PyroMark® CpG Assays: A new tool for genome-wide methylation profiling by Pyrosequencing®

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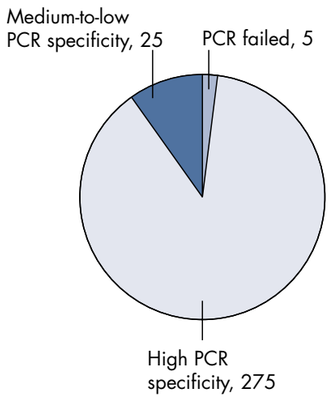
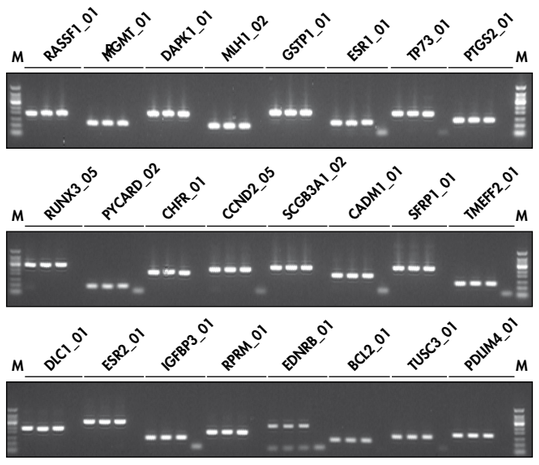
PyroMark CpG Assays

PyroMark CpG Assays enable methylation analysis of specific targets across the human genome. These predesigned methylation assays for Pyrosequencing analysis of bisulfite-converted DNA use carefully tailored algorithms to generate optimal assays. As such, PyroMark CpG Assays minimize time spent on assay optimization and maximize analysis success. PyroMark CpG Assays expand the streamlined Pyrosequencing workflow to enable methylation analysis of virtually any CpG island in the human genome.



High PCR success rate, even with bisulfite-converted DNA. The PCR performance of PyroMark CpG Assays for 310 randomly selected genes was evaluated. 305 assays (98%) resulted in the predicted PCR amplicon, usable as template for subsequent Pyrosequencing.



Primer design and PCR amplification are optimized for Pyrosequencing analysis. Dedicated buffer composition and DNA Polymerase included in the PyroMark PCR Kit enhance amplification specificity and PCR efficiency at the same time. PCR was carried out in triplicate using the PyroMark PCR Kit and bisulfite-treated leukocyte DNA as template. 5 µl of PCR product was loaded onto a 2% agarose gel. **M**: 50 bp marker.

Comparative analysis of CpG island methylation

PyroMark CpG Assays were used to profile candidate genes for CpG methylation using 4 different human cell lines. Cell line 293 was established from human embryonic kidney; whereas A549, MCF-7 and Caco-2 are human cancer cell lines established from lung, breast, and colon carcinoma, respectively. Using Pyrosequencing, we have determined the exact methylation level of all the CpG sites covered by the various assays. All genes are known from the literature to be involved in cancer development. Some of the 33 assays cover the same promoter region, but measure the methylation at a different positions within the promoter (see next panel).

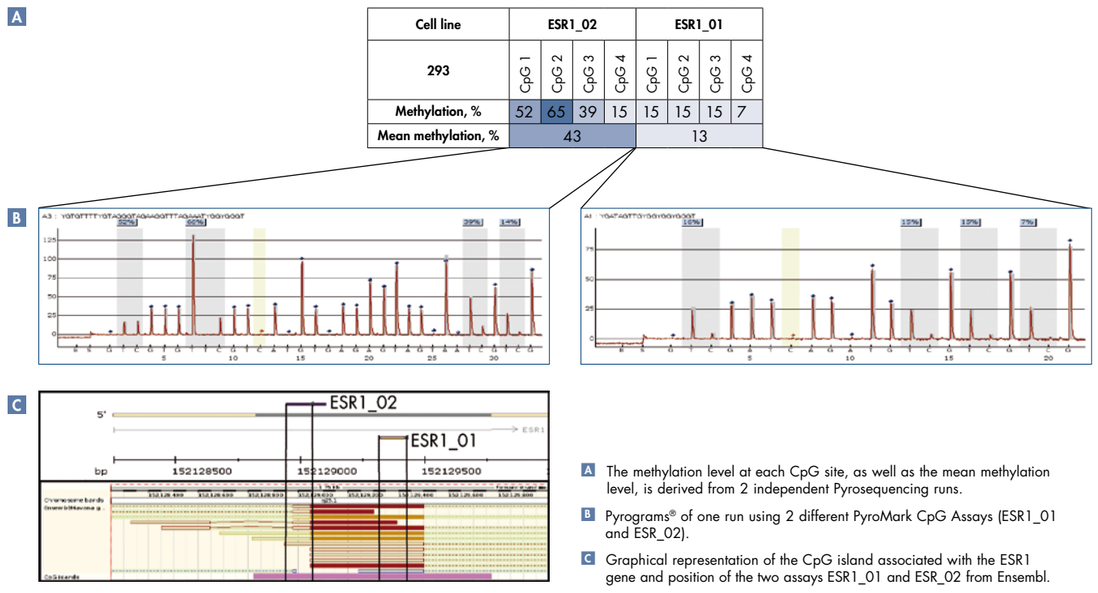
Cell line	RASSF1	MGMT	DAPK1	MLH1		GSTP1		ESR1		TP73	PTGS2	RUNX3		PYCARD	CHFR		CCND2		SCGB3A1	CADM1	SFRP1	TMEFF2		DLC1		ESR2		IGFBP3	RPRM	EDNRB	BCL2	TUSC3	PDLIM4
Assay	01	01	01	02	03	01	02	01	02	01	01	05	06	02	01	02	05	06	01	01	01	01	02	01	02	02	01	01	01	01	01	01	01
293																																	
A549																																	
MCF-7																																	
Caco-2																																	

Methylation analysis of 24 promoter regions. 4 different cell lines were analyzed for methylation of 24 promoter regions using 33 different PyroMark CpG Assays. The color scale represents the percentage of methylation levels determined by the Pyro® Q CpG Software as mean methylation levels.

- 0–5% CpG methylation
- 6–20% CpG methylation
- 21–40% CpG methylation
- 41–60% CpG methylation
- 61–80% CpG methylation
- 81–100% CpG methylation

Methylation differences within a CpG island

The methylation at different sites within a CpG island can differ significantly. Data show the analysis of two different positions within the CpG island associated with the gene ESR1. The 2 assays reveal significantly different methylation values; showing that detailed methylation analysis of individual CpG sites are needed to understand gene regulation by DNA methylation in cancer.



A The methylation level at each CpG site, as well as the mean methylation level, is derived from 2 independent Pyrosequencing runs.

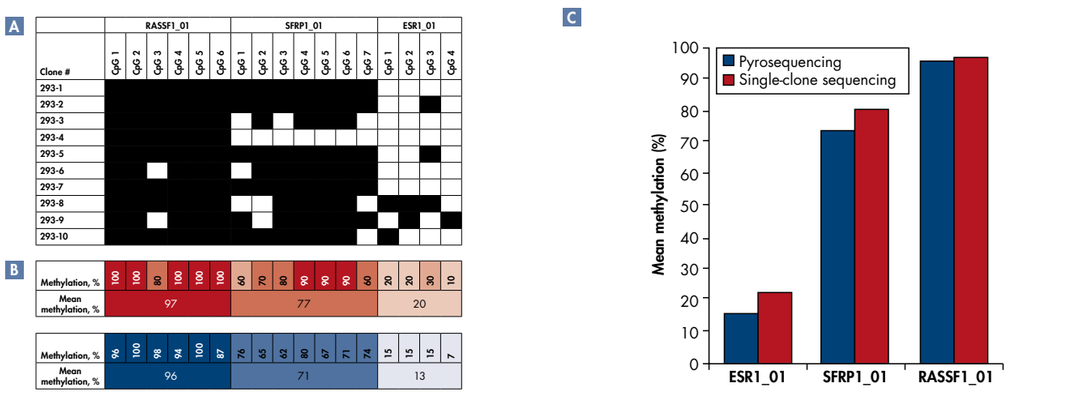
B Pyrograms® of one run using 2 different PyroMark CpG Assays (ESR1_01 and ESR_02).

C Graphical representation of the CpG island associated with the ESR1 gene and position of the two assays ESR1_01 and ESR_02 from Ensembl.

Pyrosequencing versus single-clone sequencing

Single-clone sequencing of PCR products generated from bisulfite-treated DNA has been the commonly used methodology for quantitative methylation analysis.

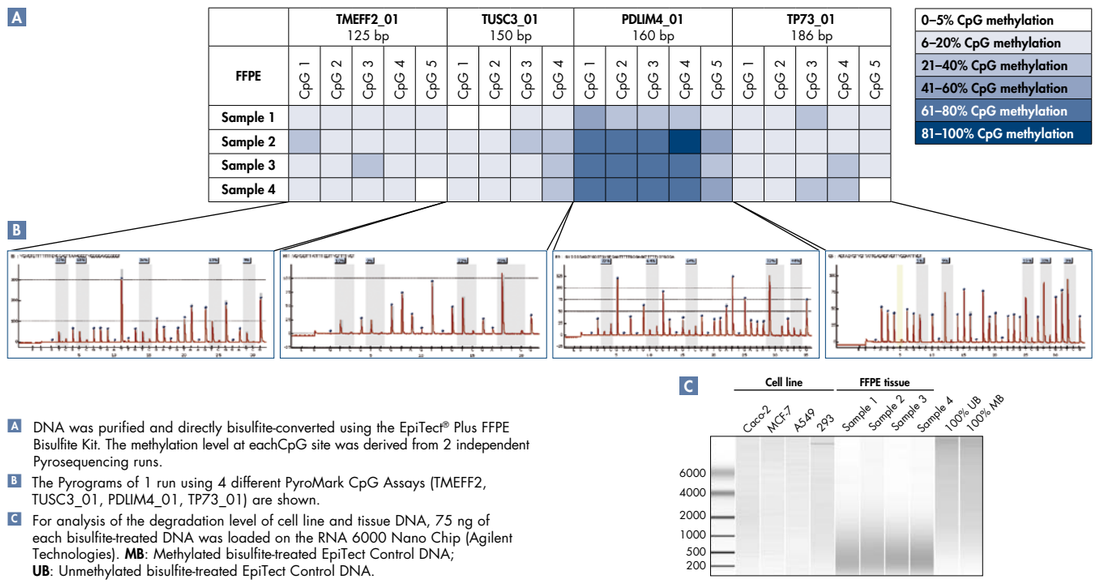
Pyrosequencing and single-clone sequencing generate comparable mean methylation levels. While the accuracy of the measured methylation level at each CpG site determined by clone sequencing depends on the number of clones processed, Pyrosequencing automatically generates reliable methylation quantification at individual CpG sites in every run, without the need for tedious cloning and expensive sequencing. Results of single-clone bisulfite sequencing and Pyrosequencing were quantitatively compared.



Results of single-clone sequencing. A PCR products were obtained with PyroMark CpG Assays for RASSF1, SFRP1, and ESR1, respectively, using bisulfite-treated genomic DNA from the cell line 293 as template. The PCR products were cloned and sequenced (10 clones/assay) on the 3730xl DNA Analyzer (Applied Biosystems®). Methylated cytosines are shown as black squares. B Comparison of methylation degree as determined by single-clone sequencing (red) and Pyrosequencing (blue). C Comparison of mean methylation of RASSF1, SFRP1, and ESR1 CpG islands. **Blue**: Pyrosequencing; **Red**: Single-clone sequencing.

DNA methylation analysis of FFPE tissue

Determination of methylation patterns in DNA from precious and limited sample materials (e.g., formalin-fixed, paraffin-embedded [FFPE] tissue) is of specific interest for the successful establishment of valuable disease-predicting biomarkers. The degraded nature and limited amounts of such DNA is especially challenging for bisulfite conversion and DNA amplification. PyroMark CpG Assays can successfully analyze even such highly degraded DNA.



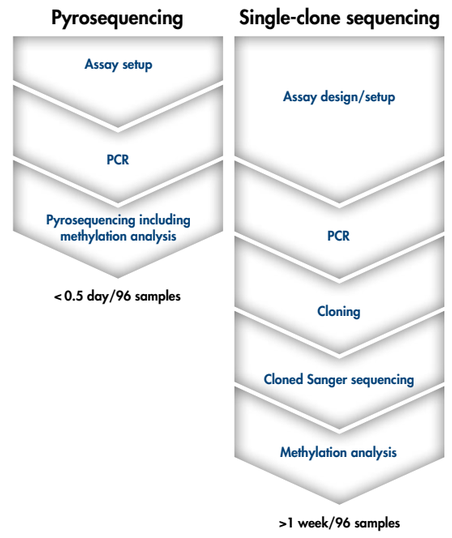
A DNA was purified and directly bisulfite-converted using the EpiTect® Plus FFPE Bisulfite Kit. The methylation level at eachCpG site was derived from 2 independent Pyrosequencing runs.

B The Pyrograms of 1 run using 4 different PyroMark CpG Assays (TMEFF2, TUSC3_01, PDLIM4_01, TP73_01) are shown.

C For analysis of the degradation level of cell line and tissue DNA, 75 ng of each bisulfite-treated DNA was loaded on the RNA 6000 Nano Chip (Agilent Technologies). **MB**: Methylated bisulfite-treated EpiTect Control DNA; **UB**: Unmethylated bisulfite-treated EpiTect Control DNA.

PyroMark CpG Assays for time- and cost-effective DNA methylation analysis

- Predesigned assays available through GeneGlobe — no tedious assay design and optimization.
- Advanced design algorithms for guaranteed results — assay primers checked against the entire bisulfitome
- Comprehensive coverage of CpG sites in human genome
- Reliable quantification of consecutive CpG sites
- Convenient download of run and analysis settings
- Proven primer quality delivers cost-effective, reliable performance
- Pyrosequencing provides fully quantitative results in under 1 hour after PCR
- Suitable for analysis of DNA from human specimens, especially fresh-frozen and FFPE tissue



The applications presented here are for research use only. Not for use in diagnostic procedures.

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