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EpiTect® Fast 96 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 in a 96-well format

QIAGEN

Contents

Kit Contents

EpiTect Fast 96 Bisulfite Kit	
Catalog no.	**59720**
No. of preps	**192**
Chemical Module	
Bisulfite Solution	12 x 1.5 ml
DNA Protect Buffer	4 x 1.9 ml
RNase-free Water	4 x 1.9 ml
EpiTect DNA Protect Buffer Reservoir	1
EpiTect 96 Conversion Plates	2
EpiTect 96 Cover Foils	2
Purification Module	
EpiTect 96 Plates	2
EpiTect 96 Elution Plates	4
Buffer BL*	4 x 31 ml
Buffer BW (concentrate)	2 x 52 ml
Buffer BD (concentrate)	2 x 3 ml
Buffer EB	15 ml
Carrier RNA	1350 µg
S-Blocks	2
AirPore Tape Sheets	25
Tape Pads	25
TopElute Fluid	2 x 1.4 ml
Quick-Start Protocol	2

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See “Safety Information”, page 6.

EpiTect Fast 96 FFPE Bisulfite Kit	
Catalog no.	**59740**
No. of preps	**192**
Lysis Kit	
Deparaffinization Solution	4 x 8 ml
Lysis Buffer FTB	4 x 0.8 ml
Proteinase K	4 x 650 µl
Chemical Module	
Bisulfite Solution	12 x 1.5 ml
DNA Protect Buffer	4 x 1.9 ml
RNase-free Water	4 x 1.9 ml
EpiTect DNA Protect Buffer Reservoir	1
EpiTect 96 Conversion Plates	2
EpiTect 96 Cover Foils	2
Purification Module	
EpiTect 96 Plates	2
EpiTect 96 Elution Plates	4
Buffer BL*	4 x 31 ml
Buffer BW (concentrate)	2 x 52 ml
Buffer BD (concentrate)	2 x 3 ml
Buffer EB	15 ml
Carrier RNA	1350 µg
S-Blocks	2
AirPore Tape Sheets	25
Tape Pads	25
TopElute Fluid	2 x 1.4 ml
Quick-Start Protocol	3

* Contains a guanidine salt. Not compatible with disinfectants containing bleach. See "Safety Information", page 6.

Shipping and Storage

The EpiTect Fast 96 Bisulfite Kit and EpiTect Fast 96 FFPE Bisulfite Kit are shipped at room temperature (15–25°C). Upon arrival, the 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 performance. All other buffers including the Bisulfite Solution should be stored at room temperature and are stable for at least 6 months under these conditions, unless otherwise indicated in the label.

Lyophilized carrier RNA can be stored at room temperature for 1 year. Carrier RNA can only be dissolved in RNase-free water. Dissolved carrier RNA should be immediately added to Buffer BL, as described in the "Things to do before starting" section in each protocol. This solution should be prepared fresh and is stable at 2–8°C for up to 48 h. Unused portions of carrier RNA dissolved in RNase-free water should be frozen in aliquots at –30 to –15°C and can be stored for up to 1 year, unless otherwise indicated in the label.

Intended Use

The EpiTect Fast 96 DNA Bisulfite Kit and EpiTect Fast 96 FFPE 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.

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 the Bisulfite Solution or 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 spilt, clean with suitable laboratory detergent and water.

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of EpiTect Fast 96 DNA Bisulfite Kit and EpiTect Fast 96 FFPE Bisulfite Kit is tested against predetermined specifications to ensure consistent product quality.

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 bisulfite conversion. Bisulfite treatment of the target DNA 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 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 procedures. Common bisulfite procedures usually require high amounts of input DNA. However, due to DNA degradation during conversion and DNA loss during purification, such procedures often lead to low DNA yield, highly fragmented DNA, and irreproducible conversion rates.

The EpiTect Fast 96 Bisulfite Conversion Kits provides a very fast and streamlined procedure for efficient conversion and purification of DNA prepared from FFPE, blood, cell, or tissue samples. The kits contain highly concentrated Bisulfite Solution, which reduces the time required to convert unmethylated cytosine residues into uracil from 5 h to as little as 30 min, as well as preparation buffers that make it unnecessary to isolate the DNA prior to bisulfite treatment. DNA fragmentation is prevented during the bisulfite conversion reaction 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 step included in the purification procedure.

Principle and procedure

The EpiTect Fast 96 Bisulfite Conversion Kit procedure comprises a few simple steps: preparation of DNA from a sample, bisulfite-mediated conversion of unmethylated cytosines, binding of the converted single-stranded DNA to the membrane of an EpiTect 96 Plate, 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 EpiTect 96 Plate. The initial sample preparation is different for FFPE slices, whole blood, and cell cultures or tissues; however, the procedure for bisulfite conversion of extracted DNA is the same for all sample types (see Figure 1, page **Error! Bookmark not defined.**). The eluted, bisulfite converted DNA is suited for all techniques currently used to analyze DNA methylation,

including PCR, real-time PCR, methylation-specific PCR, bisulfite sequencing (direct and cloning), COBRA, and Pyrosequencing®.

Bisulfite Solution

The Bisulfite Solution is conveniently provided in 12 separate aliquots (17 conversions per aliquot) that are ready to use. The 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. Bisulfite Solution can be stored at room temperature for at least 6 months.

DNA Protect Buffer

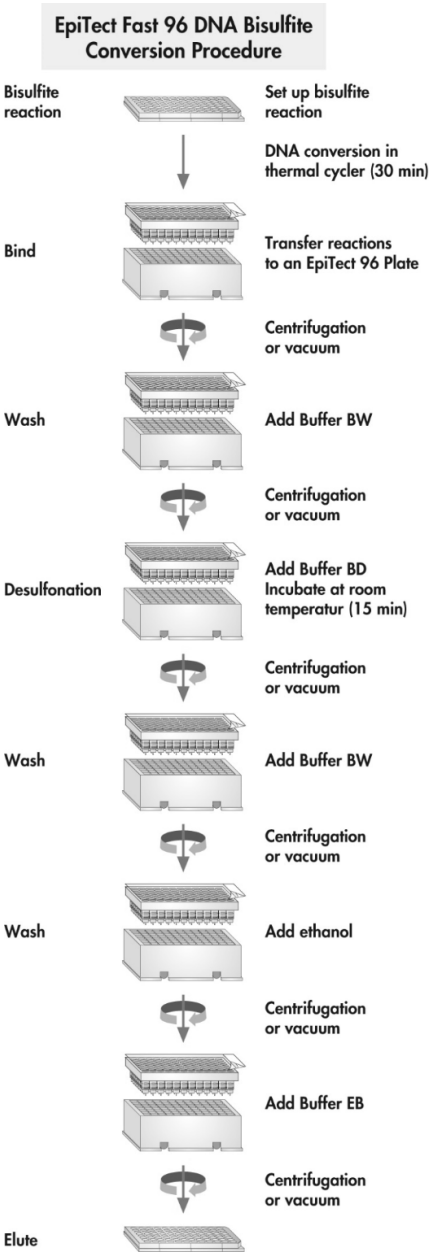
DNA Protect Buffer is uniquely formulated to prevent the DNA fragmentation usually associated with bisulfite treatment of DNA at high temperatures and low pH values. It also provides effective DNA denaturation, resulting in the single-stranded DNA necessary for complete cytosine conversion. In addition, 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 short incubation steps necessary for thermal DNA denaturation and subsequent sulfonation and cytosine deamination, enabling the highest cytosine conversion rates.

Carrier RNA

Carrier RNA is provided to enhance the binding of small quantities of DNA to the EpiTect 96 Plate. If using more than 100 ng genomic DNA template, it is not necessary to use carrier RNA, though we strongly recommend its use when processing fragmented DNA or DNA extracted from formalin-fixed, paraffin-embedded (FFPE) tissue samples. Carrier RNA does not influence downstream applications. It should be dissolved in RNase-free water before use (see “Preparation of reagents”, page 16).


EpiTect Fast 96 DNA Bisulfite Conversion Procedure
Bisulfite reaction
Set up bisulfite reaction
DNA conversion in thermal cycler (30 min)
Bind
Transfer reactions to an EpiTect 96 Plate
Centrifugation or vacuum
Wash
Add Buffer BW
Centrifugation or vacuum
Desulfonation
Add Buffer BD Incubate at room temperatur (15 min)
Centrifugation or vacuum
Wash
Add Buffer BW
Centrifugation or vacuum
Wash
Add ethanol
Centrifugation or vacuum
Add Buffer EB
Centrifugation or vacuum
Elute

Optimized buffers

EpiTect Fast 96 Bisulfite Conversion Kits contain carefully optimized buffers, enabling maximum cytosine conversion and subsequent DNA purification. Buffer BL promotes binding of the converted single-stranded DNA to the EpiTect 96 Plate. Subsequently, membrane-bound DNA is washed using Buffer BW, which efficiently removes residual Bisulfite Solution. After desulfonation using Buffer BD, the DNA is further desalted using Buffer BW. Finally, the EpiTect 96 Plate is washed with ethanol before the converted DNA is eluted using Buffer EB.

TopElute Fluid

TopElute Fluid is used in the elution step of the vacuum protocol to increase the vacuum pressure on each sample. In addition, it helps provide even pressure across the whole 96-well plate. TopElute Fluid is chemically inert and does not influence any downstream applications.

Storage stability of converted and purified DNA

DNA converted and purified using EpiTect Fast 96 Bisulfite Conversion Kits can be stored at –30 to –15°C for at least 9 months without decrease in quality or conversion. Further investigations into long-term storage are ongoing. Contact **support.qiagen.com** for more information.

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. The standard "Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA" on page 17 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 Fast 96 FFPE Bisulfite Kit and "Protocol: Sample Lysis and Bisulfite Conversion of Unmethylated Cytosines in DNA Prepared from FFPE Tissue Samples" on page 20. This kit consists of the EpiTect Fast 96 FFPE Lysis Kit, containing specialized buffers for efficient deparaffinization and lysis of the tissue sample, and the EpiTect Fast 96 Bisulfite Kit for bisulfite conversion of the extracted DNA. The protocol includes an optimized step to facilitate binding of DNA and can be used with single slices of FFPE tissue (10 µm in thickness).

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

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%)*
- Multichannel pipette and tips (we recommend pipette tips with aerosol barriers for preventing cross-contamination)
- 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)
- **Optional**: If the lid of the thermal cycler is not compatible with the provided EpiTect cover foils, cap strips can be used (available from ABgene® [8 Flat Cap Strips, cat. no. AB-0783 or 8 Domed Cap Strips, cat. no. AB-0265])†
- **Optional**: Heating block, thermomixer, or heated orbital incubator (see step 1 of each protocol)

Spin protocol

- Centrifuge with 96-well plate rotor, e.g., Centrifuge 4-16S or 4-16KS (available at **www.qiagen.com**) with Plate Rotor 2 x 96 (cat. no. 81031)

Vacuum protocol

- The QIAvac 96 can be operated using any vacuum source (e.g., a house vacuum, vacuum pump, or water aspirator). A vacuum pressure of between –800 to –900 mbar (–600 to –675 mm Hg) should be applied to the EpiTect 96 Plate.

* 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 (10 µm in thickness).

If following "Protocol: Bisulfite Conversion of Unmethylated Cytosines in DNA", page 17, the EpiTect Fast 96 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.

If purifying bisulfite-treated DNA originating from very small sample amounts, such as biopsies and FFPE tissues, we strongly recommend adding carrier RNA to Buffer BL (see "Things to do before starting", pages 17, 20, and **Error! Bookmark not defined.**).

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

Starting material

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, the DNA should be linearized before starting the procedure due to the very quick reannealing of single-stranded DNA after the denaturation step.

Handling of the EpiTect 96 Plate

Due to the sensitivity of nucleic acid amplification technologies, the following precautions are necessary when handling EpiTect 96 Plates to avoid cross-contamination between sample preps:

- Carefully pipet the sample or solution into the EpiTect 96 Plate without wetting the rim of the wells. Avoid touching the EpiTect 96 Plate membrane with the pipette tip.
- Always change pipette tips between liquid transfers. We recommend the use of aerosol-barrier pipette tips.
- Open sealed EpiTect 96 Plates carefully, 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

Centrifugation of EpiTect 96 Plates is performed at 5800 x *g* (approximately 6000 rpm) unless otherwise stated. All centrifugation steps should be carried out at room temperature.

Note: If using a centrifuge with adjustable temperature (e.g., Centrifuge 4-16KS), set the temperature to 40°C for the drying step.

Adhesive tape

To prevent cross-contamination, AirPore Tape Sheets should be used to seal EpiTect 96 Plates prior to each centrifugation step, with the exception of the drying step (step 13 of "Protocol: Cleanup of Bisulfite Converted DNA Using a Centrifuge" or step 14 of "Protocol: Cleanup of Bisulfite Converted DNA Using a Vacuum Manifold"). In addition, Tape Pads should be used to cover the plate during buffer BD incubation and prior to storage of the samples. Used wells should be labeled using a waterproof marker pen, while unused wells should be covered using an AirPore Tape Sheet cut to size. The EpiTect 96 Plate can then be stored in the blister pack

in which it was supplied. Before starting the next run, remove the tape and cover the previously used wells with fresh tape.

Preparation of reagents

Buffer BW

Add 120 ml ethanol (96–100%) to Buffer BW and store at room temperature. 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 EpiTect 96 Plate.

Carrier RNA

Add 1350 µl RNase-free water to the lyophilized carrier RNA (1350 µg) to obtain a 1 µg/µl solution. Dissolve the carrier RNA thoroughly by vortexing. Split the dissolved carrier RNA into conveniently sized aliquots (e.g., 675 µl aliquots for processing 96 reactions) and store at –30 to –15°C. Aliquots can be stored for up to 1 year. Carrier RNA enhances binding of DNA to the EpiTect 96 Fast Plate, especially if there are very few target molecules in the sample.

Add 300 µl of the dissolved carrier RNA to one bottle of Buffer BL to obtain a final concentration of 10 µg/ml. Carrier RNA is not necessary if >100 ng DNA is used. If Buffer BL contains precipitates, dissolve by heating (maximum 70°C) with gentle agitation.

Protocol: 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 (Table 1). All other protocol steps are the same.

Important points before starting

- DNA Protect Buffer should turn from green to blue after addition to DNA–Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
- Perform all centrifugation steps at room temperature.

Things to do before starting

- Prepare the kit reagents as described in “Preparation of reagents”, page 16.
- Equilibrate samples and buffers to room temperature.
- **Optional**: Set a thermomixer, heating block, or heated orbital incubator to 60°C to dissolve the Bisulfite Solution.

Procedure: Bisulfite DNA conversion

1. Thaw DNA to be used in the bisulfite reactions. Make sure the Bisulfite Solution is completely dissolved.

   Note: If necessary, heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.

   Note: Do not place dissolved Bisulfite Solution on ice.

2. Prepare the bisulfite reactions in the provided EpiTect 96 Conversion Plate according to Table 1. Add each component in the order listed.

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

   Note: If using a multichannel pipette to dispense DNA Protect Buffer, use the provided EpiTect 96 DNA Protect Buffer Reservoir. Commonly used polystyrene reservoirs are sensitive to the solvent in this buffer.

Table 1. Bisulfite reaction components

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

* The combined volume of DNA and RNase-free water must total 20 µl.
† The combined volume of DNA and RNase-free water must total 40 µl.

3. Seal the EpiTect 96 Conversion Plate using EpiTect 96 Cover Foil (provided) and mix the bisulfite reactions by vortexing thoroughly. Centrifuge the plate briefly at 650 x *g* (approximately 2000 rpm) to collect the reactions in the bottom of the wells. Store the plate at room temperature.

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

   Note: If the lid of the thermal cycler is not compatible with the provided EpiTect 96 Cover Foils, cap strips can be used (see "Equipment and Reagents to Be Supplied by User", page 13).

4. Perform the bisulfite DNA conversion using a thermal cycler. Program the cycler according to Table 2.

   The complete cycle should take approximately 30 min.

   Optional: In some cases it may be necessary to extend the 60°C cycle time up to 20 min to achieve complete bisulfite DNA conversion.

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

Table 2. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	10 min*	60°C
Denaturation	5 min	95°C
Incubation	10 min*	60°C
Hold	Indefinite†	20°C

* In some cases, it may be necessary to extend the 60°C cycle time up to 20 min to achieve complete bisulfite DNA conversion.

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

5. Place the EpiTect 96 Conversion Plate 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.

   Converted DNA can be left in the thermal cycler overnight at 20°C without any loss of performance.

6. Proceed to "Protocol: Cleanup of Bisulfite Converted DNA Using a Centrifuge" (page 25) or "Protocol: Cleanup of Bisulfite Converted DNA Using a Vacuum Manifold" (page 27) to perform the cleanup of bisulfite converted DNA.

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

This protocol is designed to be used with the EpiTect Fast 96 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.
- DNA Protect Buffer should turn from green to blue after addition to the DNA–Bisulfite Solution mixture, indicating sufficient mixing and correct pH for the bisulfite conversion reaction.
- Perform all centrifugation steps at room temperature.

Things to do before starting

- Prepare the kit reagents as described in “Preparation of reagents”, page 16.
- 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 dissolve the Bisulfite Solution.

Procedure

Deparaffinization, lysis, and decrosslinking of FFPE slice

1. Place the FFPE slice (10 µm) in a 200 µl reaction tube or 8-well strip (not provided) and add 150 µl Deparaffinization Solution.

   Optional: 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 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 addition of proteinase K.
5. Perform the lysis and decrosslinking using a thermal cycler. Program the thermal cycler according to Table 3.

   Optional: If using 1.5 ml tubes, perform the lysis and decrosslinking in a thermal block. Incubate the tubes in a thermal block at 56°C for 30 min to lyse the tissues. Ensure that tissues are completely lysed (the solution will become homogeneous); 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.

Table 3. 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 PCR tubes containing the lysis reactions into the thermal cycler. Start the incubation.

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

Bisulfite conversion of DNA

7. Make sure the Bisulfite Solution is completely dissolved.

   Note: If necessary, heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.

   Note: Do not place dissolved Bisulfite Solution 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 (see Table 4).

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

9. Prepare the bisulfite reactions in the provided EpiTect 96 Conversion Plate according to Table 4. Add each component in the order listed.

   Note: If using a multichannel pipette to dispense DNA Protect Buffer, use the provided EpiTect 96 DNA Protect Buffer Reservoir. Commonly used polystyrene reservoirs are sensitive to the solvent in this buffer.

Table 4. Bisulfite reaction components

Component	Volume per reaction (µl)
Lysis reaction	Approx. 40
Bisulfite Solution	85
DNA Protect Buffer	15
Total volume	**140**

10. Seal the EpiTect 96 Conversion Plate using EpiTect 96 Cover Foil and mix the bisulfite reactions by vortexing thoroughly. Centrifuge the plate briefly at 650 x *g* (approximately 2000 rpm) to collect the reactions in the bottom of the wells. Store the plate at room temperature.

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

    Note: If the lid of the thermal cycler is not compatible with the provided EpiTect 96 Cover Foils, cap strips can be used (see "Equipment and Reagents to Be Supplied by User", page 13).

11. Perform the bisulfite DNA conversion using a thermal cycler. Program the cycler according to Table 5.

    The complete cycle should take approximately 30 min.

    Optional: In some cases it may be necessary to extend the 60°C cycle time up to 20 min to achieve complete bisulfite DNA conversion.

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

Table 5. Bisulfite conversion thermal cycler conditions

Step	Time	Temperature
Denaturation	5 min	95°C
Incubation	10 min*	60°C
Denaturation	5 min	95°C
Incubation	10 min*	60°C
Hold	Indefinite†	20°C

* In some cases, it may be necessary to extend the 60°C cycle time up to 20 min to achieve complete bisulfite DNA conversion.

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

12. Place the EpiTect 96 Conversion Plate 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.

    Converted DNA can be left in the thermal cycler overnight at 20°C without any loss of performance.

13. Proceed to "Protocol: Cleanup of Bisulfite Converted DNA Using a Centrifuge" (page 25) or "Protocol: Cleanup of Bisulfite Converted DNA Using a Vacuum Manifold" (page 27) to perform the cleanup of bisulfite converted DNA.

Protocol: Cleanup of Bisulfite Converted DNA Using a Centrifuge

Procedure

1. Once the bisulfite conversion is complete, briefly centrifuge the EpiTect 96 Conversion Plate containing the bisulfite reactions at 650 x *g*.
2. Place an EpiTect 96 Plate on top of an S-Block. Mark the plate for later identification.
3. Dispense 310 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Preparation of reagents", page 16) into the required EpiTect 96 Plate wells.

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

   Note: Proceed with the cleanup protocol within 5 min of applying Buffer BL.
4. Transfer the complete bisulfite reactions from step 1 to the EpiTect 96 Plate and mix with Buffer BL by pipetting up and down 4 times.

   Precipitates in the bisulfite reactions will not affect performance or yield.
5. Add 250 µl ethanol (molecular biology grade, 96–100%) to each sample and mix by pipetting up and down 4 times.
6. Seal the EpiTect 96 Plate with an AirPore Tape Sheet. Load the S-Block and EpiTect 96 Plate into the centrifuge plate holder, then place the holder into the rotor bucket. Centrifuge at 5800 x *g* for 1 min.

   Note: AirPore Tape Sheets help prevent cross contamination; however, their use is optional.
7. Remove the AirPore Tape Sheet. Add 500 µl Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 x *g* for 1 min. Empty the S-Block.

8. Remove the AirPore Tape Sheet. Add 250 µl Buffer BD to each sample and seal the EpiTect 96 Plate with a Tape Pad (provided). Incubate for 15 min at room temperature.

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

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

9. Centrifuge at 5800 x *g* for 1 min.
10. Remove the Tape Pad. Add 500 µl Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 x *g* for 1 min. Empty the S-Block.
11. Remove the AirPore Tape Sheet. Add 500 µl Buffer BW to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 x *g* for 1 min.
12. Remove the AirPore Tape Sheet. Add 250 µl ethanol (96–100%) to each sample and seal the EpiTect 96 Plate with a new AirPore Tape Sheet. Centrifuge at 5800 x *g* for 1 min.
13. Dispose of the S-Block appropriately. Remove the AirPore Tape Sheet and place the EpiTect 96 Plate on top of an EpiTect Elution Plate. Centrifuge at 5800 x *g* for 15 min.

    Note: If using a centrifuge with adjustable temperature, set the temperature to 40°C. The heat generated during centrifugation ensures evaporation of residual ethanol in the sample.

14. Place the EpiTect 96 Plate on top of a new EpiTect Elution Plate.
15. To elute DNA, add 70 µl Buffer EB to each sample using a multichannel pipette.

    Important: For complete elution of bound DNA, ensure that the elution buffer is dispensed directly onto the center of each membrane on the EpiTect 96 Plate.

16. Centrifuge at 5800 x *g* for 1 min. Seal the elution plate for storage using Tape Pads.

    Note: The average eluate volume is 40–50 µl from 70 µl elution buffer. A volume of 1 µl eluate should be sufficient for PCR amplification.

    Note: We recommend storing purified DNA at 2–8°C for up to 24 h. When storing purified DNA for longer than 24 h, we recommend storage at –30 to –15°C.

Protocol: Cleanup of Bisulfite Converted DNA Using a Vacuum Manifold

Procedure

1. Briefly centrifuge the EpiTect Conversion Plate containing the bisulfite reactions at 650 x *g*.
2. Prepare the vacuum manifold (e.g., QIAvac 96) and place an EpiTect 96 Plate securely onto it.

   To prepare the QIAvac 96, place a waste tray inside the QIAvac base, then place the QIAvac Top Plate squarely over the base. Attach the QIAvac 96 to a vacuum source.

   Seal unused wells of the EpiTect 96 Plate with Tape Pads.
3. Dispense 560 µl freshly prepared Buffer BL containing 10 µg/ml carrier RNA (see "Preparation of reagents", page 16) into the required EpiTect 96 Plate wells.

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

   Note: Proceed with the cleanup protocol within 5 min of applying Buffer BL.
4. Transfer the complete bisulfite reactions from step 1 to the EpiTect 96 Plate and mix with the Buffer BL by pipetting up and down 4 times.

   Precipitates in the bisulfite reactions will not affect the performance or yield of the reaction.
5. Switch on the vacuum source. After all liquid has passed through the membrane, switch off the vacuum source.
6. Add 500 µl Buffer BW to each sample.
7. Switch on the vacuum source. After all liquid has passed through the membrane, switch off the vacuum source.

8. Add 250 µl Buffer BD to each sample and seal the EpiTect 96 Plate using a Tape Pad (provided). Incubate for 15 min at room temperature. Remove the Tape Pad.

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

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

9. Switch on the vacuum source. After all liquid has passed through the membrane, switch off the vacuum source.
10. Add 500 µl Buffer BW to each sample.
11. Switch on the vacuum source. After all liquid has passed through the membrane, switch off the vacuum source.
12. Repeat steps 10 and 11 once.
13. Add 250 µl ethanol (96–100%) to each sample.
14. Switch on the vacuum source. After ethanol in all wells has passed through the membrane, apply maximum vacuum for an additional 10 min to dry the membrane.

    Important: This step removes residual ethanol from the membrane. The removal is only effective when maximum vacuum is used (i.e., turn off vacuum regulator valves or leakage valves if they are used), allowing maximum airflow to go through the wells.

15. Switch off the vacuum source and ventilate the vacuum manifold slowly. Lift the QIAvac 96 Top Plate from the base (do not lift the EpiTect 96 Plate from the top plate), vigorously tap the top plate on a stack of absorbent paper until no drops come out, and blot the nozzles of the EpiTect 96 Plate with clean absorbent paper.

    This step removes residual ethanol from around the outlet nozzles and collars of the EpiTect 96 Plate. Residual ethanol may inhibit subsequent downstream reactions.

16. Remove the waste tray and insert the vacuum manifold adapter for elution plates (see “Equipment and Reagents to Be Supplied by User”, page 13). Place an elution plate directly onto the adapter and the top plate back onto the base.

17. To elute DNA, dispense 70 µl of Buffer EB and 10 µl TopElute Fluid into each sample well of the EpiTect 96 Plate. Switch on the vacuum source a maximum of 1 min. Switch off the vacuum source and ventilate the vacuum manifold slowly.

    Important: For complete elution of bound DNA, ensure that the elution buffer is dispensed directly onto the center of each membrane on the EpiTect 96 Plate.

    Note: The average eluate volume is 40–50 µl from 70 µl elution buffer. A volume of 1 µl eluate should be sufficient for PCR amplification.

    Important: Small amounts of TopElute Fluid can be eluted together with the DNA. TopElute Fluid is clearly visible as a small bubble on top of the eluate. TopElute Fluid is chemically inert and does not influence any downstream applications. However, TopElute Fluid should not be transferred to the downstream reaction since it will reduce the amount of DNA intended to be transferred into the downstream reaction.

    Note: Seal the elution plate for storage using Tape Pads. We recommend storing purified DNA at 2–8°C for up to 24 h. When storing purified DNA for longer than 24 h, we recommend storage at –30 to –15°C.

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 or protocols in this handbook (for contact information, visit **support.qiagen.com**).

	Comments and suggestions
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 16.
b) Buffer BW or Buffer BD prepared incorrectly	Check that Buffer BW and Buffer 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.
c) Buffer BW or Buffer BD prepared with 70% ethanol	Check that Buffer BW and Buffer 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 are 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 Solution, and DNA Protect Buffer are added in the order indicated in Table 1, Table 4, or Table 6.
b) Incorrect thermal cycling conditions used	Use the thermal cycling conditions given in Table 2, Table 5, or Table 7.
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.

		Comments and suggestions
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 – 2 µg for purified DNA. For FFPE samples, cells, tissue, or blood, refer to the indicated sample amounts on page 11.
e)	DNA Protect Buffer not added	Upon addition of DNA Protect Buffer, the DNA–Bisulfite Solution mixture should turn from green to blue indicating sufficient mixing and the correct pH for DNA binding to the EpiTect 96 Plate membrane. If this color change does not occur, repeat the reaction ensuring that DNA Protect Buffer has been added.
f)	Bisulfite Solution contains precipitates	Heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.
g)	Insufficient time for bisulfite DNA conversion	Extend thermal cycling step of 60°C from 10 min to 20 min.
Incomplete deparaffinization of FFPE tissue		
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 pipette 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 21 (FFPE slices) or page **Error! Bookmark not defined.** (whole blood, cell culture, or tissues).
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.

		Comments and suggestions
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 EpiTect 96 Plate.
c)	Bisulfite Solution contains precipitates	Heat the Bisulfite Solution to 60°C and vortex until all precipitates are dissolved again.

Ordering Information

Product	Contents	Cat. no.
EpiTect Fast 96 Bisulfite Kit	2 x EpiTect 96-well Plates, Bisulfite Solution, DNA Protect Buffer, Carrier RNA, Buffers	59720
EpiTect Fast 96 FFPE Bisulfite Kit	Deparaffinization Solution, Lysis Buffer, Proteinase K, 2 x EpiTect 96-well Plates, Bisulfite Solution, DNA Protect Buffer, Carrier RNA, Buffers	59740
Related products		
EpiTect Fast DNA Bisulfite Kit (50)	For 50 preps: Bisulfite Solution, DNA Protect Buffer, MinElute® DNA Spin Columns, Carrier RNA, and Buffers	59824
EpiTect Fast FFPE Bisulfite Kit (50)	For 50 preps: Deparaffinization Solution, Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59844
EpiTect Fast LyseAll Bisulfite Kit (50)	For 50 preps: Lysis Buffer, Proteinase K, Bisulfite Solution, DNA Protect Buffer, MinElute DNA Spin Columns, Carrier RNA, and Buffers	59864
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

Product	Contents	Cat. no.
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 MSP Kit — for highly accurate methylation-specific PCR without optimization		
EpiTect MSP PCR Kit (100)	EpiTect MSP Master Mix for 100 x 50 µl reactions	59305
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 x 50 µl reactions	59436
EpiTect MethyLight PCR + ROX™ Vial Kit (200)	Master mix without ROX for methylation-specific real-time PCR analysis, 200 x 50 µl reactions	59496

For up-to-date licensing information and product-specific disclaimers, see the respective QIAGEN kit handbook or user manual. QIAGEN kit handbooks and user manuals are available at **www.qiagen.com** or can be requested from QIAGEN Technical Services or your local distributor.

Document Revision History

Date	Changes
07/2020	Corrected the volume of carrier RNA to be added to 1 bottle of Buffer BL to obtain a concentration of 10 µg/ml. Changed precise storage temperatures to temperature ranges. Updated quantities of Buffer BL and Proteinase K in "Kit Contents". Deleted all references to discontinued cat. no. 59760, the EpiTect Fast 96 LyseAll Bisulfite Kit. Updated recommended centrifuge models. Deleted all discontinued products from "Ordering Information".

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