DNA methylation analysis by pyrosequencing

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Pyrosequencing is a sequencing-by-synthesis method that quantitatively monitors the real-time incorporation of nucleotides through the enzymatic conversion of released pyrophosphate into a proportional light signal. Quantitative measures are of special importance for DNA methylation analysis in various developmental and pathological situations. Analysis of DNA methylation patterns by pyrosequencing combines a simple reaction protocol with reproducible and accurate measures of the degree of methylation at several CpGs in close proximity with high quantitative resolution. After bisulfite treatment and PCR, the degree of each methylation at each CpG position in a sequence is determined from the ratio of T and C. The process of purification and sequencing can be repeated for the same template to analyze other CpGs in the same amplification product. Quantitative epigenotypes are obtained using this protocol in approximately 4 h for up to 96 DNA samples when bisulfite-treated DNA is already available as the starting material.

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INTRODUCTION

Sequencing-by-synthesis methods rely on sequential addition and incorporation of nucleotides in a primer-directed polymerase extension. Only if the added nucleotide is complementary to the template DNA is it incorporated by a DNA polymerase, and this event can be monitored in real time. The pyrosequencing technology makes use of the release of PP_i molecules during the iterative incorporation of unmodified nucleotides that are quantitatively converted into a bioluminometric signal[1,2]. Only one of the four nucleotides is present at any time in the reaction vessel, and the biochemical reactions are carried out with a balanced mixture of four enzymes (**Fig. 1**): the Klenow fragment of the DNA polymerase I from *Escherichia coli*, an ATP sulfurylase (*Saccharomyces cerevisiae*), the luciferase (*Photinus pyralis*) and an apyrase (*Solanum tuberosum*). To prohibit primer degradation and thus out-of-phase signals, an exonuclease-deficient DNA polymerase is used for nucleotide incorporation. The PP_i is converted into ATP by the ATP sulfurylase using adenosine 5′ phosphosulfate as substrate. This reaction provides the energy for the luciferase to oxidize D-luciferin. The product oxyluciferin is generated in an excited state, which decays to the ground state with the emission of a photon ($\lambda = 560$ nm) detected by a charge-coupled device (CCD) camera. As the nucleotide dATP acts as a natural substrate for luciferase, the modified α-S-dATP is used as the nucleotide for primer extension as it is equally well incorporated by the polymerase. Unincorporated nucleotides are degraded before adding the next nucleotide by the apyrase, allowing iterative addition of nucleotides by an inkjet-type cartridge into an agitated and temperature-controlled microtiter plate. Nucleotides are dispensed into alternating wells with a pulse delay to minimize cross-talk of generated light between adjacent wells. One pmole of DNA yields approximately 6×10^{11} ATP molecules generating 6×10^{9} photons. A lens array focuses the light signals generated in case of successful incorporation from each reaction well to a specific locus of a CCD camera imaging the plate every second to follow the pyrosequencing reaction in real time.

Its ease of use, high reliability and flexibility have made pyrosequencing an analysis platform that has been widely used for various diagnostic applications such as routine (multiplex) genotyping[3], sensitive detection of mutations[4] and microbacterial identification[5]. The pyrosequencing technology is also used for massively parallel sequencing on the 454 sequencing systems[6]. One of its major strengths is the quantitative nature of the results. The bioluminometric response is linear ($R^2 > 0.99$) for the sequential addition of up to five identical nucleotides (C, G, T) or three α-S-dATPs. Pyrosequencing has therefore been used in a variety of applications where quantitative assessment of the relative abundance of two individual nucleotides or short sequences is required such as genotyping of polyploid samples[7], determination of single nucleotide polymorphism (SNP) allele frequencies in pooled samples[8,9], analysis of copy number variation[10] and karyotyping by paralogous sequence quantification[11].

Accurate quantitative analysis is very important for DNA methylation analysis. This epigenetic modification occurs at the 5′ position of cytosine in the context of the dinucleotide CpG and is crucial for proper development[12]. Aberrant methylation patterns are found in many diseases, especially cancer, where an overall decrease in DNA methylation is accompanied by hypermethylation of CpG islands in the promoter regions of the genes involved in DNA repair, detoxification, cell cycle regulation and apoptosis[13]. Analysis of DNA methylation patterns has proven useful as biomarker for the early diagnosis, classification, prognosis and therapy of human cancers[14]. Several methods for the analysis of DNA methylation at either the genome-wide or gene-specific level have been devised[14,15]. Most methods for the analysis of DNA methylation in a target region rely on bisulfite treatment of the genomic DNA, converting unmethylated cytosine to uracil (thymine after PCR amplification) while methylated cytosine are refractory to the treatment. The chemical reaction thus translates epigenetic information into differential sequence information, and the positions exhibiting potentially variable degrees of methylation can be analyzed as virtual C/T polymorphisms in the bisulfite-treated DNA. Direct fluorescent Sanger sequencing of PCR products amplified from bisulfite-treated DNA can be used for detailed analysis of DNA methylation. However, direct sequencing has proven to be technically demanding in regions with heterogeneous or low methylation levels, and quantitation of the read-out is imprecise and difficult. Therefore, in most cases PCR products are cloned and multiple colonies are subsequently sequenced. Because of this the procedure is expensive,

time-consuming and cumbersome when applied in high-throughput, and only a small number of alleles, on the order of approximately 10–20 colonies, is usually analyzed. From this it is difficult to infer statistically meaningful results. Methods with improved quantitative resolution based on matrix-assisted laser desorption/ionization mass spectrometry[16] or pyrosequencing[17–19] therefore represent valuable alternatives.

Pyrosequencing combines the ability of direct quantitative sequencing, reproducibility, speed and ease-of-use and is becoming more widely used. It is a flexible analysis platform as it can be applied for the analysis of DNA methylation in CpG-rich regions as well as CpG-poor regions, where the number of required CpGs within the primer binding sites might be insufficient for alternative methods such as methylation-specific PCR approaches. The successive use of several sequencing primers on the same DNA template (serial pyrosequencing) significantly reduces cost, labor and analysis time, as well as saving precious DNA samples for the analysis of a specific region amplified in a single PCR[20]. Besides the identification of genes aberrantly silenced by promoter hypermethylation in cancer[21,22], pyrosequencing has been used as a reference method for the validation of newly developed methods for DNA methylation analysis[23], to monitor chemically induced demethylation in leukemia patients[24], as well as for a diagnostic test for aberrant methylation in the imprinting disorders Prader–Willi and Angelman syndrome[25]. Pyrosequencing can also be used to monitor allele-specific methylation patterns if the sequencing primer overlaps at its 3′-terminus with a heterozygous SNP[26] or global methylation changes by either analyzing the methylation degree in repetitive elements[27] or after restriction digestion (LUminometric Methylation Assay)[28] demonstrating the wide range of applications of the pyrosequencing technology in DNA methylation analysis in addition to the above-mentioned genetic applications. Several of the mentioned applications use an approach for which one of the amplification primers is tailed with a sequence complementary to a universal biotinylated primer, thereby slightly reducing the cost per assay as amplification primers can be purchased without modifications[17,24]. The pyrosequencing platform is very flexible, and individual wells are as easily analyzed as a complete plate as no reference samples are required. The same assay can be used to analyze all templates or every sample can be analyzed with different assays. In addition to the analysis of the methylation level, information on polymorphisms such as the SNPs retained after bisulfite treatment can be obtained.

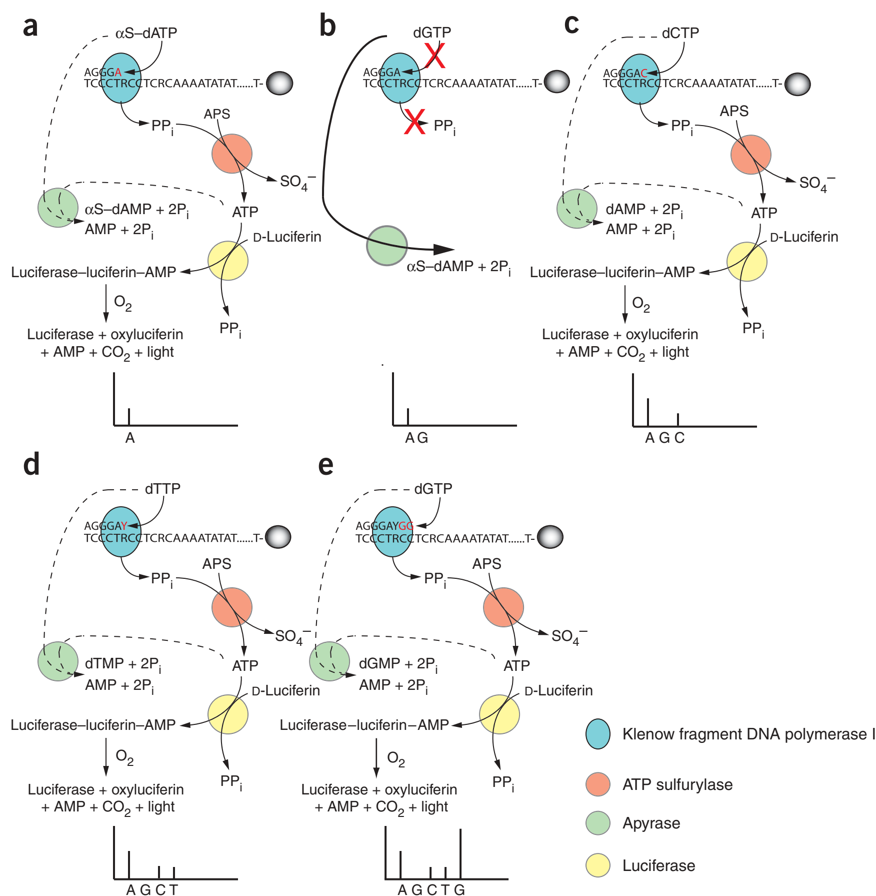

Figure 1 | Enzymatic cascade of the pyrosequencing reaction in the example of a bisulfite-treated template sequence, including a CpG position that is methylated on approximately 50% of all molecules. This situation corresponds to CpGs in the differentially methylated region of an imprinted gene. (**a**) α-S-dATP is added as nucleotide for extension. This modified nucleotide is used instead of dATP, which would act as a direct substrate of the luciferase and thereby uncouple the generation of a bioluminometric signal from the release of PP_i. The incorporation of the complementary α-S-dATP by the Klenow fragment of DNA polymerase I at the 3′-end of the pyrosequencing primer results in the release of PP_i, which is in turn used to convert adenosine phosphosulfate (APS) into ATP. The ATP provides the energy to form an unstable luciferase–luciferin–AMP complex, which in the presence of oxygen results in the release of light in a proportional amount to the available ATP and thus PP_i. Unincorporated α-S-dATP as well as to a small extent ATP is degraded to the mononucleotides by apyrase. Carefully balanced proportions of the respective enzymes ensure the preferential incorporation of the nucleotide instead of degradation. (**b**) dGTP is added as second nucleotide. As it is not complementary to the template sequence, no PP_i is released and the nucleotide is degraded. (**c**,**d**) The 'R' in the template sequence corresponds to a potential methylation variable position as it is part of a CpG (on the complementary strand). Thus C (methylated cytosine before bisulfite treatment) or T (unmethylated cytosine before bisulfite treatment) can be incorporated and the respective ratio yields the methylation degree at this CpG position. In the example, 50% of the molecules incorporate a cytosine (C), which can be seen as peak of half of the intensity compared to first A peak corresponding to a single nonpolymorphic nucleotide incorporation. (**d**) The other half of the molecules is complementary to the T added after the dispensation of the C nucleotide and the degradation of any unincorporated Cs. (**e**) The following two nucleotides in the template sequence are identical, thereby resulting in a peak of twice the height compared with the first A peak when dispensing the complementary dGTP.

Outline of the assay procedure

Pyrosequencing-based methylation analysis investigates quantitatively the degree of methylation at CpG positions in close proximity after bisulfite treatment of genomic DNA. The procedure for a

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pyrosequencing assay can be divided into several steps, as shown in **Figure 2** and described in detail later. The preparative steps include the design of the assay and treatment of the samples of interest and references with sodium bisulfite. A target region of up to 350 bp is then amplified by PCR using a pair of primers complementary to the bisulfite-treated DNA sequence, amplifying all states irrespective of methylation status. One of these two amplification primers carries a biotin label at its 5′-terminus. The incorporated biotinylated primer is subsequently immobilized on streptavidin-coated beads used to purify and render the PCR product single-stranded (as only one strand is biotinylated). A pyrosequencing primer complementary to the single-stranded template is then hybridized to the template, and the pyrosequencing reaction is performed by the sequential addition of single nucleotides in a predefined order. After pyrosequencing, the template can be repurified to analyze a second part of the template with a new sequencing primer (serial pyrosequencing).

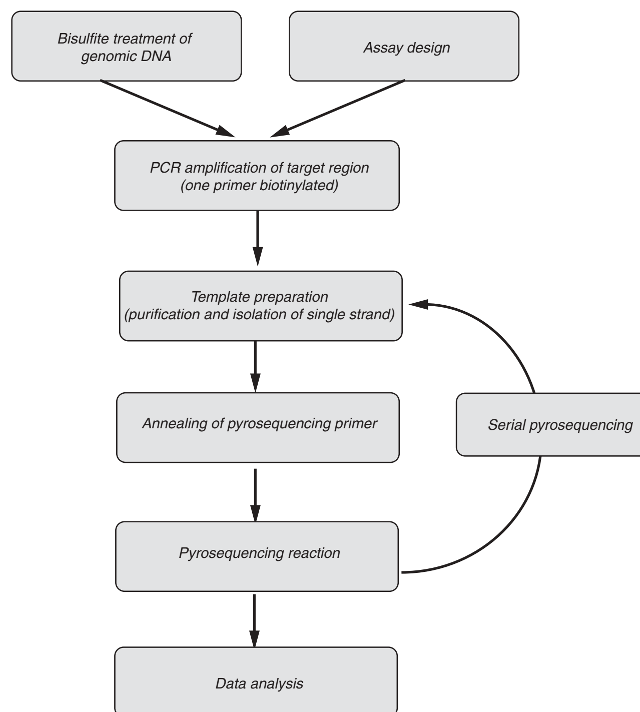

Figure 2 | Outline of the procedure for the pyrosequencing assay for DNA methylation analysis.

Assay design

Analysis of DNA methylation by pyrosequencing is a very straightforward process if the PCR amplification yields a strong and specific PCR product, and there are no similar annealing sites for the pyrosequencing primer in the amplification product. The design of the pyrosequencing assay is the most crucial point for successful and accurate quantitative analysis. Pyrosequencing provides commercial software that automatically performs the assay design including PCR primers and pyrosequencing primers. This software has, however, been developed for the design of SNP genotyping assays, and the reduced complexity of the bisulfite-treated DNA often leads to failure or a large list of potential errors and complications of the assay design as the required quality criteria might be too stringent for bisulfite-treated DNA. Unfortunately, there is no complete software solution available that designs pyrosequencing assays for the analysis of multiple CpG positions on bisulfite-treated DNA. We therefore use different tools for PCR primer design and pyrosequencing primer design, and have found that the design of PCR primers using MethPrimer is much more successful.

For the design of amplification primers, several software tools are available free of charge on the internet such as MethPrimer[29] (http://www.ucsf.edu/urogene/methprimer/index1.html), which is based on the widely used Primer3 algorithm, Bisearch[30] (http://bisearch.enzim.hu/) or MethylPrimer express from Applied Biosystems (http://www.appliedbiosystems.com/methylprimerexpress). We routinely use MethPrimer in our laboratory. The thermal instability of the luciferase requires pyrosequencing to be carried out at a low temperature (28 °C), which imposes some limitations on assay design. The size of the amplification product should be restricted to 350 bp or less, as otherwise secondary structures such as loops can form in the single-stranded template that interfere with or inhibit the sequencing reaction or increase the background signal due to the extension of the 3′-end of the terminus. Capture efficiency of the biotinylated amplification product decreases with size.

PCR primers should be designed according to the following guidelines:

- They should be complementary to a region with at least 4 cytosine outside of CpG positions that have been converted during bisulfite treatment to ensure that they are only complementary to completely converted DNA as the chemical treatment is rarely quantitative.
- Primers should be devoid of palindromes (within primers) and complementary sequences between primers to ensure specific amplification.
- Primers should not overlap with any CpG position as this might induce preferential amplification of a subset of molecules. We recommend moving the target region slightly within the CpG island of interest rather than using degenerate positions in the amplification primers. In most cases, this will not lead to a loss of information. If the incorporation of degenerate bases cannot be avoided, accurate quantification needs to be verified carefully using mixtures with a known degree of methylation, and the degenerate positions (including the use of inosine, a nucleoside found in tRNAs that base pairs equally well with adenine, cytosine and thymine) should be as far as possible toward the 3′-terminus. It should be noted that this might bias the results even if the degenerate position is at the 5′-end. We strongly discourage the users from using degenerate bases within amplification primers.

After bisulfite conversion, the two strands of genomic DNA are no longer complementary as unmethylated cytosine are deaminated to uracil, which do no longer base pair with the unmodified guanine in the formerly complementary strands. Both strands can be used for amplification. MethPrimer takes only the forward strand into account, and the reverse strand has to be created manually or using a variety of freely available software tools and used for an alternative design of the amplification product. Once the amplification product is successfully designed, the corresponding genomic sequence should be compared against the genomic reference sequence using a basic local alignment search tool search to detect

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any known SNP or other polymorphism underlying the primer annealing sites or within the amplification product. If SNPs are found in the sequences complementary to the amplification primers, the amplification product needs to be redesigned as polymorphism even at the 5′-end of the primer might bias the amplification.

There are then two approaches to design the sequencing primer. First, the user can fix the designed PCR primers in the pyrosequencing software and design only the pyrosequencing primer with the help of the software. In most cases, this procedure yields sequencing primers that work in practice although the design software shows many potential problems. A second possibility is to identify sequences where a sequencing primer could be positioned, and at least the last five bases from the 3′-terminus do not overlap with any other potentially variable position including CpGs and SNPs that are retained after bisulfite treatment. Then the sequence (most importantly the last four or five bases from the 3′-terminus) is verified to be unique in the amplicon manually, for example, using the Microsoft (MS) Word find tool, and the possibility for primer dimers or possible hairpin structures is checked. As few as four consecutive nucleotides complementary to a sequence in the amplification product might add to background signal confounding precise quantification. As the read-length of pyrosequencing is approximately 150 bases, primers can also be positioned in non-polymorphic regions next to the variable region. At least 1 cytosine not followed by a guanine should be included in the dispensation order to control for complete bisulfite conversion.

Template preparation

DNA extraction, determination of the concentration and bisulfite treatment can be performed by a variety of methods. The protocol described below works well in our laboratory, but other methods might be equally well suited to prepare the starting material for pyrosequencing analysis. It is critical to include a proteinase K incubation step in the DNA extraction procedure to destroy any chromatin structure associated with the DNA that might hamper the dissociation of the two DNA strands, which is a prerequisite for successful bisulfite conversion. We also prefer to adjust the DNA concentration after measurement with fluorescent dyes specific for DNA rather than optical density, which might be influenced by a variety of biomolecules as well as dust particles. Bisulfite conversion remains a critical step for many methods for DNA methylation analysis. Small deviations from optimized protocols might lead to incomplete conversion, which results in an over-representation of methylation in the final output. Especially, incomplete denaturation of the double-stranded DNA before conversion leads to this artifact as only cytosine in single-stranded DNA molecules are deaminated. Various improvements or modifications have been reported, which allow bisulfite treatment of samples that are only available in a limited quantity such as incorporation of the DNA in beads of low-melting agarose[31] or high recovery rates through the use of concentrating centrifuge filters[32]. Specialized protocols permit the extraction, conversion and subsequent analysis of DNA from formalin-fixed paraffin-embedded (FFPE) archival specimens[33], as well as DNA from a small number of cells obtained by laser capture microdissection[34]. The ability to convert DNA in a significantly shorter period streamlines the process of DNA methylation analysis, but might also lead to greater damage of the DNA as the reaction is carried out at high temperatures[35]. Several kits for bisulfite treatment can be purchased, which perform equally well as the below described protocol and might facilitate bisulfite conversion especially when performed in high-throughput, as some of the kits are available in 96-well format. Examples are the MethylEasy high-throughput bisulphite modification kits, which can be purchased from Human Genetic Signatures, and the different EZ-96 DNA methylation kits from Zymo Research.

PCR amplification and pyrosequencing

To generate a sufficient amount of template and reduce the complexity of the sequence context, a PCR is performed, with one of the two amplification primers labeled with biotin. Because biotinylated template strands as well as unincorporated biotinylated primers will be captured on streptavidin-coated beads, only a small amount of primer (5 pmol) is used in the PCR amplification and a large number of amplification cycles (usually 50) is performed to exhaust the primers. These amplification conditions are necessary; otherwise, the biotinylated primer might act as an additional sequencing primer and thereby interfere with the subsequent sequencing reaction. A minimum of 10 ng of template DNA is necessary to achieve high reproducibility for each CpG methylation analysis as reproducibility decreases strongly with reduced amounts of template DNA[36]. For a successful pyrosequencing reaction, it is absolutely crucial to have a single strong band of the amplification product when 5 μl are deposited on an agarose gel. We also strongly advise to include one or several negative controls in the PCR amplification as the large number of cycles might amplify even small amounts of 'contaminating' nucleic acids. Physical separation of pre- and post-PCR manipulation is essential to reduce the risk of contamination. For the PCR setup, we advise to perform a temperature gradient and use several methylation standards (calibration mixtures) with a known degree of methylation for the first experiments on the pyrosequencer to detect any preferential amplification as well as unexpected sequence pattern. Bisulfite-treated DNA is also prone to biased amplification during PCR, as the sequence difference of formerly methylated and unmethylated molecules induced by the conversion might result in different melting behavior[37]. Several methods have been devised to overcome preferential amplification of a subset of molecules such as the incorporation of bases complementary to methylated molecules only to counterbalance the preferential amplification of unmethylated molecules[38] or the careful optimization of the annealing temperature[39]. The single-stranded DNA template is prepared using immobilization on streptavidin-coated sepharose beads and subsequent alkali treatment. This step also serves to remove salts that inhibit subsequent enzymatic reactions. This preparation step is best carried out with a vacuum preparation tool (Biotage), which captures the beads and holds them during the different purification steps, whereas the solution easily passes through the filters. After template preparation, a sequencing primer is hybridized to the template several bases 5′ to the site of interest sequencing 30–120 bases dependent on the sequence context.

The limitation in the read-length of pyrosequencing is mainly due to the dilution effects and incomplete incorporation of dispensed nucleotides, leading to a negative frame-shift in the pyrogram, that is, some molecules lag behind others in the incorporation of the expected nucleotide. On the other hand, incomplete degradation of unincorporated nucleotides can lead to positive frame-shift of pyrograms, that is, some molecules

incorporate nucleotides in addition to those incorporated by the majority of molecules, resulting in increased background signals. This limitation of the read-length constitutes the main disadvantage of the use of the pyrosequencing technology for DNA methylation analysis. While sequencing and cloning permits the analysis of CpGs in a sequence of up to 600 nt in a single sequencing run, several PCR amplicons each with two to three different sequencing primers are required to analyze a genomic region of comparable size, which makes pyrosequencing less suitable as discovery tool. Nonetheless, cloning and sequencing of multiple clones rapidly becomes cost- and time-inhibitive when performed at high-throughput while pyrosequencing is very well suited as a rapid technology to screen a large number of samples quantitatively in a target region of limited size when preliminary data indicate the presence of variable methylation patterns. Sequencing and cloning as well as pyrosequencing can be used to define allele-specific methylation patterns, but sequencing and cloning provide additional information on which CpGs are methylated in phase on the same molecule while pyrosequencing provides an average of the many molecules analyzed simultaneously. However, a further non-negligible advantage of the pyrosequencing technology is the analysis of the quantitative surrounding nonpolymorphic sequence, assuring that the right sequence has successfully been amplified in the preceding PCR. The degree of methylation is calculated as the quotient of the peak height of the methylated peak and the sum of the peaks corresponding to the methylated and the unmethylated peaks. The sum has also to correspond to the average signal of the nonpolymorphic reference sequence.

Serial pyrosequencing

If all CpGs in the amplicon of up to 300 bases are of interest, several sequencing primers have to be used. However, it is not necessary to perform additional PCR amplifications. Serial pyrosequencing is a recent improvement of the conventional pyrosequencing protocol, permitting the sequencing of an entire PCR amplification product by successive annealing of different sequencing primers and washing away of the *de novo* synthesized template[20]. This process can be repeated up to seven times, but in most cases two or three pyrosequencing primers are sufficient to sequence the entire amplification product.

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MATERIALS

REAGENTS

- Genomic DNA (1 μg in a volume of 20 μl) (for extraction see REAGENT SETUP)
- SssI methylase (New England Biolabs, cat. no. M0226L)
- Proteinase K (New England Biolabs, cat. no. P8102S)
- REPLI-g Mini Kit (Qiagen, cat. no. 150025)
- Quant-iT dsDNA broad-range assay kit (Invitrogen, cat. no. Q33130)
- Sodium bisulphite (Sigma, cat. no. S-9000) ▲ CRITICAL Prepare freshly a 5 M solution.
- Sodium hydroxide (NaOH) pellets (Aldrich, cat. no. 480878), prepare a 5 M stock solution and store in plastic bottles
- Hydroquinone (Sigma, cat. no. H-9003) (see REAGENT SETUP) ! CAUTION Harmful and dangerous for the environment. Handle with care, wear suitable protective clothing, gloves and eye protection, and avoid release into the environment.
- 2-Butanol (isopropanol; Aldrich, cat. no. B8,591-9)
- Ethanol (Prolabo, cat. no. 20 821,321)
- Phenol (ultra pure buffer saturated phenol; Invitrogen, cat. no. 15513) ! CAUTION Toxic. Handle with care, wear suitable protective clothing and gloves, avoid skin contact.
- Chloroform (Fluka, cat. no. 25690) ! CAUTION Harmful. Handle with care, wear suitable protective clothing and gloves.
- Ammonium acetate (Riedel de Häen, cat. no. 32301)
- Tween 20 (Fluka, cat. no. 93773)
- Hydrochloric acid (HCl; Aldrich, cat. no. 32,033-1) ! CAUTION Corrosive. Handle with care, wear suitable protective clothing, gloves and eye protection.
- Acetic acid (Riedel de Häen, cat. no. 27225) ! CAUTION Corrosive. Handle with care, wear suitable protective clothing, gloves and eye protection.
- Magnesium acetate (Mg-acetate; Merck, cat. no. 1.05818.1000)
- Wizard DNA clean-up system (Promega, cat. no. A7280)
- Primers for PCR amplification and sequencing primers for pyrosequencing (Biotez)
- Biotinylated primers for PCR amplification, HPLC-purified (Biotez) ▲ CRITICAL Free biotin in the primer solution will block the binding sites of the sepharose beads during purification and diminish the efficiency of template preparation. The quality of the biotinylated primer might vary significantly from one commercial supplier to another, and we recommend testing several suppliers when starting off with pyrosequencing.
- Platinum Taq DNA polymerase (Invitrogen Life Technologies, cat. no. 10966-026) or HotStar Taq DNA polymerase (Qiagen, cat. no. 203205)
- dNTPs [GE Healthcare, cat. nos. 27-2050-01 (dATP), 27-2060-01 (dCTP), 27-2070-01 (dGTP), 27-2080-01 (TTP)]
- Thermo-Fast 96-skirted 96-well plates (Abgene, cat. no. AB-0800)
- Ethidium bromide 0.1% wt/vol (Sigma, cat. no. E8751) ! CAUTION Very toxic. Possibility of irreversible changes. Handle with care, wear suitable protective clothing and gloves. Take off clothes that have been contaminated and wash skin with large amount of water.
- Bromphenol blue sodium salt (Sigma, cat. no. 8826)
- 100-bp DNA ladder (Invitrogen, cat. no. 15628)
- Streptavidin sepharose HP beads (GE Healthcare, cat. no. 17-5113-01)
- Pyrosequencing Kit: PyroGold SQA reagent kit 1 × 96 (Pyrosequencing AB, cat. no. 40-0045)
- Binding buffer (see REAGENT SETUP)
- Denaturing solution (see REAGENT SETUP)
- Washing buffer (see REAGENT SETUP)
- Annealing buffer (see REAGENT SETUP)
- Loading buffer (see REAGENT SETUP)

EQUIPMENT

- SpectraMAX Gemini XPS microplate (Molecular Devices) spectrofluorometer or similar
- NanoDrop spectrophotometer or similar for measuring optical density after bisulfite treatment
- Eppendorf 96 Gradient Mastercycler (Eppendorf) or any other thermocycler
- Thermomixer or any device for shaking at more than 1,000 r.p.m. at room temperature (20–25 °C) ▲ CRITICAL Slower shaking speeds will lead to sedimentation and potential loss of the beads.
- Vacuum preparation tool (Pyrosequencing AB) with the corresponding filter probes (Pyrosequencing AB, cat. no. 60-0180)
- Troughs for sample preparation (Pyrosequencing AB, cat. no. 60-0182)
- Heating device [e.g., heating plate or thermoblock (80 °C)]
- Thermoplate for sample preparation (Pyrosequencing AB, cat. no. 60-0092)
- PSQ 96 plate low (plate for pyrosequencing analysis; Pyrosequencing AB, cat. no. 40-0010)
- PSQ 96MA System SNP/SQA (Pyrosequencing AB, cat. no. 60-0140)
- Cartridge for reagent dispensation, (PSQ 96 reagent cartridge; Pyrosequencing AB, cat. no. 40-0022)
- Q-CpG software (Pyrosequencing AB, cat. no. 60-260)
- Thermowell sealing tape (Corning incorporated Costar, cat. no. 6569)
- Falcon tube

REAGENT SETUP

Extraction of DNA from tissues Excise 0.5 cm^3 of liquid nitrogen frozen tissue and add it to 500 μl lysis buffer (Tris 50 mM, EDTA 10 mM, SDS 1% wt/vol, NaCl 200 mM; pH 8.0), add 20 μl of proteinase K (10 mg ml^{-1}) and incubate

overnight at 37 °C with gentle shaking. Add 500 μl phenol and 300 μl chloroform and shake gently for 10 min at room temperature. Centrifuge for 2 min at 16,000*g*, collect the supernatant, add 1 volume (500–800 μl) of chloroform, centrifuge again for 2 min at 16,000*g* and collect the supernatant. Precipitate DNA with 2.5 volumes of 100% ethanol, discard supernatant and wash the pellet with 80% ethanol. Dissolve the pellet in 50–100 μl water depending on the size of the pellet.

Determination of DNA concentration The concentration of DNA is determined using the Quant-iT dsDNA broad-range assay kit according to the manufacturer's instructions on a SpectraMAX Gemini XPS microplate spectrofluorometer or similar. In brief, prepare a 1:200 dilution of the fluorescent dye with the provided buffer and add 2 μl of the isolated genomic DNA. A calibration curve is established using different concentration of the provided λ DNA standard. DNA is adjusted to a final concentration of 50 ng μl^{-1} using high-purity water. **! CAUTION** As the measurement of DNA concentration relies on an intercalating fluorescent dye, necessary precautions for handling should be taken.

Preparation of calibration standards Highly methylated DNA is prepared by the incubation of genomic DNA with the CpG methylase SssI. 7.5 μl NE-buffer 2, 10 nmol *S*-adenosylmethionine (SAM) and 6 U *Sss*I are added to 4.5 μg of human genomic DNA in a final volume of 67.5 μl. The solution is incubated at 37 °C in a water bath. After 3 h and again after additional 2 h, 10 nmol SAM and 6 U *Sss*I are added, and the reaction is incubated overnight at 37 °C. The enzyme is inactivated at 95 °C for 5 min and DNA stored at –20 °C until further use. DNA is stable for approximately 6 months.

Unmethylated DNA is prepared using the REPLI-g Kit following the manufacturer's instructions. DNA concentration is determined and adjusted as described earlier. Completely *in vitro* methylated and unmethylated DNAs are bisulfite-treated using the procedure described in Steps 2–5, and normalized to a concentration of 20 ng μl^{-1} using a NanoDrop spectrophotometer. Completely methylated DNA is diluted into the unmethylated DNA to create mixtures with a methylation degree of 10 or 25% increments to control for preferential amplification.

Binding buffer 10 mM Tris, 2 M NaCl, 1 mM EDTA, 0.1 % Tween 20; pH 7.6 (adjust with 1 M HCl).

Denaturing solution 0.2 M NaOH.

Washing buffer 10 mM Tris; pH 7.6 (adjust with 4 M acetic acid).

Annealing buffer 20 mM Tris, 2 mM Mg-acetate; pH 7.6 (adjust with 4 M acetic acid).

Preparation of the hydroquinone/bisulfite solution for bisulfite conversion Dissolve 90 mg of hydroquinone in 10 ml H_2O, add 333 μl of this solution to 2.7 g of sodium bisulfite, add 400 μl of 5 M NaOH and adjust to a final volume of 5 ml with H_2O (the volume has to be respected, otherwise sodium bisulphite will not dissolve completely).

Loading buffer for gel electrophoresis Water, 50% glycerol, 0.25% bromphenol blue.

PROCEDURE

Bisulfite treatment ● TIMING 28 h

1| Reduce the size of the genomic DNA either by enzymatic digestion or heat-induced fragmentation (100 °C, 5 min).

2| Dilute 1 μg DNA into 48 μl H_2O and incubate with 2 μl of 5 M NaOH (final concentration 0.2 M) for 15 min at 37 °C.
▲ **CRITICAL STEP** Incomplete denaturation results in incomplete bisulfite conversion.

3| Add 550 μl of the bisulphite/hydroquinone solution to the 50 μl of freshly denatured DNA, and incubate for 16 h in a water bath at 50 °C in darkness.

4| Purify the bisulphite-treated DNA as follows: add 1 ml of Wizard DNA clean-up resin to each bisulphite-treated sample and mix gently. Attach a 2-ml syringe barrel to a Promega mini-column above a Falcon tube, transfer the sample into the syringe and apply it slowly onto the column with the help of the piston. Wash the column with 2 ml isopropanol (80%). Transfer the mini-column to a 1.5-ml microcentrifuge tube. Centrifuge the mini-column at 12,000*g* in a microcentrifuge for 20 s to remove residual isopropanol. Transfer the mini-column to a new microcentrifuge tube and elute the DNA from the resin by adding 50 μl of pre-warmed H_2O (70–80 °C) water. After incubating for 1 min, centrifuge the mini-column for 20 s at 12,000*g*. Add 5.5 μl of 3 M NaOH (0.3 M final) and incubate for 5 min at room temperature. Add 5.6 μl of 3 M ammonium acetate and 200 μl ethanol (100%) to precipitate the DNA. Dissolve the precipitated DNA in 25 μl H_2O. To increase stability of the DNA and minimize repeated cycles of freezing and thawing, aliquots of 5–10 μl can be prepared at this stage.
■ **PAUSE POINT** DNA can be stored at –80 °C for at least 12 months and at –20 °C for approximately 6 months.

5| Verify completeness of the bisulfite conversion by amplification with the primers complementary to completely converted DNA as well as a pair of primers complementary to unconverted genomic DNA (following the example PCR protocol in Steps 6–9). Add a successfully converted DNA as well as unconverted genomic DNA as positive controls for the amplifications.
? TROUBLESHOOTING

PCR ● TIMING 2–3 h

6| Amplify the region of interest in the bisulfite-converted samples by PCR according to the reaction mix below in a 96-well plate (or microtubes):

Reagent	Volume (μl)	Final concentration
Bisulfite-treated DNA (40 ng μl^{-1})	1	40 ng
10× PCR buffer	2.5	1× (contains 1.5 mM $MgCl_2$)
$MgCl_2$ (25 mM)	1.6	1.6 mM
dNTP mix (8 mM)	1.25	200 μM
Primer forward (10 pmol μl^{-1})	0.5	0.2 μM
Primer reverse (10 pmol μl^{-1})	0.5	0.2 μM
HotStar Taq DNA polymerase (5U μl^{-1})	0.4	2 U
High-purity water	—	up to 25 μl

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7| Add a positive control to your samples such as bisulfite-treated mixed human DNA or a cell line and at least one negative control to detect any contamination.

8| Place the PCR in a thermalcycler and activate the polymerase by incubating at 95 °C for 15 min. Carry out the amplification reaction for 50 cycles with 30 s denaturation at 95 °C, annealing at the respective temperature approximately 60 °C for 30 s, and extension for 10 s at 72 °C.

9| Verify the specificity of the PCR amplification as well as the absence of product in the negative controls by mixing 5 µl of the amplification product and the positive and negative controls with 7 µl of loading buffer and separating the amplification products for 25 min at 110 mA on a 2% agarose gel.
▲ CRITICAL STEP Any signals in the negative controls, as well as weak or unspecific bands in the lanes of the gel corresponding to the amplified samples, will compromise the success and the quantitative accuracy/reproducibility of the subsequent pyrosequencing reaction. Only single, strong bands should be further processed.
■ PAUSE POINT PCR products can be stored for several weeks at −20 °C or up to a week at 4 °C.
? TROUBLESHOOTING

Template preparation ● TIMING 30 min

10| Fill three troughs with 180 ml of 70% ethanol, washing buffer and water, respectively, and a fourth trough for denaturing with 120 ml of the denaturing solution.
▲ CRITICAL STEP The level of the denaturing solution must be below the other solutions to ensure complete removal of any NaOH residue sticking to the filter tips, which might otherwise inhibit the ensuing enzymatic reactions.

11| Prepare a pyrosequencing plate by dispensing 15 pmol of pyrosequencing primer diluted in 40 µl of annealing buffer into each well of a new PSQ 96 plate low.

12| Resuspend the sepharose beads by turning the vial gently and prepare a reaction mixture of 51 µl of binding buffer and 4 µl of sepharose beads per sample to be analyzed. Add 25 µl of each PCR product to be analyzed by pyrosequencing to an individual well of a PCR plate, and add 55 µl of the reaction mixture to each well.
▲ CRITICAL STEP Do not vortex beads as this might damage the bead structure.

13| Seal the PCR plate (from Step 12) with the thermowell sealing tape and incubate for 10 min at room temperature under constant mixing (1,400 r.p.m.).
▲ CRITICAL STEP The plate must be kept under constant agitation before performing the below described purification steps, otherwise the beads might sediment to the bottom of the wells and cannot be recovered.

14| Create vacuum in the aspiration device (450 mmHg) and aspirate the binding mix from the wells of the PCR plate to capture the beads on the filter tips. Immerse the tips of the filters in three successive baths for 5 s each: ethanol 70%, denaturing solution and washing solution. Turn over the tool and release vacuum. Immerse the tip of the filters in the annealing mix of the pyrosequencing plate (from Step 11) and shake gently to release the beads into the wells. The solution should look cloudy.
■ PAUSE POINT The prepared pyrosequencing plate products can be stored at 4 °C for 48 h.
? TROUBLESHOOTING

15| Place the vacuum tool in the trough filled with water and turn on the vacuum again, wash tips extensively by aspirating the entire contents of the water trough, aspirate air for a few minutes to facilitate drying of the filter tips before turning off the vacuum.

16| Incubate the pyrosequencing plate for 2 min at 80 °C on the thermoplate placed on a heating device. Sealing of the plate is not necessary. Allow the plate to cool to room temperature for annealing of the primers to the PCR product.

Pyrosequencing ● TIMING 1 min per nucleotide dispensation

17| Program the sequencing run on the pyrosequencer using the Q-CpG software, enter the respective assays and sample names per well or copy it from a MS Excel template.

18| Dispense the reagents (nucleotides, substrate and enzyme mix) into the appropriate wells of the cartridge according to the volume calculation of the software. Place the pyrosequencing plate and the reagents cartridge in the pyrosequencer and run the system.
? TROUBLESHOOTING

19| Analyze the sequence runs by the Q-CpG software. Manually verify wells that are indicated as problematic by the software. Export the quantitative methylation values as 'csv file' to be further treated with statistical or graphical software.

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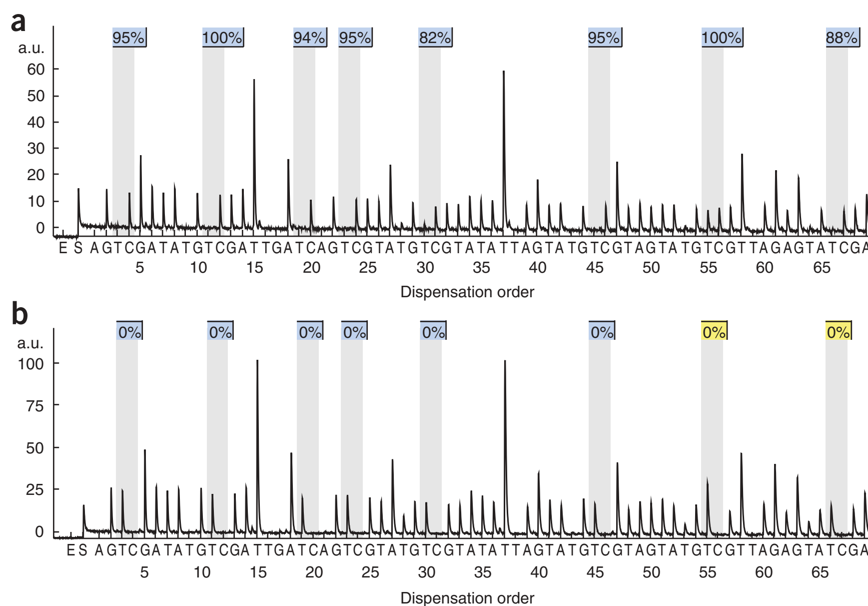

Figure 3 | Two pyrograms analyzing 8 CpGs in the CpG island spanning the transcription start site of the DNA repair gene *MLH1*. The *y*-axis represents the signal intensity in arbitrary units (a.u.) while the *x*-axis shows the dispensation order. Panel (**a**) depicts the analysis for DNA extracted from a microsatellite instable colon cancer while panel (**b**) shows the same region analyzed in a distant peritumoral colon tissue sample of the same patient. Dispensations corresponding to the potentially methylated cytosine (C or T after bisulfite treatment) are highlighted in gray. The percentage of methylation at individual CpG positions is shown as the percentage of methylation above the respective positions. Perfect calls (i.e., correlation between expected and observed pyrograms) are shown in blue, minor deviations from the expected patterns are underlain with a yellow.

Serial pyrosequencing (optional)
● TIMING **30 min + time for pyrosequencing**

20| Add 20 µl of binding buffer to each well of the plate subjected to pyrosequencing in Step 18, and pipette the solution vigorously up and down to resuspend sedimented beads. Transfer the solution to a PCR microtiter plate with V-bottom-shaped wells.

21| Add another 20 µl of binding buffer to each well of the pyrosequencing plate, and pipette and add this solution to the first transferred mixture.
■ **PAUSE POINT** The plate can be stored at 4 °C for 48 h.

22| Before repurification of the template strand, resuspend the beads by pipetting the solution up and down.

23| Render the template single-stranded, anneal a new sequencing primer and pyrosequence the additional part of the amplification product by repeating Steps 14–19.

● **TIMING**
Steps 1–4, bisulfite treatment of genomic DNA: 28 h
Step 5, verification of complete bisulfite conversion: 2–3 h
Steps 6–8, PCR amplification: 2–3 h
Step 9, verification of PCR amplification: 30 min
Steps 10–16, template preparation for pyrosequencing: 30 min
Steps 17–19, pyrosequencing: 1–2 h
Steps 20–23, template preparation for serial pyrosequencing: 30 min

? TROUBLESHOOTING
Troubleshooting advice can be found in **Table 1**.

TABLE 1 | Troubleshooting table.

Step	Problem	Possible reason	Possible solution
5	Amplification with primers complementary to genomic sequence	Bisulfite conversion failed; DNA not sufficiently denatured is the most common reason	Bisulfite treatment has to be repeated; use only freshly prepared solutions for bisulfite treatment; specialized protocols permit to freeze the DNA in a single-stranded state[31]
9	No, weak or unspecific PCR product	No PCR product, but amplification of the positive control	Measure the concentration of the bisulfite-treated DNA on a spectrophotometer such as the NanoDrop, which minimizes the loss of sample through measurement—if DNA concentration is sufficient, control DNA and bisulfite conversion quality by amplifying several regions with the primers complementary to converted

TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Possible solution
			or to unconverted genomic sequences, respectively. Repeat bisulfite treatment if necessary
		Unspecific PCR products	Reoptimize PCRs according to standard procedures; reduce extension time and/or reduce magnesium and/or amount of used polymerase. Verify that the polymerase and buffer components are compatible with the amplification of bisulfite treatment (no uracil *N*-glycosylase treatment, no polymerases from *Archaea*)
		Weak PCR products	Reoptimize PCRs following the recommendations above. In general, it is not worth continuing with the pyrosequencing analysis if the PCR displays potential problems
9	Negative controls of the PCR amplification are positive	Contamination	Repeat PCR, do not continue with the analyses as the results will be meaningless. Change primer aliquots and/or other PCR ingredients. Separate pre- and post-PCR manipulation areas. As 50 cycles of PCR amplification are performed, the smallest contamination with the respective amplification product might result in a potential erroneous amplification of the contamination
14	Sepharose bead/PCR product mixture is not aspirated at all from some wells	The filter tip is blocked	Continue the purification of the aspirated wells while sealing and continuing to mix the PCR plate at 1,400 r.p.m. After the aspirated beads have been released into the pyrosequencing plate, change blocked filter tips and repeat Step 14 and release the beads in the same pyrosequencing plate
			Verify regularly, by aspirating a plate filled with water, whether all filters are clean and functional. Exchange filter tips if they do not perform optimally
14	Beads are visible at the bottom of the PCR plate after aspiration	Sedimentation of beads due to insufficient agitation of PCR plate or time delay between agitation of PCR plate and aspiration of wells	Add 40 μl of binding buffer to the wells where beads are still present and resuspend the beads by vigorous pipetting. Repeat Step 14 and release beads into the same pyrosequencing plate
18	Pyrosequencing: no signal (from the beginning)	Wrong pyrosequencing primer	Verify that the correct sequencing primer was added corresponding to the sequence to be analyzed. Verify that all nucleotides and the substrate and enzyme mix were added correctly into the respective cartridge compartments
		The plate has not been heated to 80 °C so that the sequencing primer does not anneal to the template	Repurify the pyrosequencing plate following Steps 20–23
		Blocked cartridge: if there is a small peak at the substrate dispensation, the substrate and enzyme work well, and the problem is related to either a blocked needle of the cartridge or a noncomplementary sequencing prime; if not, substrate and/or enzyme have not been dispensed, due to a blocked cartridge	Blocked cartridges may be cleaned in most cases by placing the cartridge overnight in a warm water bath (∼37 °C) and manually pressing deionized water through the needles
18	Pyrosequencing weak signals	Pyrosequencing primer has not been correctly annealed	Verify the heating device

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TABLE 1 | Troubleshooting table (continued).

Step	Problem	Possible reason	Possible solution
		Pyrosequencing primer did not hybridize well to the template	Redesign the pyrosequencing primer that does not hybridize to the template well
		Weak PCR product	Reoptimize PCR to get a stronger amplification signal
		Low-quality biotin primer	Biotinylated primers should be ordered HPLC-purified to minimize free biotin
18	Pyrosequencing: missing peaks	Presence of an SNP	Could the pattern be explained by an SNP? If yes, include potential polymorphic positions in the sequence-to-analyze
		Dispensation cartridge blocked during the sequencing run	Dispensation cartridge blocked during the sequencing run, clean the cartridge and prepare the pyrosequencing plate using the protocol for serial pyrosequencing (Steps 20–23)
		Weak PCR product	Reoptimize PCR to get a stronger amplification signal
18	Pyrosequencing: wrong reference sequence	Wrong pyrosequencing primer or misannealing of the pyrosequencing primer	Verify the use of the correct pyrosequencing primer and sequence-to-analyze. Check for additional binding sites of the PCR primers, redesign PCR
18	Large differences (greater than 5%) in the quantitative methylation degree between replicates	Too little DNA (less than 15–20 ng) input for PCR amplification	Quantify DNA after bisulfite treatment using, for example, a NanoDrop spectrophotometer. If persistent, redesign PCR

ANTICIPATED RESULTS

Pyrogram intensity should be at least at 10 arbitrary units for a peak corresponding to a single nucleotide incorporation to ensure accurate quantification. **Figure 3** shows a typical program analyzing eight CpGs in the CpG island of *MLH1*. Quantitative reproducibility of technical replicates amplified by the same PCR is approximately 2%, variation induced by different bisulfite treatments and/or separate PCR amplifications is approximately 5%. In the example presented in **Figure 3**, the theoretical sequence corresponds perfectly to the sequence found by pyrosequencing. **Figure 4** shows the example of a so far unknown C/T polymorphism SNP, creating an additional CpG position as it is located directly 5′ to a G. This effect, which is not uncommon, is easily identifiable by the decrease of signal intensity at position

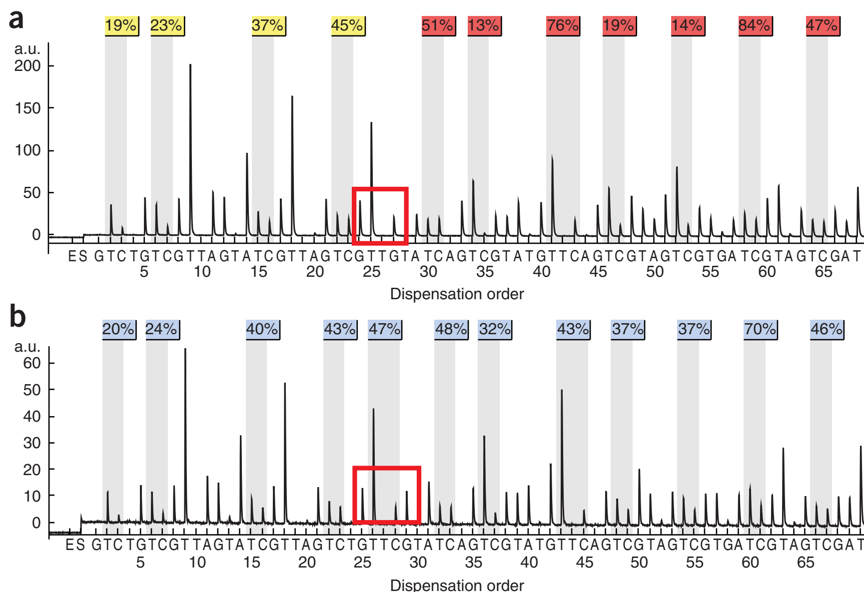

Figure 4 | Potential complications during the pyrosequencing reaction. Panels **a** and **b** show the same sequence analyzed from the same tumor sample. In dispensation panel **a**, the sequence corresponds to the expected sequence pattern up to dispensation 27. As the sequence after position 27 no longer corresponds to the expected one, only the first CpG positions gets the yellow quality score for minor deviation from the expected pattern, but the following CpG positions are highlighted in red indicating a problem (in comparison to the expected pyrogram) and requiring manual verification of the sequence. The G dispensation for a peak at position 27 is only half of the size compared with the peak at position 24 indicating the presence of a so far unknown single nucleotide polymorphism in the sequence. In panel **b**, the sequence is accordingly modified to accommodate the sequence change and the sample is again analyzed. High reproducibility of the quantitative methylation values between the two analyses of the same sample is clearly visible when comparing the percentage values for the first four CpG positions demonstrating the accuracy of the pyrosequencing platform for DNA methylation analysis.

27 in **Figure 4a** by half. Modification of the sequence to accommodate the change permits the accurate quantification of the created CpG position as well as the following CpG positions. Read-lengths of up to 120 nt can be achieved with one pyrosequencing primer, but the sequence context and possible formation of secondary structures can have negative impact. By successively annealing several sequencing primers, the analysis of the entire amplification product of 300 bases can be achieved (usually two to three sequencing primers).

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