Biotage

Pyro Q-CpG™: quantitative analysis of methylation in multiple CpG sites by Pyrosequencing®

Pyro Q-CpG from Biotage gives a new dimension to DNA methylation studies by quantitatively measuring the individual degree of methylation of consecutive CpG sites consistently over time. This reveals previously unseen patterns of methylation.

Methylation of cytosines in CpG dinucleotides is an important regulator of gene expression in the human genome. Changes in methylation are now known to have a fundamental role in the development of a variety of tumors. Quantitative measurement of variation of methylation over time and among tissues will reveal important correlations with other biological phenomena.

Pyro Q-CpG is attractive for the analysis of CpG methylation because it is capable of quantifying methylation in explicit sequence context, and is fast and easy to perform. Assay design is flexible, as the distance from the first base to be sequenced can be varied, and therefore, the primer can usually be positioned in a region free of CpG sites. In addition, there are four options for design: the assay can be performed in forward or reverse orientations, and on either the top or the bottom strands.

The approach uses bisulfite treatment and PCR to differentiate methylated cytosine (^{m}C) from unmethylated cytosine (C), and Pyrosequencing to quantify the ratio ^{m}C:C at each site[1–5]. It was first reported in 2002 (ref. 1) and independently developed by other groups[2–5].

Quantification by Pyrosequencing

Pyrosequencing analyzes single-stranded DNA templates by synthesizing complementary strands. Briefly, the four nucleotides (A, T, G and C) are added sequentially by a Pyrosequencing instrument to DNA templates. For every successful nucleotide incorporation, pyrophosphate (PPi) is released. PPi is converted in enzyme-catalyzed reactions to drive light emission in a quantity that is proportional to the number of incorporations[6] (**Fig. 1**). Therefore, peak heights in the Pyrogram™ inform on homopolymeric sequences and allele frequencies.

Principle of analysis

As with most methods for quantitative analysis of CpG methylation, CpG sites of genomic DNA are first chemically converted by bisulfite treatment and then amplified by PCR. In this process, C is converted to uracil (U), whereas ^{m}C remains unchanged. In the subsequent PCR, U is amplified as thymine (T), and ^{m}C is amplified as C. In the Pyrogram, ^{m}C and C are therefore represented as C and T peaks, respectively. These peak heights are proportional to the number of methylated alleles at each CpG site (**Fig. 2**).

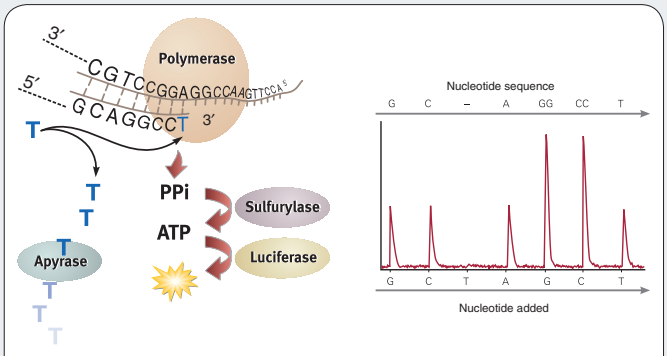

Figure 1 | The principle of Pyrosequencing and the output Pyrogram™. Double peak heights indicate incorporations of two nucleotides in a row.

Experimental considerations

Sequence context is an important control because bisulfite-treated, PCR-amplified DNA is A-T-rich, which decreases sequence variation. Pyro Q-CpG therefore guarantees that the correct sequence was analysed.

Pyro Q-CpG assays can contain an internal control for bisulfite treatment. C that is not followed by G in sequence is not methylated, and should be fully converted to T by bisulfite and PCR. To confirm this, all templates should show only T and zero C in this position (**Fig. 2**).

Pyro Q-CpG is practical in terms of starting material and throughput. DNA is readily analyzed from both fresh frozen tissue as well as the short PCR fragments that are typical of paraffin-embedded tissue, in which restriction fragment analysis would be difficult. The analysis takes about one hour for 96 specimens in parallel, at a fraction of the cost and time of equivalent dideoxy sequencing reactions.

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APPLICATION NOTES

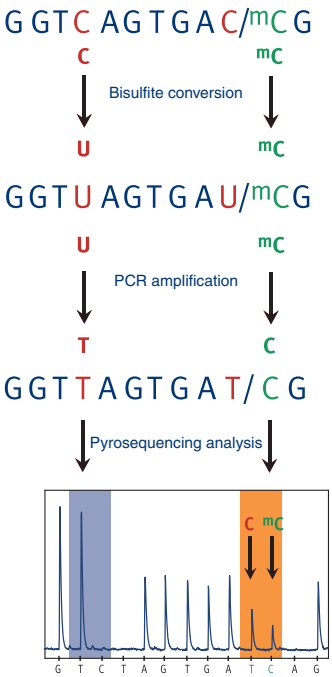

Figure 2 | Principle of analysis. Unmethylated C (red) and methylated C (green) are differentiated by bisulfite treatment and PCR. The ratio C:^{m}C at each CpG site (peaks in orange column) is measured in sequence context. C not followed by G acts as control for the bisulfite step (blue column).

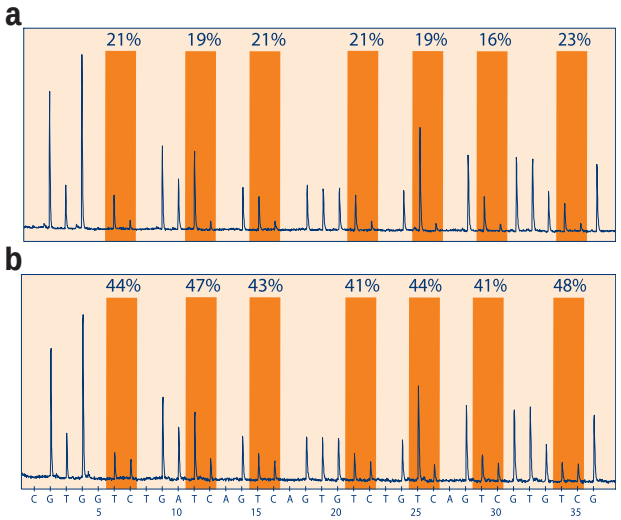

Figure 3 | Data output from analysis of seven CpG sites in the *p16^{INK4A}* promoter. Methylation levels in primary tumor (**a**) and metastatic lymphnodes (**b**) in head and neck cancer. Data courtesy of R. Krahe, M.D. Anderson Cancer Center.

Workflow

Commercial reagent kits are available for bisulfite treatment of human genomic DNA. The PCR is performed with one of the PCR primers biotinylated. This is required for the conversion of the PCR product to single-stranded DNA templates. A sequencing primer is then added, which anneals to the single-stranded DNA template. Analyses are automated, allowing routine processing of 96 samples in parallel.

Pyro Q-CpG

Pyro Q-CpG is a complete solution from Biotage for methylation analysis that exploits the advantages of Pyrosequencing technology. Pyro Q-CpG comprises PyroMark™ instrumentation and optimized software for CpG methylation analysis, as well as validated Research-Use-Only (RUO) tests for measuring methylation in specific genes. The PyroMark RUO tests include assays for methylation in p*16^{INK4A} (CDKN2A)*, *MLH1*, *MGMT*, *RASSF1A*, as well as Prader-Willi and Angelman syndromes, with more tests in the pipeline. Instrument users can access over 1,000 assays in the PyroMark online assay database, which contains assays for single nucleotide polymorphism (SNP), mutation and methylation analysis. For researchers who prefer to outsource methylation analysis, Biotage offers consultation for custom methylation assay design and laboratory services for sample analysis, including bisulfite treatment.

Summary

Pyro Q-CpG from Biotage offers several advantages for methylation analysis. Primarily, reproducible quantification of consecutive CpG sites is easy and fast on 96 samples in parallel. Assay design is flexible. The method is versatile for a range of analyses, from single and multiple consecutive CpGs to estimations of global methylation. As methylation of each site is measured in the context of the DNA sequence, software automatically performs quality control of the raw data to ensure that the expected sites were analyzed. Furthermore, Cs not followed by a G are used as quality control to evaluate whether the bisulfite treatment went to completion, thereby ensuring reliable data. The method is suitable for analysis of fresh -frozen, fixed and paraffin-embedded specimens.

Pyro Q-CpG is a valuable approach for acquiring quantitative methylation data that are comparable over time. Pyro Q-CpG can thus further our understanding on the variability of methylation with external variables such as treatment, individual and tissue sample (**Fig. 3**), which is a prerequisite for developing models describing methylation, gene expression and drug response.

1. Uhlmann, K., Brinckmann, A., Toliat, M.R., Ritter, H. & Nürnberg, P. Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. ***Electrophoresis* 23**, 4072–4079 (2002).
2. Colella, S., Shen, L., Baggerly, K.A., Issa, J.-P.J. & Krahe, R. Sensitive and quantitative universal Pyrosequencing methylation analysis of CpG sites. ***Biotechniques* 35**, 146–150 (2003).
3. 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).
4. Dupont, J.M., Tost, J., Jammes, H., & Gut, I.G. **De novo** quantitative bisulfite sequencing using the pyrosequencing technology. ***Anal. Biochem.* 333**, 119–27 (2004).
5. Yang, A.S. *et al.* A simple method for estimating global DNA methylation using bisulfite PCR of repetitive DNA elements. ***Nucleic Acids Res.* 32**, e38 (2004).
6. Ronaghi, M. Pyrosequencing sheds light on DNA sequencing. ***Genome Res.* 11**, 3–11 (2001).