December 2014

EpiTect® Plus Bisulfite Conversion Handbook

For sample lysis and complete bisulfite conversion/cleanup of DNA from FFPE, blood, cultured cells, or tissue samples, optimized for methylation analysis

epigenomics

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Contents

Kit Contents

EpiTect Plus DNA Bisulfite Kit	**(48)**
Catalog number	**59124**
Number of preps	**48**
Bisulfite Mix (aliquots for 8 reactions)	6 tubes
DNA Protect Buffer	1.9 ml
RNase-Free Water	3 x 1.9 ml
MinElute® DNA Spin Columns	48
Collection Tubes (2 ml)	96
Buffer BL*	31 ml
Buffer BW (concentrate)	2 x 13 ml
Buffer BD (concentrate)	3 ml
Buffer EB	15 ml
Carrier RNA	310 µg
Handbook	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See "Safety Information" (page 8).

EpiTect Plus FFPE Bisulfite Kit	**(48)**
Catalog number	**59144**
Number of preps	**48**
EpiTect Plus FFPE Lysis Kit	
Deparaffinization Solution	8 ml
Lysis Buffer FTB	0.8 ml
Proteinase K	1.4 ml
EpiTect Plus DNA Bisulfite Kit	
Bisulfite Mix (aliquots for 8 reactions)	6 tubes
DNA Protect Buffer	1.9 ml
RNase-Free Water	3 x 1.9 ml
MinElute DNA spin columns	48
Collection Tubes (2 ml)	96
Buffer BL*	31 ml
Buffer BW (concentrate)	2 x 13 ml
Buffer BD (concentrate)	3 ml
Buffer EB	15 ml
Carrier RNA	310 µg
Handbook	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See "Safety Information" (page 8).

EpiTect Plus LyseAll Bisulfite Kit	**(48)**
Catalog number	**59164**
Number of preps	**48**
EpiTect Plus LyseAll Lysis Kit	
Buffer EL	25 ml
Lysis Buffer FTB	0.8 ml
Proteinase K	1.4 ml
EpiTect Plus DNA Bisulfite Kit	
Bisulfite Mix (aliquots for 8 reactions)	6 tubes
DNA Protect Buffer	1.9 ml
RNase-Free Water	3 x 1.9 ml
MinElute DNA spin columns	48
Collection Tubes (2 ml)	96
Buffer BL*	31 ml
Buffer BW (concentrate)	2 x 13 ml
Buffer BD (concentrate)	3 ml
Buffer EB	15 ml
Carrier RNA	310 µg
Handbook	1

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See "Safety Information" (page 8).

Shipping and Storage

The EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, and EpiTect Plus LyseAll Bisulfite Kit are shipped at room temperature (15–25°C). Upon arrival, the MinElute DNA spin columns, DNA Protect Buffer, and Buffer BD should be stored at 2–8°C. However, short-term storage (up to 4 weeks) at room temperature does not affect their performance. All other buffers and the Bisulfite Mix should be stored at room temperature and are stable for at least 6 months under these conditions. Dissolved Bisulfite Mix can be stored at –15 to –30°C for up to 4 weeks.

Lyophilized carrier RNA can be stored at room temperature for 1 year. Carrier RNA should only be dissolved in RNase-free water. Dissolved carrier RNA should be added immediately to Buffer BL, as described in "Preparation of reagents" on page 19. This solution should be prepared fresh, and is stable at 2–8°C for up to 48 hours. Unused portions of carrier RNA dissolved in RNase-free water should be frozen in aliquots at –15 to –30°C and can be stored for up to 1 year.

Product Use Limitations

The EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, and EpiTect Plus LyseAll Bisulfite Kit are intended for molecular biology applications. These products are not intended for the diagnosis, prevention, or treatment of a disease.

All due care and attention should be exercised in the handling of the products. We recommend all users of QIAGEN® products to adhere to the NIH guidelines that have been developed for recombinant DNA experiments, or to other applicable guidelines.

Product Warranty and Satisfaction Guarantee

QIAGEN guarantees the performance of all products in the manner described in our product literature. The purchaser must determine the suitability of the product for its particular use. Should any product fail to perform satisfactorily due to any reason other than misuse, QIAGEN will replace it free of charge or refund the purchase price. We reserve the right to change, alter, or modify any product to enhance its performance and design. If a QIAGEN product does not meet your expectations, simply call your local Technical Service Department or distributor. We will credit your account or exchange the product — as you wish. Separate conditions apply to QIAGEN scientific instruments, service products, and to products shipped on dry ice. Please inquire for more information.

A copy of QIAGEN terms and conditions can be obtained on request, and is also provided on the back of our invoices. If you have questions about product specifications or performance, please call QIAGEN Technical Services or your local distributor (see back cover or visit www.qiagen.com).

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, and EpiTect Plus LyseAll Bisulfite Kit is tested against predetermined specifications to ensure consistent product quality.

Technical Assistance

At QIAGEN, we pride ourselves on the quality and availability of our technical support. Our Technical Service Departments are staffed by experienced scientists with extensive practical and theoretical expertise in sample and assay technologies and the use of QIAGEN products. If you have any questions or experience any difficulties regarding the EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, EpiTect Plus LyseAll Bisulfite Kit, or QIAGEN products in general, please do not hesitate to contact us.

QIAGEN customers are a major source of information regarding advanced or specialized uses of our products. This information is helpful to other scientists as well as to the researchers at QIAGEN. We therefore encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

For technical assistance and more information, please see our Technical Support Center at www.qiagen.com/Support or call one of the QIAGEN Technical Service Departments or local distributors (see back cover or visit www.qiagen.com).

Safety Information

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, please consult the appropriate safety data sheets (SDSs). These are available online in convenient and compact PDF format at www.qiagen.com/safety where you can find, view, and print the SDS for each QIAGEN kit and kit component.

CAUTION: DO NOT add bleach or acidic solutions directly to waste containing Buffer BL.

Buffer BL contains a guanidine salt, which can form highly reactive compounds when combined with bleach. If liquid containing this buffer is spilled, clean with suitable laboratory detergent and water.

Introduction

Epigenetics describes the study of heritable changes in gene function that occur without a change in the nuclear DNA sequence. In addition to RNA-associated silencing and histone modification, a major epigenetic mechanism in higher-order eukaryotes is DNA methylation.

Methylation of DNA occurs on cytosine residues, especially on CpG dinucleotides enriched in small regions of DNA (<500 bp). These regions, with a GC content greater than 55%, are known as CpG islands. They are usually clustered around the regulatory region of genes and can affect the transcriptional regulation of these genes. Methylation of CpG islands by DNA methylases has been shown to be associated with gene inactivation and plays an important role in the development of cancer and cell aging. Reversal of DNA methylation at these sites is a potential therapeutic strategy as this reversal may restore expression of transcriptionally silenced genes. In addition to CpG, methylated cytosine residues are also found at CpNpG or CpNpN sites (N = A, T, or C) in plants.

The methylation status of a DNA sequence can best be determined using sodium bisulfite. Incubation of the target DNA with sodium bisulfite results in conversion of unmethylated cytosine residues into uracil, leaving the methylated cytosines unchanged. Therefore, bisulfite treatment gives rise to different DNA sequences for methylated and unmethylated DNA (see below).

	Original sequence	**After bisulfite treatment**
Unmethylated DNA	N-**C**-G-N-**C**-G-N-**C**-G-N	N-**U**-G-N-**U**-G-N-**U**-G-N
Methylated DNA	N-**C**-G-N-**C**-G-N-**C**-G-N	N-**C**-G-N-**C**-G-N-**C**-G-N

The most critical step for correct determination of a methylation pattern is the complete conversion of unmethylated cytosines. This is achieved by incubating the DNA in high bisulfite salt concentrations at high temperature and low pH. These harsh conditions usually lead to a high degree of DNA fragmentation and subsequent loss of DNA during purification. Purification is necessary to remove bisulfite salts and chemicals used in the conversion process that inhibit sequencing. Common bisulfite procedures usually require high amounts of input DNA to compensate for DNA degradation during conversion and DNA loss during purification that often lead to low DNA yield, highly fragmented DNA, and irreproducible conversion rates.

The EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, and EpiTect Plus LyseAll Bisulfite Kit now provide a fast and streamlined procedure for efficient conversion and purification of DNA prepared from FFPE, blood, cell, or

tissue samples. The kits contain preparation buffers that make it unnecessary to isolate the DNA prior to bisulfite treatment. DNA fragmentation is prevented during the bisulfite conversion by the unique DNA Protect Buffer, which contains a pH-indicator dye as a mixing control in reaction setup, allowing confirmation of the correct pH for cytosine conversion.

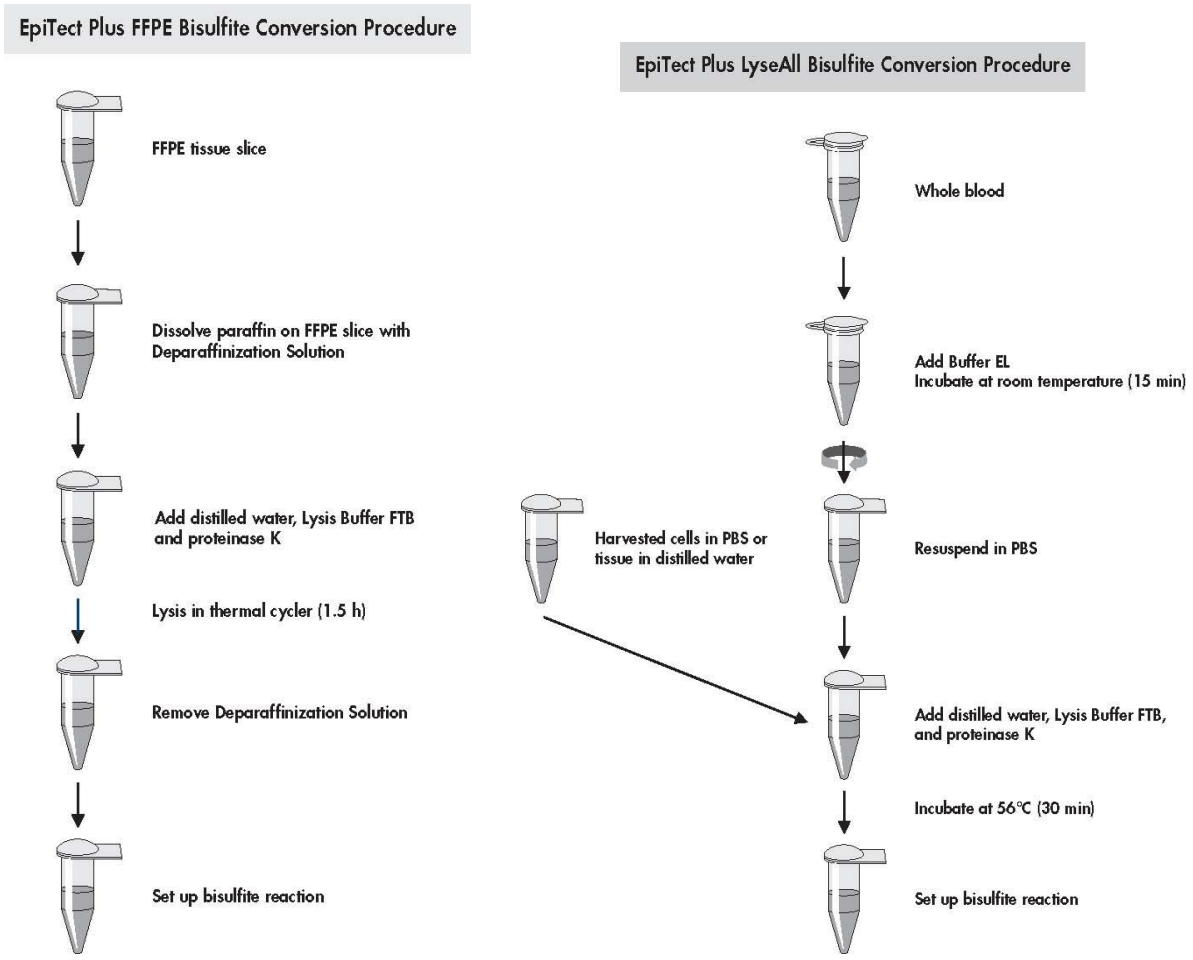
Furthermore, the bisulfite thermal cycling program provides an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent sulfonation and cytosine deamination, enabling high cytosine conversion rates of over 99%. Desulfonation, the final step in chemical conversion of cytosines, is achieved by a convenient on-column step included in the purification procedure. The final elution volume can be as low as 10 µl though this may result in a yield reduction.

Principle and procedure

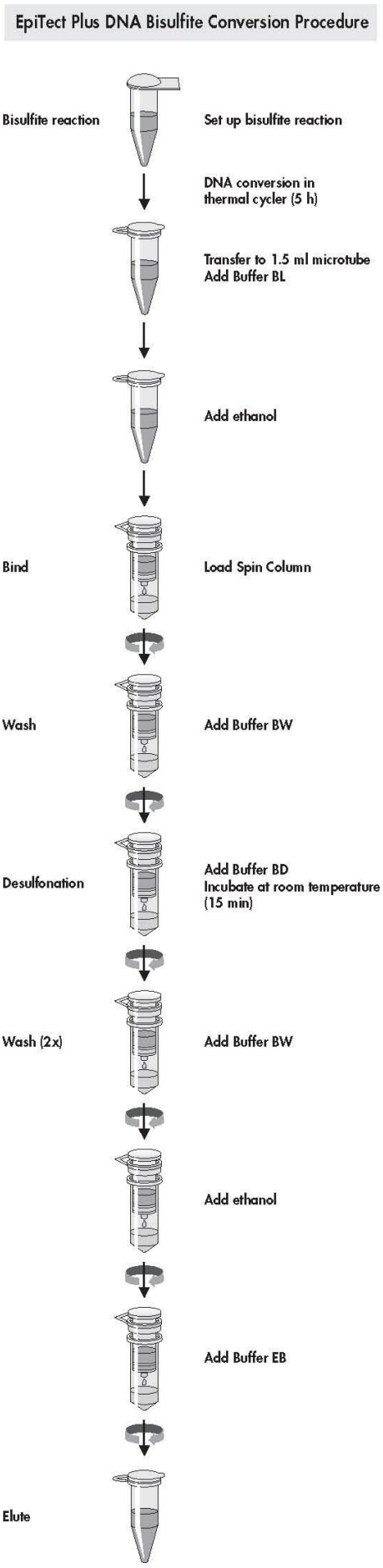
The EpiTect Plus bisulfite conversion procedure comprises a few simple steps: preparation of DNA from sample, bisulfite-mediated conversion of unmethylated cytosines; binding of the converted single-stranded DNA to the membrane of an MinElute DNA spin column; washing; desulfonation of membrane-bound DNA; washing of the membrane-bound DNA to remove the desulfonation agent; and elution of the pure, converted DNA from the spin column. Sample preparation is different for FFPE slices, whole blood, and cell cultures or tissues, whereas the procedure for bisulfite conversion of extracted DNA is the same for all sample types (see flowcharts on pages 12–13). The eluted, bisulfite converted DNA is suited for all techniques currently used for the analysis of DNA methylation, including PCR, real-time PCR, MSP-PCR, bisulfite sequencing (direct and cloning), COBRA, and Pyrosequencing®.

Bisulfite Mix

The Bisulfite Mix is conveniently provided in separate aliquots (8 conversion reactions per aliquot). The sodium bisulfite in each aliquot is supplied in a unique formulation that provides the optimal pH for complete conversion of cytosine to uracil, without the need for tedious pH adjustment. The Bisulfite Mix must be dissolved in 800 µl RNase-free water before use (see "Preparation of reagents", page 19). Dissolved Bisulfite Mix can be stored at –15 to –30°C for up to 4 weeks.



After setting up the bisulfite reaction, continue with the procedure shown in the flowchart “EpiTect Plus DNA Bisulfite Conversion Procedure”, page 13).


EpiTect Plus DNA Bisulfite Conversion Procedure
Bisulfite reaction
Set up bisulfite reaction
DNA conversion in thermal cycler (5 h)
Transfer to 1.5 ml microtube
Add Buffer BL
Add ethanol
Bind
Load Spin Column
Wash
Add Buffer BW
Desulfonation
Add Buffer BD
Incubate at room temperature (15 min)
Wash (2x)
Add Buffer BW
Add ethanol
Add Buffer EB
Elute

DNA Protect Buffer

DNA Protect Buffer is uniquely formulated to prevent the fragmentation usually associated with bisulfite treatment of DNA at high temperatures and low pH values. It also provides effective DNA denaturation, resulting in the single-stranded DNA necessary for complete cytosine conversion. In addition, the DNA Protect Buffer contains a pH indicator dye as a mixing control and to allow confirmation of the correct pH for cytosine conversion.

Bisulfite thermal cycling

The thermal cycling program provides an optimized series of incubation steps necessary for thermal DNA denaturation and subsequent sulfonation and cytosine deamination, enabling highest cytosine conversion rates.

Carrier RNA

Carrier RNA is provided to enhance the binding of small quantities of DNA to the MinElute DNA spin-column membrane. If using more than 100 ng of genomic DNA template, it is not necessary to use carrier RNA, though we strongly recommend its use when processing fragmented DNA or DNA isolated from formalin-fixed, paraffin-embedded (FFPE) tissues. Carrier RNA should be dissolved in RNase-free water before use (see "Preparation of reagents", page 19).

Optimized buffers

The EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, and EpiTect Plus LyseAll Bisulfite Kit contain carefully optimized buffers enabling effective lysis of samples (FFPE, cells, tissue, whole blood) in DNA isolation procedures, deparaffinization of FFPE tissues, and maximum cytosine conversion and subsequent DNA purification. Buffer BL promotes binding of the converted single-stranded DNA to the MinElute DNA spin-column membrane. Subsequently, the membrane-bound DNA is washed using Buffer BW, which efficiently removes residual sodium bisulfite. After desulfonation using Buffer BD, the DNA is further desalted using Buffer BW before elution from the spin-column membrane using Buffer EB.

Storage stability of converted and purified DNA

DNA converted and purified using the EpiTect Plus DNA Bisulfite Kit, EpiTect Plus FFPE Bisulfite Kit, or EpiTect Plus LyseAll Bisulfite Kit can be stored at –15 to –30°C for at least 18 months without decrease in quality or conversion.

Description of protocols

The choice of kit and the corresponding protocol to use is determined by the type of starting material. The protocols are interconnected and the actual bisulfite conversion reaction is the same for all samples, as illustrated in Figure 1. The standard protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA", on page 21, can be used for conversion of 1 ng – 2 µg DNA in a volume of up to 20 µl or 1–500 ng in a maximum volume of 40 µl.

Formalin-fixed, paraffin-embedded (FFPE) tissues are processed with the EpiTect Plus FFPE Bisulfite Kit and the protocol "Sample Lysis and Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Prepared from FFPE Tissue Samples", on page 26. This kit consists of the EpiTect Plus FFPE Lysis Kit, containing specialized buffers for efficient deparaffinization and lysis of the tissue sample, and the EpiTect Plus DNA Bisulfite Kit for the bisulfite conversion of the extracted DNA. The protocol includes an optimized step to facilitate binding of DNA and is used with single slices of FFPE tissue. Each slice should be approximately10 µm thick and have a surface area no greater than 100 mm^2.

Whole blood, cultured cells, or tissue samples are processed with the EpiTect Plus LyseAll Bisulfite Kit and the protocol "Sample Lysis and Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Prepared from Whole Blood, Cultured Cells, or Tissue", on page 29. This kit consists of the EpiTect Plus LyseAll Lysis Kit, including an innovative lysis buffer, and the EpiTect Plus DNA Bisulfite Kit for the bisulfite conversion of the extracted DNA. The protocol includes an optimized step to facilitate binding of DNA and can be used with 0.5–20 µl blood or 10–10^5 cells (as little as 60 pg of DNA).

All protocols achieve the same cytosine conversion rates and lead to equal DNA recoveries after purification of converted DNA, independent of DNA starting amounts.

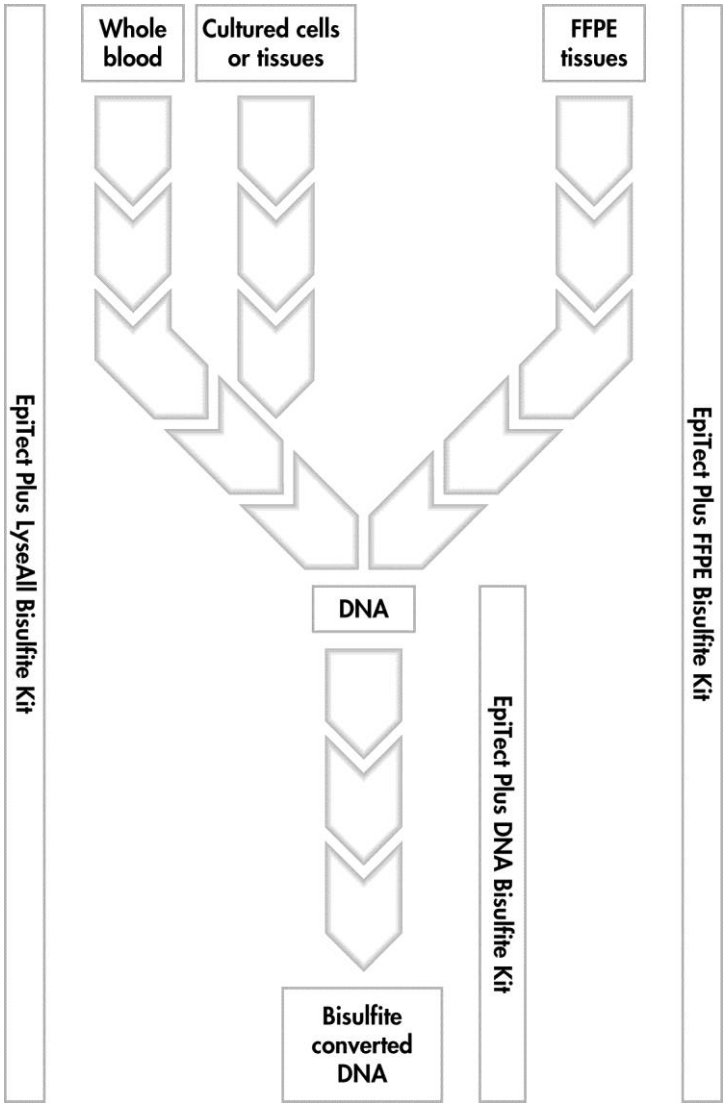


Figure 1. Interconnected protocols of EpiTect Plus Bisulfite Kits. Depending on sample type, the bisulfite conversion of DNA calls for the use of a specific kit and protocol. Kits contain buffers optimized for DNA extraction and all reagents for the DNA bisulfite conversion.

Equipment and Reagents to Be Supplied by User

When working with chemicals, always wear a suitable lab coat, disposable gloves, and protective goggles. For more information, consult the appropriate safety data sheets (SDSs), available from the product supplier.

- Ethanol (molecular biology grade, 96–100%)*
- Pipets and pipet tips (we recommend pipet tips with aerosol barriers for preventing cross-contamination)
- 0.2 ml reaction tubes or 8-well strips
- Thermal cycler with heated lid (since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure)
- 1.5 ml microcentrifuge tubes for elution steps (available from Brinkmann [Safe-Lock, cat. no. 022363204], Eppendorf® [Safe-Lock, cat. no. 0030 120.086], or Sarstedt [Safety Cap, cat. no. 72.690])†
- Microcentrifuge
- **Optional**: Heating block, thermomixer, or heated orbital incubator

* Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

† This is not a complete list of suppliers and does not include many important vendors of biological supplies.

Important Notes

Yield and size of DNA

The yield of DNA purified after bisulfite conversion depends on the amount of DNA and source of the starting material. DNA can be prepared from FFPE tissue (a single slice 10 µm thick, with a surface area no greater than 100 mm^2), 0.5–20 µl blood, or 10–10^5 cells.

The EpiTect Plus DNA Bisulfite Kit is suited for DNA input amounts ranging from 1 ng to 2 µg, with high levels of DNA recovery throughout this range.

The size of the template DNA can vary between 500 bp (in laser microdissections) and 30 kb (fresh samples or blood). DNA purified from serum, urine, or FFPE tissue may be <500 bp in length.

Note: The purified sample will contain considerably more carrier RNA than DNA. Carrier RNA does not influence downstream applications.

If purifying bisulfite-treated DNA originating from very small sample amounts or that is very fragmented (e.g., from biopsies or FFPE tissues), we strongly recommend adding carrier RNA to Buffer BL (see "Preparation of reagents", page 19).

Starting material

The bisulfite conversion also depends on the nature of DNA used as starting material. Genomic DNA should be used for bisulfite treatment without any previous restriction digest step.

If working with plasmid DNA, linearize the DNA first due to the very quick reannealing of the single-stranded DNA after the denaturation step.

Handling of MinElute DNA spin columns

Due to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling MinElute DNA spin columns to avoid cross-contamination between sample preps:

- Carefully pipet the sample or solution into the MinElute DNA spin column without wetting the rim of the column. Avoid touching the MinElute DNA spin column membrane with the pipet tip.
- Always change pipet tips between liquid transfers. We recommend the use of aerosol-barrier pipet tips.
- Open one MinElute DNA spin column at a time, and take care to avoid generating aerosols.
- Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.

Centrifugation

MinElute DNA spin columns will fit into most standard 1.5–2 ml microcentrifuge tubes. A set of 2 ml collection tubes is supplied for the dry centrifugation step.

All centrifugation steps should be carried out at room temperature (15–25°C).

Processing MinElute DNA spin columns in a microcentrifuge

- Always close MinElute DNA spin columns before placing them in the microcentrifuge.
- For efficient parallel processing of multiple samples, we recommend filling a rack with the collection tubes into which the MinElute DNA spin columns can be transferred after centrifugation. Collection tubes can be used several times.

Preparation of reagents

Buffer BW

Add 30 ml ethanol (96–100%) to Buffer BW and store at room temperature (15–25°C). Invert the bottle several times before starting the procedure.

Buffer BD

Add 27 ml ethanol (96–100%) to Buffer BD and store at 2–8°C. Invert the bottle several times before starting the procedure and make sure to close the bottle immediately after use. White precipitates may form in the Buffer BD–ethanol mix after some storage time. These precipitates will not affect the performance of Buffer BD. However, avoid transferring precipitates to the MinElute DNA spin column.

Carrier RNA

Add 310 µl RNase-free water to the lyophilized carrier RNA (310 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing. When processing 48 samples in parallel, add the complete volume of dissolved carrier RNA to the bottle of Buffer BL, and check the box on the bottle lid label. If processing fewer samples, split the dissolved carrier RNA into conveniently sized aliquots (e.g., 50 µl) and store at –15 to –30°C. Aliquots can be stored for up to 1 year. If fewer than 48 conversions will be performed in a 2-week period, only make enough Buffer BL–carrier RNA solution as required (see Table 1, page 20, for example volumes). Carrier RNA enhances binding of DNA to the spin-column membrane, especially if there are very few target molecules in the sample. Carrier RNA is not necessary if >100 ng DNA is used.

Add dissolved carrier RNA to Buffer BL. Calculate the volume of Buffer BL and dissolved carrier RNA required for the number of samples to be processed (see Table 1 for example volumes). If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.

Table 1. Carrier RNA and Buffer BL volumes

Number of samples	1	4	8	16	24	48
Volume of Buffer BL*	620 µl	2.5 ml	5 ml	10 ml	15 ml	31 ml
Volume of carrier RNA solution†	6.2 µl	25 µl	50 µl	100 µl	150 µl	310 µl

* The volumes given contain a 10% surplus for pipetting inaccuracies.

† Resulting in a final concentration of 10 µg/ml carrier RNA in Buffer BL.

Protocol: Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA

This protocol enables bisulfite conversion of DNA amounts of 1 ng–2 µg in a volume of up to 20 µl (high concentration), or 1–500 ng in a maximum volume of 40 µl (low concentration). Conversion of high-concentration or low-concentration DNA samples differs only in the setup of the bisulfite reactions (see Table 2, page 22). All other protocol steps are the same.

Important points before starting

- Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, dissolved Bisulfite Mix can be stored at –15 to –30°C for up to 4 weeks without any loss of performance.
- DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
- Perform all centrifugation steps at room temperature (15–25°C).

Things to do before starting

- Prepare the kit reagents as described in "Preparation of reagents", page 19.
- Equilibrate samples and buffers to room temperature.
- **Optional:** Set a thermomixer, heating block, or heated orbital incubator to 60°C to enable the Bisulfite Mix to dissolve.

Procedure

Bisulfite conversion of DNA

1. **Thaw DNA to be used in the bisulfite reactions. Dissolve the required number of aliquots of Bisulfite Mix by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.**

   Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.

   Note: Do not place dissolved Bisulfite Mix on ice.

2. **Prepare the bisulfite reactions in 200 µl PCR tubes (not provided) according to Table 2, page 22. Add each component in the order listed.**

   Note: The combined volume of DNA solution and RNase-free water must total 20 µl for high-concentration samples, and 40 µl for low-concentration samples.

Table 2. Bisulfite reaction components

Component	High-concentration samples (1 ng – 2 µg) Volume per reaction (µl)	Low-concentration samples (1–500 ng) Volume per reaction (µl)
DNA solution	Variable* (maximum 20 µl)	Variable† (maximum 40 µl)
RNase-free water	Variable*	Variable†
Bisulfite Mix (dissolved), see step 1	85	85
DNA Protect Buffer	35	15
Total volume	**140**	**140**

* The combined volume of DNA solution and RNase-free water must total 20 µl.

† The combined volume of DNA solution and RNase-free water must total 40 µl.

3. **Close the PCR tubes and mix the bisulfite reactions thoroughly. Store the tubes at room temperature (15–25°C).**

   Note: DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.

4. **Perform the bisulfite DNA conversion using a thermal cycler. Program the thermal cycler according to Table 3, page 23.**

   The complete cycle should take approximately 5 h.

   Note: If using a thermal cycler that does not allow you to enter the reaction volume (140 µl), set the instrument to the largest volume setting available.

Table 3. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	25 min	60°C
Denaturation	5 min	95°C
Incubation	85 min (1 h 25 min)	60°C
Denaturation	5 min	95°C
Incubation	175 min (2 h 55 min)	60°C
Hold	Indefinite*	20°C

* Converted DNA can be left in the thermal cycler overnight without any loss of performance.

5. **Place the PCR tubes containing the bisulfite reactions into the thermal cycler. Start the thermal cycling incubation.**

   IMPORTANT: Since the bisulfite reaction is not overlaid with mineral oil, only thermal cyclers with heated lids are suitable for this procedure. It is important to use PCR tubes that close tightly.

   Converted DNA can be left in the thermal cycler overnight without any loss of performance.

Cleanup of bisulfite converted DNA

6. **Once the bisulfite conversion is complete, briefly centrifuge the PCR tubes containing the bisulfite reactions, and then transfer the complete bisulfite reactions to clean 1.5 ml microcentrifuge tubes.**

   Transfer of precipitates in the solution will not affect the performance or yield of the reaction.

7. **Add 310 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Preparation of reagents", page 19) to each sample. Mix the solutions by vortexing and then centrifuge briefly.**

   Note: Carrier RNA is not necessary when using >100 ng DNA.

8. **Add 250 µl ethanol (96–100%) to each sample. Mix the solutions by pulse vortexing for 15 s, and centrifuge briefly to remove the drops from inside the lid.**

9. **Place the necessary number of MinElute DNA spin columns and collection tubes in a suitable rack. Transfer the entire mixture from each tube from step 8 into the corresponding MinElute DNA spin column.**
10. **Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
11. **Add 500 µl Buffer BW (wash buffer) to each spin column, and centrifuge at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
12. **Add 500 µl Buffer BD (desulfonation buffer) to each spin column, and incubate for 15 min at room temperature (15–25°C).**

    If there are precipitates in Buffer BD, avoid transferring them to the spin columns.

    IMPORTANT: The bottle containing Buffer BD should be closed immediately after use to avoid acidification from carbon dioxide in air.

    Note: It is important to close the lids of the spin columns before incubation.
13. **Centrifuge the spin columns at maximum speed for 1 min. Discard the flow-through, and place the spin columns back into the collection tubes.**
14. **Add 500 µl Buffer BW to each spin column and centrifuge at maximum speed for 1 min. Discard the flow-through and place the spin columns back into the collection tubes.**
15. **Repeat step 14 once.**
16. **Add 250 µl ethanol (96–100%) to each spin column and centrifuge at maximum speed for 1 min.**
17. **Place the spin columns into new 2 ml collection tubes, and centrifuge the spin columns at maximum speed for 1 min to remove any residual liquid.**

    Optional: Place the spin columns with open lids into a clean 1.5 ml microcentrifuge tube (not provided) and incubate the columns for 5 min at 60°C in a heating block. This step enables the evaporation of any remaining liquid.
18. **Place the spin columns into clean 1.5 ml microcentrifuge tubes (not provided). Add 15 µl Buffer EB (elution buffer) directly onto the center of each spin-column membrane and close the lids gently.**
19. **Incubate the spin columns at room temperature for 1 min.**

20. Centrifuge for 1 min at 15,000 x *g* (12,000 rpm) to elute the DNA.

Note: As little as 10 µl Buffer EB can be used for elution if a higher DNA concentration is required, but the yield will be reduced by approximately 20%. Do not elute with less than 10 µl Buffer EB as the spin column membrane will not be sufficiently hydrated.

Note: If the purified DNA is to be stored for up to 24 h, we recommend storage at 2–8°C. For storage longer than 24 h, we recommend storage at –15 to –30°C.

Protocol: Sample Lysis and Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Prepared from FFPE Tissue Samples

This protocol is designed to be used with the EpiTect Plus FFPE Bisulfite Kit for processing DNA from formalin-fixed, paraffin-embedded (FFPE) tissue samples.

Important points before starting

- If using FFPE samples on slides, scrape the FFPE slice from the slide and proceed with step 1.
- Deparaffinization Solution solidifies at temperatures below 18°C. Incubate at 30°C to resolve.
- Precipitates may form in Lysis Buffer FTB. Make sure all precipitates are dissolved at 30°C.
- Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, reconstituted Bisulfite Mix can be stored at –15 to –30°C for up to 4 weeks without any loss of performance.
- DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
- Perform all centrifugation steps at room temperature (15–25°C).

Things to do before starting

- Prepare the kit reagents as described in “Preparation of reagents”, page 19.
- Equilibrate samples and buffers to room temperature.
- **Optional**: If performing the deparaffinization, lysis, and decrosslinking of the FFPE slice in a 1.5 ml tube (see step 1), set a heating block to 56°C.
- **Optional:** Set a thermomixer, heating block, or heated orbital incubator to 60°C to enable the Bisulfite Mix to dissolve.

Procedure

Lysis of FFPE slice

1. **Place a FFPE slice in a 200 µl reaction tube or 8-well strip (not provided) and add 150 µl Deparaffinization Solution.**

   The slice should be 10 µm thick, with a surface area ≤ 100 mm^2.

   Optional: The deparaffinization, lysis, and decrosslinking of the FFPE slice (steps 1–5) can be performed in a 1.5 ml tube (not provided).

2. **Flick or vortex the tube until all paraffin is dissolved.**
3. **Add 20 µl distilled water, 15 µl Lysis Buffer FTB, and 5 µl proteinase K.**

   Note: A master mix comprising of distilled water, Lysis Buffer FTB, and proteinase K may be prepared in advance.
4. **Vortex and briefly centrifuge the samples.**

   Note: The Deparaffinization Solution will form a layer above the Lysis Buffer FTB with the added proteinase K.
5. **Perform the lysis and decrosslinking using a thermal cycler. Program the thermal cycler according to Table 4.**

   Optional: If using 1.5 ml tubes, perform the lysis and decrosslinking in a thermal block. Incubate the tubes in a thermal block set to 56°C for 30 min to lyse the tissues. Ensure that tissues are completely lysed; if not, incubate the tubes for an additional 30 min at 56°C. Once all tissues are lysed, increase the temperature of the heating block to 95°C for 60 min for the decrosslinking step. Samples should be kept at room temperature (15–25°C). Proceed as soon as possible with the bisulfite conversion.

Table 4. Lysis thermal cycling conditions

Step	Time	Temperature
Lysis	30 min*	56°C
Decrosslinking	60 min	95°C

* Ensure that the tissue is completely lysed; if not, add an additional lysis step (30 min at 56°C).

6. **Place the PCR tubes containing the lysis reactions into the thermal cycler. Start the thermal cycling incubation.**

   Note: Samples should be kept at room temperature. Proceed as soon as possible with bisulfite conversion.

Bisulfite conversion of DNA

7. **Dissolve the required number of Bisulfite Mix aliquots by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.**

   Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.

   Note: Do not place dissolved Bisulfite Mix on ice.

8. **Remove the Deparaffinization Solution (approximately 130 µl) from the lysis reactions.**

   Remove as much Deparaffinization Solution as possible without disturbing the lysed sample material to make sufficient space in the reaction tube for the bisulfite reaction components (Table 5).

   Note: Small amounts of remaining Deparaffinization Solution have no effect on the bisulfite reaction.

9. **Prepare the bisulfite reactions by adding the reagents listed in Table 5. Add each component in the order listed.**

   Optional: If using 1.5 ml tubes for the deparaffinization, lysis, and decrosslinking, transfer the lysis reactions remaining from step 8 to 200 µl tubes. Add the reagents to the 200 µl tubes in the order listed in Table 5.

Table 5. Bisulfite reaction components

Component	Volume per reaction (µl)
Lysis reaction	Approx. 40
Bisulfite Mix (dissolved), see step 7	85
DNA Protect Buffer	15
Total volume	**140**

10. **Proceed to Protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA", starting with step 3 on page 22, to perform the bisulfite DNA conversion.**

Protocol: Sample Lysis and Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA Prepared from Whole Blood, Cultured Cells, or Tissue

This protocol is designed to be used with the EpiTect Plus LyseAll Kit for processing DNA from whole blood, cultured cells, or tissue samples.

Important points before starting

- Precipitates may form in Lysis Buffer FTB. Make sure all precipitates are dissolved at 30°C.
- Each aliquot of Bisulfite Mix is sufficient for 8 conversion reactions. If converting fewer than 8 DNA samples, reconstituted Bisulfite Mix can be stored at –15 to –30°C for up to 4 weeks without any loss of performance.
- DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Mix, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
- Perform all centrifugation steps at room temperature (15–25°C).

Things to do before starting

- Prepare the kit reagents as described in “Preparation of reagents”, page 19.
- Equilibrate samples and buffers to room temperature.
- **Optional:** Set a thermomixer, heating block, or heated orbital incubator to 60°C to enable the Bisulfite Mix to dissolve.

Procedure

Sample lysis

1. **If working with whole blood samples, follow steps 2–11; if working with cultured cells, follow steps 12–17; if working with tissue samples, follow steps 18–22.**
2. **Dilute the blood sample (maximum 20 µl) 1:20 with Buffer EL (e.g., add 19 µl Buffer EL to 1 µl blood).**
3. **Incubate at room temperature (15–25°C) for 10–15 min.**

   Note: Invert tubes several times during incubation.
4. **Centrifuge at maximum speed for 5 min.**

5. **Discard supernatant and add an additional 125 µl Buffer EL.**
   Make sure to not disturb the blood cell pellet while removing supernatant.
6. **Centrifuge at maximum speed for 1 min and discard the supernatant.**
7. **Resuspend pellet in 10 µl PBS and transfer into a 200 µl reaction tube or into 8-well strips (not provided).**
   IMPORTANT: Do not place cells on ice, as this will cause Lysis Buffer FTB to precipitate.
8. **Add 10 µl distilled water, 15 µl Lysis Buffer FTB, and 5 µl proteinase K.**
   Note: A master mix comprising of distilled water, Lysis Buffer FTB, and proteinase K may be prepared in advance.
9. **Vortex and briefly centrifuge the samples.**
10. **Incubate samples for 30 min at 56°C.**
11. **Proceed as soon as possible with bisulfite conversion, step 23.**
    Note: Samples should be kept at room temperature.
12. **Harvest cells according to your current protocol.**
13. **Resuspend cells in 10 µl PBS.**
    Note: Do not use more than 1 x 10^5 cells per 10 µl PBS.
    IMPORTANT: Do not place cells on ice, as this will cause Lysis Buffer FTB to precipitate.
14. **Add 10 µl distilled water, 15 µl Lysis Buffer FTB, and 5 µl proteinase K.**
    Note: A master mix comprising of distilled water, Lysis Buffer FTB, and proteinase K may be prepared in advance.
15. **Vortex and briefly centrifuge the samples.**
16. **Incubate samples for 30 min at 56°C.**
17. **Proceed as soon as possible with bisulfite conversion, step 23.**
    Note: Samples should be kept at room temperature (15–25°C).
18. **Add 20 µl distilled water to the tissue sample.**
    Note: Do not use more than 100 µg tissue per 20 µl distilled water.
    IMPORTANT: Do not place tissues on ice, as this will cause Lysis Buffer FTB to precipitate.
19. **Add 15 µl Lysis Buffer FTB and 5 µl proteinase K.**
    Note: A master mix comprising of Lysis Buffer FTB and proteinase K may be prepared in advance.
20. **Vortex and briefly centrifuge the samples.**
21. **Incubate samples for 30 min at 56°C.**

22. Proceed as soon as possible with bisulfite conversion, step 23.

Note: Samples should be kept at room temperature (15–25°C).

Bisulfite conversion

23. Dissolve the required number of Bisulfite Mix aliquots by adding 800 µl RNase-free water to each aliquot. Vortex until the Bisulfite Mix is completely dissolved. This can take up to 5 min.

Note: If necessary, heat the Bisulfite Mix–RNase-free water solution to 60°C and vortex again.

Note: Do not place dissolved Bisulfite Mix on ice.

24. Prepare the bisulfite reactions in 200 µl PCR tubes (not provided) according to Table 6. Add each component in the order listed.

Table 6. Bisulfite reaction components

Component	Volume per reaction (µl)
Lysis reaction	40
Bisulfite Mix (dissolved), see step 23	85
DNA Protect Buffer	15
Total volume	**140**

25. Proceed to Protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines in DNA", starting with Step 3 on page 22, to perform the bisulfite DNA conversion.

Troubleshooting Guide

This troubleshooting guide may be helpful in solving any problems that may arise. For more information, see also the Frequently Asked Questions page at our Technical Support Center: www.qiagen.com/FAQ/FAQList.aspx. The scientists in QIAGEN Technical Services are always happy to answer any questions you may have about either the information and protocols in this handbook or sample and assay technologies (for contact information, see back cover or visit www.qiagen.com).

	Comments and suggestions
Incomplete deparaffinization	
a) Insufficient time for deparaffinization	Vortex the sample in Deparaffinization Solution until paraffin is visibly dissolved.
b) Tissue slice not exposed to Deparaffinization Solution	Vortex and invert tube to ensure entire slice is uniformly exposed to Deparaffinization Solution.
Incomplete lysis	
a) FFPE tissue slice not deparaffinized	Ensure paraffin is completely dissolved before adding Lysis Buffer FTB and proteinase K.
b) Sample not exposed to Lysis Buffer FTB and proteinase K	Ensure tissue is covered by lysis reagents (e.g., tissue should not be stuck to the tube cap). It may be necessary to fold the tissue into the solution using a clean pipet tip.
c) Insufficient time for lysis	Incubate the sample at 56°C for an additional 30 min.
d) Lysis reaction prepared incorrectly	Make sure to add all necessary components of the lysis reaction, as described on page 26 (FFPE slices) or page 29 (whole blood, cell culture, or tissues).
Little or no DNA recovery in purification step	
a) Carrier RNA not added to Buffer BL	Prepare carrier RNA and add to Buffer BL, as described in "Preparation of reagents", page 19.
b) Buffer BW or Buffer BD prepared incorrectly	Check that Buffer BW and BD concentrates were diluted with the correct volumes of ethanol (96–100%). Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.

	Comments and suggestions
c) Buffer BW or BD prepared with 70% ethanol	Check that Buffer BW and BD concentrates were diluted with 96–100% ethanol. Do not use denatured alcohol, which contains other substances such as methanol or methylethylketone.
d) Buffer BW and Buffer BD used in the wrong order	Ensure that Buffer BW and Buffer BD are used in the correct order in the protocol.
e) Sample not completely passed through the membrane	Centrifuge for 1 min at full speed or until the entire sample has passed through the membrane.
f) Buffer BL contains precipitates	Check Buffer BL for precipitate. Dissolve by heating (maximum 70°C) with gentle agitation.
Low conversion rate	
a) Bisulfite reaction components not added in the correct order	Ensure that DNA, Bisulfite Mix, and DNA Protect Buffer are added in the order indicated in Table 2 (page 22), Table 5 (page 28), or Table 6 (page 31).
b) Incorrect thermal cycling conditions used	Use the thermal cycling conditions given in Table 3 (page 23).
c) Poor DNA quality (i.e., protein contamination)	Check that the A_{260}/A_{280} ratio of the sample DNA is between 1.7 and 1.9.
d) Amount of DNA used outside recommended range	Increase or decrease the amount of starting DNA material to stay within the range of 1 ng to 2 µg for purified DNA. For FFPE samples, cells, tissue, or blood, please refer to the indicated sample amounts on page 15.
e) Bisulfite Mix stored incorrectly	Dissolved Bisulfite Mix can be stored at –15 to –30°C for 4 weeks.
f) Presence of a special CpG region with high sequence representation of CpGs	Extend the bisulfite conversion thermal-cycling conditions by adding the following step: Denaturation for 5 min at 95°C and 2 h at 60°C; then hold at 20°C.

	Comments and suggestions
g) DNA Protect Buffer not added	Upon addition of DNA Protect Buffer, the DNA–Bisulfite Mix solution should turn from green to blue indicating sufficient mixing and the correct pH for DNA binding to the MinElute DNA spin column. If this color change does not occur, repeat the reaction ensuring that DNA Protect Buffer has been added.
Poor results in downstream PCR applications	
a) Little or no PCR product even in control reaction	If performing hot-start PCR, confirm that the initial enzyme activation step was performed. Ensure that all PCR components were added and that suitable cycling conditions were used.
b) Failure of conversion reaction	The starting DNA was not sufficiently pure. Ensure that only high-quality DNA is used for the conversion reaction. Ensure that all steps of the modification and cleanup protocol were followed. Sample DNA was degraded before modification reaction. Ensure that sample DNA is handled and stored correctly. PCR primers were not appropriate or incorrectly designed. Check primer design. Amount of template DNA used in PCR was insufficient. Increase amount of template DNA.
Unexpected findings in buffers	
a) Color of DNA Protect Buffer changes from light green to olive during storage	DNA Protect Buffer is stable at 2–8°C for one year, and a change in color within this time has no influence on performance.
b) Precipitates in Buffer BD	There may be slight clouding and/or insoluble precipitates in Buffer BD during storage. Buffer BD is stable at 2–8°C for one year, and a precipitate within this time has no influence on performance. Precipitates should not be transferred onto the membrane of the spin column.

References

QIAGEN maintains a large, up-to-date online database of scientific publications utilizing QIAGEN products. Comprehensive search options allow you to find the articles you need, either by a simple keyword search or by specifying the application, research area, title, etc.

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Ordering Information

Product	Contents	Cat. no.
EpiTect Plus DNA Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers	59124
EpiTect Plus FFPE Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers, Deparaffinization Solution, Lysis Buffer FTB	59144
EpiTect Plus LyseAll Bisulfite Kit (48)	48 MinElute DNA spin columns, Bisulfite mix, DNA Protect Buffer, Carrier RNA, Buffers, Lysis Buffer FTB	59164
Related products		
EpiTect Bisulfite Kit (48)	48 EpiTect Bisulfite Spin Columns, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59104
EpiTect 96 Bisulfite Kit (2)	2x EpiTect Bisulfite 96-well Plates, Reaction Mix, DNA Protect Buffer, Carrier RNA, Buffers	59110
EpiTect Whole Bisulfitome Kit — for amplification of bisulfite converted DNA		
EpiTect Whole Bisulfitome Kit (25)	REPLI-g® Midi DNA Polymerase, EpiTect WBA Reaction Buffer, Nuclease free water	59203
EpiTect Whole Bisulfitome Kit (100)	REPLI-g Midi DNA Polymerase, EpiTect WBA Reaction Buffer, Nuclease free water	59205
EpiTect MSP Kit — for highly accurate methylation-specific PCR without optimization		
EpiTect MSP PCR Kit (25)	EpiTect MSP Master Mix for 25x50 µl reactions	59303
EpiTect MSP PCR Kit (100)	EpiTect MSP Master Mix for 100x50 µl reactions	59305
EpiTect MSP PCR Kit (400)	EpiTect MSP Master Mix for 400x50 µl reactions	59307

Product	Contents	Cat. no.
EpiTect MethyLight PCR Kit — for real time quantification of methylation status		
EpiTect MethyLight PCR Kit (200)	Master Mix for Methylation-specific Real-Time PCR analysis, 200 x50 µl reactions	59436
EpiTect MethyLight PCR Kit (1000)	Master Mix for Methylation-specific Real-Time PCR analysis, 1000 x50 µl reactions	59438
EpiTect MethyLight PCR + ROX Vial Kit (200)	Master Mix without ROX for Methylation-specific Real-Time PCR analysis, 200 x50 µl reactions	59496
EpiTect MethyLight PCR + ROX Vial Kit (1000)	Master Mix without ROX for Methylation-specific Real-Time PCR analysis, 1000 x50 µl reactions	59498

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Notes:

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