

Quantitative CpG Methylation Analysis using Pyrosequencing™

Investigation into CpG methylation levels may provide more detailed knowledge of genetic modifications associated with disease development and progression in mammals. Pyrosequencing™ offers a powerful sequence-based analysis method to determine the level of methylation at CpG sites.

INTRODUCTION

Methylation of DNA plays a crucial role in chromatin structure and gene expression. The human genome can be composed of 16 possible dinucleotide combinations (AA, AT, AC, AG, TT, TA, TC, TG, CC, CA, CT, CG, GG, GA, GT and GC), all having an equal probability of occurring with the exception of CG which, possibly due to the hypermutability of methylated Cytosine, has a significantly lower frequency of occurrence (<1%). However, small stretches of CpG-rich DNA (where p represents phosphodiester bond linking the two bases) can be found, the majority of which are located within the promoter regions of human genes (Strathdee and Brown, 2002). These dense regions are called CpG islands. Cytosine is only capable of becoming methylated when followed with a Guanine base; however, unlike standard DNA regions, CpG islands are

typically not methylated. Methylation within the islands has been shown to be associated with transcriptional repression of certain genes, notably tumor suppressor genes, and is thought to play a key role in tumorigenesis.

Pyrosequencing's unique combination of sequencing and quantification enables CpG sites to be rapidly analysed to give the individual degree of methylation, which is then presented in the context of the underlying DNA sequence, making it possible to easily communicate and compare methylation data. The application of the technology for CpG methylation analysis has been verified and further developed in a number of independent studies (Colella *et al.*, 2003; Dupont *et al.*, 2004; Tost *et al.*, 2003; Uhlmann *et al.*, 2002; Yang *et al.*, 2004).

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PRINCIPLE OF ANALYSIS

In most quantitative methods for the analysis of CpG methylation, CpG sites of genomic DNA undergo bisulfite treatment, which converts unmethylated Cytosine (C) to Uracil (U), whereas methylated Cytosine (mC) remains unchanged. The treated DNA is then amplified by PCR, so that unmethylated C (now U) are converted to Thymine (T) and methylated C remain unchanged. The degree of methylation is therefore represented by the allele frequency C/T. Discrimination between mC and C is thereby achieved by transforming mC and C to appear as a C/T SNP (figure 1).

INBUILT QUALITY CONTROLS

The data generated by Pyrosequencing contain unique features that act as quality controls. Firstly, the sequence data gives confirmation that the analysis was made at the correct sites. Secondly, as methylation of C can only occur when followed by G, if the assay includes analysis of a C not followed by a G, that unmethylated C should be fully converted to T. This acts as a useful quality control for the full conversion of unmethylated C to T by the bisulfite treatment and subsequent PCR reaction.

PREPARATION OF SINGLE-STRANDED DNA (ssDNA) FOR PYROSEQUENCING

The preparation of high quality genomic DNA is essential in achieving accurate reproducible results. Biotage use the EZ DNA Methylation Kit™ from Zymo Research for bisulfite treatment of human genomic DNA.

The subsequent PCR reaction is performed with one biotinylated PCR primer (Biomers.net), which enables the conversion of the PCR product to a ssDNA template suitable for Pyrosequencing. A sequencing primer is added, which anneals to the ssDNA template. The time taken to prepare the DNA for Pyrosequencing is about 15 minutes and can be performed in parallel on 96 samples.

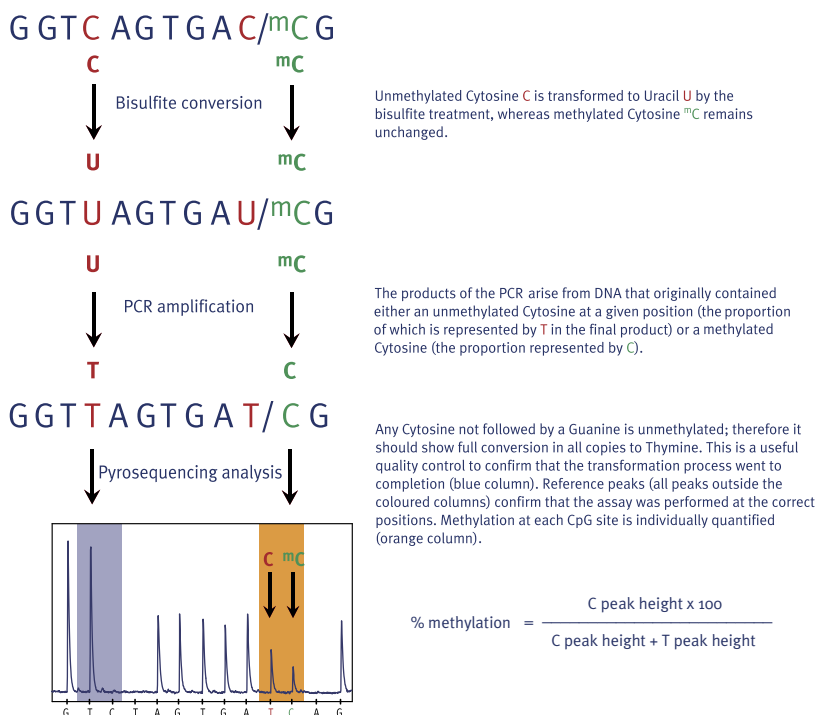


Figure 1: Principles of Pyrosequencing: An example of a DNA sequence and its conversion by bisulfite treatment and further amplification by PCR. By Pyrosequencing, unmethylated Cytosine (C) is measured as the relative content of T at the CpG site, and methylated Cytosine (mC) is measured as the relative content of C at the CpG site

PYROSEQUENCING

Pyrosequencing can accurately and reproducibly quantify the C/T ratio (obtained from bisulfite-treated and PCR-amplified DNA) by sequential addition of nucleotides (figure 2). At the C/T site, Cytosine is dispensed to the DNA template. If incorporation occurs, pyrophosphate (PPi) is released in a quantity equimolar to the amount of incorporated nucleotide. ATP sulfurylase quantitatively converts PPi to ATP in the presence of adenosine 5' phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. A CCD camera detects the light produced in the luciferase-catalysed reaction. The procedure is repeated, this time dispensing Thymine and measuring the intensity of light produced. Each light signal is proportional to the number of nucleotides incorporated.

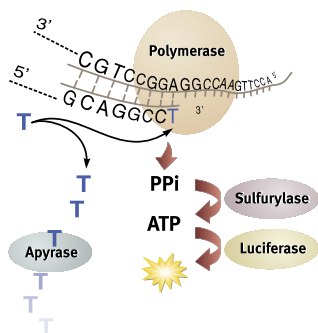


Figure 2: The Pyrosequencing reaction cascade generates light for every incorporated nucleotide, the intensity of which is proportional to the number of bases incorporated.

Pyrosequencing of the PCR product generates a so-called Pyrogram™, a pictorial representation of sequential nucleotide dispensations plot-ted against the resultant measured light intensity (figure 3). The Pyrogram™ displays both the nucleotide sequence (as peak sequence) as well as a quantitative representation of the incorporation events (as peak heights). The degree of methylation is calculated from the peak heights of C and T:

$$\% \text{ methylation} = \frac{\text{C peak height} \times 100}{\text{C peak height} + \text{T peak height}}$$

“Among the numerous technologies for methylation analysis, Pyrosequencing represents a breakthrough by combining the processivity of PCR-based technologies with the ability to analyze all the individual CpGs of a given region.”

Jean-Michel Dupont, Jörg Tost, Hélène Jammes, and Ivo Glynn Gut, Centre National de Genotypage, Evry, France. (Extracted from “De novo quantitative bisulfite sequencing using the Pyrosequencing technology”, *analytical biochemistry*; 333 (2004): 119-127)

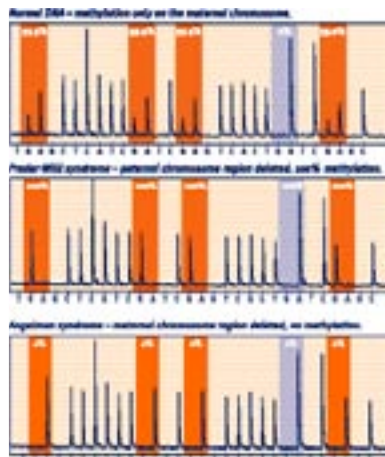


Figure 3: Methylation in normal DNA compared to individuals with Prader-Willi and Angelman syndromes

ASSAY RESULTS

Results of a CpG methylation analysis are presented in figure 3. This particular assay is designed to analyse methylation in Prader-Willi (PWS) and Angelman (AS) Syndromes, genetic aberrations caused by imprinting of the paternal gene. Different degrees of methylation distinguish PWS and AS from normal individuals. If the maternal chromosome region is deleted, methylation is close to 0%, and if the paternal chromosome region is deleted, methylation is close to 100%. The orange shaded regions highlight the peaks resulting from sequential dispensations of C and T from which methylation is assessed. The blue bar highlights a C not followed by a G, which shows, as expected, quantitative conversion to T. The results show a clear difference in methylation pattern between the two syndromes.

SUMMARY

Pyrosequencing offers a fast and accurate sequence-based analysis method to determine the level of methylation at CpG sites. Used with ABgene®'s Thermo-Start® DNA Polymerase an unprecedented level of resolution in CpG methylation analysis can be achieved.

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RELIABLE SYNTHESIS OF ssDNA FOR PYROSEQUENCING USING THERMO-START® DNA POLYMERASE

Biotage R&D has historically enjoyed success in developing assays using ABgene®'s Thermo-Start® DNA Polymerase, for both SNP assays as well as CpG methylation assays. CpG methylation assays based on Pyrosequencing are fully quantitative and should function with a range of tissue samples and assay conditions. For all assays, ABgene® Thermo-Start® performed well and showed good reproducibility between runs and with different starting materials (measured as the standard deviation in mean methylation among replicates). Furthermore, Thermo-Start® appeared to be more robust compared to other polymerases giving a PCR product where some other enzymes failed. The signal levels obtained (measured as Pyrosequencing peak heights) were as good as, if not better than, that obtained using other polymerases.

CAT. NO.	DESCRIPTION	QUANTITY
AB-0908/a	Thermo-Start® DNA Polymerase	250 units
For further information, please visit www.abgene.com .		

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