EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit

Base Catalog # P-1048

PLEASE READ THIS ENTIRE USER GUIDE BEFORE USE

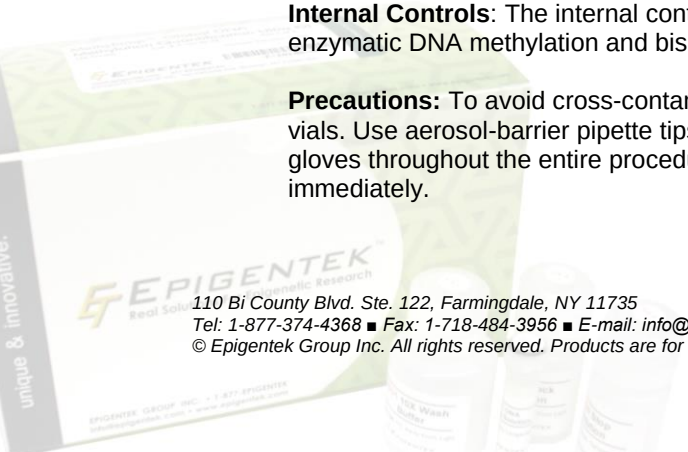
Uses: The EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit is a complete set of optimized reagents designed for conducting a gene-specific or genome-wide analysis of both chromatin accessibility and DNA methylation simultaneously from various biological samples at a single molecule or population level, followed by bisulfite-sequencing or next generation sequencing.

Starting Materials: Starting materials can include various mammalian tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, etc.

Input Amount of Cell/tissues: The amount of cell/tissues for each reaction can be from 1 x 10^5 cells or 2 mg tissues to 1 x 10^6 cells or 20 mg tissues. For an optimal reaction, the input chromatin amount should be about 0.5 x 10^6 cells or 10 mg tissues.

Internal Controls: The internal control primers are included in the kit for validating whether the proper enzymatic DNA methylation and bisulfite modification are achieved.

Precautions: To avoid cross-contamination, carefully pipette the sample or solution into the tube or vials. Use aerosol-barrier pipette tips and always change pipette tips between liquid transfers. Wear gloves throughout the entire procedure. In case of contact between gloves and sample, change gloves immediately.



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KIT CONTENTS

Component	48 reactions Cat. #P-1048-48‡	Storage Upon Receipt
LB (10X Lysis Buffer)	11 ml	RT
WB (10X Wash Buffer)	10 ml	4°C
PIC (1000X Protease Inhibitor Cocktail)*	50 µl	4°C
MRB (10X Methyl Reaction Buffer)	3 ml	4°C
SAM (Adomet, 30 mM)*	100 µl	-20°C
Mse (Methylase 4 U/µl)*	50 µl	-20°C
PK (Proteinase K,10 mg/ml)*	55 µl	4°C
CS (Conversion Solution)	11 ml	RT
CP (Conversion Powder)	5 vials	RT
DBS (DNA Binding Solution)	15 ml	RT
BS (Balance Solution)	400 µl	RT
ES (Elution Solution)	1 ml	RT
Control Primer-F (20 µM)*	10 µl	-20°C
Control Primer-R (20 µM)*	10 µl	-20°C
F-Spin Column†	50	RT
F-Collection Tube	50	RT
User Guide	1	RT

* *Spin the solution down to the bottom prior to use.*

† *Always cap spin columns before placing them in the microcentrifuge.*

‡ *For 24 samples and 24 No-Mse controls (negative control).*

SHIPPING & STORAGE

The kit is shipped in two parts: the first part at ambient room temperature and the second part on frozen ice packs at 4°C.

Upon receipt: (1) Store **SAM**, **Mse**, **Control Primer-F**, and **Control Primer-R** at –20°C away from light; (2) Store **WB**, **PIC**, **MRB** and **PK** at 4°C away from light; (3) Store remaining components at room temperature away from light.

All components of the kit are stable for 6 months from the date of shipment, when stored properly.

*Note: Check whether **WB** (10X Wash Buffer) contains salt precipitates before use. If so, briefly warm at room temperature or 37°C and shake the buffer until the salts are re-dissolved.*

MATERIALS REQUIRED BUT NOT SUPPLIED

- ☐ Vortex mixer
- ☐ Dounce homogenizer
- ☐ Centrifuge including desktop centrifuge (up to 14,000 rpm)
- ☐ Incubator for 37°C and 60°C temperatures
- ☐ 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.*

- ☐ Pipette and pipette tips
- ☐ 0.2 ml PCR tubes
- ☐ 1.5 ml microcentrifuge tubes
- ☐ 90% ethanol
- ☐ Cells or tissues
- ☐ Cell culture medium
- ☐ 37% formaldehyde
- ☐ 1.25 M Glycine solution
- ☐ 1X PBS
- ☐ Distilled water

GENERAL PRODUCT INFORMATION

Quality Control: Each lot of EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit is tested against predetermined specifications to ensure consistent product quality. Epigentek guarantees the performance of all products in the manner described in our product instructions.

Product Warranty: If this product does not meet your expectations, simply call our technical support unit or your regional distributor. We also encourage you to contact us if you have any suggestions about product performance or new applications and techniques.

Safety: Suitable lab coat, disposable gloves, and proper eye protection are required when working with this product.

Product Updates: Epigentek reserves the right to change or modify any product to enhance its performance and design. The information in this User Guide is subject to change at any time without notice. Thus, only use the User Guide that was supplied with the kit when using that kit.

Usage Limitation: The EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit is for research use only and is not intended for diagnostic or therapeutic application.

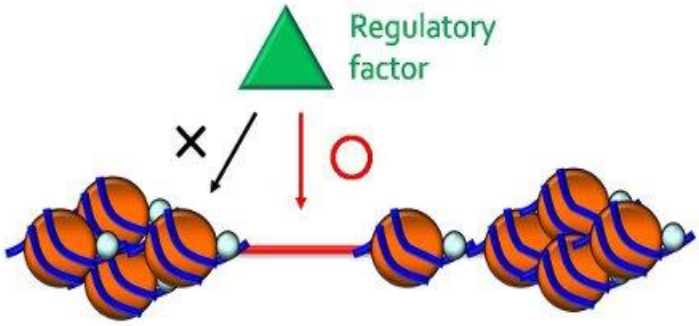
Intellectual Property: The EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit and methods of use contain proprietary technologies by Epigentek.

A BRIEF OVERVIEW

The accessibility of regulatory elements in chromatin is critical for many aspects of gene regulation. Nucleosomes positioned over regulatory elements inhibit access of transcription factors to DNA. The nucleosome is a fundamental unit of chromatin that is comprised of approximately 147 bases of DNA wrapped around a histone octamer consisting of two copies each of the core histones H2A, H2B, H3 and H4. To elucidate the role of the interactions between chromatin and transcription factors, it is crucial to determine the chromatin accessibility through mapping the nucleosome positioning along the genome. In general, the more condensed the chromatin, the harder it is for transcription factors and other DNA binding proteins to access DNA and carry out their tasks. The more accessible the DNA, the more likely surrounding genes are actively transcribed. The presence (or the absence) of nucleosomes directly or indirectly affects a variety of other cellular and metabolic processes like recombination, replication, centromere formation, and DNA repair.

DNA methylation occurs by the covalent addition of a methyl group at the 5-carbon of the cytosine ring, resulting in 5-methylcytosine. Three Dnmts (Dnmt1, Dnmt3a and Dnmt3b) are required for establishment and maintenance of DNA methylation patterns. Two additional enzymes (Dnmt2 and Dnmt3L) may also have more specialized but related functions. When a CpG island in the promoter region of a gene is methylated, expression of the gene is repressed. A number of human diseases, such as cancer, are known to occur when DNA methylation is not properly established and/or maintained.

It has been shown that DNA methylation induces a more compact and rigid nucleosome structure. Nucleosomes containing methylated DNA stabilize *de novo* DNA methyltransferases, Dnmt3A/3B, allowing few free Dnmt3A/3B enzymes to exist in the nucleus. Stabilization of Dnmt3A/3B on nucleosomes in methylated regions further promotes propagation of DNA methylation. It has also been demonstrated that nucleosome positioning influences DNA methylation patterning throughout the genome and that DNA methyltransferases preferentially target nucleosome-bound DNA. Thus, obtaining DNA methylation and nucleosome positioning information from the same DNA molecule would enable researchers to better understand how DNA methylation and nucleosome positioning work together to control chromatin accessibility which regulates gene expression. This information can also be useful in monitoring disease progression and responses to therapy.

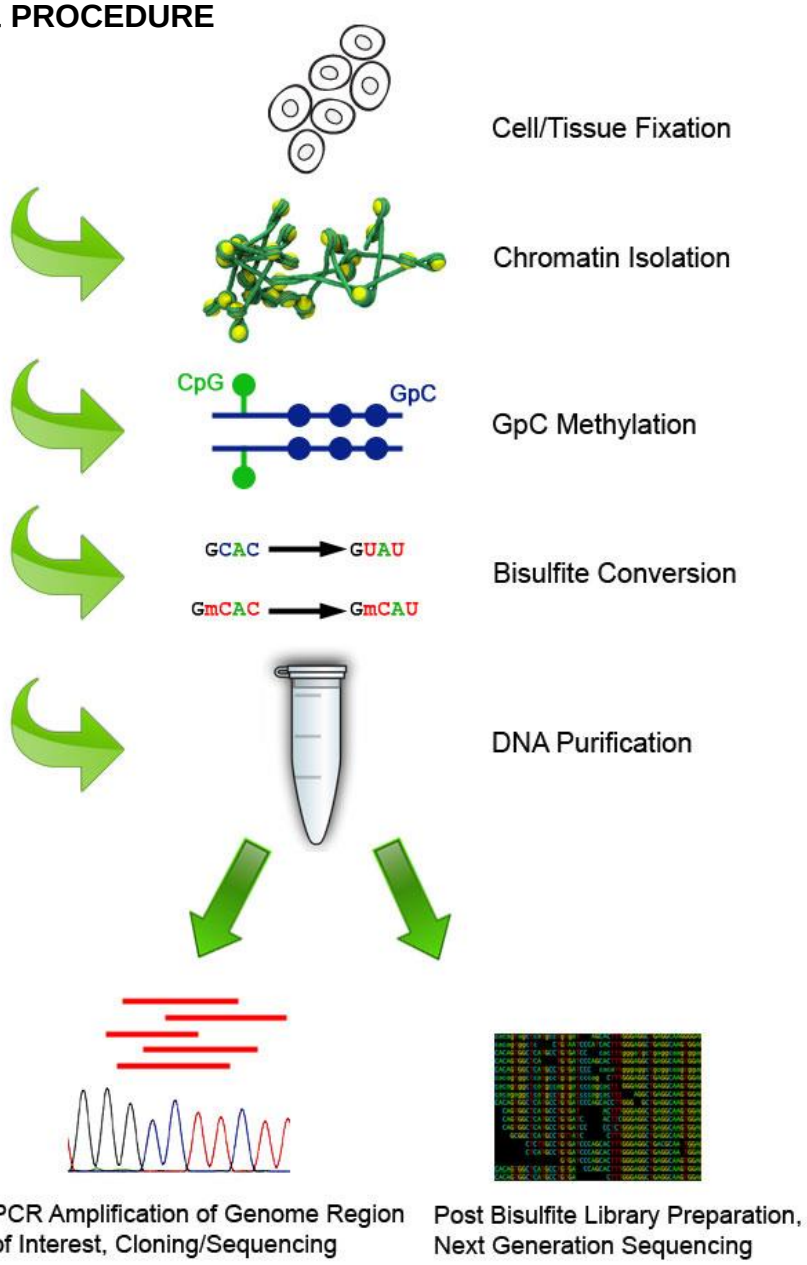


Tanaka, Y. et al. *Genome Informatics* (2009): The nucleosome limits the accessibility of many regulatory factors including Dnmts. Chromatin accessibility is one of several important clues towards clarifying the mechanism of regulation for various nuclear events (transcription, replication, DNA repair etc).

There are several methods currently used for detecting chromatin accessibility or DNA methylation separately. The traditional method is a nuclease digestion of chromatin DNA followed by qPCR, microarray, or sequencing. However, this method may cause DNA damage and destroy the integrity of a DNA molecule. It also needs a significant amount of starting materials and introduces assay bias because of the difficult control of enzyme concentrations and digestion time. The prominent method for DNA methylation analysis is bisulfite modification of DNA followed by MS-qPCR or sequencing, which allows gene-specific or genome-wide analysis of DNA methylation to be carried out. Most recently, two methods, MAPit and NoMe-Seq, were developed to analyze chromatin accessibility (nucleosome positioning) and DNA methylation simultaneously. These methods utilize GpC-methyltransferase to methylate GpC sites not protected by nucleosomes and allow the endogenous CpG site to remain unaffected. After bisulfite conversion, the DNA is amplified, cloned, and sequenced. The sequencing results reveal CpG methylation status and nucleosome positioning on the same DNA molecule. However, major disadvantages for these methods include being time-consuming (3-4 days), labor-intensive, and low throughput with high costs. To address these problems, Epigentek developed the EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit that provides both a chromatin accessibility profile and a DNA methylation footprint for the same DNA molecules. The kit has the following advantages and features:

- Extremely fast and convenient protocol that allows the entire procedure (from cell tissue sample to ready-to-use DNA for cloning/library preparation) to be finished in as short as 5 hours.
- Simultaneously detect chromatin accessibility (nucleosome/transcription factor positioning) and CpG methylation at a base-resolution level.
- Internal control primers are included in the kit for validating whether the proper enzymatic DNA methylation and bisulfite modification are achieved.
- Recommended procedures for cloning and sequencing are included.

PRINCIPLE & PROCEDURE



Schematic procedure of the EpiQuik™ Chromatin Accessibility and Methylome Sequencing (CAMe-Seq) Kit.

The CAMe-Seq Kit contains all the necessary reagents required for the assay. In CAMe-Seq, the cells/tissues are fixed with formaldehyde. Chromatin is isolated and treated with the included GpC DNA methyltransferase that only methylates GpC sites unprotected by nucleosomes or DNA/protein complexes without affecting endogenous CpG sites. DNA is then bisulfite modified after reversal of cross-linking. The bisulfite-converted DNA can be PCR amplified with or without cloning followed by

sequencing for region-specific analysis of chromatin accessibility and DNA methylation. The C to T conversion in the GpC sites indicates accessible euchromatin regions while unchanged GpC sites represent chromatin regions that are not accessible because of the presence of nucleosome or DNA/protein complexes. The GpC methylated and bisulfite-converted DNA obtained with this kit can also be used in next generation sequencing to analyze genome-wide chromatin accessibility profiles and DNA methylation footprints.

REACTION PROTOCOL

For the best results, please read the protocol in its entirety prior to starting your experiment.

Starting Materials

Starting materials can include various mammalian tissue or cell samples such as cells from flask or microplate cultured cells, fresh and frozen tissues, etc. Input Amount of Cell/tissues for each reaction can be from 1 x 10^5 cells or 2 mg tissues to 1 x 10^6 cells or 20 mg tissues. For an optimal reaction, the input amount should be about 0.5 x 10^6 cells or 10 mg tissues.

1. Buffer/Solution Preparation

a. **1X LB Lysis Buffer:** Adding 1 ml of **10X Lysis Buffer** and 10 µl of **Protease Inhibitor Cocktail** to every 9 ml of distilled water.

b. **Cross-linking Solution**: Add 270 µl of 37% formaldehyde to every 10 ml of cell culture medium.

c. **1X WB Wash Buffer**: Add 5 ml of **10X Wash Buffer** to every 45 ml of distilled water.

d. **1X MRB Buffer:** Add 1 ml of **10X Methyl Reaction Buffer** to every 9 ml of distilled water.

2. Cell/tissue Collection and In Vivo Cross-linking

For Monolayer or Adherent Cells:

a. Grow cells (treated or untreated) to 80%-90% confluence on a 100 mm plate (about 2x10^6 to 4x10^6 cells; 0.5x10^6 cells are required for each reaction), then trypsinize and collect them into a 15 ml conical tube. Count the cells in a hemocytometer.

b. Centrifuge the cells at 1000 rpm for 5 min. Discard the supernatant.

c. Wash cells with 10 ml of PBS once by centrifugation at 1000 rpm for 5 min. Discard the supernatant.

d. Add 5 ml **Cross-linking Solution** to 2 X 10^6 cells.

e. Incubate at room temperature (20-25°C) for 10 min on a rocking platform (50-100 rpm).

For Suspension Cells:

a. Collect cells (treated or untreated) into a 15 ml conical tube. (1x 10^6 cells are required for each reaction). Count cells in a hemocytometer.

b. Centrifuge the cells at 1000 rpm for 5 min. Discard the supernatant.

c. Wash cells with 10 ml of PBS once by centrifugation at 1000 rpm for 5 min. Discard the supernatant.

d. Add 5 ml **Cross-linking Solution** per 2 X 10^6 cells.

e. Incubate at room temperature (20-25°C) for 10 min on a rocking platform (50-100 rpm).

For Tissues:

a. Put the tissue sample into a 60 or 100 mm plate. Remove unwanted tissue such as fat and necrotic material from the sample.

b. Weigh the sample and cut the sample into small pieces (1-2 mm^3) with a scalpel or scissors.

c. Transfer tissue pieces to a 15 ml conical tube (10 mg tissue is required for each reaction).

d. Add 5 ml of **Cross-linking Solution** per 40 mg tissues.

e. Incubate at room temperature for 15-20 min on a rocking platform.

f. Add 0.6 ml of 1.25 M glycine per 5 ml of **Cross-linking Solution** to stop cross-linking reaction.

g. Mix and centrifuge at 800 rpm for 5 min. Discard the supernatant.

h. Wash tissues with 10 ml of ice-cold PBS once by centrifugation at 800 rpm for 5 min. Discard the supernatant.

i. Transfer tissue pieces to a Dounce homogenizer.

j. Add 800 µl of **1X LB Lysis Buffer** for every 40 mg tissues.

k. Disaggregate tissue pieces by 10-20 strokes.

l. Transfer homogenized mixture to a 15 ml conical tube and centrifuge at 3000 rpm for 5 min at 4°C. If total mixture volume is less than 2 ml, transfer mixture to a 2 ml vial and centrifuge at 5000 rpm for 5 min at 4°C and then go directly to Step 3g under **Cell Lysis and Chromatin Extraction**.

3. Cell Lysis and Chromatin Extraction

a. Add 0.6 ml of 1.25 M glycine per 5 ml of **Cross-linking Solution** to stop cross-linking reaction.

b. Mix and centrifuge at 1000 rpm for 5 min.

c. Remove medium and wash cells once with 10 ml of ice-cold PBS by centrifuging at 1000 rpm for 5 min.

d. Add **1X LB Lysis Buffer** to re-suspend the cell pellet (400 µl/1x10^6).

e. Transfer 200 µl of cell suspension to a 1.5 ml vial as a sample and 200 µl of cell suspension to another 1.5 ml vial as a No-Mse control for treatment at the GpC methylation step.

f. Incubate the cell suspension on ice for 10 min.

g. Vortex vigorously for 10 sec and centrifuge at 5000 rpm for 5 min.

h. Carefully remove supernatant and wash chromatin pellet one time with 1 ml of **1X Wash Buffer** by resuspending the chromatin pellet and centrifuging at 3000 rpm for 5 min at 4°C (in bench top centrifuge).

4. GpC Methylation

a. Wash chromatin pellet one time with 0.5 ml of **1X MRB Buffer** by resuspending the chromatin pellet and centrifuging at 3000 rpm for 5 min at 4°C (in bench top centrifuge).

b. Carefully remove supernatant.

c. Prepare the GpC methylation reaction mixture as follows. For each reaction:

	Sample	**No-Mse Control**
Distilled H2O	19.8 µl	21.8 µl
10 X MRB	2.5 µl	2.5 µl
SAM	0.7 µl	0.7 µl
Mse	2 µl (final added)	--
Total	25 µl	25 µl

Mix and add 25 µl of the mixture to the chromatin sample and unmodified control vials. No-Mse control is used for determining endogenous CpG methylation.

d. Re-suspend the chromatin pellet by pipetting 3 to 4 times and transfer the suspension to a 0.2 ml PCR tube or 96-well PCR plate.

e. Tightly cap the tube/wells and incubate at 37°C (waterbath or thermal cycler) for 60 min.

f. After 60 min reaction, place the sample at 95°C for 15 min to reverse cross-linked chromatin. Cool down to room temperature. Add 1 µl of **PK** to each vial and incubate at 60°C for 15 min.

5. Bisulfite DNA Conversion

a. Add 1.0 ml of **CS Conversion Solution** to 1 vial of **CP Conversion Powder**. Mix by inverting and shaking the vial repeatedly for 2 min. Add 40 µl of **BS Balance Solution** to the vial and mix by inverting and shaking for an additional 2 min (trace amount of undissolved **CP** may remain, which is normal as **CP** is saturated in solution).

b. Add 110 µl of the mixed **CS/CP/BS** solution into the sample and No-Mse control tubes.

*Prepared **CS/CP/BS** solution can be stored at -20°C for up to 2 weeks without significant loss of efficiency. For the best results, the mixed solution should be used immediately.*

Tightly close the PCR tubes and place them in a thermal cycler with a heated lid. Program and run the thermal cycler with the following program:

95°C 4 min
65°C 30 min
95°C 4 min
65°C 30 min
95°C 4 min

65°C 60 min
Hold 18-20°C up to 6 h

Meanwhile, insert the number of F-Spin Columns ("column") into F-Collection Tubes ("collection tube") as needed by your experiment.

6. Converted DNA Clean-Up

a. Add 250 µl of **DBS DNA Binding Solution** to each column. Then transfer the samples from each PCR tube to each column containing the **DBS**. Centrifuge at 12,000 rpm for 30 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

b. Add 200 µl of 90% ethanol solution to each column. Centrifuge at 12,000 rpm for 20 sec.

c. Prepare final desulphonation buffer by adding 20 µl of **BS Balance Solution** to every 1 ml of 90% ethanol, and mix. Add 100 µl of the final desulphonation buffer (**BS** and 90% ethanol mixture) to each column. Allow columns to sit for 15 min at room temperature, then centrifuge at 12,000 rpm for 20 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes.

d. Add 200 µl of 90% ethanol to each column. Centrifuge at 12,000 rpm for 20 sec. Remove columns from collection tubes and discard the flowthrough. Place columns back into collection tubes. Add 200 µl of 90% ethanol to each column again and centrifuge at 12,000 rpm for 30 sec.

e. Insert each column into a new 1.5 ml tube. Add 20 µl of **ES Elution Solution** directly to each column's filter membrane. Centrifuge at 12,000 rpm for 30 sec to elute the converted DNA.

Modified DNA is now ready for use, or storage at or below –20°C for up to 6 months.

7. Confirmation of GpC Methylation and Bisulfite Modification

To confirm if the samples are successfully methylated on GpC sites and bisulfite converted, a methylation specific-qPCR (MS-qPCR) can be performed with use of the internal control primers included in the kit. MS-qPCR could be performed by using your own successful method. For your convenience and the best results, Epigentek offers the Methylamp™ MS-qPCR Fast Kit (Cat. No. P-1028) that is optimized for fast methylation specific qPCR reactions in 70 minutes.

a. Prepare the PCR Reactions:

Component	Size (µl)	Final Concentration
Methylamp Master Mix (2X)	10 µl	1X
Forward Primer	1 µl	0.4-0.5 µM
Reverse Primer	1 µl	0.4-0.5 µM
DNA Template	1-2 µl	0.1 ng-0.1 µg
DNA/RNA-free H_2O	6-7 µl	
Total Volume	**20 µl**	

For the negative control, use DNA/RNAse-free water instead of DNA template.

b. Program the PCR Reactions:

Cycle Step	Temp	Time	Cycle
Activation	95°C	7 min	1
Cycling	95°C 55°C 72°C	10 sec 10 sec 12 sec	40-45
Final Extension	72°	1 min	1

c. For data analysis, fold enrichment (FE) can be calculated by simply using a ratio of amplification efficiency of the Mse-methylated DNA sample over that of the No-Mse control sample.

$$FE\% = 2^{(\text{No-Mse CT} - \text{Mse CT})} \times 100\%$$

For example, if CT for No-Mse is 38 and the Mse sample is 32, then:

FE% of Mse-methylated sample = $2^{(38-32)} \times 100\% = 6400\%$

FE% of Mse-methylated sample should generally be greater than 1600% for successful methylation of GpC sites and bisulfite conversion.

8. Bisulfite Sequencing

PCR Amplification of Selected Genomic Region of Interest

Bisulfite DNA-Specific Primer Design: A methylation-specific PCR can be carried out to amplify the selected genomic region of interest by using bisulfite DNA-specific primers. Bisulfite DNA-specific primers can be designed to anneal to a sequence specific for GpC methylated gDNA, or unmethylated gDNA. The MethylViewer (http://dna.leeds.ac.uk/methylviewer/) (Pardo et al., 2010) is a suitable computer program for helping to design such primers, and provides an integrated solution for primer design and rapid, accurate, and detailed analysis of bisulfite sequencing. In principle, the primer covered region of interest should have a high GpC density to increase the resolution of chromatin accessibility analysis. The primer sequence itself should not contain GpC or CpG sites. The amplicon generated with the primers should be 300-400 bps in length, which enables a long enough sequence to be read to confirm the nucleosome positioning without affecting complete reads by single-pass sequencing.

Other important criteria for bisulfite DNA-specific primer design include: (1) Look for two asymmetric Cs very close to the 3' end (at least 2 in the last five nucleotides) and make one of these the last nucleotide in your primer; this will select for converted DNAs. Ideally, your primer will have two asymmetric Cs right at the end; (2) If possible, avoid polyT and A runs near the 3' end (i.e., include some G's). This will prevent amplification of simple sequences (and primer dimer with similar sequences in the bottom strand primer); (3) Change all Cs to Ts except Cs at GpC or CpG sites. Leave Cs in GpC or CpG sites unchanged; and (4) Adjust the length so the Tm is around 65 degrees in order to run the PCR at or near to 60°C. Running the PCR in this temperature range is important to ensure the specificity of the PCR reaction.

The designed primers should be tested to confirm their specificity against bisulfite-converted DNA. Confirmation can be achieved by the paired use of bisulfite-untreated DNA and bisulfite treated DNA for PCR with the primers. The primers should only amplify bisulfite treated DNA and not generate amplicons for bisulfite-untreated DNA.

Methylation-Specific PCR: As the bisulfite converted DNA is in a single-stranded form, 'hot-start' PCR amplification should be used. You can use your own successful MS-PCR amplification methods.

Here is a suggested protocol for rapid PCR amplification of bisulfite-converted DNA by using JumpStart Polymerase:

1. Prepare PCR Master Mix:

10 X JumpStart Reaction Buffer	10 µl
10 mM dNTP	2.5 µl
H2O	76 µl
MgCl2 (100 mM)	2.5 µl
Bisulfite DNA Specific Primer-forward (20 µM)	3 µl
Bisulfite DNA Specific Primer-reverse (20 µM)	3 µl
JumpStart Taq (2.5 U/µl)	3 µl
Total	**100 µl**

2. For each 0.2 ml PCR tube, add 19 µl of PCR Master Mix and 1 µl of the bisulfite-treated or untreated DNA. The PCR Master Mix should be mixed well before use.

3. Tightly close the PCR tubes and place them in a thermal cycler with a heated lid. Program and run the thermal cycler with the following program:

Cycle Step	Temp	Time	Cycle
Activation	94°C	3 min	1
Cycling	95°C 55°C 72°C	45 sec 45 sec 1 min	45
Final Extension	72°C	5 min	1

PCR amplification conditions must be optimized to eliminate non-full-length products. PCR products should be analyzed by agarose gel electrophoresis for a pure product of the correct size. The product can also be gel purified if PCR optimization does not result in a product of high purity. For analysis of the relative amount of 5mC and chromatin accessibility of each target site at population level, direct thermal cycle sequencing of the PCR product can be performed using a primer specific to the strand of interest. The best results are usually obtained with primers that are internal to the original PCR amplification primer. For analysis at single molecule level, PCR products should be cloned into plasmids for sequencing.

Cloning and Sequencing of PCR Amplicons

Cloning and Sequencing: Purify the PCR products and clone fresh PCR products into plasmids for sequencing (e.g., use the TOPO TA Cloning Kit from Invitrogen or p-GEM-T Easy Vector System from Promega). Make sure to spread enough plates so that you will get enough clones for your analyses (typically ~20 to 30 colonies can be obtained per plate). Colonies can be analyzed by PCR to determine if the proper inserts present prior to sequencing. Isolate plasmid DNA from at least 10 colonies after confirmation for sequencing (i.e., use ABI cycle sequencing reagents and automated sequencing with Big Dye chemistry) using primers specific to the cloning vectors of choice.

Data Analysis: Compare sequences of Mse-methylated DNA to No-Mse control references to separately obtain GpC and CpG methylation profiles and identify if methylation at GpCpG site is endogenous methylation or due to the artificial results.

a. If cytosine at the GpC site is unmethylated in a region of GpC sequence over a distance of 147 bps or greater, it indicates nucleosome positioning;

b. If cytosine at the GpC site is unmethylated in a region of GpC sequence over a distance of 10-80 bps, it indicates regulator factor binding;

c. If cytosine at the GpC site is methylated in a region of GpC sequence, it indicates an area of the chromatin that is accessible to regulatory factors or nucleosome depletion;

d. If there is one unmethylated GpC site within a stretch of methylated GpC sites, it would still indicate a chromatin area that is accessible to regulatory factors or nucleosome depletion;

e. If there is one methylated GpC site within a stretch of unmethylated GpC sites, it would still indicate nucleosome positioning;

f. Endogenous methylation frequencies are obtained by summing the number of 5-mC residues at each site in sequenced templates;

g. The relationship between chromatin accessibility and DNA methylation can be determined by overlaying GpC and CpG methylation profiles together;

Next Generation Sequencing

For genome-wide analysis of chromatin accessibility and DNA methylation, next-generation sequencing can be used after CAMe-Seq assay. After purification of bisulfite DNA, post bisulfite adaptor tagging can be performed. After size fractionation, selected DNA could be used for next-generation sequencing with an Illumina platform (i.e., HiSeq 2000). A protocol for bisulfite-next generation sequencing is available on the Epigentek website at www.epigentek.com/bisulfite-seq.

TROUBLESHOOTING

Problem	Possible Causes	Suggestions
Low yield of chromatin	Insufficient amount of samples.	To obtain the best results, the amount of samples should be 0.5 x10^6 to 1x10^6 cells, or 10 to 20 mg tissues per reaction.
	Insufficient chromatin extraction.	Ensure that all reagents have been added with the correct volume and in the correct order based on the sample amount.
		Check for sample lysis under microscope after the tissue/cell lysis step.
		Ensure that the cell or tissue species are compatible with this extraction procedure.
	Lysis or extraction reagents have expired. Expired reagents may cause inefficient extraction.	Ensure that the kit has not exceeded the expiration date of the kit. Standard shelf life, when stored properly, is 6 months from date of receipt.

	Incorrect temperature and/or insufficient incubation time during extraction.	Ensure the incubation time and temperature described in the protocol are followed correctly.
Poor GpC methylation	Insufficient amount of GpC methylase added to the well in Step 2c.	Ensure a sufficient amount of **Methylase** is added.
	The quality of the GpC methylase has been degraded due to improper storage conditions.	Follow the Shipping & Storage guidance for storage instructions of **Methylase.**
	Excessive amount of cells used per reaction and/or reagents are not added incorrectly.	Use 0.5-1 X 10^6 cells per reaction and confirm whether the reagents added for methylation reaction are correct.
DNA is Poorly Modified	Temperature or thermal cycling condition is incorrect.	Check for appropriate temperature or thermal cycling conditions.
	Insufficient DNA clean-up.	Ensure that 20 µl of **BS** is added into every 1 ml of 90% ethanol in Step 5 of Converted DNA Clean-up.
	Kit is not stored or handled properly.	Store all kit components it at room temperature. Tightly cap the **CS** vial after each opening or use.
Eluate Contains Little or No DNA	Buffer **DBS** (DNA Binding Solution) is not added into the sample.	Ensure that **DBS** is added in Step 3.
	Concentration of ethanol solution used for DNA clean-up is not correct.	Use 90% ethanol for DNA clean-up.
	Sample is not completely passed through the filter membrane of column.	Centrifuge for 1 min at 12,000 rpm or until the entire sample has passed through the filter membrane.
	Little or no PCR product in Mse-methylated samples.	Ensure that all PCR components are added and a suitable PCR program is used (PCR cycle should be >40).
Poor Results in Downstream Methylation-Specific PCR	Little or no PCR product in Mse-methylated samples; or little or no PCR product in Mse-methylated samples with control primers.	PCR primers and probes were not appropriate or were incorrectly designed. Ensure the primer and probes are suitable for MS-PCR and the target regions to be amplified are less than 250 bps.
		Ensure the amount of template DNA used in PCR was sufficient.
		Failed bisulfite conversion. Ensure that all steps of the modification and cleanup protocol were followed and that input DNA amount is within the recommended range.
	Little or no PCR product in Mse-methylated samples with control primers.	Failed methylase reaction. See problem note for “Poor GpC Methylation” in this troubleshooting section.

RELATED PRODUCTS

Chromatin Sample Preparation

P-2001	ChromaFlash™ Chromatin Extraction Kit
P-2023	ChromaFlash™ Chromatin Isolation & Shearing Kit

DNA Bisulfite Modification

P-1002	Methylamp™ Coupled DNA Isolation & Modification Kit
P-1008	Methylamp™-96 DNA Modification Kit
P-1016	Methylamp™ Whole Cell Bisulfite Modification Kit
P-1026	BisulFlash™ DNA Modification Kit

DNA Methylation Analysis

P-1005	TuMinute™ PCR Clean-Up Kit
P-1011	Methylamp™ Universal Methylated DNA Kit
P-1019	Methylamp™ Universal Methylated DNA Preparation Kit
P-1028	Methylamp™ MS-qPCR Fast Kit

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