Improved DNA methylation analysis through prevention of DNA fragmentation during bisulfite treatment

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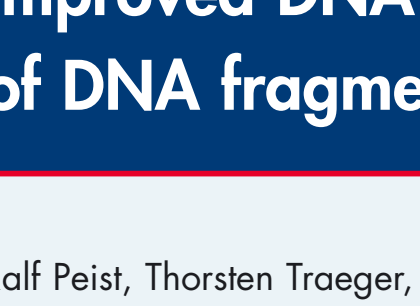
Introduction

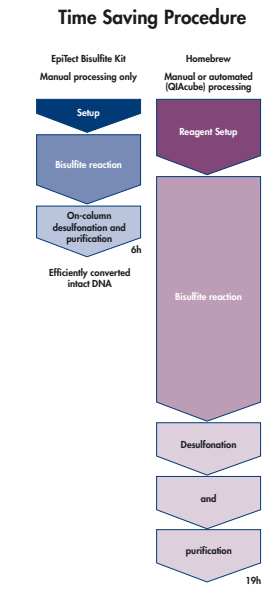
QIAGEN has developed a fast and streamlined 6-hour procedure for complete bisulfite DNA conversion and purification of 2 µg down to as little as 1 ng DNA. The new EpiTect® Bisulfite Kit prevents DNA fragmentation during the bisulfite conversion reaction using an innovative and unique DNA Protect Buffer.

The streamlined purification procedure utilizes EpiTect spin columns and optimized buffers, and includes a convenient on-column desulfonation step for easier handling. This procedure can be easily processed either manually or automated using the QIAcube.* High recovery rates and reproducible yields of efficiently converted pure DNA are provided. The DNA is suited for direct use in all techniques currently utilized for DNA methylation analysis, even after long-term DNA storage.



A DNA Protect Buffer: A unique solution preventing DNA fragmentation during bisulfite treatment of DNA. The innovative formulation protects the DNA from the harsh conditions required for conversion (i.e., low pH, high temperature, and high bisulfite salt concentrations) and facilitates the formation of single-stranded DNA, enabling complete bisulfite conversion. B The pH-indicator dye in the DNA Protect Buffer enables visualization of complete reaction mixing thereby ensuring the correct pH is achieved for complete cytosine conversion.

* QIAcube procedure coming soon.



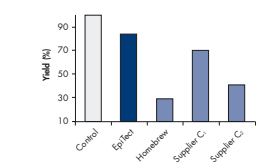
Comparison of a typical "homebrew" bisulfite conversion and the EpiTect Bisulfite Kit procedure

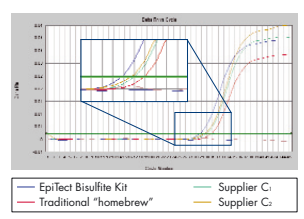
High yields of converted DNA and fragmentation prevention

Each EpiTect Bisulfite Kit reaction converts 1 ng – 2 µg of DNA with equal efficiency. The novel bisulfite treatment and purification procedure, with convenient on-column desulfonation, and optimized buffers for purification of single-stranded DNA enable the recovery of high yields of converted DNA. The highly efficient cytosine conversion is apparent by the low C_T values obtained when amplifying converted DNA using real-time PCR.

The DNA Protect Buffer-mediated prevention of fragmentation during the bisulfite treatment procedure enables subsequent amplification and analysis of large PCR fragments.

High Yields of Converted DNA

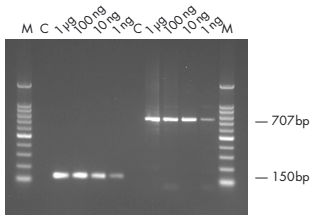




The EpiTect Bisulfite Kit was compared with a number of sodium bisulfite conversion techniques. A 1 µg genomic DNA was treated with sodium bisulfite and purified (according to manufacturer's instructions). DNA yield was determined using OD_{260} measurement. Control: 1 µg untreated genomic DNA. B 1 ng converted DNA was used in a real-time PCR assay that only detects converted DNA. Assays were performed using the QuantiTect® Probe PCR Kit on an ABI® 7500 real-time system. Lower C_T values generated using the EpiTect Bisulfite Kit indicate higher conversion efficiency compared with all other methods tested.

Bisulfite conversion method	C_T value
EpiTect Bisulfite Kit	29.26
Homebrew	31.25
Supplier C_1	30.75
Supplier C_2	30.36

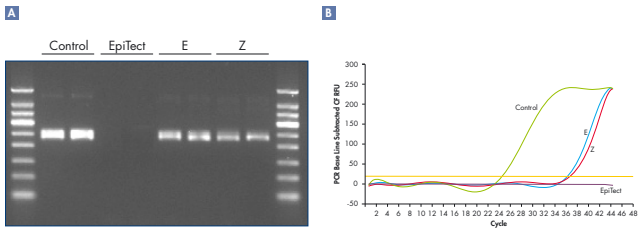
Amplification of large PCR Products from Minimal Amounts of Template DNA



Human genomic DNA was purified from blood using the QIAamp® DNA Blood Mini Kit, and various amounts (1 ng – 1 µg) were converted using the EpiTect Bisulfite Kit. PCR was performed using the HotStarTaq® Master Mix Kit and 2 sets of primers designed to amplify converted DNA. 5 µl of each PCR was loaded onto a 1.3 % agarose gel. As little as 1 ng DNA is sufficient for conversion using the EpiTect Bisulfite Kit. **C**: untreated genomic DNA (negative control). **M**: marker.

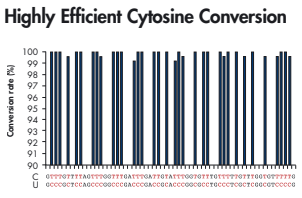
Complete conversion

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 (which provides the optimum pH for complete conversion without the need for tedious pH-adjustment) and DNA Protect Buffer (facilitating the single-strand DNA formation necessary for bisulfite conversion) enables maximal conversion rates to be achieved.



1 µg genomic DNA was converted using the EpiTect Bisulfite Kit or competitor kits (E and Z) according to the manufacturers' instructions. Comparable amounts of converted DNA were analyzed either by A end-point PCR or B SYBR® Green based real-time PCR. The PCR assays specifically amplified a 244 bp amplicon from untreated or unconverted genomic DNA. A The expected amplicon was amplified from the control DNA (untreated genomic DNA). Lanes E and Z show the same amplicons, clearly indicating a significant proportion of unconverted DNA is present after bisulfite treatment with the respective competitor kits. EpiTect converted DNA is not amplified indicating complete DNA conversion. B The green line correlates to the genomic DNA control. The blue and red lines correspond to competitor E and Z respectively. The pink line from the EpiTect treated DNA again indicates complete DNA conversion.

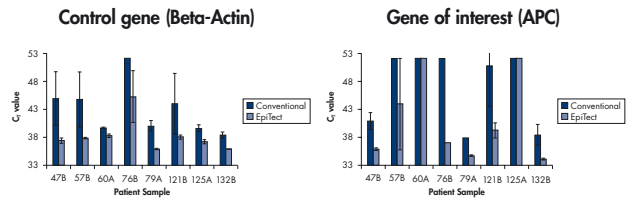
Highly Efficient Cytosine Conversion



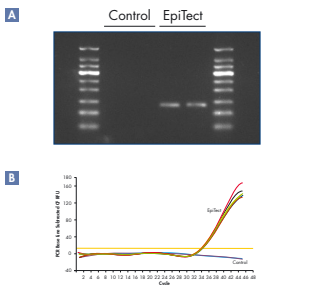
Cytosine to thymine conversion rates of 99.4–99.8% were obtained using the EpiTect Bisulfite Kit. Over 900 clones (generated by PCR from 6 independent EpiTect Bisulfite reactions [1 µg DNA each] and cloned using the QIAGEN PCR Cloning Kit) were analyzed. Over 150,000 nt of the 450,000 nt sequenced contained an unmethylated cytosine (lower sequence) that was converted into a thymine (upper sequence). (The sequence shown is a small portion of the total sequence analyzed.) **C**: converted DNA; **U**: untreated DNA.

Optimized protocols for bisulfite treatment of DNA from FFPE or microdissected tissue samples

Determination of methylation patterns in DNA from precious and limited sample materials (e.g., microdissected and FFPE [formalin-fixed, paraffin-embedded] tissue) is of specific interest for the successful establishment of valuable disease predicting biomarkers. These sample types are especially challenging for bisulfite conversion due to the limited amount of DNA available (caused by the degraded nature of the DNA and/or limited number of cells). The EpiTect Bisulfite Kit addresses these challenges with optimized protocols for these sample types.



The methylation status of DNA from laser-microdissections from paraffin slices of oesophagus tissue samples from 8 patients was determined. DNA was isolated, divided in 2 aliquots and each aliquot was bisulfite treated using either a conventional bisulfite treatment method or a combination of the EpiTect Bisulfite Kit protocols for "low concentration DNA solutions" and "FFPE samples". C_T values in a control gene (beta actin) and the gene of interest (APC) were determined in parallel real-time PCR assays for both aliquots. The assays detected methylated and converted DNA only. In the control, the results with the EpiTect Bisulfite Kit were clearly improved as indicated by lower C_T values and reduced error bars. In sample 76B, the conventional method failed whereas the EpiTect treated sample still gave a measurable C_T value. With APC, the results were even more convincing. Again all EpiTect treated samples showed lower C_T values. Non-methylation of the APC gene was detected in samples 60A and 125A after treatment with both bisulfite methods, however only after EpiTect conversion was the methylation status was detectable in samples 57B and 76B. Data kindly provided by Ute Warnecke-Eberz, Dept. Visceral and Vascular Surgery, University Hospital Cologne, Germany.



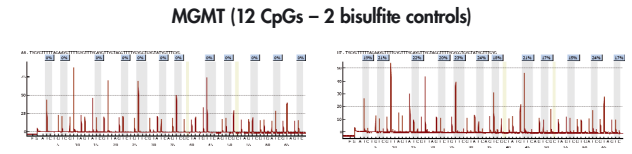
1 µg DNA isolated from an FFPE sample using the QIAamp DNA Mini Kit, was converted with the EpiTect Bisulfite Kit using the FFPE protocol. 1 µl of the 40 µl eluate was analyzed by A end-point PCR and B TaqMan® probe-based real-time PCR. Both assays for the Glutathione S-Transferase gene, were specific for converted DNA. In each application the unconverted genomic DNA was not amplified.

Downstream applications and long-term storage of converted DNA

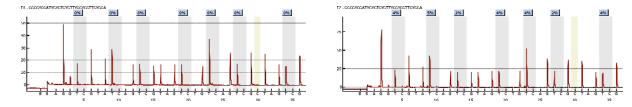
A combination of the gentle DNA protection provided by DNA Protect Buffer and the optimized purification procedure (which efficiently removes impurities that inhibit downstream applications) enables long-term storage of the converted DNA for at least 12 months without affecting the DNA quality.

EpiTect Bisulfite converted DNA can be used in all downstream applications, including methylation-specific PCR, real-time PCR analysis, bisulfite sequencing, COBRA, and Pyrosequencing®.

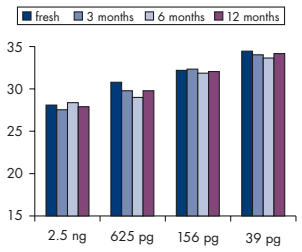
MGMT (12 CpGs – 2 bisulfite controls)



p16 (7 CpGs – 1 bisulfite control)



To develop lung cancer-specific biomarkers 1.5 µg DNA, isolated from frozen biopsy materials from lung cancer tumour patients was bisulfite treated using the EpiTect Bisulfite Kit, PCR amplified, and successfully pyrosequenced for 2 loci. The MGMT locus was analyzed for 12 CpGs and the p16 locus for 7 CpGs. Each locus contained 1 or 2 additional bisulfite controls (non-CpG cytosines that are always unmethylated; highlighted in yellow). The missing peaks in these positions of the pyrograms indicate 100% conversion of these control cytosines. Data kindly provided by T. Liloglou, Cancer Research Centre, University of Liverpool, UK



A real-time PCR assay using the QuantiTect Probe Kit and converted DNA-specific primers was performed on various amounts of DNA converted using the EpiTect Bisulfite Kit before and after DNA storage in elution buffer at –20°C for 3, 6, and 12 months. Even low concentrations of DNA converted using the EpiTect Bisulfite Kit show no loss of DNA quality upon storage.

Summary and future developments

EpiTect Bisulfite Kits provide:

- **Fast and reliable results** — streamlined 6-hour procedure and convenient prealiquoted buffers save time and resources
- **Complete DNA conversion** — conversion of ≥99% unmethylated cytosines
- **Unique DNA protection** — innovative DNA Protect Buffer limits DNA degradation for increased sensitivity
- **Single column-based desulfonation and purification procedure** — fast high yields and pure DNA enabling long-term storage of converted DNA
- **Optimized protocols** — for the conversion of DNA from FFPE tissue samples and low concentration DNA solutions

New features soon to be added to EpiTect Bisulfite Kits:

- **Vacuum protocol for manual processing** — a centrifugation-free alternatively to the current spin column protocol
- **Walkaway automation using the new QIAcube** — complete automation of the existing kit format saves time and requires no SOP changes
- **Increase throughput with a 96-well EpiTect procedure** — high throughput spin or vacuum processing using our established 96-well purification technology

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