April 2008

EpiTect® Whole Bisulfitome Handbook

For whole genome amplification of bisulfite converted DNA for PCR analysis

QIAGEN®

Sample & Assay Technologies

QIAGEN Sample and Assay Technologies

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- microRNA research and RNAi
- Automation of sample and assay technologies

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Contents

Kit Contents

EpiTect Whole Bisulfitome Kit	**(25)**
Catalog No.	**59203**
Number of 40 µl reactions (approximately 1–3 µg yield)	**25**
REPLI-g® Midi DNA Polymerase (blue lid)	25 µl
EpiTect WBA Reaction Buffer (yellow lid)	725 µl
RNase-Free Water	1.9 ml
Handbook	1

EpiTect Whole Bisulfitome Kit	**(100)**
Catalog No.	**59205**
Number of 40 µl reactions (approximately 1–3 µg yield)	**4 x 25**
REPLI-g® Midi DNA Polymerase (blue lid)	4 x 25 µl
EpiTect WBA Reaction Buffer (yellow lid)	4 x 725 µl
RNase-Free Water	4 x 1.9 ml
Handbook	1

Shipping and Storage

The EpiTect Whole Bisulfitome Kit is shipped on dry ice. The kit should be stored immediately upon receipt at –20°C in a constant-temperature freezer. When stored under these conditions and handled correctly, this product can be kept for at least 6 months after shipping without showing any reduction in performance.

Product Use Limitations

The EpiTect Whole Bisulfitome Kit is intended for research use. No claim or representation is intended to provide information 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).

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 Whole Bisulfitome 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 material safety data sheets (MSDSs). These are available online in convenient and compact PDF format at www.qiagen.com/ts/msds.asp where you can find, view, and print the MSDS for each QIAGEN kit and kit component.

24-hour emergency information

Emergency medical information in English, French, and German can be obtained 24 hours a day from:

Poison Information Center Mainz, Germany

Tel: +49-6131-19240

Quality Control

In accordance with QIAGEN's ISO-certified Quality Management System, each lot of the EpiTect Whole Bisulfitome Kit is tested against predetermined specifications to ensure consistent product quality.

Introduction

The EpiTect Whole Bisulfitome Kit utilizes proven REPLI-g technology to allow reproducible and representative amplification of bisulfite converted genomic DNA. The kit provides all reagents required for highly reliable results including DNA polymerase, buffers, and reagents.

This unique kit allows informative methylation analysis of DNA derived from precious samples and can be used to generate unlimited amounts of bisulfite treated DNA that can be used for subsequent downstream applications.

Principle and procedure

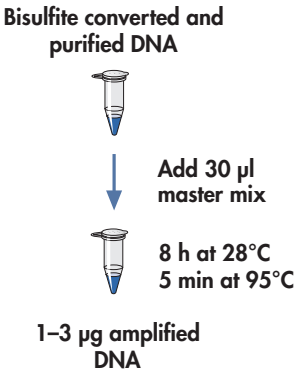
Methylation analysis of genomic DNA sequences can be limited by the small amount of sample available. The EpiTect Whole Bisulfitome Kit provides highly uniform amplification across the entire bisulfite converted genome, with negligible sequence bias. The method is based on Multiple Displacement Amplification (MDA) technology, which carries out isothermal genome amplification utilizing a uniquely processive DNA polymerase capable of replicating up to 100 kb without dissociating from the genomic DNA template. The DNA polymerase has a 3'–5' exonuclease proofreading activity to maintain high fidelity during replication and is used in the presence of exonuclease-resistant primers to achieve high yields of converted DNA products for methylation analysis.

The sodium bisulfite DNA conversion step traditionally used in DNA methylation analysis, converts non-methylated cytosines to uracil, while methylated cytosines are not converted. During this conversion process, DNA quality is often compromised due to DNA fragmentation. Both DNA fragmentation and the change in nucleotide composition affects the amplification of DNA when using conventional whole genome amplification methods. Problems associated with high levels of DNA fragmentation can be overcome by using EpiTect Bisulfite Kits for DNA conversion. These kits contain a unique DNA protection buffer that prevents excessive DNA degradation. The EpiTect Whole Bisulfitome Kit has been specially developed to amplify bisulfite converted DNA of smaller fragment size and changed nucleotide composition while maintaining the converted sequence representation. Typically, DNA yields of 1–3 µg are generated per reaction using the EpiTect Whole Bisulfitome Kit.

The DNA amplified by the EpiTect Whole Bisulfitome Kit is highly suited for use in 100–300 real-time PCR assays (e.g., using EpiTect MethyLight PCR Kits) or end-point PCRs (e.g., using EpiTect MSP Kits) if using 10 ng of amplified DNA.

For more information about bisulfite conversion and subsequent PCR-based methylation analysis, visit www.qiagen.com/goto/Epigenetics .

Amplification Procedure



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 material safety data sheets (MSDSs), available from the product supplier.

- Microcentrifuge tubes
- Microcentrifuge
- Thermal cycler, water bath, or heating block (see steps 5 and 6, page 11)
- Vortexer
- Pipets and pipet tips
- Ice

Protocol: Amplification of bisulfite converted DNA using the EpiTect Whole Bisulfitome Kit

This protocol allows amplification of previously bisulfite converted and purified DNA (e.g., DNA prepared using EpiTect Bisulfite Kits).

Important points before starting

- This protocol is optimized for the amplification of >50 ng bisulfite converted DNA by the EpiTect Bisulfite Kit (see Appendix A, page 14), fresh or stored frozen at –20°C not exceeding 12 weeks*. The DNA should be resuspended in TE or nuclease-free water. Lower quality of DNA or smaller amounts of bisulfite converted DNA may result in less than 1 µg of amplified DNA and loss of sequence representation.
- For sodium bisulfite conversion of DNA, we recommend EpiTect Bisulfite Kits (see Ordering Information, page 18).
- This protocol has been developed to provide DNA suitable for use in downstream PCR and real-time PCR applications. Amplified DNA may be successfully used in other applications; however, this has not been tested by QIAGEN.
- REPLI-g Midi DNA Polymerase should be thawed on ice (see step 2). EpiTect WBA Reaction Buffer should be thawed at room temperature.
- EpiTect WBA Reaction Buffer should be thoroughly mixed prior to use by vortexing for at least 10 seconds.

Procedure

1. **Place >50 ng bisulfite converted template DNA in 1–10 µl TE buffer or Buffer EB into a microcentrifuge tube. Adjust the volume to 10 µl using nuclease-free water.**

   Optimal results are obtained using DNA converted with EpiTect Bisulfite Kits (see Ordering Information, page 18).

2. **Thaw REPLI-g Midi DNA Polymerase on ice. Thaw all other components at room temperature, vortex, then centrifuge briefly.**

   The EpiTect WBA Reaction Buffer may form a precipitate after thawing. The precipitate will dissolve by vortexing for 10 s.

3. **Prepare an EpiTect Amplification Master Mix on ice according to Table 1. Mix and centrifuge briefly.**

   Important: Add the EpiTect Amplification Master Mix components in the order listed in Table 1. The EpiTect WBA Reaction Buffer should be vortexed for at least 10 s before use. The EpiTect Amplification Master Mix should be kept on ice and used immediately upon addition of the REPLI-g Midi DNA Polymerase.

* Investigations into longer storage of converted DNA are ongoing.

Table 1. Preparation of EpiTect Amplification Master Mix

Component	Volume/reaction
EpiTect WBA Reaction Buffer	29 µl
REPLI-g Midi DNA Polymerase	1 µl
Total volume	**30 µl**

4. **Add 30 µl of the EpiTect Amplification Master Mix to 10 µl of bisulfate converted DNA (step 1).**
5. **Incubate the solution at 28°C for 8 h.**

   Place the reaction tubes into a waterbath or heating block at 28°C.

   If a thermal cycler is used with a heated lid, the temperature of the lid should be set to 70°C.
6. **Inactivate REPLI-g Midi DNA Polymerase by heating the sample for 5 min at 95°C.**

   If the amplified DNA will be quantified using PicoGreen® reagent, please note that the reagent only binds double-stranded DNA efficiently. Therefore, quantify the DNA before proceeding with the 95°C incubation, or remove an aliquot (taken after step 5 and cooled to 4°C) for later quantification. If the DNA was quantified after denaturation at 95°C using PicoGreen, multiply the yield by a factor of 2 to compensate for the use of single-stranded DNA.
7. **Store amplified DNA at 4°C for short-term storage or –20°C for long-term storage.**

   If performing PCR analysis, see guidelines in Appendix A (page 14).

   Note: The amplified DNA should be treated as genomic DNA (i.e., minimize the number of freeze–thaw cycles). Storage of nucleic acids at low concentration over a long period of time may result in acid hydrolysis. We therefore recommend storage of the amplified DNA without dilution.

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
Reduced or no high-molecular-weight product in agarose gel in some or all EpiTect whole bisulfitome amplification samples		
a)	Reaction temperature is too high	Check the incubator for correct reaction temperature (28°C) during the EpiTect whole bisulfitome amplification reaction. If a cycler with a heated lid is used, set the temperature to 70 °C. Alternatively, the EpiTect whole bisulfitome amplification reaction can be performed at room temperature, which should give the appropriate yield.
b)	Poor DNA quality	Converted DNA is too fragmented. Use EpiTect Bisulfite Kits including DNA Protect Buffer for conversion of DNA (see Ordering Information, page 18) and use a larger amount of converted DNA (up to 200 ng) for the EpiTect whole bisulfitome amplification reaction.
c)	Carryover of alcohol in converted DNA sample	Residual alcohol in the converted DNA sample may reduce the yield of the EpiTect whole bisulfitome amplification reaction. When using the EpiTect Bisulfite Kit to convert DNA, ensure the duration of the drying step is sufficient. If using a centrifuge with adjustable temperature settings, set the temperature to 40°C. If a temperature controlled centrifuge is not available, we recommend placing the EpiTect 96 plate or EpiTect columns in an incubator at 65°C for 15 min to evaporate residual ethanol.

DNA yields of approximately 1–3 µg but varying or negative result in downstream assay (e.g., PCR)		
a)	Poor DNA quality	DNA purified after conversion using the bisulfite method is too fragmented. Use EpiTect Bisulfite Kits for DNA conversion. Use larger amount of bisulfite converted DNA. Increase the amount up to 200 ng.
b)	PCR cycling conditions not optimal	Prolonged extension times are not recommended. Set the extension time no longer than 30 seconds for PCR fragments of up to 500 bp. A reduction of the PCR extension time may increase the yield of specific PCR products.
Multiple bands in end-point PCR		
	Non–specific amplicons are produced during PCR	Extension time too long. Prolonged extension times are not recommended. Set the extension time not longer than 30 seconds for PCR fragment of up to 500 bp. A reduction of the PCR extension time may increase the yield of specific PCR products.
DNA yields of approximately 1–3 µg in negative (no-template) controls and positive result in downstream assay (e.g., PCR)		
	Contaminating DNA templates	Decontaminate all laboratory equipment, and take all necessary precautions to avoid contamination of reagents and samples with extraneous DNA. If possible, work in a laminar-flow hood. Use sterile equipment and barrier pipet tips only, and store amplification chemistry and DNA templates in separate locations.
Downstream application results not optimum		
	Sensitive downstream applications may require DNA cleanup after amplification using the EpiTect Whole Bisulfitome Kit	Contact QIAGEN Technical Services or visit www.qiagen.com for DNA cleanup recommendations suitable for your application.

Appendix A: PCR of DNA Amplified Using the EpiTect Whole Bisulfitome Kit

Amount of amplified DNA for end-point PCR and real-time PCR

If analyzing the amplified DNA by PCR, we recommend dilution of the EpiTect whole bisulfitome amplification reaction with water or TE buffer to a final concentration of 5 ng/µl. For example, if the concentration of DNA after the EpiTect whole bisulfitome amplification reaction is 50 ng/µl (corresponding to a yield of 2 µg), dilute your reaction 1/5 (e.g., add 2 µl amplified DNA to 8 µl water). Use 2–3 µl of the diluted DNA in each PCR reaction.

Comparative determination of methylation in reference and test sample

Typically, reference and test DNA samples are compared in methylation specific PCR to determine the grade of methylation at the sequence of interest.

It is necessary that both DNAs – reference and test DNA – are treated in the same way:

1) Reference and test DNA are converted by the same bisulfite reagents (EpiTect Bisulfite Kit).
2) Reference and test DNA are both amplified by the EpiTect Whole Bisulfitome Kit.
3) The concentration of the amplified DNA is determined by the same procedure (see Appendix C, page 15).

Appendix B: Starting Material

The success of downstream applications depends on the starting material used in the EpiTect whole bisulfitome amplification procedure. The EpiTect whole bisulfitome amplification procedure starts with purified DNA converted by sodium bisulfite. During bisulfite conversion of genomic DNA, fragmentation occurs and the sequence is changed due to the conversion of non-methylated cytosines to uracil. The degree of DNA fragmentation may vary depending on the bisulfite conversion method chosen. The higher the degree of DNA fragmentation, the lower the DNA copy number. This means a large number of DNA sequences are broken and therefore cannot be amplified by the EpiTect Whole Bisulfitome Kit. Thus, a higher degree of fragmentation of converted DNA can be compensated by larger amounts of starting DNA. For optimal results, we recommend DNA conversion using EpiTect Bisulfite Kits, which contain a unique DNA protect mechanism thereby preventing excessive DNA degradation during bisulfite conversion. Thus, converted DNA from paraffin–embedded tissue is not usable for EpiTect whole bisulfitome amplification reactions due to its higher degree of fragmentation. If using the EpiTect 96 Bisulfite Kit, we strongly recommend the protocol "Sodium Bisulfite Conversion of Unmethylated Cytosines Using a Centrifuge" (page 15, EpiTect 96 Bisulfite Handbook).

Appendix C: Determination of DNA Concentration and Yield

Quantification of DNA yield

A 40 µl EpiTect Whole Bisulfitome Kit reaction typically yields approximately 1–3 µg of DNA. However, if a more accurate quantification of DNA is required, it is important to utilize a DNA quantification method that is specific for double-stranded DNA, since EpiTect Whole Bisulfitome Kit amplification products contain unused reaction primers and dNTPs. PicoGreen® reagent displays enhanced binding to double-stranded DNA and may be used, in conjunction with a fluorometer, to quantify the double-stranded DNA product.

Quantification of locus representation

Locus representation for each sample can be quantified by real-time PCR. Contact QIAGEN Technical Services or visit our website at www.qiagen.com for a protocol.

Appendix D: PicoGreen Quantification of EpiTect Whole Bisulfitome Amplified DNA

This protocol is designed for quantification of double-stranded amplified DNA using PicoGreen reagent.

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

Equipment and reagents to be supplied by user

- Quant-iT™ PicoGreen dsDNA reagent (Invitrogen, cat. no. P7581)
- TE buffer (10 mM Tris·Cl; 1 mM EDTA, pH 8.0)
- Human genomic DNA (e.g., Promega, cat. no. G3041)
- 2 ml microcentrifuge tube
- 96-well plates (suitable for use in a fluorescence microplate reader)
- Fluorescence microplate reader (e.g., Tecan® Ultra)

Procedure

1. **In a 2 ml microcentrifuge tube, make a 1/150 dilution of PicoGreen stock solution in TE buffer. Each quantification reaction requires 20 µl. Cover the microcentrifuge tube in aluminum foil or place it in the dark to avoid photodegradation of the PicoGreen reagent.**

   For example, to prepare enough PicoGreen working solution for 100 samples, add 13.3 µl PicoGreen to 1986.7 µl TE buffer.

   Important: Prepare the PicoGreen/TE solution in a plastic container as the PicoGreen reagent may adsorb to glass surfaces.

2. **Prepare a 16 µg/ml stock solution of genomic DNA in TE buffer.**
3. **Make 200 µl of 1.6, 0.8, 0.4, 0.2, and 0.1 µg/ml DNA standards by further diluting the 16 µg/ml genomic DNA with TE buffer.**
4. **Transfer 20 µl of each DNA standard in duplicate into a 96-well plate labeled A (see figure below).**

   Note: The 96-well plate must be suitable for use in a fluorescent microplate reader.

96-Well Plate

	1	2	3	4	5	6	7	8	9	10	11	12
A												
B												
C												
D												
E												
F												
G												
H			1.6	0.8	0.4	0.2	0.1	1.6	0.8	0.4	0.2	0.1

Gray squares: genomic DNA standard (µg/µl).

5. **Place 2 µl of each EpiTect reaction for quantification into a new 96-well plate and add 48 µl TE buffer to make a 1/25 dilution. Store the remaining amplified DNA at –20°C.**
6. **Place 20 µl diluted DNA (from step 5) into an unused well of 96-well plate A.**

   The remaining 30 µl of the 1/25 dilutions can be stored at –20°C and used for future downstream sample analysis.

7. **Add 20 µl PicoGreen working solution (from step 1) to each sample (amplified DNA and DNA standards) in 96-well plate A. Gently shake the plate on the bench top to mix the samples and reagent.**
8. **Centrifuge the 96-well plate briefly to collect residual liquid from the walls of the wells.**
9. **Measure the sample fluorescence using a fluorescence microplate reader and standard fluorescence filters (excitation approximately 480 nm; emission approximately 520 nm).**

   To ensure that the sample readings remain in the detection range of the microplate reader, adjust the instrument's gain so that the sample with the highest DNA concentration yields fluorescence intensity near the fluorimeter's maximum.

Calculation of DNA concentration and yield

10. **Generate a standard curve by plotting the concentration of DNA standards (µg/ml) (x-axis) against the fluorescence reading generated by the microplate reader (y-axis). Plot an average of the fluorescence recorded for each DNA standard of the same concentration.**
11. **Use the standard curve to determine the concentration (µg/ml) of the diluted amplified DNA sample. This is achieved by plotting the fluorescence reading of the sample against the standard curve and reading the DNA concentration on the x-axis.**

    Note: The calculation of DNA concentration depends on the standard curve and the determination of the slope. For accurate results, the standard curve should be a straight line. Any deviation from this may cause inaccuracies in the measurement of amplified DNA concentrations.
12. **Multiply the value determined in step 11 by 25 to give the concentration of undiluted sample DNA (as the sample DNA measured by PicoGreen fluorescence had been diluted 1 in 25).**
13. **To determine the total amount of DNA in your sample, multiply the concentration of undiluted sample DNA (µg/ml) (step 12) by the reaction volume in milliliters (i.e., for a 40 µl reaction, multiply by 0.04).**

Ordering Information

Product	Contents	Cat. no.
Related products		
EpiTect Bisulfite Kits — for complete bisulfite conversion and cleanup of DNA for methylation analysis		
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 Control DNA — for evaluation of PCR primers used for methylation analysis		
EpiTect Control DNA, methylated (100)	Methylated and bisulfite converted human control DNA for 100 control PCRs	59655
EpiTect Control DNA, unmethylated (100)	Unmethylated and bisulfite converted human control DNA for 100 control PCRs	59665
EpiTect Control DNA (1000)	Unmethylated human control DNA for 1000 control PCRs	59568
EpiTect PCR Control DNA Set (100)	Human control DNA set (containing both bisulfite converted methylated and unmethylated DNA and unconverted unmethylated DNA) for 100 control PCRs	59695
EpiTect MSP Kit — for highly accurate methylation-specific end-point PCR without optimization		
EpiTect MSP Kit (25)	EpiTect MSP Master Mix for 25 x 50 µl reactions	59303
EpiTect MSP Kit (100)	EpiTect MSP Master Mix for 100 x 50 µl reactions	59305
EpiTect MSP Kit (400)	EpiTect MSP Master Mix for 400 x 50 µl reactions	59307

Ordering Information

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 x 50 µl reactions	59436
EpiTect MethyLight PCR Kit (1000)	Master Mix for methylation-specific real-time PCR analysis, 1000 x 50 µl reactions	59438
EpiTect MethyLight PCR + ROX Vial Kit (200)	Master Mix without ROX for methylation-specific real-time PCR analysis, 200 x 50 µl reactions	59496
EpiTect MethyLight PCR + ROX Vial Kit (1000)	Master Mix without ROX for methylation-specific real-time PCR analysis, 1000 x 50 µl reactions	59498

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

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