Reliable Methylation Analysis for Epigenetic Research

Novel Technologies offering a complete and standardized workflow

Gerald Schock, Thorsten Träger, Christian Korfhage, Ralf Peist, Nicolas Rudinger, Nan Fang, Doris Jansen, and Dirk Löffert

QIAGEN GmbH, Germany

Introduction

With implications in cancer and heritable diseases, the study of epigenetic mechanisms is becoming increasingly important for cancer research, identification of biomarkers, predisposition factors and potential drug targets. Epigenetic mechanisms involve an interdependence between both DNA and histone modification. The acetylation status of histones alters chromatin structure, subsequent DNA packaging and therefore, access to genes for transcription. The modification of the DNA sequences themselves by methylation at CpG dinucleotides can restrict or permit gene expression from promoter sites.

However, the analysis of changes in DNA methylation is challenging due to the lack of standardized methods for providing reproducible data, particularly from limited sample material. QIAGEN has developed the first product line for standardized solutions for DNA methylation analysis. Following DNA collection and stabilization, EpiTect offers a complete solution from bisulfite conversion to PCR driven methylation analysis.

Sample collection and stabilization	DNA purification	Bisulfite treatment	Analysis
■ PAXgene Blood™ DNA Kits	■ QIAamp® Kits ■ DNeasy® Kits	■ EpiTect® Bisulfite Kit ■ EpiTect® Bisulfite 96 Kit ■ EpiTect® Whole Bisulfitome Kit	■ EpiTect® Control DNA Set ■ EpiTect® MSP Kit ■ EpiTect® MethyLight PCR Kit ■ EpiTect® MethyLight Assays ■ EpiTect® Bisulfite Sequencing Service

QIAGEN Solutions for Epigenetics. In addition to the EpiTect product range, QIAGEN's sample preparation technologies complete the workflow from sample collection and stabilization to downstream analysis. For more information, visit www.qiagen.com/goto/epigenetics.

Complete bisulfite conversion and Whole Bisulfitome Amplification

The most critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines to uracil. The combination of EpiTect Bisulfite Mix and DNA Protect buffer facilitates the optimum pH and the formation of single-stranded DNA necessary for complete conversion (Figure 1). In addition, a unique DNA Protect buffer prevents DNA fragmentation during bisulfite conversion. The prevention of fragmentation enables subsequent amplification of large DNA fragments by whole bisulfitome amplification (WBA) while maintaining the converted sequence representation. EpiTect Whole Bisulfitome Kit uses proven isothermal Multiple Displacement Amplification (MDA) technology (REPLI-g) and has been adapted to the special requirements of bisulfite converted DNA. Amplified DNA is suitable for end-point PCR (Figure 2), real-time PCR, Pyrosequencing, and MALDI-TOF analysis (Figure 7).

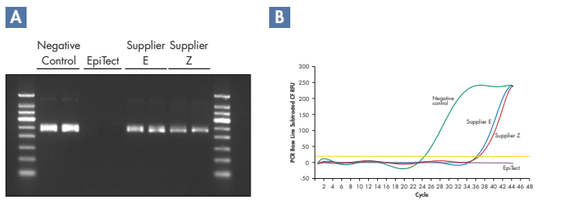


Figure 1. Complete cytosine conversion. One microgram of genomic DNA was converted using the EpiTect Bisulfite Kit or bisulfite kits from alternative suppliers (Supplier E and Supplier Z) according to the manufacturers' instructions. Next, the presence of unconverted DNA in comparable amounts of each sample was determined by A end-point PCR using the HotStarTaq *Plus* Master Mix Kit and B SYBR® Green based real-time PCR using the QuantiTect SYBR Green PCR Kit. The EpiTect Bisulfite Kit treated DNA was completely converted, while, DNA treated using kits from Suppliers E and Z contained a significant proportion of unconverted DNA.

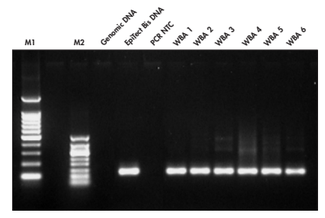


Figure 2. Reliable end-point PCR. EpiTect Whole Bisulfitome amplified DNA was compared to non-amplified DNA by PCR specific for bisulfite converted DNA. Fifty nanograms of genomic DNA was bisulfite converted in 6 independent reactions using the EpiTect Bisulfite Kit. Ten nanograms of converted DNA (EpiTect Bis DNA) and 10 ng of amplified DNA (WBA 1-6) were used for PCR using the EpiTect MSP Kit. PCR bands obtained are comparable in size and intensity. Genomic DNA and non template control gave no PCR result. Marker 1:100 bp Ladder Marker 2:50 bp Ladder.

Improved primer discrimination in MSP

Methylation specific PCR (MSP) is a particularly demanding application as, in order to provide reliable results, it requires primer extension only from those primers that bind either to unmethylated or methylated sequences after bisulfite conversion. Methylation-specific primers also often bind to the unmethylated converted DNA with a mismatch or several mismatches at the 3' end of the primer. Conventional *Taq* DNA Polymerase can efficiently elongate these primers regardless of the primer mismatch leading to false-positive methylation results.

The unique and novel genetically engineered HotStarTaq® d-Tect Polymerase prevents elongation of mismatched primer–template complexes located at the 3′ end (Figure 3). Therefore, it improves PCR specificity and reduces false-positive results in MSP reactions (Figure 4).

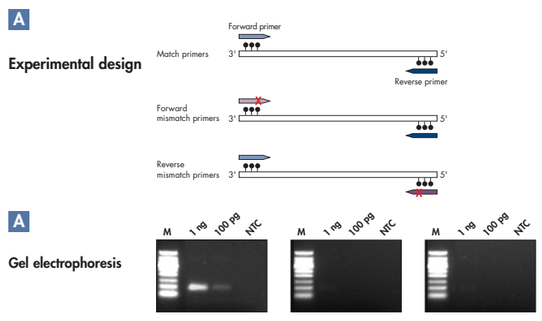
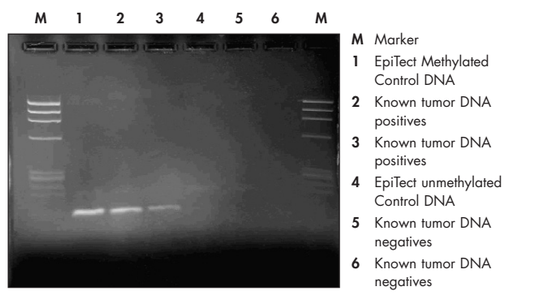


Figure 3. Single base discrimination of HotStarTaq® d-Tect Polymerase. A Both primers used for methylated LDOC1 PCR were modified such that a mismatch was introduced at the 3' end of either the forward or the reverse primer. This modification mimics the situation of unchanged primers annealing to bisulfite converted DNA which differs due to a missing methylation at one CpG site. B A single mismatch is sufficient for correct MSP results.



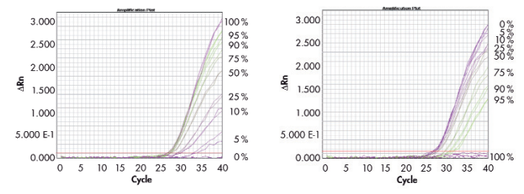
M Marker
1 EpiTect Methylated Control DNA
2 Known tumor DNA positives
3 Known tumor DNA positives
4 EpiTect unmethylated Control DNA
5 Known tumor DNA negatives
6 Known tumor DNA negatives

Figure 4. MSP analysis of p16 methylation. Methylation-specific PCR for the p16 locus was performed by using the EpiTect MSP Kit. Tumor DNA previously analyzed by Pyrosequencing (data not shown) was compared to EpiTect methylated Control DNA and EpiTect unmethylated Control DNA (both pre-bisulfite converted). The results show that the methylation status could be confirmed by p16 MSP and that EpiTect Control DNA gives reliable results in MSP. Data kindly provided by Triantafillos Liloglou, Roy Castle International Centre for Lung Cancer Research, Liverpool, UK.

Reliable quantification of methylation sites

MethyLight assays, probe-based real-time PCR for methylation analysis, are often used for sensitive quantification of the methylation pattern. The EpiTect MethyLight PCR Kit enables sensitive and reliable quantification for all TaqMan or dual-labeled probe-based analysis of methylation sites. MethyLight assays can be performed in both, a quantitative or semi quantitative format.

Quantitative MethyLight assays (e.g., EpiTect MethyLight Assays), consisting of two probes, one methylation specific, and the other nonmethylation specific, can be used in a single real-time PCR reaction. This enables highly accurate quantitative methylation analysis, due to the simultaneous detection of methylated and unmethylated DNA.



Signal methylated:	Signal unmethylated:	Percentage of methylation:
$C_{T\,(CG)}$ (FAM) – represents the threshold cycle of the CG reporter (FAM channel)	$C_{T\,(TG)}$ (VIC) – represents the threshold cycleof the TG reporter (VIC channel)	$C_{meth} = 100/[1+2^{(C_{T\,meth}-C_{T\,unm})}]\%$

Defined methylation degree in 10 ng control DNA mixes	Mean $C_{T\,(CG)}$ value (FAM probe)	Mean $C_{T\,(TG)}$ value (VIC probe)	Calculated methylation degree in % per sample
0% methylation	40.00	27.45	0.02
5% methylation	32.99	27.43	2.40
10% methylation	30.92	27.46	8.36
25% methylation	29.30	27.88	27.26
50% methylation	28.22	28.61	56.62
75% methylation	27.72	29.73	80.13
90% methylation	27.46	31.13	92.71
95% methylation	27.29	32.25	96.87
100% methylation	27.12	40.00	99.99

Figure 5. Sensitive real-time PCR quantification of small changes in the methylation status. EpiTect MethyLight Assays enable the direct quantification of the methylation degree in a sample by taking the threshold cycles (C_T) determined in the FAM channel with the probe detecting methylated DNA or in the VIC channel with the probe detecting unmethylated DNA. Ten nanograms of bisulfite converted, methylated and unmethylated human control DNA, or defined mixtures of both DNAs resulting in 95% to 5% methylated DNA, as indicated in the figure, were used in a methylation quantification experiment on the ABI PRISM 7900 instrument, applying the EpiTect MethyLight Assay HS_CDKN2A in combination with the EpiTect MethyLight PCR Kit. The methylation degree was calculated from the C_T values in FAM ($C_{T\,(CG)}$) and VIC ($C_{T\,(TG)}$) channel, obtained in quantitative real-time PCR using the formula described in [2].

Pre-converted Control DNA for methylation analysis

QIAGEN provides ready-to-use human control DNAs for standardized and reliable control reactions in methylation experiments. EpiTect PCR Control DNAs are suitable for end-point MSP (Figure 4), real-time MethyLight assays (Figure 5 and 6), Pyrosequencing, and MALDI-TOF analysis (Figure 7). They enable the validation of primer specificity used for methylation analysis and can be used as quantification standards and internal controls (positive, negative).

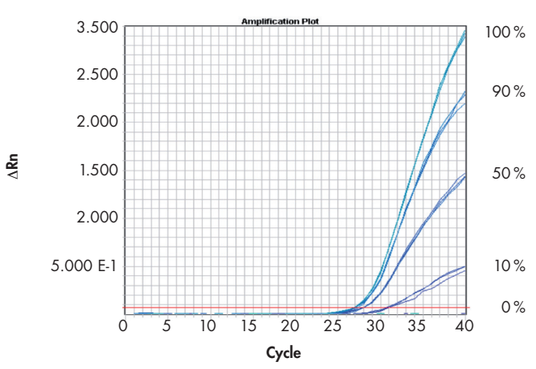


Figure 6. Quantitation standards for quantitative, real-time PCR analysis using EpiTect Control DNA. EpiTect Methylated Control DNA and EpiTect Unmethylated Control DNA (both pre-bisulfite converted) were mixed resulting in 100 %, 90 %, 50%, 10 %, and 0 % methylated DNA. MethyLight assays for the human PITX2 gene were run in triplicate using 10 ng of each DNA samples. The results show that EpiTect Control DNA can be used as a methylation standard for the quantification of unknown DNA samples.

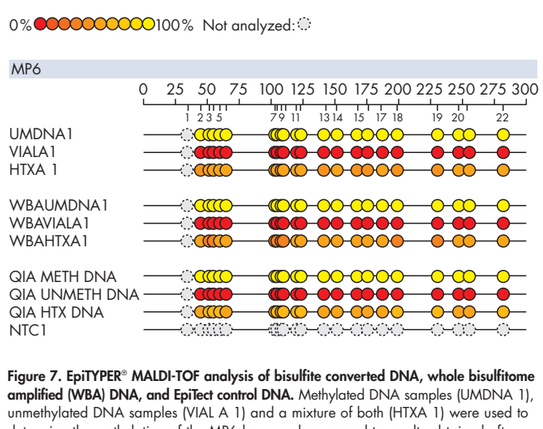


Figure 7. EpiTYPER® MALDI-TOF analysis of bisulfite converted DNA, whole bisulfitome amplified (WBA) DNA, and EpiTect control DNA. Methylated DNA samples (UMDNA 1), unmethylated DNA samples (VIAL A 1) and a mixture of both (HTXA 1) were used to determine the methylation of the MP6 locus and compared to results obtained after Whole Bisulfitome amplification (WBAUMDNA1, WBAHTXA 1, WBAVIAL A 1) using the EpiTect Whole Bisulfitome Kit. The methylation pattern is closely resembled by the WBA-DNA. As a control EpiTect Methylated Control DNA, EpiTect unmethylated Control DNA (both pre-bisulfite converted) and a mixture of both were used, showing absolutely identical methylation patterns. Data kindly provided by Hany Ezzeldin, Mayo Clinic, Rochester, USA.

Summary

QIAGEN offers the first complete solution for standardized DNA methylation analysis.

- Fast and complete bisulfite conversion with minimal DNA damage through a unique DNA Protect mechanism
- Reliable whole bisulfitome amplification of bisulfite treated DNA to overcome limitations in DNA amounts
- Flexible primer design for highly specific MSP through a novel genetically engineered HotStarTaq® d-Tect Polymerase
- Sensitive detection of small changes in DNA methylation by optimized MethyLight PCR reaction chemistry
- Standardization through ready-to-use pre-converted control DNAs, also suitable as quantitative DNA standards
- Highest quality standard Bisulfite Sequencing Service providing sequence information and methylation status quantification

With its newly introduced EpiTect solutions, QIAGEN makes available standardized, pre-analytical and analytical solutions from DNA sample collection, stabilization and purification, to bisulfite conversion and real-time or end-point PCR methylation analysis or sequencing.

Learn more about QIAGEN's portfolio for epigenetics. Visit www.qiagen.com/epigenetics.

References
1. Eads, C. A. et al. (2000). MethyLight: A high-throughput assay to measure DNA methylation. Nucleic Acids Res. 28, e32.
2. Cottrell et al. (2007). Discovery and validation of 3 novel DNA methylation markers of prostate cancer prognosis. The Journal of Urology. 177, 1753.

All kits are intended for research use. No claim or representation is intended to provide information for the diagnosis, prevention, or treatment of a disease.

1053230 05/2008