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CpG METHYLATION ANALYSIS BY PYROSEQUENCING – BENCHMARKS AND APPLICATION

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Executive Summary

- The complexities associated with methylation research demand powerful analytical methods that can accurately quantify multiple CpG sites with high resolution and reproducibility.
- Comparative studies with other technologies show that Pyrosequencing® technology combines the advantages of sequence-based approaches (quality control, resolution of individual CpG sites and quantification of methylation level) with high-throughput (amenable to automation and low cost) and the advantages of PCR-based technologies (smaller amounts of lower quality DNA required).
- Pyrosequencing technology facilitates studies of the relation of methylation to disease and response to treatment. Results are presented where the technology has been used for the analysis of methylation of several genes, and also global methylation.

Introduction

DNA methylation is now widely recognized as a fundamental epigenetic mechanism that is essential for normal embryonic development, with dysfunction leading to a range of genetic diseases including neoplasia. Methylation of cytosines that are followed by guanines (annotated CpG methylation) is the most widely investigated epigenetic modification in humans. The appreciation of its fundamental role in development, gene silencing and genomic stability has subjected the changes in DNA methylation status to ever-increasing scrutiny over the last 20 years.

In cancer, it is generally accepted that CpG hypermethylation in upstream promoter regions is associated with the silencing of tumor suppressor genes and that the genomes of cancer cells are hypomethylated in repetitive elements relative to their normal counterparts (Robertson, 2005). However, detailed knowledge of the relations between methylation and gene expression, and the triggers for the changes in methylation associated with tumorigenesis and disease progression for the multitudes of cancers remain largely uncharted. Methylation research is confounded by the definition of limits of what is hypomethylated and hypermethylated (relative to what, and the acceptable margins of variation), the reliance on archived tissues with poor quality DNA, and the perhaps significant variation of methylation from site to site as well as from tissue to tissue (Ehrlich, 2002). Progress has been hindered by the lack until recently of a powerful approach that can accurately quantify multiple alleles of large sample cohorts with high resolution and reproducibility, which is probably necessary to reveal the relatively subtle differences among tumor and normal tissues, and before and after treatment.

Although a variety of methods are available to assess methylation status, studying methylation is limited by the low sensitivity and/or the high consumption of time and labor of existing protocols. Restriction enzyme-based techniques often require large amounts of DNA and the loci which can be investigated are limited to those sites recognized by the enzymes. Several techniques have been applied to analyze bisulfite-modified DNA, but these suffer from either a low throughput or a labor-intensive setup.

Pyrosequencing is a fast, simple and quantitative method for analysis of CpG methylation in multiple sites in a single assay and eliminates the reliance on restriction sites, large quantities of DNA, gels, dyes and a mandatory separation step. For a technical description of Pyrosequencing in CpG methylation analysis, see England & Pettersson (2005).

Early Benchmarking of Pyrosequencing in Methylation Analysis

Uhlmann and co-workers adapted Pyrosequencing and SNaPShot™ technologies for methylation analysis of a single CpG site, which they called PyroMeth and SNaPmeth, respectively. They found that both methods quantified methylation, but PyroMeth showed less variation among replicates (Table 1). In a calibration experiment in which cloned PCR fragments containing either 100% or 0% methylated alleles were mixed in varying proportions, the standard deviation obtained with SNaPmeth ranged between 0 and 3.7%, and in the PyroMeth assay between 0.2 and 1%. SNaPmeth gave consistently higher values of methylation grade (on average 8% higher), which may be due to the competition among the 4 nucleotides for incorporation in SNaPmeth, which is circumvented in the PyroMeth approach.

<table>
<thead>
<tr>
<th>Difference in methylation between replicates / % C allele</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Greatest</strong></td>
<td><strong>Average</strong></td>
</tr>
<tr>
<td>SNaPmeth</td>
<td>15.9</td>
</tr>
<tr>
<td>PyroMeth</td>
<td>3.6</td>
</tr>
</tbody>
</table>

*Table 1. Comparison of variation in methylation values between independently generated and analyzed PCR amplicon replicates, measured by SNaPmeth and PyroMeth (from Uhlmann et al. 2002).*
Colella and co-workers (2003) and Tost and co-workers (2003) independently verified and expanded upon this approach with similar comparisons of Pyrosequencing against other methylation analysis methods. Colella and co-workers compared Pyrosequencing assays to a COBRA (combined bisulfite restriction enzyme analysis) assay for the p16 promoter of the CDKN2A gene, and tested the feasibility of using a universal biotinylated primer to lower the cost per test, calling the methods PyroMethA and universal PyroMethA. They found that there was complete concordance between COBRA and both PyroMethA methods, and that the two PyroMethA methods showed strong correlation ($R^2 > 0.99$) and better reproducibility than COBRA. Unlike COBRA, Pyrosequencing assays are not limited by restriction sites and are capable of analyzing multiple consecutive CpG sites, which is important since methylation levels in neighboring CpG sites can differ significantly. Pyrosequencing assays work better on small PCR products (100-150 bp), which is an advantage when working with archived DNA since partial DNA degradation can be a major problem for techniques requiring large amplicons.

Tost and co-workers applied Pyrosequencing to facilitate the study of multiple consecutive CpG sites in the transcription start region of GSTP1. Unmethylated in normal prostate tissue, CpG sites in this gene segment are highly methylated in tumors. They compared the Pyrosequencing assay to their existing primer extension-based assay using MALDI (matrix-assisted laser desorption ionization) mass spectroscopic detection. To quantify consecutive CpG sites, primer extension/MALDI-MS assays require multiple primers, making design of such assays difficult or impossible in CpG islands that are densely populated by CpG sites. Using Pyrosequencing, they were able to quantify 6 CpG sites in a single assay. Less than 5% differences were found compared to the 2 sites that were quantified by primer extension and MALDI-MS. Standard deviations were 2% for MALDI-MS and 1% for Pyrosequencing in the methylation range 30-90%. Pyrosequencing analyses were found to be highly reproducible and accurate when calibration was carried out properly (to detect or compensate for bias in PCR amplification). PCR bias can be a potential source of error in quantification when using any PCR-based methylation analysis. Designing assays without PCR bias is discussed later, using the PyroMark p16 test as an example.

Improving the technology

The possibility of analyzing multiple CpG sites with Pyrosequencing technology encouraged Dupont and co-workers (2004) to extend the read length of the assay to map uncharted regions of CpG islands as well as to circumvent the difficulty of designing assays in CpG-rich regions. They targeted 61 CpGs in 4 clusters of 9, 17, 19 and 16 consecutive CpGs in the human imprinted genes IGF2 and H19, implicated in Beckwith-Wiedemann Syndrome, an imprinting disorder.

Two major improvements were made compared to previous protocols: the Biotage PSQ SQA kit, optimized for sequencing applications, was used, and single stranded DNA binding protein (SSB) was added to the assay. SSB was previously shown to improve the sequencing reaction through increased efficiency of the enzymes, reduced non-specific signals, increased signal intensity and longer read length (Ronaghi, 2000). With SSB added at an optimal level, they were able to read 75 bases containing 10 CpG sites. Since this publication, Biotage has released Pyro Gold, a new chemistry formulation for Pyrosequencing that includes optimized quantities of SSB in the enzyme mix. The authors also expressed a need for software dedicated to methylation analysis that could quantify more sites in a single analysis. Biotage released Pyro Q-CpG™ software in early 2006. The use of Pyro Q-CpG software is exemplified below using analysis of the p14 promoter.

Dupont and co-workers tested the reproducibility of methylation quantification in each step of the process (i.e. PCR reaction and Pyrosequencing). They found that when sequencing the same PCR product several times, very good reproducibility was obtained with a mean SD of 1.3 (range of 0.6 to 2.4). The minimum quantity of template DNA required for high reproducibility in methylation analysis by Pyrosequencing is assay-specific. This study showed that analysis of lower quantities of genomic DNA gave larger variation between replicates.

The authors conclude that Pyrosequencing technology is excellent for de novo methylation analysis with simultaneous quantification. The relatively low work load of this method facilitates the analysis of a statistically sound number of samples. Pyrosequencing is called a breakthrough by combining the processivity of PCR based technologies with the ability to analyze all the CpGs of a given region. The main advantage is the quantification of the methylation status of each individual CpG in the sequence. This point is of particular interest because within a CpG island, successive CpGs can display significantly different levels of methylation. This accentuates the importance of studying several CpGs in preference to a single site which may not be sufficiently representative. This point is illustrated well by a Pyrosequencing assay for RASSF1A described on the next page.
The Ras-associated domain family 1A (RASSF1A) is a tumor suppressor gene on chromosome 3p21.3. RASSF1A is inactivated in a variety of tumors by hypermethylation and/or allelic loss of its locus.

Quantification of methylation of CpGs in RASSF1A by Pyrosequencing demonstrates that methylation levels vary significantly among consecutive sites, and that these patterns may be different among tumors (Figure 1). This underlines the importance of individually quantifying a number of CpGs when a single site may not be representative.

Estimation of Global Methylation

There have been two approaches to the application of Pyrosequencing technology in determination of global methylation levels: analysis of repetitive DNA elements, and analysis of CpG sites sensitive to cleavage by restriction enzymes. Yang and co-workers (2004) evaluated a method for assessing genome-wide changes in methylation based upon bisulfite treatment and PCR amplification of repetitive DNA elements. Alu and LINE-1 elements are numerous in the genome and are usually heavily methylated, with more than one-third of genomic DNA methylation estimated to occur in repetitive elements.

They designed PCR primers that amplified ~150 bp fragments of Alu or long interspersed nucleotide element (LINE-1) sequences, placing primers in regions free of CpG sites to avoid bias towards methylated or unmethylated DNA. To test the sensitivity of the approach for detecting changes in methylation, 3 colon cancer cell lines were treated with the methylation inhibiting agent 5-aza-2’dexoytidine (DAC). Comparing COBRA, direct sequencing and Pyrosequencing, they found general agreement in the change in methylation between control and treated cells. Although most CpG dinucleotides in repetitive elements are mutated so that they are no longer a target for DNA methylation, Yang and co-workers calculated that a sufficient proportion remain to act as a reliable surrogate marker for global methylation studies in normal and cancer tissues. The approach offers a major advantage of being less labor intensive and requiring less DNA than previous methods since it is PCR based, thus enabling experiments on larger cohorts.

Jeong and Lee reported a comparable method applicable to mice (Jeong and Lee, 2005). In this case the B1 repetitive element was used since, with 30 000 copies, it is the most common repetitive element in the mouse genome. The 163 bp B1 element contains 6 CpG dinucleotides. The Pyrosequencing assay gave results comparable in value and reproducibility to those obtained using methylation-specific PCR, and both methods indicated the same pattern of decrease in the global methylation level of a fibroblast cell-line on exposure to the methylation inhibitor 5-AzaC.

The second, innovative approach to analysis of global methylation levels was developed by Karimi and coworkers (Karimi et al, 2006). It involves analyzing CpG sites globally through cleavage of genomic DNA by the CpG methylation-sensitive restriction enzyme HpaII and its methylation-insensitive isoschizomor MspI in parallel reactions. EcoRI is included in all reactions as a control. MspI and HpaII leave 5'-CG overhangs that can be extended in the Pyrosequencing reaction to give GC peaks. The EcoRI enzyme generates 5'-AATT overhangs to give TTAA peaks as controls for variation in DNA amount. Both overhangs are then analysed using Pyrosequencing technology and the software generates peak height values that can be used to calculate the degree of global methylation. The excellent performance of the method was demonstrated using dilutions of in vitro methylated DNA, and also cell-lines treated with the methylation inhibitor 5-AzaC.
Reproducible, Quantitative Methylation Data in Clinical Research

An increasing number of publications demonstrate how Pyrosequencing is playing an important role in revealing how differences in methylation are associated with disease or response to treatment. Here are just a few examples.

The Effect of Decitabine Dosage on the Dynamics of Hypomethylation

The prognosis for patients with chronic myelogenous leukaemia (CML) who develop resistance to conventional therapy with imatinib is poor. A novel therapy is decitabine, a cytosine analog that incorporates into DNA and depletes DNA methyltransferase protein levels, resulting in DNA hypomethylation. The hypomethylation reactivates tumor-suppressor genes, and its consequent effect on gene expression appears to be the mechanism of drug response in patients with CML. At high doses, decitabine is cytotoxic, and studies show that decitabine is equally (or perhaps more) effective at relatively low doses compared with high doses. Issa and co-workers (2005) studied the effects of two dosage levels of decitabine on the levels of hypomethylation and response to treatment in patients with CML who had developed resistance to imatinib.

Pyrosequencing analysis enabled the tracking of the methylation dynamics in individuals, and showed significant differences in methylation dynamics with respect to dose (P = 0.1 at day 5 and P < 0.008 at day 12) and between responders to therapy vs. non-responders (P = 0.007).

Global methylation in patients was estimated across LINE-1 repetitive elements using a Pyrosequencing assay on bisulfite-treated DNA. First, the reproducibility of the assay was evaluated by measuring in duplicate the methylation of 105 samples with values ranging from 32.1% and 81.3%. The correlation between duplicates was R² = 0.82 (P < 0.001). The reproducibility over time of the Pyrosequencing LINE-1 assay was found to be higher than that of other methods; measurements of independent samples over time by real-time MSP have a typical correlation around R² = 0.4 (private communication with author). The reproducibility of the LINE-1 assay, is also demonstrated below (see Products).

This research group has performed two additional studies on the effect of decitabine on patients with CML and acute myelogenous leukemia (AML; Yang et al, 2006), and myelodyplastic syndrome (MD) and chronic myelomonocytic leukemia (CML; Kantarjian et al, 2007). Pyrosequencing technology was used to determine global methylation by the LINE-1 assay, and also gene-specific methylation of the p15 tumor suppressor gene. In the first study they showed that global methylation decreases rapidly on treatment but that methylation of p15 was aberrant in some cases, suggesting that analysis of this gene may be confounded by other factors. The second study involved a clinical trial to determine an optimal treatment schedule. They concluded that a low overall dose presented at a high dose-intensity gave optimal epigenetic modulation of hypomethylation induction and also activation of p15, together with optimal clinical response.

Cr(III) Exposure and Hypermethylation in rRNA

Occupational exposure of parents to metals has frequently been associated epidemiologically with increased risk of cancers in children, though mechanisms remain unclear. In animal studies, an epigenetic mechanism has been hypothesized to lie behind the increased tumor risk in offspring from parents exposed to Cr(III). Shiao and co-workers (2005) found genes in mice sperm where methylation patterns or levels were altered by Cr(III) exposure. Specifically, several regions of the rRNA gene were found to be hypomethylated, predominantly in the gene's upstream spacer promoter.

They performed DNA cloning to obtain individual sequence clones of the rRNA gene, which were subsequently bisulfite-treated and analyzed by Sanger dideoxy DNA sequencing. A total of 27 CpG sites were examined in each cloned sequence. They also performed Pyrosequencing analysis using four sequencing primers to analyze CpG sites in the cloned sequence.

Pyrosequencing analysis revealed with high reproducibility that neighboring CpG sites showed different degrees of average methylation, and importantly that Cr(III)-treated mice were significantly hypomethylated in sites 18-26 compared to controls (P<0.0001). In Cr(III)-treated mice, methylation was consistently lower by about 20-25%, in agreement with the findings from the Sanger sequencing approach.

In particular, CpG site 19 was significantly more highly methylated than sites 18, 20 and 21, whereas methylation of the sites of the core region of the spacer promoter was quite uniform. This is another example that illustrates the importance of Pyrosequencing analysis for individual quantification of multiple CpG sites; assessing methylation in single CpG sites or averaging several sites may obscure changes in methylation. In principle, Pyrosequencing analysis could be used to quantify methylation in all 27 CpG sites in this study, and simultaneously genotype the gene polymorphism in the spacer promoter region that the researchers also discovered.

Changes in DNA methylation patterns in subjects exposed to low-dose benzene

Another paper that illustrates new findings in the relationship between environmental exposure and disease progression involved the use of Pyrosequencing assays to study the possible relationship between DNA methylation and exposure to benzene (Bollati et al, 2007). This carcinogen has been consistently associated with acute myelogenous leukemia (AML), which in turn is linked to aberrant methylation patterns such as global hypomethylation, gene-specific hypermethylation/hypomethylation, and loss of imprinting. The study involved healthy individuals exposed to low levels of benzene. Pyrosequencing assays were used to determine global methylation through the surrogates Alu and LINE-1, and also the methylation of the promoters of MAGE-1, a gene hypomethylated in malignant cells, and the tumor-suppressor gene p15. The results indicated that exposure lead to decreased methylation of Alu and LINE-1 repeated sequences, and an increase in methylation of the p15 promoter: alterations in DNA methylation that were qualitatively comparable to changes seen in AML and other malignancies. Another assay for loss of imprinting gave no clear results. This is the first human study linking changes in methylation patterns to exposure to low levels of a carcinogen.
**Dissecting the variation in methylation throughout gene promoters**

Shaw and co-workers have used Pyrosequencing technology to analyze CpG methylation sites in promoters of a number of genes in connection with oral cancer (Shaw et al, 2006b). The methylation status of 4-5 CpG site in each promoter of p16, RARß, E-cadherin, CYGB and CYCA1 were analyzed in tumor and normal tissue. Significant and reproducible differences in the level of methylation were noted for all genes except RARß.

**Detection of genomic imprinting through allele-specific DNA methylation analysis**

Genomic imprinting is a form of allele-specific expression where only one chromosomal (=parental) allele is expressed. There are few methods available to detect parent-specific expression. Sanger sequencing of bisulfite-treated DNA from multiple alleles is one, rather tedious method. An innovative alternative has been developed by Wong and co-workers (Wong et al, 2006). They took bisulfite-treated DNA, amplified a region containing the H19 imprinting centre, and then sequenced the CpG methylation sites using a Pyrosequencing assay based on allele-specific sequencing primers that are placed over a heterozygous SNP. They demonstrated that the method correctly identified the percentage of allele-specific methylation within 3%. Results were confirmed using COBRA and direct sequencing. This method could be very useful when classifying persons in terms of loss-of-imprinting in epidemiological studies.

**Performance of Pyrosequencing technology Compared to Other Technologies**

Essentially, Pyrosequencing combines the advantages of sequence-based approaches (quality control, resolution for individual CpG sites and quantification of methylation levels) with those of high-throughput (amenable to automation and low cost) and the advantages of PCR-based technologies (smaller amounts of lower quality DNA required).

Pyrosequencing analysis gives a reproducible measurement of average methylation in several CpG sites in sequence directly from a PCR product, rapidly and accurately for many samples at a time. It is therefore well suited, for example, to clinical research applications where the methylation levels of several individuals need to be measured accurately over time for correlation to a disease status or to response to treatment. Pyrosequencing reproducibility and accuracy result in low measurement variance, thereby increasing the likelihood of early detection of small changes in methylation levels that may become apparent in response to treatment. Relatively low cost and easy automation allows the researcher to increase the experiment's sample population to detect trends that would otherwise not have a sufficient sampling basis for statistical significance.

Accuracy in quantification by Pyrosequencing technology is primarily limited by variation in PCR amplification. The variation between repeat assays performed on the same PCR product is very low (for example Tost et al, 2003). The relatively low sensitivity in detecting the methylated allele is a consequence of the fact that PCR assays are designed to be quantitative, i.e. to amplify methylated and unmethylated alleles at each CpG site in an unbiased fashion. Naturally, the PCR prior to Pyrosequencing can be designed to exclusively amplify the methylated allele. In such cases, the sensitivity of Pyrosequencing to detect the methylated allele would be equivalent to that of methylation specific PCR (MSP), but the ability of the assay to quantify methylation, and thereby a lot of useful information, would be consequently lost.

One concern when using MSP is the risk of false positives due to the absence of controls for complete bisulfite treatment. Another concern is the risk of false positives due to miss-priming, especially when many cycles are used to amplify from limited amounts of DNA. Shaw and co-workers have addressed this problem with a novel method called ‘methylation enrichment Pyrosequencing’ (MEP), which combines the sensitivity of MSP with validation of the PCR product by Pyrosequencing analysis (Shaw et al, 2006a). Their tests on clinical samples involved analysis in triplicate where the results of two or all three replicates indicated the final result. This gave an analytical sensitivity of 91% and analytical specificity of 100%. Furthermore, their results suggested that miss-priming may cause up to 10% false positives in MSP, which would be picked up by Pyrosequencing validation in MEP. This group is using the technique to detect minimum residual disease in head and neck cancers.

Methylation analysis using Pyrosequencing assays certainly facilitates the accurate analysis of individual CpG sites, but there is always a desire to analyse more sites, and with less material. Tost and coworkers (Tost et al, 2006) have demonstrated that a PCR product bound to a solid-phase can be analyzed repeatedly by one (or more) Pyrosequencing assays up to seven times without significant loss of accuracy. This opens up the possibility of extending the analysis range beyond one sequencing primer. Indeed it could permit the analysis of all CpG sites on a relatively long PCR product. The possibility of analyzing many methylation sites using a limited number of Pyrosequencing assays has been elegantly demonstrated by Brakensiek and co-workers (Brakensiek et al, 2007). They used Pyrosequencing technology to measure the methylation level at 48 CpG sites in the CpG island of the CDKN2B gene, which is frequently hypermethylated in myeloid malignancies. Assays were performed on 82 samples from patients and 32 control samples, making a total of 7762 successful analyses, with each assay covering up to 15 CpG sites over approximately 100 bases. The most interesting result was the detection of distinct regions with high discriminatory power for disease status. This clearly demonstrates the utility of Pyrosequencing assays in scanning large regions to determine specific sites or groups of sites that should be subjected to a limited, high-throughput analysis.

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**CpG Methylation Analysis by Pyrosequencing – Benchmarks and Application - WP 010**

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Products for Methylation Analysis

Biotage has developed a number of products to facilitate analysis of CpG methylation. These include software, Research-Use-Only tests and also assays in an Assay Database.

Pyro Q-CpG Software

Pyro Q-CpG Software has been developed to address applications that benefit from high-quality and high-throughput analysis of DNA methylation. This is achieved by quantitative sequence-based analysis of multiple, consecutive CpG sites in up to 96 samples in parallel, enabling an individual to analyze and process many thousands of samples and CpG sites to provide statistically reliable data.

Functions that are essential for high throughput and data reliability have been incorporated into Pyro Q-CpG Software. After the Pyrosequencing run, analysis of ninety-six samples takes less than one minute, even when each sample contains 20 or more CpG sites.

Another key improvement is the automatic quality control of each sample for completion of bisulfite treatment, eliminating the need for manual inspection and estimation of levels of non-converted DNA.

With the overview feature, the user can easily navigate through methylation results, mean values, methylation ranges and quality scores for each sample as well as individual CpG sites (Figure 2). Pyro Q-CpG software is compatible with all existing Pyrosequencing instrument platforms. It can be installed and run in parallel with the current instrument control software.

Figure 2a.  
Sequencing primer

13 CpG sites analyzed using a single sequencing primer

Figure 2b.

Figure 2: Quantification of 13 CpG sites in the promoter region of p14 using Pyro Q-CpG Software.

2a) Arrangement of primers in relation to CpG sites.

2b) The resulting Pyrogram. Grey areas indicate the CpG sites that were analyzed. The yellow regions indicate controls regions for automatic assessment of bisulfite conversion (unmethylated C should be fully converted to T).

PyroMark Research-Use-Only Tests for methylation analysis

PyroMark tests include PCR and Pyrosequencing primers and validated protocols that are optimized for accurate and reproducible quantification.

p16 (CDKN2A)

The p16 gene (CDKN2A) is located on chromosome 9 (9p21). The gene is frequently mutated or deleted in a wide variety of tumors and is known to be an important tumor suppressor gene. It is also inactivated by promoter hypermethylation in a number of different cancers. PyroMark p16 assay detects the level of methylation in a region +148 to +174 in exon 1 of the gene. (Ensembl gene: ENSG00000147889).

In product development, each PyroMark test is assessed for PCR bias (i.e. the disproportional amplification of one allele over another) and if bias is present, it is eliminated by redesigning the test. We present an example of attained linearity from the PyroMark p16 test. Stepwise increments of methylated DNA were prepared by mixing unmethylated DNA with in vitro methylated DNA (80% methylated) in various proportions. The mixtures were then bisulfite treated and subjected to PCR using the PCR primer design supplied in the PyroMark p16 test, followed by Pyrosequencing analysis. The strong linear correlation (R^2 > 0.99) demonstrates the zero bias in PCR amplification of the alleles as well as the capability of Pyrosequencing for absolute quantification of methylation.
LINE-1 elements

LINE-1 retrotransposable elements make up about 15% of human genome. DNA methylation within the promoter region of human LINE-1 elements is important for maintaining transcriptional inactivation and for inhibiting transposition. Genome-wide losses of DNA methylation within the promoter region of human LINE-1 elements have been regarded as a common epigenetic event in malignancies and may play crucial roles in carcinogenesis. This methylation assay amplifies a region of the LINE-1 element and serves as a marker for global methylation. (Pos 305 to 331 in Acc. no. X58075).

High reproducibility of the LINE-1 assay is important for detecting the relatively small daily changes in methylation levels associated with hypomethylation, and to detect differences in patterns in normal and tumor tissues. Figure 2 shows the results of LINE-1 assays repeated on the same samples at 4 separate occasions. The results on each occasion were nearly identical, with replicate measurements at each CpG site showing small variation. The methylation patterns (CpG sites 1-4) are repeatedly different between normal and tumor samples.

Prader-Willi & Angelman Syndromes – reverse strand assay

PyroMark PWS/AS exploits the quantitative properties of Pyrosequencing by quantifying methylation to identify Prader-Willi and Angelman Syndromes. Prader-Willi syndrome (PWS) is caused by loss of expression of genes located on segment q11-13 on the paternally derived chromosome 15. When the expression from the same segment is lost from the maternally derived chromosome 15, Angelman syndrome (AS) arises. This test detects the effect on methylation of deletion of segment q11-13 on chromosome 15.

The assay illustrates the flexibility of Pyrosequencing assay design, which can be made on the top and bottom strands, and in the forward and reverse directions. This analysis is a reverse assay of the top strand, so CpGs are represented by G/A polymorphisms instead of C/T polymorphisms. Bisulfite conversion is consequently represented by 100% A instead of T.
Figure 5. Illustration of PyroMark Prader-Willi/Angelman Syndromes assay results. Orange columns indicate quantified CpG sites. Blue columns indicate QC for completion of the bisulfite treatment (a C followed by an A in the original sequence). Pyrogram trace shows the reverse assay of the top strand.

**MLH1**
The human mutL homologue hMLH1 is located on chromosome 3 (3p21.23). The gene is inactivated by promoter hypermethylation in a number of different cancers, such as colon, endometrial and gastric cancers. PyroMark MLH1 detects the level of methylation in a region -209 to -188 from the transcription start site. (Ensembl gene: ENSG00000076242).

**MGMT**
The O\(^6\)-Methylguanine DNA methyltransferase (MGMT) gene is located at chromosome 10(10q26). Loss of gene expression frequently occurs via hypermethylation of the CpG sites in the promoter region. This product can be used to detect the level of methylation in positions 17 to 39 in exon 1 of the MGMT gene (Ensembl ID: OTTHUMT00000051009).

**Assay database**
Containing almost 1000 designs, the Assay database is an important addition to the PyroMark product range. All assays are optimized and wet-tested to reduce the time to get the assay up and running in your lab. At the time of writing, the database contained the methylation assays shown in Table 2. Full details of the assays can be found in the Assay Database.

<table>
<thead>
<tr>
<th>Genetic region</th>
<th>GSTP1 M-5,-6,-7 (R)</th>
<th>NORE1A</th>
</tr>
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<tbody>
<tr>
<td>Alu repetitive elements</td>
<td>GSTP1 M1,2,3 (F)</td>
<td>p14 (CDKN2A)</td>
</tr>
<tr>
<td>APC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B1 repetitive elements</td>
<td>GSTP1 M10,9, 8, 7, 6, 5 (R)</td>
<td>p15 (CDKN2B)</td>
</tr>
<tr>
<td>CDKN2A promoter</td>
<td>GSTP1 M3, 1, 2, 1 (R)</td>
<td>PTEN</td>
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<td>H19, DMR, Region 4</td>
<td>RARbeta2</td>
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<td>p18 (CDKN2C)</td>
<td>RASSF1A</td>
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<td>p27 (CDKN1B)</td>
<td>TCL-1 promoter</td>
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<td>SHP1</td>
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<tr>
<td>GSTP1 M-7, -6, -5 (F)</td>
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<td>GSTP1 M-4,-3,-2 (F)</td>
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</table>

**Important note:** A patent or patent applications may cover the assays in the PyroMark Assay Database. These assays are made available for scientific research only. Third party licenses may be required and are the responsibility of the user of the database.
Summary

Pyrosequencing technology 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. Since 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, C's not followed by a G are utilized 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.

Methylation analysis by Pyrosequencing is valuable for acquiring quantitative data that are comparable over time. This can help further understanding on the variability of methylation with external variables such as treatment, individual and tissue sample, which is a prerequisite for developing models describing changes in methylation, the relation of methylation to other biological phenomena e.g. gene expression, and clinical studies of methylation levels in disease and in response to treatment.

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References

NOTE: A complete, updated list of literature concerning Pyrosequencing technology is available at our website


Pyrosequencing Systems are designed for Laboratory Use Only which means that they may be used for either research purposes or by high complexity CLIA certified labs.

Pyrosequencing sequencing by synthesis systems enable DNA to be analyzed either bound to solid support or in solution. A review of DNA purification methods can be found in Fakhrai-Rad, et al. Hum Mutation (2002) and other papers listed on the Biotage web site.


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