. A rapid, quantitative, non-radioactive bisulfite-SNuPE- IP RP HPLC assay for methylation analysis at specific CpG sites. *Nucleic Acids Res.* **30**, e25 (2002).
42. Gitan, R.S., Shi, H., Chen, C.M., Yan, P.S. & Huang, T.H. Methylation-specific oligonucleotide microarray: a new potential for high-throughput methylation analysis. *Genome Res.* **12**, 158–164 (2002).
43. Adorjan, P. *et al.* Tumour class prediction and discovery by microarray-based DNA methylation analysis. *Nucleic Acids Res.* **30**, e21 (2002).
44. Thomassin, H., Kress, C. & Grange, T. MethylQuant: a sensitive method for quantifying methylation of specific cytosines within the genome. *Nucleic Acids Res.* **32**, e168 (2004).
45. Bibikova, M. *et al.* High-throughput DNA methylation profiling using universal bead arrays. *Genome Res.* **16**, 383–393 (2006).
46. Herman, J.G., Graff, J.R., Myohanen, S., Nelkin, B.D. & Baylin, S.B. Methylation-specific PCR: a novel PCR assay for methylation status of CpG islands. *Proc. Natl. Acad. Sci. USA* **93**, 9821–9826 (1996).
47. Eads, C.A. *et al.* MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* **28**, E32 (2000).
48. Rand, K., Qu, W., Ho, T., Clark, S.J. & Molloy, P. Conversion-specific detection of DNA methylation using real-time polymerase chain reaction (ConLight-MSP) to avoid false positives. *Methods* **27**, 114–120 (2002).
49. Zeschnigk, M. *et al.* A novel real-time PCR assay for quantitative analysis of methylated alleles (QAMA): analysis of the retinoblastoma locus. *Nucleic Acids Res.* **32**, e125 (2004).
50. Cottrell, S.E. *et al.* A real-time PCR assay for DNA-methylation using methylation-specific blockers. *Nucleic Acids Res.* **32**, e10 (2004).
51. Rand, K.N. *et al.* Headloop suppression PCR and its application to selective amplification of methylated DNA sequences. *Nucleic Acids Res.* **33**, e127 (2005).
52. Shaw, R.J., Akufo-Tetteh, E.K., Risk, J.M., Field, J.K. & Liloglou, T. Methylation enrichment pyrosequencing: combining the specificity of MSP with validation by pyrosequencing. *Nucleic Acids Res.* **34**, e78 (2006).
53. Rand, K., Mitchell, S., Clark, S. & Molloy, P. Bisulphite differential denaturation PCR for analysis of DNA methylation. *Epigenetics* **1**, 94–100 (2006).
54. Warnecke, P.M. *et al.* Detection and measurement of PCR bias in quantitative methylation analysis of bisulphite-treated DNA. *Nucleic Acids Res.* **25**, 4422–4426 (1997).