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## Sensitive and quantitative universal Pyrosequencing™ methylation analysis of CpG sites

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DNA methylation is implicated in multiple normal cellular processes, including the regulation of tissue- and development-specific gene expression, imprinting, X-chromosome inactivation, DNA repair, and the suppression of parasitic DNA sequences. Aberrant gene promoter methylation and resulting altered gene expression have been associated with cancers (1,2). Altered methylation patterns have been described in human sporadic cancers: genome-wide hypomethylation and localized hypermethylation have been observed, and both alterations can be present in the same tumor (3). Together with loss-of-heterozygosity and gene mutations, transcriptional silencing by methylation has been shown to be a major inactivating event of tumor suppressor genes in accordance with Knudson's two-hit hypothesis (4). Gene inactivation by promoter hypermethylation and resulting loss of function have been shown for several cancer genes involved in DNA repair, cell cycle control, apoptosis, angiogenesis, differentiation, metastasis/invasion, transcription, and signal transduction (1).

A wide range of methods exists to detect genomic DNA methylation, including approaches to detect genome-wide and gene-specific methylation levels (5). Most methods used to analyze the methylation status of a specific sequence are based on bisulfite modification of the DNA. Following the treatment, the methylation status can be assessed as a sequence difference by sequencing, methylation-specific PCR, methylation-sensitive single-stranded conformational polymorphism, methylation-sensitive single nucleotide primer

extension (Ms-SNuPE), or restriction enzyme digestion. Recently, high-throughput methods to detect site-specific methylation have been developed using the TaqMan® technology (6,7)

We developed a highly quantitative method to assess DNA methylation levels at specific sites using the Pyrosequencing™ technology (Pyrosequencing AB, Uppsala, Sweden). Pyrosequencing methylation analysis (PyroMethA) is a modification of the combined bisulfite restriction analysis (COBRA), where the restriction analysis is substituted with the highly quantitative Pyrosequencing reaction. Recently, Uhlmann et al. (8) reported the independent development of a Pyrosequencing-based method to detect methylation. Our assay designed for a different gene further validates the use of the Pyrosequencing technology to detect methylation at specific CpG sites. In addition, we developed and validated universal PyroMethA as an approach for high-throughput methylation detection. Standard PyroMethA and universal PyroMethA were equally robust and quantitative.

Figure 1A compares the experimental approaches for PyroMethA and COBRA for the *CDKN2A* (p16) promoter region tested. Although methylation per se does not affect the primary genomic DNA sequence, after bisulfite treatment all unmethylated cytosines are converted into uracil, while the methylated cytosines remain unchanged. Thus, the presence of methylation in a CpG island can be detected and quantified as a chemically induced C-to-T transition. Pyrosequencing is a real-time sequencing technique based

on the detection of the release of inorganic pyrophosphate during nucleotide incorporation (9). The pyrophosphate released in the DNA synthesis reaction is quantified by monitoring a luciferase reaction. The luciferase reaction produces a signal proportional to the number of pyrophosphate molecules released (i.e., to the nucleotides incorporated in the DNA). Using the Pyrosequencing allele quantification software (Pyrosequencing AB), the sequence and allelic contribution is depicted as a quantitative Pyrogram™. This allows highly accurate determination of the frequency of polymorphic sites. The technology has been used successfully to determine mutant to wild-type allele ratios (10), to quantitatively determine allelic states of polyploid organisms (11), and to analyze DNA pools for the determination of allele frequencies in population-based studies (12,13). Here we show that Pyrosequencing can be used to successfully quantify the methylation status of specific CpG sites. In addition, to facilitate high-throughput determination of site-specific methylation levels for multiple genes, we developed a PCR amplification strategy using a tailed reverse primer in combination with a biotin-labeled universal primer in the same reaction (Figure 1B).

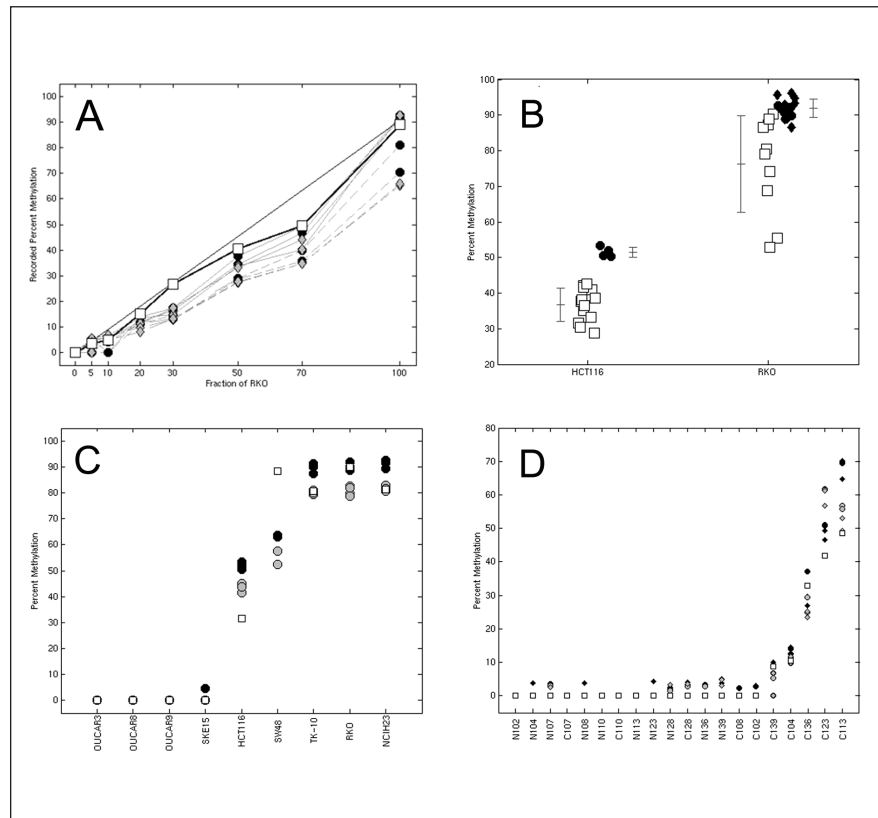
Briefly, *CDKN2A* promoter PCR amplification was performed in a 50- $\mu$ L reaction containing HotStarTaq™ Master Mix (Qiagen, Valencia, CA, USA), 0.1  $\mu$ M forward primer *CDKN2A-F*, 5'-GGTTGTTTTYGGT-TGGTGT-TTT-3', and biotinylated reverse primer *CDKN2A-R*, 5'-ACCC-TATCCCTCAAATCCTCTAAAA-3'. The amplification was carried out according to the general guidelines suggested by Pyrosequencing: denaturation at 95°C for 5 min, followed by 50 cycles at 95°C for 30 s, 56°C for 1 min, 72°C for 45 s, and a final extension at 72°C for 7 min. To reduce the cost/assay, we developed an amplification protocol using a universal primer approach (Figure 1B). Briefly, the biotinylated reverse primer was substituted with a 5' tailed (5'-gacgggacaccgct-gatcgttta-3') unlabeled reverse primer, *CDKN2A-UNIVR*, 5'-gacgggacaccgct-gatcgtttaACCCTATCCCTCAAATCCTCTAAAA-3', and a biotinylated



the method of quantification. These data indicate that PyroMethA/universal PyroMethA and COBRA always give concordant calls for the methylation status when a 10% threshold to declare methylation is used. (Figure 2, A–D).

Several techniques have been developed to assess gene-specific methylation events. However, most of them do not lend themselves to high-throughput screening. COBRA has been used extensively in methylation studies, but it is especially laborious. Thus, the development of a reliable quantitative method with high reproducibility, ease of use, and potential for high-throughput analysis is highly de-

sirable. Here we developed and validated PyroMethA as a new method to quantitatively determine methylation levels. PyroMethA is similar to the Ms-SNuPE technique (15), except that Pyrosequencing allows the interrogation of multiple consecutive sites and does not require radioactivity. Like COBRA (14) and Ms-SNuPE (15), PyroMethA is a PCR-based method. It works better on small PCR products (100–150 bp), which is advantageous when working with DNA from clinical or archival samples, since partial DNA degradation can be a major problem for some of the other techniques. Like other site-specific methylation meth-



**Figure 2. Methylation levels for the gene promoter regions of *CDKN2A*.** (A) Comparison of COBRA (open squares) with PyroMethA (filled circles) in a dilution series of RKO genomic DNA with specific levels of *CDKN2A* promoter methylation. The straight line through the origin represents the expected results for the dilution series at the first CpG site based on RKO cell line methylation levels previously determined by COBRA. Symbols connected by straight lines represent observed data at the first CpG site; symbols connected by stippled lines represent data observed at the second CpG site. (B) Comparison of COBRA (open squares), PyroMethA (filled circles), universal PyroMethA (filled diamonds) for cell lines HCT116 and COBRA (open squares), PyroMethA (filled circles), universal PyroMethA (filled diamonds) for cell line RKO, to determine reproducibility.  $\bar{x} \pm s_D$  levels are indicated. (C) COBRA (open squares) and PyroMethA results (site 1, filled circle; site 2, shaded circle) for a panel of nine cancer cell lines in ascending order of methylation levels, as determined by PyroMethA. (D) COBRA (open squares), PyroMethA (circles; site 1, filled; site 2, shaded), and universal PyroMethA (diamonds; site 1, filled; site 2, shaded) results for a panel of 10 pairs of matched normal/colorectal cancer samples in ascending order of methylation levels, as determined by COBRA.



ods, PyroMethA can be applied only to known sequences. Although the design of the assay for the CpG sites we studied was relatively straightforward, assay design and quantification can be affected by sequence context.

Our data indicate that both PyroMethA and universal PyroMethA compare favorably to COBRA in terms of sensitivity, specificity, and robustness. Moreover, validating and implementing the universal-primer strategy in the initial PCR, we substantially reduced the cost/assay. In addition, with the assay we developed for *CDKN2A*, we demonstrated that PyroMethA provides a more comprehensive tool to determine methylation levels at multiple CpG sites throughout a given CpG island. Since our method is not dependent on informative restriction sites, the spectrum of target sequences that can be assayed is broader than that for COBRA. Our data indicated that methylation of neighboring CpG sites within the same CpG island can be variable. Differential levels of methylation at different CpG sites in a given promoter could underlie differential expression of affected genes and may thus be of biological significance.

With the completion of the sequence of the human genome, gene promoter sequences and regulatory CpG islands can be readily identified *in silico* (16). The development of new methods, such as PyroMethA/universal PyroMethA, will enable the more detailed study of candidate genes affected by methylation. Large-scale screening for promoter methylation at specific sites will provide a better understanding of the epigenetic and epigenomic contributions to the molecular processes of development and disease. For example, high-throughput studies targeted at promoter regions of candidate tumor suppressor genes subject to epigenetic inactivation by methylation can be easily designed and carried out. To determine the efficacy of de-methylating agents in clinical trials, PyroMethA would also constitute a suitable high-throughput platform. In conclusion, PyroMethA/universal PyroMethA is a new, highly sensitive method to quantify CpG site methylation, which can be easily adopted for high-throughput studies of gene-specific methylation events.

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## REFERENCES

1. Costello, J.F. and C. Plass. 2001. Methylation matters. *J. Med. Genet.* 38:285-303.
2. Paz, M.F., M.F. Fraga, S. Avila, M. Guo, M. Pollan, J.G. Herman, and M. Esteller. 2003. A systematic profile of DNA methylation in human cancer cell lines. *Cancer Res.* 63:1114-1121.
3. Ehrlich, M. 2002. DNA methylation in cancer: too much, but also too little. *Oncogene* 21:5400-5413.
4. Knudson, A.G. 2000. Chasing the cancer demon. *Ann. Rev. Genet.* 34:1-19.
5. Fraga, M.F. and M. Esteller. 2002. DNA methylation: a profile of methods and applications. *BioTechniques* 33:632-649.
6. Eads, C.A., K.D. Danenberg, K. Kawakami, L.B. Saltz, C. Blake, D. Shibata, P.V. Danenberg, and P.W. Laird. 2000. MethyLight: a high-throughput assay to measure DNA methylation. *Nucleic Acids Res.* 28:E32.
7. Akey, D., J. Akey, K. Zhang, and L. Jin. 2002. Assaying DNA methylation based on high-throughput melting curve approaches. *Genomics* 80:376.
8. Uhlmann, K., A. Brinckmann, M.R. Toliat, H. Ritter, and P. Nurnberg. 2002. Evaluation of a potential epigenetic biomarker by quantitative methyl-single nucleotide polymorphism analysis. *Electrophoresis* 23:4072-4079.
9. Ronaghi, M., B. Pettersson, M. Uhlen, and P. Nyren. 1998. PCR-introduced loop structure as primer in DNA sequencing. *BioTechniques* 25:876-884.
10. Ahmadian, A., J. Lundeberg, P. Nyren, M. Uhlen, and M. Ronaghi. 2000. Analysis of the p53 tumor suppressor gene by pyrosequencing. *BioTechniques* 28:140-147.
11. Rickert, A.M., A. Premstaller, C. Gebhardt, and P.J. Oefner. 2002. Genotyping of SNPs in a polyploid genome by pyrosequencing. *BioTechniques* 32:592-600.
12. Wasson, J., G. Skolnick, L. Love-Gregory, and M.A. Permutt. 2002. Assessing allele frequencies of single nucleotide polymorphisms in DNA pools by pyrosequencing technology. *BioTechniques* 32:1144-1150.
13. Neve, B., P. Froguel, L. Corset, E. Vaillant, V. Vatin, and P. Boutin. 2002. Rapid SNP allele frequency determination in genomic DNA pools by pyrosequencing. *BioTechniques* 32:1138-1142.
14. Xiong, Z. and P.W. Laird. 1997. COBRA: a sensitive and quantitative DNA methylation assay. *Nucleic Acids Res.* 25:2532-2534.
15. Gonzalgo, M.L. and P.A. Jones. 1997. Rapid quantitation of methylation differences at specific sites using methylation-sensitive single nucleotide primer extension (Ms-SNuPE). *Nucleic Acids Res.* 25:2529-2531.
16. Davuluri, R.V., I. Grosse, and M.Q. Zhang. 2001. Computational identification of promoters and first exons in the human genome. *Nat. Genet.* 29:412-417.

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