FFPE Bisulfite Conversion

(version A1)

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Overview

For the investigation of DNA methylation patterns, bisulfite conversion and subsequent DNA sequencing is the gold standard because it provides detailed information on the methylation pattern of individual DNA molecules at single-base-pair resolution. Bisulfite conversion is based on the deamination of cytosine residues into uracils in the presence of sodium bisulfite. Since methylated cytosine residues (both 5-mC and 5-hmC) are not converted under these conditions, the original methylation state of the DNA can be determined. Converted DNA can be analyzed by PCR amplification followed by DNA sequencing (gene-specific or genome-wide), array-based methods, or other downstream applications.

Active Motif's FFPE Bisulfite Conversion Kit is designed specifically for DNA methylation analysis of formalin-or paraformalin-fixed, paraffin-embedded (FFPE) tissues. First, DNA is extracted from FFPE tissue blocks or unsectioned core samples. Then, the DNA is heated to reverse protein-DNA cross-links caused by fixation. Samples are then treated with a protease solution to degrade proteins and the DNA is column purified prior to bisulfite conversion. Finally, Active Motif's optimized bisulfite conversion reagents and protocol are used to convert cytosine residues not protected by methylation into uracils to provide a DNA methylation profile at single-base-pair resolution. The kit also includes a positive control conversion-specific PCR primer pair that is specific for bisulfite-converted human or mouse DNA. Because the primer pair produces a PCR product only if conversion has occurred, you can confirm the procedure worked before starting sequencing or other analysis methods.

The FFPE Bisulfite Conversion Kit contains enough reagents to perform 40 DNA isolations from up to four 20 µm FFPE tissue sections, or up to 35 mg of unsectioned, core samples per reaction and 40 bisulfite conversion reactions.

product	format	catalog no.
FFPE Bisulfite Conversion Kit	40 rxns	55021

Flow Chart of Process

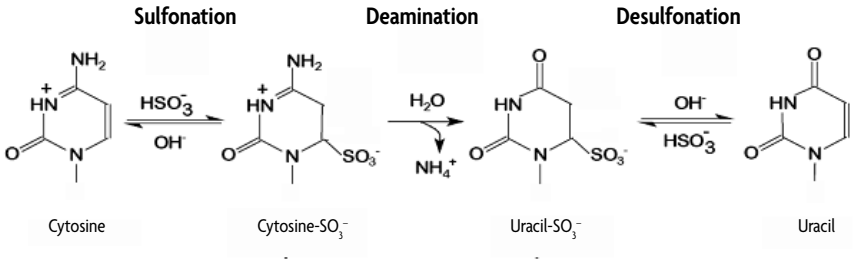

Figure 1: Schematic of the bisulfite conversion reaction of unmethylated cytosine to uracil.

The first step in the bisulfite conversion reaction is sulfonation, where sodium bisulfite is added to the double bond of cytosine to form a cytosine-bisulfite derivative. This reaction is reversible, with the forward reaction being favored by high temperature and low pH. The second step is an irreversible hydrolytic deamination of the cytosine-bisulfite derivative that results in a uracil-bisulfite derivative. This reaction is also favored by low pH. The final step involves desulfonation of the uracil-bisulfite to uracil under high pH conditions. Only unmethylated cytosines are susceptible to the bisulfite reaction. Methylated (5-mC and 5-hmC) cytosines do not undergo conversion.

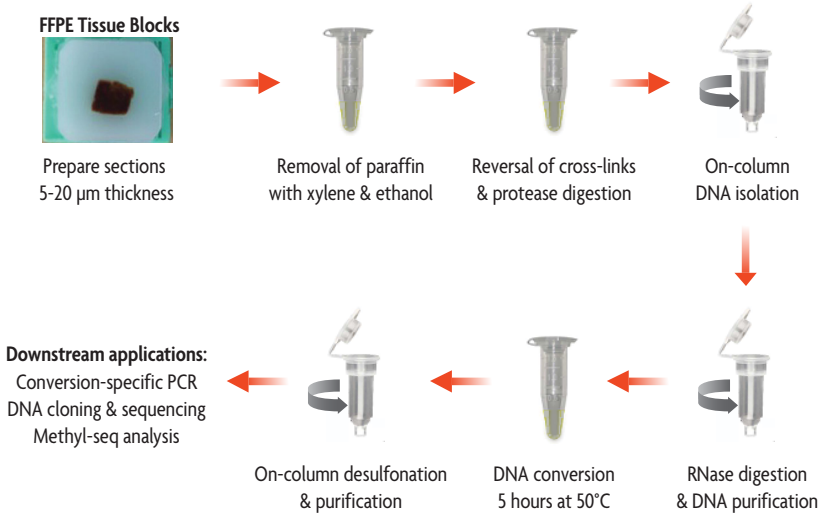

Figure 2: Flow chart of the FFPE Bisulfite Conversion Kit.

In the FFPE Bisulfite Conversion Kit, genomic DNA of interest is isolated from FFPE tissue sections. The DNA is heated to reverse protein-DNA cross-links and treated with protease solution. The DNA is then column purified prior to bisulfite conversion. Following a 5 hour DNA conversion reaction, the sample is added to the included DNA purification columns, and a simple on-column desulfonation is performed. DNA is then eluted from the column and is ready for use in PCR.

Introduction

DNA methylation is an essential epigenetic modification that plays a key role in transcriptional regulation and assures the proper regulation of gene expression and stable gene silencing in normal cells. DNA methylation is the covalent addition of a methyl group to the fifth position of the cytosine pyrimidine ring in DNA and usually occurs within CpG dinucleotides, although in some cases the DNA methylation can also be found in a non-CpG context[1, 2]. CpG dinucleotides are concentrated in large clusters called CpG islands, which are often positioned in the promoter regions of many genes and are usually unmethylated[3]. It is estimated that the human genome contains around 29,000 CpG islands and that almost 60% of all human genes are associated with CpG islands[4]. Aberrant cytosine methylation is associated with silencing of tumor suppressor genes[5] and plays a decisive role in the development of many cancers[6-14]. Alterations in the methylation status of DNA are promising candidates for highly specific and sensitive indicators of cancer diagnosis and prognosis[15-17]. Apart from carcinogenesis, DNA methylation is crucial for a variety of processes, such as genomic imprinting, X-chromosome inactivation, and suppression of repetitive elements[4, 18]. Thus profiling DNA methylation across the genome is vital to understanding the influence of epigenetics.

The bisulfite reaction was first described in the early 1970s[19] and was used by Frommer *et al*.[20], and Clark *et al*.[21] to distinguish between cytosine and 5-methylcytosine (5-mC) in DNA. It is now known that both 5-methylcytosine and 5-hydroxymethylcytosine (5-hmC) remain unchanged during conversion and therefore this method cannot be used to distinguish between the different methyl modifications[22-24]. In the bisulfite conversion reaction, DNA is first treated with sodium bisulfite to convert cytosine residues into uracil in denatured (single-stranded) DNA, under conditions whereby 5-mC and 5-hmC remain essentially non-reactive. The DNA is then amplified by PCR where the uracils are converted to thymines. Bisulfite converted DNA can be analyzed for gene- or allele-specific methylation patterns, or adapted for genome-wide DNA methylation analysis. A methylation profile of the sample can then be created by comparing the sequence of the converted DNA to untreated DNA.

Active Motif's FFPE Bisulfite Conversion Kit simplifies analysis of DNA methylation from FFPE samples by providing DNA isolation reagents, optimized conversion reagents and an easy-to-use protocol. Conversion conditions have been optimized to work with FFPE DNA, which is often highly fragmented due to the harsh chemicals needed to preserve tissue structure and prevent putrefaction. The FFPE Bisulfite Conversion Kit has minimal DNA degradation and >99% conversion efficiency of unmethylated cytosines to uracils. The kit includes a positive control conversion-specific PCR primer pair that is specific for bisulfite-converted human and mouse DNA. Because the primer pair produces a 220 bp PCR product only if conversion has occurred, you can validate the success of the conversion reaction before spending extra time and money on sequencing.

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Kit Performance and Benefits

The FFPE Bisulfite Conversion Kit is for research use only. Not for use in diagnostic procedures.

DNA Yield: The DNA yield and quality will depend on the tissue and fixing procedure. We have recovered 0.1 - 3.5 µg DNA per mg FFPE tissue sample.

Sensitivity: FFPE Bisulfite Conversion can be performed on 5 pg - 2 µg of DNA, with the optimal range of 500 ng to 2 µg.

Conversion efficiency: >99%

DNA Recovery: >80%

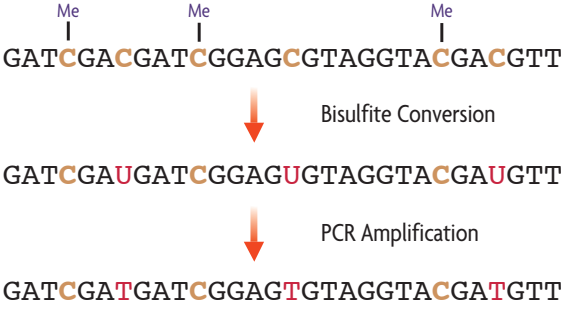

Figure 3: DNA sequence following bisulfite conversion and PCR amplification.

This figure shows an example of the effect of bisulfite treatment on a DNA sequence. The original DNA sequence contains 6 cytosine residues, 3 methylated (Me) and 3 unmethylated. Following bisulfite conversion the unmethylated cytosines are converted into uracil while the methylated cytosines remain as cytosine. The methylation profile of the DNA can then be determined by PCR amplification followed by DNA sequencing, where the uracils will be detected as thymine. This is why it is often stated that bisulfite treatment converts C to T.

Kit Components and Storage

FFPE Bisulfite Conversion Kit

FFPE Bisulfite Conversion Kit components arrives in boxes with two different storage temperatures. We recommend storing each component at the temperatures listed in the table below. Components are guaranteed stable for 6 months from date of receipt when stored properly.

Reagents	Quantity	Storage
Protease Solution	160 µl	-20°C
RNase A	400 µl	-20°C
Glycogen (20 mg/ml)	10 µl	-20°C
Conversion-specific PCR primer pair (4 µM)	200 µl	-20°C
Digestion Buffer AM3	10 ml	RT
Wash Buffer 1	60 ml	RT
Wash Buffer 2/3	60 ml	RT
Isolation Additive	15 ml	RT
Elution Buffer AM5	4 ml	RT
Filter cartridges	2 x 20 ea	RT
Collection tubes	2 x 40 ea	RT
Conversion Reagent	4 tubes	RT
Hydroquinone	4 tubes	RT
Buffer A	1.5 ml	RT
Buffer B	1 ml	RT
DNA Binding Buffer	2 x 15 ml	RT
DNA Wash Buffer	20 ml	RT
DNA Elution Buffer	2.5 ml	RT
DNA purification columns & collection tubes	40 ea	RT

Additional Materials Required

DNA Isolation from FFPE samples

- 100% xylene, ACS grade or higher quality
- 100% ethanol, ACS grade or higher quality
- Microtome for tissue sectioning
- Microcentrifuge tubes and microcentrifuge

- Mortar and pestle with liquid nitrogen to crush and grind samples
- Thermocycler or heat blocks set at 50°C and 95°C
- Spectrophotometer (or Nanodrop) for DNA quantification

Bisulfite Conversion and PCR Analysis

- Purified sample DNA
- 0.2 ml PCR tubes and caps
- 100% isopropanol
- Microcentrifuge tubes and microcentrifuge
- dNTP mixture (10 mM each)
- Thermocycler
- Hot Start *Taq* polymerase (5 U/µl) and PCR reaction buffer recommended for use with bisulfite converted DNA (*e.g.* Qiagen HotStarTaq DNA polymerase, Catalog No. 203203)
- Methylation-specific PCR primers for the gene of interest
- 2% agarose TAE gel
- Gel apparatus and power supply
- 6x Loading dye
- Molecular weight marker

DNA Clean Up

- (Optional) DNA purification kit (*e.g.* QIAquick PCR purification kit, Qiagen Cat. No. 28104)
- Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v)
- 3 M Sodium acetate, pH 5.5
- Glycogen, molecular biology grade (20 ng/µl) or tRNA (10 µg/µl)
- 100% ethanol

DNA Cloning and Gene-specific Sequencing

- Ligation vector (*e.g.* Topo TA Cloning Kit for sequencing,Thermo Fisher Cat. No. 450030)
- Competent *E.coli* cells (*e.g.* RapidTrans™ TAM 1, Active Motif Cat. No. 11096)
- Bacterial growth media and plates with appropriate selection
- DNA miniprep materials
- DNA sequencing reagents

Genome-wide sequencing analysis

- Methyl-Seq Library Preparation Kit and Index primers (e.g. Accel-NGS® Methyl-Seq DNA Library Kit, Swift Biosciences, Cat No. 30024)

PLEASE READ THE ENTIRE PROTOCOL BEFORE STARTING!

Buffer Preparation for DNA Isolation from FFPE Samples

Wash Buffer 1

On the first use of the kit, add 42 ml of 100% ethanol to the bottle labeled Wash Buffer 1. Mix well and mark the bottle as ethanol added.

Wash Buffer 2/3

On the first use of the kit, add 48 ml of 100% ethanol to the bottle labeled Wash Buffer 2/3. Mix well and mark the bottle as ethanol added.

Digestion Buffer AM3

Is supplied ready to use.

Isolation Additive

Is supplied ready to use.

Protease Solution

Is supplied ready to use.

RNase A

Is supplied ready to use.

Elution Buffer AM5

Is supplied ready to use.

Buffer Preparation for Bisulfite Conversion

Input DNA

Input DNA should be in the range of 5 pg to 2 µg, with the optimal range being 500 ng to 2 µg.

Preparation of Desulfonation Buffer

For each reaction, combine 22 µl Buffer B with 88 µl dH_2O and 110 µl 100% isopropanol. If this reagent will not be used immediately, it can be stored for up to one week at -20°C.

Diluted Buffer B

Prepare a 3-fold dilution of Buffer B by adding 10 µl Buffer B to 20 µl dH_20. Store at RT.

Preparation of Conversion Buffer

Four tubes of Conversion Reagent are provided in the Bisulfite Conversion Kit. Each vial is sufficient for performing 10 bisulfite conversions. Prepare the Conversion Buffer by resuspending one of the Conversion Reagent tubes with 875 µl dH_2O and 350 µl Buffer A. Mix at room temperature for 10 minutes with intermittent vortexing.

NOTE: This is a saturated solution and it is normal for the Conversion Reagent to not dissolve completely. The Conversion Buffer is stable for 1 week at -20°C. Conversion Reagent is sensitive to air and moisture. Keep cap on tight and re-cap immediately after use.

Preparation of Hydroquinone

Hydroquinone is an alkalizing agent and prevents DNA from strand breakage because of depurination. Each tube provided can be used for 10 conversion reactions. Prepare a working stock of Hydroquinone by resuspending one tube with 100 µl dH_2O. Keep mixture in dark and discard after use.

DNA Binding Buffer / DNA Wash Buffer / DNA Elution Buffer

Are supplied ready to use.

Glycogen (20 mg/ml)

Prepare a 1 mg/ml working solution of glycogen by adding 2 µl Glycogen stock to 38 µl dH_2O. The diluted stock may be stored at -20°C for future use.

Positive control Conversion-specific PCR primer pair

The Bisulfite Conversion Kit's positive control Conversion-specific PCR primer pair (4 µM) is specific for amplification of bisulfite converted human or mouse DNA. This primer pair should amplify DNA that has been successfully bisulfite converted to generate a 220 bp PCR product. It should not generate a PCR product with untreated human or mouse DNA. If using a different species of DNA besides human or mouse, the included positive control Conversion-specific PCR primer pair will not work and conversion-specific primers will need to be designed to your species of interest.

Design of PCR primers

PCR primer design is critical for successful analysis of converted DNA. There are several sites that offer information on designing methylation-specific PCR primers. The MethPrimer website: http:// www.urogene.org/methprimer/ is a free site that contains several useful tools for the design of bisulfite-conversion-based Methylation PCR Primers. Additional programs are also available. We suggest designing both bisulfite conversion specific and untreated PCR primer pairs for each gene of interest to validate the success of the conversion reaction prior to downstream analysis. Since FFPE DNA will tend to be highly fragmented, we recommend designing primers to generate PCR amplicons between 100-200 bp. For design of primers to be used in the same PCR reaction as the positive control Conversion-Specific PCR primer pair we recommend an annealing temperature between 56-60°C.

This protocol is designed to isolate DNA from formalin- or paraformalin-fixed, paraffin-embedded (FFPE) tissues. Up to four 20 µm sections, or up to 35 mg of unsectioned core samples can be processed per reaction. Please refer to page 8 of the manual to prepare reagents.

Section A: FFPE Sample Preparation

1. Prepare FFPE samples for DNA isolation:

   FFPE Sections: Cut 5- 20 µm sections from FFPE tissue blocks using a microtome. Use samples cut from the interior of the paraffin block to minimize nucleic acid damage caused by exposure to the atmosphere during storage. Place up to 80 µm of tissue slices in a single microcentrifuge tube (e.g. four 20 µm, eight 10 µm or sixteen 5 µm slices)

   Unsectioned Core Samples: Cut an unsectioned core sample, up to 35 mg, from a paraffin block. Use samples that were cored from the interior of the paraffin block to minimize nucleic acid damage caused by exposure to the atmosphere during storage. Crush or grind the sample in liquid nitrogen or on dry ice and place in a 1.5 ml microcentrifuge tube.

2. Add 1 ml 100% xylene to each sample. Vortex briefly to mix. Quick spin to collect liquid to the bottom of the tube.

   NOTE: Xylene is a toxic substance. Handle only in well ventilated area using personal protection equipment. Dispose of xylene waste (including solutions containing trace amounts of xylene) according to applicable regulations.

3. Heat the sample for 3 minutes at 50°C to melt the paraffin.
4. Centrifuge the sample at maximum speed for 2 minutes at room temperature to pellet the tissue. If the sample does not form a tight pellet following the first spin, repeat the centrifugation for an additional 2 minutes at maximum speed.
5. Carefully remove the xylene without disturbing the pellet. The tissue pellet is usually clear and can be difficult to see. If the pellet is loose, you may need to leave some residual xylene to avoid sample loss.
6. To wash the pellet, add 1 ml 100% ethanol (room temperature) and vortex to mix. The tissue pellet should turn opaque.
7. Centrifuge the sample at maximum speed for 2 minutes at room temperature to wash the pellet. Carefully remove and discard the ethanol without disturbing the pellet. Ethanol will contain trace amounts of xylene an should be discarded accordingly.
8. Repeat Step 6-7 above to wash a second time with 1 ml of 100% ethanol.
9. Quick spin the tube to collect any remaining liquid to the bottom of the tube. Remove as much residual ethanol as possible without disturbing the pellet.
10. Air dry the pellet for 15-45 minutes at room temperature. For larger tissue sections, we recommend using a centrifugal vacuum concentrator at medium (40-45°C for < 20 minutes) or low (37-40°C for 20-40 minutes) heat.

Section B: Reversal of Cross-links and Protease Digestion

1. Using the table below, prepare Digestion Buffer AM3 based on the sample size. Add the recommended amount of Digestion Buffer to each sample.

Sample Size	Digestion Buffer AM3 per Sample
< 40 µm tissue slices	100 µl
40-80 µm tissue slices	200 µl

2. Swirl the tube gently to mix and immerse the tissue pellet. If needed, use a pipet tip to push the tissue pellet down from the side of the tube and back into solution.
3. Heat the sample for 30 minutes at 95°C to reverse the formaldehyde cross-links.
4. Quick spin the tube to collect liquid to the bottom. Add 4 µl Protease Solution to each tube.
5. Incubate the sample for 16 hours at 50°C.

   Note: Most samples will clarify after 16 hour Protease Solution treatment. If the sample does not clarify, it may be heavily oxidized and therefore resistant to protease digestion. Samples that do not clarify may have slightly lower yields and smaller DNA fragments. Increasing the incubation time at 50°C to 48 hours may be beneficial in obtaining larger DNA fragments.

Section C: DNA Isolation

1. Using the table below, prepare Isolation Buffer based on the sample size. Add the recommended amount of Isolation Additive and ethanol to each sample.

	< 40 µm tissue slices	40-80 µm tissue slices
Isolation Additive	120 µl	240 µl
100% Ethanol	275 µl	550 µl
TOTAL	**395 µl**	**790 µl**

2. Add the appropriate volume of Isolation Buffer to each sample. Mix by pipetting up and down. Samples may appear white and cloudy after mixing.
3. For each sample place a filter cartridge into one of the supplied collection tubes.
4. Add up to 700 µl of the sample onto each filter cartridge and close the lid. Avoid pipetting large pieces of undigested tissue onto the filter cartridge as this may clog the filter.
5. Centrifuge 10,000 x g for 30 seconds to filter the mixture.
6. Discard the flow-through and replace the filter cartridge back inside the same collection tube.

7. If necessary, repeat Steps 4-6 until all the sample mixture has passed through the filter.
8. On the first use of the kit, prepare Wash Buffer 1 and Wash Buffer 2/3 by adding the recommended amount of ethanol as described in the Buffer Preparation section on page 8.
9. Add 700 µl Wash Buffer 1 to the filter cartridge.
10. Centrifuge 10,000 x g for 30 seconds to wash the cartridge.
11. Discard the flow-through and replace the filter cartridge back inside the same collection tube.
12. Add 500 µl Wash Buffer 2/3 to the filter cartridge.
13. Centrifuge 10,000 x g for 30 seconds to wash the cartridge.
14. Discard the flow-through and replace the filter cartridge back inside the same collection tube.
15. Centrifuge 10,000 x g for 30 seconds to remove any residual liquid.

Section D: RNase Digestion and DNA Purification

1. Using the table below, prepare the RNase A mix. A master mix can be used if there is more than one sample.

	RNase A Mix per Sample
RNase A	10 µl
Nuclease-free Water	50 µl
TOTAL	**60 µl**

2. Add 60 µl RNase A mix to the center of each filter cartridge. Cap the tube and incubate for 30 minutes at room temperature.
3. Before the end of this incubation, place Elution Buffer AM5 at 95°C to preheat.
4. Add 700 µl Wash Buffer 1 to the filter cartridge. Incubate for 1 minute at room temperature.
5. Centrifuge 10,000 x g for 30 seconds to wash the cartridge.
6. Discard the flow-through and replace the filter cartridge back inside the same collection tube.
7. Add 500 µl Wash Buffer 2/3 to the filter cartridge.
8. Centrifuge 10,000 x g for 30 seconds to wash the cartridge.
9. Discard the flow-through and replace the filter cartridge back inside the same collection tube.
10. Repeat Steps 7-9 to wash a second time with 500 µl Wash Buffer 2/3.
11. Centrifuge 10,000 x g for 30 seconds to remove any residual liquid.

12. Transfer the filter cartridge to a fresh collection tube.
13. Add 60 µl of preheated (95°C) Elution Buffer AM5 to the center of each filter and close the cap. Incubate for 1 minute at room temperature.
14. Centrifuge at maximum speed for 1 minute to collect eluate.
15. Determining DNA concentration by spectrophotometry, such as a NanoDrop. Measure the absorbance at 260 nm. Make sure your samples fall within the range of detection for your spectrophotometer or use a more sensitive fluorescence-based quantitation.
16. Store DNA at -20°C.

In this protocol, the isolated FFPE DNA will undergo bisulfite conversion to determine the DNA methylation status at single-base-pair resolution. The FFPE Bisulfite Conversion Kit can efficiently convert DNA samples containing between 5 pg to 2 µg of DNA, with the optimal input range of 500 ng to 2 µg. Please refer to pages 8-9 of the manual to prepare reagents.

Section E: Conversion Reaction

1. Prepare Conversion Buffer, Hydroquinone and Buffer B dilution as described in the Buffer Preparation Section on pages 8-9.
2. Set up reactions in the order listed below into PCR tubes.

Reagent	Quantity
DNA (5 pg – 2 µg)	X µl
dH_2O	adjust volume to 10 µl
Diluted Buffer B	3 µl
Total Volume	**13 µl**

3. Incubate samples at 90°C for 2 minutes. Immediately transfer to ice.
4. Quick spin the samples to collect liquid to the bottom of the tube. Place on ice.
5. Set up conversion reactions on ice for each sample by adding reagents in the order listed below into each PCR tube. Protect conversion reactions from light. Mix well by pipetting. The final volume should be 140 µl.

Reagent	Quantity
DNA mixture	13 µl
Conversion Buffer	120 µl
Hydroquinone	7 µl
Total Volume	**140 µl**

6. Place tubes in the thermocycler and set the following program. To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.

   94°C for 3 minutes
   50°C for 5 hours
   Hold at 4°C

7. Remove tubes and continue with Section F. The protocol can also be stopped here and DNA samples can be kept at 4°C in the dark for up to 5 days.

Section F: On-column Desulfonation and DNA Purification

1. Prepare Desulfonation Buffer as described in the Buffer Preparation section on page 8.
2. For each conversion reaction, aliquot 500 µl DNA Binding Buffer and 1 µl Glycogen (1 mg/ml) into a 1.5 ml microcentrifuge tube. Add the entire conversion reaction mixture to the DNA Binding Buffer in the tube and vortex.
3. Remove the desired number of DNA purification columns and place each one in a collection tube.
4. Pipet each DNA Binding Buffer/conversion reaction mixture into one of the DNA Purification columns and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
5. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge.
6. Add 200 µl Desulfonation Buffer to each column and incubate 20 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
7. Add 200 µl DNA Wash Buffer to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge. Empty the collection tube and place the column back on the collection tube.
8. To remove any residual wash buffer, spin at 10,000 rpm for 30 seconds in a microcentrifuge. Remove the column and place in a new 1.5 ml microcentrifuge tube.
9. Add 20 µl DNA Elution Buffer directly to the filter of the column.
10. Incubate 3 minutes at room temperature. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. The eluate will contain the converted DNA.
11. Store DNA at -20°C.

Protocols – Optional Downstream Analysis

The following protocols are provided as guidelines to perform downstream analysis of the bisulfite treated DNA. Protocols are available for quantification of DNA yield (Section G), for evaluation of bisulfite conversion efficiency using conversion-specific PCR (Sections H-I), for cloning and sequencing of gene-specific regions (Sections H-L) and to perform genome-wide Methyl-seq analysis (Section M).

Section G: Quantification of Bisulfite Converted DNA

Following bisulfite treatment of DNA, the original base-pairing of DNA no longer exists since the unmethylated cytosine residues are converted into uracil. If using a spectrophotometer, set the unit to measure RNA absorption (using a conversion of 40 µg/ml for A260 nm = 1.0) because bisulfite converted DNA resembles RNA since it contains uracil and is largely single stranded. Alternatively, use a fluorescence-based Qubit ssDNA Assay or check the quality of the bisulfite converted DNA using Agilent Bioanalyzer with RNA 6000 Pico Kit or similar detection methods. However, if the bisulfite DNA is PCR amplified prior to quantification, traditional methods to quantify DNA can be used.

Section H: Conversion-Specific PCR

Determine the number of conversion-specific PCR reactions to perform. The included positive control Conversion-specific PCR primer pair can be used to quickly assess the success of the bisulfite conversion reaction using human or mouse DNA samples. For conversion-specific gene analysis, use a conversion-specific PCR primer pair and an untreated PCR primer pair (if desired) for each gene locus of interest. Follow the recommendation listed on page 9 to design bisulfite conversion-specific primers for your gene of interest. We recommend an annealing temperature between 56-60°C if the primers are to be used in the same PCR reaction as the positive control Conversion-Specific PCR primer pair.

We also recommend performing a Hot Start PCR reaction when working with bisulfite converted DNA to ensure reliable and consistent amplification of the DNA template.

DNA Template	Positive Control Conversion-specific Primer Pair	Gene-specific Converted Primer Pair	Gene-specific Untreated Primer Pair
Bisulfite Converted DNA	Rxn 1	Rxn 4	Rxn 7
Untreated DNA	Rxn 2	Rxn 5	Rxn 8
H_2O control	Rxn 3	Rxn 6	Rxn 9

1. Label the desired number of PCR tubes and place in a PCR tube rack on ice.
2. Make a 20 ng/µl dilution of unconverted genomic DNA (the same Input DNA that was used for the conversion reaction).
3. Prepare a PCR master mix for each of the primer sets to be tested in 1.5 ml microcentrifuge tubes as described below. DNA templates will be added later directly to the PCR tubes. Combine components on ice and mix by vortexing. Four reactions are prepared to account for any loss during pipetting. The table below is provided as an example, please adjust as needed to follow the recommendation of the Hot Start PCR polymerase to be used.

Reagent	One Rxn	Master Mix
dH_2O	13.55 µl	54.2 µl
PCR primer pair (4 µM)	2 µl	8 µl
dNTP mixture (10 mM each dNTP)	0.25 µl	1 µl
10X PCR Buffer	2 µl	8 µl
Hot Start *Taq* (5 U/µl)	0.2 µl	0.8 µl
Total Volume (Not including DNA template)	18 µl	72 µl

4. Aliquot 18 µl of this PCR cocktail into the appropriately labeled tubes on ice.
5. Add template DNA or water to the PCR tubes as indicated below and mix. Ensure that the entire 20 µl PCR reaction is on the bottom of the tube. Centrifuge tubes if necessary.

PCR Rxn #	PCR Master Mix	DNA Template	dH_2O
1, 4 & 7	18 µl	2 µl Bisulfite Converted DNA	–
2, 5 & 8	18 µl	2 µl Untreated DNA (20 ng/µl)	–
3, 6 & 9	18 µl	–	2 µl

6. Place tubes in PCR cycler and program the thermocycler as below. (To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.)

   94°C for 15 minutes
   (94°C for 30 seconds, 58°C for 30 seconds, 72°C for 1 minute) for 30–35 cycles
   72°C for 10 minutes
   Hold at 4°C

Section I: Agarose Gel Analysis of PCR Products

1. Pour a 2% agarose gel in 1X TAE.
2. Add 5 µl from each of the PCR reactions to 1 µl 6X DNA loading dye and load sample on a gel. Use a 100 bp or 50 bp DNA ladder. Run the gel until the marker is near the bottom of the gel and the markers are well separated. Only the PCR reactions containing bisulfite converted DNA should show a PCR product when using conversion-specific primer sets. Untreated DNA

and the H_2O control PCR reactions should not have any PCR amplification. For the positive control Conversion-specific PCR primer pair, the bisulfite converted DNA should generate a 220 bp amplicon as shown in Figure 4.

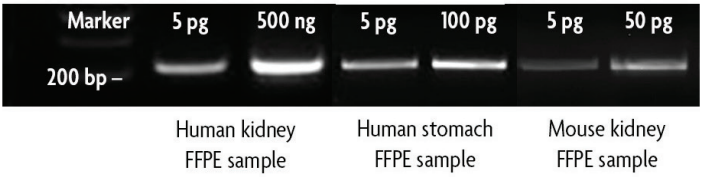

Figure 4: Positive Control Conversion-specific PCR primer pair is specific for converted human or mouse DNA.
The figure above depicts the PCR results of bisulfite converted DNA from FFPE tissue for human kidney, human stomach and mouse kidney samples. DNA was isolated according to the FFPE Bisulfite Conversion Kit assay protocol and the DNA quantity listed was bisulfite converted and PCR amplified using the included positive control Conversion-specific PCR primer pair. The primer pair is specific towards bisulfite converted DNA and therefore a 220 bp PCR amplicon is present in the bisulfite treated DNA samples.

Section J: DNA Clean Up

Prior to cloning it is necessary to clean up the DNA. Use the following protocol to perform a phenol/chloroform extraction followed by ethanol precipitation. Alternatively, DNA can be purified using columns such as QIAquick PCR purification kit (Qiagen part no. 28104). Elute in 30-50 µl volume. If more than one band is amplified by the PCR reaction in Section I, the DNA band of the expected size should be gel-purified using a gel extraction kit such as QIAEX II gel extraction kit (Qiagen part no. 20021).

1. To the remaining PCR reaction from Section I, add the necessary amount of sterile water to make the total volume 100 µl.
2. Add an equal volume of Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v/v) to the sample.
3. Vortex the tube at maximum speed for 15 seconds.
4. Centrifuge the tube for 5 minutes at 12,000 x *g* at room temperature.
5. Carefully transfer the top aqueous phase to clean microcentrifuge tube without collecting any of the lower organic phase or precipitate that may occur between the phases.
6. To each sample add:

   1 µl Glycogen (20 ng/µl)

   1/10 sample volume of 3 M sodium acetate pH 5.5

   2.5 sample volumes of 100% ethanol
7. Mix well and incubate at -80°C for at least 2 hours.
8. Centrifuge the tube for 20 minutes at 12,000 x *g*, 4°C.
9. Carefully discard the supernatant without disturbing the pellet.

10. Add 500 µl of cold 70% ethanol.
11. Centrifuge the tube for 10 minutes at 12,000 x *g*, 4°C.
12. Carefully discard the supernatant without disturbing the pellet.
13. Air-dry the pellet for 5-10 minutes (do not completely dry the pellet).
14. Resuspend the DNA pellet in 30 µl sterile DNase-free water.
15. This eluted DNA can be used immediately in cloning or stored at -20°C. If the DNA is stored at -20°C, it will need to be reheated at 37°C for 10 minutes prior to use to ensure DNA does not stick to the walls of the tube.

Section K: DNA Cloning

1. The cleaned PCR product can be cloned into a DNA vector of choice according to the instructions of the manufacturer (*e.g.* Topo TA Cloning Kit for sequencing, Thermo Fisher Cat. No. 450030). If possible, plasmids containing the *Lac*Z gene for blue/white screening are recommended. An example ligation reaction is shown below, but please follow the recommendations associated with the DNA vector of choice.

Reagent	Quantity
DNA template	4 µl
Ligation Buffer	1 µl
DNA vector	1 µl
Total Volume	**6 µl**

Incubate 5 minutes at room temperature.

2. Transform competent bacterial cells, such as RapidTrans™ TAM 1 (Active Motif Cat. No. 11096), with 2 µl of the ligation reaction. Follow the appropriate protocol for the competent cells used. The RapidTrans™ TAM 1 chemically competent cell protocol is listed below as an example.
    a. Remove required number of transformation reaction tubes from –80°C storage and place on ice to thaw.
    b. Add 1-5 µl of plasmid DNA/ligation to thawed cells. Mix by tapping tubes gently and replace on ice immediately. Do not mix by vortexing or pipetting. Do not add more than 5 µl (10% of competent cell volume) to reaction.
    c. Incubate transformation reactions on ice for 30 minutes.
    d. Heat-shock the tubes by immersing in a 42°C water bath for exactly 30 seconds.
    e. Replace transformation reactions on ice for 2 minutes.
    f. Aseptically add 250 µl SOC medium to each reaction.

g. Incubate tubes at 37°C for 1 hour with shaking at 225-250 rpm.

h. Using a sterile spreader, plate out 20-200 µl of each transformation on pre-warmed LB agar plates. If using blue/white screening, ensure that the selective plate contains X-Gal. For preparation of X-gal Stock solution and plates see note below.

Note: Make a 40 mg/ml solution of X-gal in dimethylformamide (DMF). Use a glass or polypropylene tube and wrap in aluminum foil to prevent damage by light. Store at –20°C. Plate 40 µl of X-gal stock solution on top of the LB agar and let stand for approximately 1 hour. Alternatively, add the X-gal to the molten LB agar prior to pouring plates.

i. Allow plates to completely absorb any excess media.

j. Incubate inverted plates overnight at 37°C.

3. Select 10 colonies for screening. Select white colonies if using blue/white screening as the expression of *Lac*Z has been interrupted with the insertion of the ligation product.

4. Isolate plasmid DNA of the 10 selected colonies using any commercially available kit or published protocol.

5. Analyze the isolated DNA using restriction enzyme analysis to confirm the presence of the correct insert, or by PCR using region-specific primers or primers corresponding to sequences within the vector (*e.g.* M13 forward and reverse primers)

6. Submit DNA from the positive clones for sequencing. Use forward and reverse primers based on the DNA vector used for cloning. It is recommended to submit 10 colonies for sequencing to ensure representative results. It is also necessary to submit unconverted DNA for sequencing comparison.

Section L: Analyzing the Sequencing Results

1. If the CpG dinucleotide was methylated in the original DNA, the sequencing read will show a CG at that location. If the CpG dinucleotide was unmethylated in the original DNA, the bisulfite conversion will have converted the cytosine to uracil and the sequencing read will show a TG at that location. All unmethylated cytosines should be converted to thymines. If any of the sequenced clones show a high level of unconverted cytosines (especially at non CpG sites) this indicates incomplete conversion and that clone should be discarded.

2. Compare the bisulfite converted sequence data against the unconverted DNA sequence.

3. Quantify the number of methylation sites for the region of interest to determine the percentage of methylation.

Section M: Methyl-Seq Library preparation

Commercially available kits are available for preparation of NGS libraries to sequence bisulfite converted DNA such as Accel-NGS® Methyl-Seq DNA Library Kit (Swift Biosciences, Cat No. 30024). Ensure that the selected library kit will work with the amount of bisulfite converted DNA recovered from the FFPE Bisulfite Conversion Kit (*e.g.* if using 5 pg of input DNA for bisulfite conversion there may not be enough material for library preparation). Follow the recommendations of the manufacturer for library preparation. If desired, libraries can be quantified prior to sequencing using the KAPA Library Quantification Kits (KAPA Biosystems, Cat. No. KK4824) to ensure sufficient material is present for sequencing. If optimization of the library preparation is needed, we suggest repeating the library preparation and increasing the amount of template DNA or increasing the number of PCR cycles during the index PCR to improve recovery.

Appendix

Section N. Troubleshooting Guide

Problem/question	Recommendation
Extracted DNA is highly fragmented	Sample fixation, embedding and storage have a strong impact on the integrity of DNA in FFPE samples. Samples may be highly fragmented upon extraction. As long as sample yield is greater than 5 pg following DNA isolation, proceed with bisulfite conversion.
Solution does not clarify during protease digestion	If the solution does not clarify during protease digestion this could indicate that the sample is oxidized. This may result in slightly lower yields. Increase the incubation time at 50°C to up to 48 hours. This usually results in the recovery of functional DNA.
Poor elution from the filter cartridge	It is important to allow the sample to sit for one full minute in Elution Buffer AM5. This will allow the eluant to permeate the entire filter before centrifugation and improve sample recovery.
Starting DNA volume is greater than 13 µl	DNA volumes up to 20 µl can be used without affecting the protocol
Do I need to use Glycogen with my conversion reaction?	When converting a small amount of DNA (less than 100 ng) we recommend to include 1 µl Glycogen (1 mg/ml) as a carrier to the 500 µl DNA Binding Buffer for each reaction during the on-column desulfonation and DNA purification step to minimize sample loss. If using larger amounts of DNA for conversion, this step can be omitted.
Conversion time	Large quantities of DNA may require longer conversion time to achieve complete conversion. For highly fragmented FFPE DNA we recommend performing a 5 hour incubation at 50°C to efficiently convert DNA samples. For DNA sequences that are GC-rich and/or contain extensive secondary structures we suggest a 9 hour conversion at 50°C. Longer conversion times, up to 16 hours, may be used if noticing incomplete conversion, however, this may result in degradation of the DNA. If noticing a loss of DNA after the conversion reaction, the conversion time can be reduced to 3 hours at 50°C.
No PCR product	Conversion reagent is sensitive to air and moisture. If conversion reagent was not properly sealed and stored, repeat conversion reaction using a new tube of conversion reagent.
	Test primers on control bisulfite converted DNA. Evaluate the optimal PCR conditions including annealing temperature, primer and magnesium concentrations. Consider designing new primers.
	We recommend performing a Hot Start PCR reaction using a polymerase that has been validated to work with bisulfite converted DNA. Follow the recommendations of the manufacturer for the preparation of the PCR reaction.
	If working with low amounts of Input DNA, such as 200 pg, it may be difficult to PCR amplify the bisulfite converted DNA for visualization on an agarose gel. Concentrate DNA prior to PCR to use more DNA per reaction, or use a different analysis method.
Can I store my conversion reaction samples for longer than 5 days before performing the DNA purification and desulfonation steps?	The DNA is recommended to be stored at 4°C for 5 days. If longer storage is needed, store samples at -20°C. As precipitates can form during -20°C, long term storage is not recommended.
Storage of converted DNA	Once DNA is prepared using Bisulfite Conversion Kit, samples may be stored at -20°C prior to PCR analysis
How do I quantify my bisulfite converted DNA?	Bisulfite converted DNA will resemble RNA as it is single stranded and contains uracil in place of the unmethylated cytosines. Use a spectrophotometer set to measure RNA with a conversion factor of 40 µg/ml for A260nm = 1.0. However, if the bisulfite converted DNA has been PCR amplified prior to quantification, traditional methods to quantify DNA can be used.

Section O. Related Products

DNA Methylation	Format	Catalog No.
Bisulfite Conversion Kit	50 rxns	55016
FFPE Bisulfite Conversion Kit	40 rxns	55021
MethylCollector™ Ultra	30 rxns	55005
HypoMethylCollector™	30 rxns	55004
MeDIP	10 rxns	55009
Hydroxymethyl Collector™	25 rxns	55013
hMeDIP	10 rxns	55010
PvuRts1I restriction enzyme	50 units	55011
β-Glucosyltransferase enzyme	500 units	55012
Recombinant TET1 protein, active	25 µg	31363
DNMT Activity / Inhibition Assay	1 x 96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
5-Carboxylcytosine DNA Standard	0.5 µg	55014
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Antibodies	Application	Format	Catalog No.
3-Methylcytosine rabbit pAb	DB	100 µg	61111
5-Carboxylcytosine rabbit pAb	DB, IF	100 µl	61225
5-Formylcytosine rabbit pAb	DB, IF	100 µl	61223
5-Hydroxymethylcytidine mouse mAb	DB, MeDIP	100 µg	39999
5-Hydroxymethylcytidine rabbit pAb	DB, IF, IHC, MeDIP	100 µl	39769
5-Methylcytosine mouse mAb	DB, FACS, IHC, IP, MeDIP	50 µg	39649
5-Methylcytosine rabbit pAb	DB, IP, MeDIP	100 µg	61255
CGBP rabbit pAb	WB	200 µl	39203
DNMT1 mouse mAb	ChIP, IHC, IP, WB	100 µg	39204
DNMT2 rabbit pAb	WB	100 µg	39205
DNMT3A mouse mAb	ChIP, IF, IHC, WB	100 µg	39206
DNMT3B mouse mAb	ChIP, IF, IP, WB	100 µg	39207
DNMT3L rabbit pAb	WB	100 µl	39907
Kaiso mouse mAb	WB	200 µg	39365
MBD1 mouse mAb	WB	100 µg	39215
MBD2 rabbit pAb	WB	200 µl	39547
MBD3 mouse mAb	WB	100 µg	39216
MBD4 rabbit pAb	WB	100 µg	39217
MeCP2 rabbit pAb	WB	100 µg	39218
MeCP2 mouse mAb	ChIP, IF, IHC, IP, WB	100 µg	61291
Tet1 rabbit pAb	ChIP, WB	100 µl	61443
Tet2 mouse mAb	IP, WB	100 µg	61389
Tet3 rabbit pAb	WB	100 µl	61395
Ubiquitin mouse mAb	IF, IHC, IP, WB	100 µg	39741
Uhrf1 rabbit pAb	WB	200 µl	39625

For more information or to see a complete list of DNA methylation antibodies, please visit our website at **www.activemotif.com/methylabs**.

Technical Services

If you need assistance at any time, please call Active Motif Technical Service at one of the numbers listed below.

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