NOMe-Seq

Catalog No. 54000

(version A2)

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Overview

Understanding the relationship between nucleosome occupancy, transcription factor binding and DNA methylation profiles is a complex system that is not well understood. Epigenome mapping studies have shown nucleosome depleted regions at transcription start sites and enhancers, but their role in gene regulation is not clear. To better interrogate the function of nucleosome occupancy in the context of DNA methylation, Active Motif offers a high resolution single-molecule Nucleosome Occupancy and Methylome sequencing (NOMe-Seq) Kit. The NOMe-Seq assay allows for simultaneous analysis of nucleosome occupancy and CpG methylation on the same DNA strand[1,2,3].

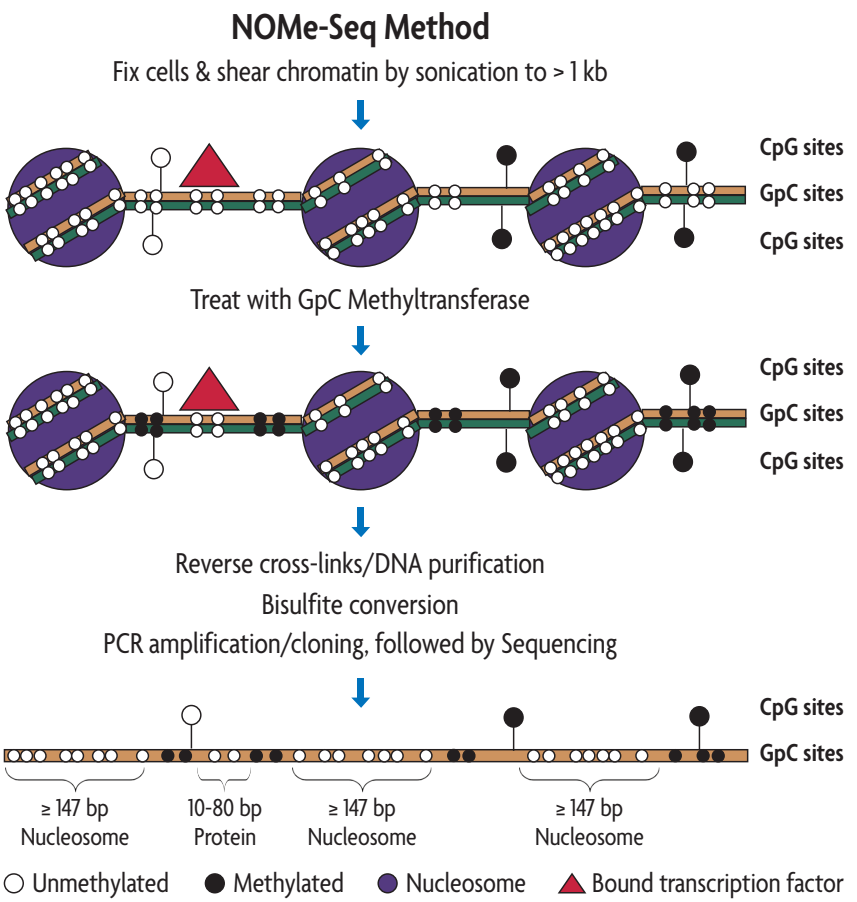
The NOMe-Seq assay works by treating fixed chromatin with a GpC DNA methyltransferase enzyme to artificially methylate GpC dinucleotides that are not protected by nucleosomes or protein-DNA binding. Following DNA extraction and bisulfite conversion, genomic regions of interest are PCR amplified, cloned and sequenced. Alternatively, for larger genome analysis, Next-Gen libraries can be generated and sequenced[4]. The readout of the GpC methylation provides a footprint of nucleosome occupancy in combination with the endogenous CpG DNA methylation profile for the same DNA strand. By utilizing fixed chromatin in which all protein-DNA binding interactions are preserved, NOMe-Seq also offers the added benefit of providing preliminary information regarding transcription factor binding sites.

Enough reagents are provided in the NOMe-Seq Kit to perform 10 enzymatic treatments and 10 negative control reactions. Each GpC Methyltransferase treatment is performed on chromatin from 250,000 cell equivalents.

product	format	catalog no.
NOMe-Seq	10 rxns*	54000

* Licensed technology. Patent pending.

Flow Chart of Process



Flow Chart of NOMe-Seq Methodology

In NOMe-Seq, cells are formaldehyde fixed to cross-link protein/DNA interactions and preserve the location of the nucleosomes. The chromatin is then sonicated to generate fragments of 1 kb or greater for analysis. Treatment of chromatin with a GpC Methyltransferase enables artificial methylation of GpC dinucleotides that are not protected by nucleosomes or DNA-bound proteins. Following enzymatic treatment, the chromatin is subjected to a reversal of cross-links, treatment by Proteinase K, RNase A and DNA purification. Bisulfite conversion is then performed on the DNA to provide single-nucleotide resolution of the methylation profile. For locus-specific analysis, bisulfite converted PCR primers are used to PCR amplify the region of interest which is cloned and sequenced. Alternatively, Next-Gen sequencing methods can be used. By plotting the GpC regions of the locus of interest and analyzing the methylation profile, areas in which GpC methylation occurred (black circles) will represent open chromatin regions that were accessible to enzymatic treatment. GpC sites that were not methylated (white circles) represent regions of the chromatin that were protected from enzymatic treatment by the presence of nucleosomes or protein. To overlap the chromatin state with the DNA methylation profile for the same gene, the endogenous CpG methylation from the bisulfite sequencing data can be analyzed. The NOMe-Seq method therefore allows for information to be simultaneously determined regarding nucleosome occupancy and CpG methylation for the same DNA strand.

Introduction

Nucleosome occupancy and gene regulation

The genomes of eukaryotic organisms are packaged into chromatin. The nucleosome is the smallest subunit of chromatin and consists of 147 base pairs of DNA wrapped around an octamer of core histone proteins (two each of Histone H2A, Histone H2B, Histone H3 and Histone H4). Histone H1 exists as a linker histone present at the interface between the nucleosome core and DNA entry/exit points.

Chromatin is subject to a variety of chemical modifications, including post-translational modifications of the histone proteins and the methylation of cytosine residues in the DNA. These modifications play a major role in regulating gene expression by modifying the accessibility of promoter regions to transcriptional machinery[5,6]. Transcriptional co-activator complexes interact with nucleosomes, and these interactions induce nucleosomal rearrangements to unfold the nucleosome completely or disassemble it at the transcriptional start site (TSS) to allow for transcription initiation[7,8,9,10]. The presence of the nucleosome depleted region (NDR) upstream of the TSS is critical for gene activation[11].

DNA methylation in mammalian cells is generally associated with gene silencing, either directly by inhibiting binding of transcription factors to their recognition sequences[12], or indirectly by preventing transcription factors from accessing their target sites through attachment of methyl-CpG binding proteins (MBD) that recruit histone deacetylases and histone methyltransferases. Mechanisms such as ATP-dependent chromatin remodeling work to alter chromatin structure in order to regulate DNA accessibility[13].

Classical techniques used to understand nucleosome positions, such as nuclease digestion and primer extension[14], often destroy the physical linkages between binding sites and prevent direct analysis of single molecule promoter function. Instead these technologies look at the average distribution across a panel of remodeled nucleosomes[15]. Active Motif's NOMe-Seq Kit provides a method to obtain single molecule analysis of nucleosome positioning.

In NOMe-Seq, a GpC methyltransferase enzyme is used to obtain nucleosome positioning information. The enzyme artificially methylates GpC residues not protected by nucleosomes or transcription factor binding. Due to the abundance of GpC dinculeotides in the genome, their methylation profile enables high resolution of nucleosome position. Additionally, GpCs are not endogenously methylated in mammalian genomes, so artificial methylation does not interfere with the ability to establish an endogenous methylation profile. Following enzymatic treatment, the chromatin is subjected to a reversal of cross-links, treatment by RNaseA, Proteinase K and DNA purification. The purified DNA undergoes bisulfite conversion to obtain the methylation profile of each DNA strand. The NOMe-Seq reactions are then PCR amplified to analyze the region of interest, cloned and sequenced. The sequencing results provide information on both nucleosome position and CpG methylation to reveal the relationship between multiple epigenetic modifications on the same DNA molecule. A comparison to negative control reactions will resolve if the methylation status of GpCpG trinucleotides is due to artificial or endogenous methylation.

Traditional Methods to Study Nucleosome Positioning or DNA Methylation

To date, there are several methods used to study nucleosome positioning or DNA methylation independently:

1. **Nuclease Digestion:** The use of enzymes, such as micrococcal nuclease (MNase) or DNaseI, to digest DNA followed by sequencing or PCR, is often used to map the position of nucleosomes. The technique is based on the fact that protein bound to DNA will often protect the DNA from enzymatic cleavage. Therefore, enzymes will digest linker DNA connecting two nucleosomes and the resulting digestion pattern reveals nucleosome positioning. However, these methods require significant amounts of material, are variable based on enzyme concentration and can introduce bias into the system based on cutting preferences.
2. **FAIRE:** Formaldehyde-Assisted Isolation of Regulatory Elements (FAIRE) is a method used to enrich for nucleosome depleted DNA using formaldehyde fixation and phenol-chloroform extraction[16]. This method relies on the idea that formaldehyde cross-linking is more efficient in nucleosome-bound DNA, therefore DNA cross-linked to nucleosomes is sequestered in the interphase, whereas DNA recovered in the aqueous phase corresponds to nucleosome-depleted regions of the genome. The nucleosome-free DNA can be sequenced to identify regions that correspond with transcriptional start sites, enhancers and active promoters.
3. **Bisulfite conversion:** Bisulfite conversion consists of the treatment of genomic DNA with sodium bisulfite, leading to deamination of unmethylated cytosines into uracil. PCR is then performed with primers that differentiate between methylated and unmethylated sequences (Methylation-specific PCR (MSP)), or the bisulfite converted DNA is sequenced to reveal the methylation profile at single-nucleotide resolution. One draw back of this technique is that bisulfite conversion cannot distinguish between 5-methylcytosine and 5-hydroxymethylcytosine.
4. **MAP**: Methyltransferase Accessibility Protocol (MAP) utilizes DNA methyltransferases as a means to map nucleosomes and non-histone protein-DNA interactions[17]. The accessibility of the DNA for the methyltransferase is obtained through thermal cycle sequencing with a primer specific to the strand of interest[18]. The result is the relative frequency of accessibility across a population. The MAP technology has also been expanded to MAP for individual templates (**MAPit**), which is a single-molecule approach to determine the CpG methylation of mammalian genes and their overlaying chromatin structure[18, 19]. MAP-IT uses a combination of a cytosine DNA methyltransferase enzyme and bisulfite sequencing to map protein-DNA interactions.

Kit Performance and Timeline

NOMe-Seq is intended for locus-specific analysis of nucleosome positioning in combination with the DNA methylation profile of the locus of interest. PCR is used to amplify the region of interest for Sanger sequencing analysis. However, if a larger portion of the genome is to be analyzed, the NOMe-Seq assay can be combined with Next-Gen sequencing using the adapter ligation protocol provided at www.activemotif.com/nome-seq.

Day 1

- Cell fixation with formaldehyde
- Cell lysis and nuclei collection
- Prepare 750,000 cell equivalents for sonication
- Sonicate chromatin into fragments greater than 1 kb
- Treat 250,000 cell equivalents with GpC methyltransferase for 4 hrs at 37 ° C
- Overnight reversal of cross-links

Day 2

- Treat with RNaseA and Proteinase K
- Purify genomic DNA
- Bisulfite conversion of DNA for 5 hrs at 50 ° C

Day 3

- Desulfonation and DNA purification of bisulfite treated DNA
- PCR amplification of genomic region of interest with bisulfite-specific PCR primers
- Purify PCR amplicon of interest

Day 4 +

- Ligation of PCR product into cloning vector
- Transformation of ligated DNA vector
- Selection of colonies for screening
- PCR amplify to screen for correct insert
- Isolation of plasmid DNA from correct clones
- Submit DNA samples for **Sanger sequencing**

- Perform end repair
- Concatemerization of amplicons
- Sonication of concatemerized sample
- Perform end repair
- Add A overhang to 3' ends
- Ligate adapters
- Size select library
- Enrich the adapter-modified DNA
- Validate the library
- Submit DNA samples for **Next-Gen sequencing**

NOMe-Seq Kit Components and Storage

NOMe-Seq Kits are for research use only. Not for use in diagnostic procedures. Kits are shipped in two boxes and contain reagents with multiple storage temperatures inside. Please store each component at the temperature listed below. The product is stable for 6 months when stored properly.

Reagents	Quantity	Storage
10X PBS	100 ml	4°C
10X Glycine	40 ml	-20°C
100 mM PMSF	500 µl	-20°C
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C
Lysis Buffer	20 ml	-20°C
10X NE Wash Buffer	5 ml	4°C
10X Reaction Buffer AM2	500 µl	4°C
1 M DTT	100 µl	-20°C
100X AdoMet	120 µl	-20°C
GpC Methyltransferase enzyme (4 U/µl)	50 µl	-20°C
Stop Solution AM2	1 ml	RT
RNase A (10 mg/ml)	2 x 40 µl	-20°C
Proteinase K (10 mg/ml)	200 µl	-20°C
DNA Purification Binding Buffer (AM #103496)	50 ml	RT
DNA Purification Wash Buffer (AM # 103497)*	50 ml	RT
DNA Purification Elution Buffer (AM #103498)	5 ml	RT
3 M Sodium Acetate**	500 µl	RT
Genomic DNA purification columns (AM # 103945)	20 ea	RT
Conversion Reagent	5 ea	RT
Buffer A	2 ml	RT
Denaturation Reagent	1 ml	RT
Hydroquinone	5 ea	RT
Buffer B	1 ml	4°C
DNA Purification Columns (AM #101037)	50 ea	RT
DNA Binding Buffer (AM #101232)	15 ml	RT
DNA Wash Buffer (AM #101233)	25 ml	RT
DNA Elution Buffer (AM #100762)	3 ml	RT

* DNA Purification Wash Buffer must be reconstituted to a final concentration of 80% ethanol prior to use.

** Do not store the 3M Sodium Acetate at 4°C.

Additional materials required but not supplied

- Cell culture reagents for growing cell lines
- 37% Formaldehyde
- Microprobe tip sonicator (Active Motif's Epishear Sonicator, Catalog No. 53051 and EpiShear Cooled Sonication Platform, Catalog No. 53073)
- Hemocytometer & Trypan Blue for cell counting
- Cell scraper (rubber policeman)
- Dounce homogenizer (Active Motif, Catalog No. 40401)
- Incubators for 37°C, 50°C, 65°C and 95°C temperatures
- Ethanol, 100%
- Isopropanol, 100%
- 1.5 and 2 ml microcentrifuge tubes and microcentrifuge
- Thermocycler
- 0.2 ml PCR tubes and caps
- PCR reagents (Bioline 2X MyTaq™ Master Mix, Catalog # BIO-25041, or individual PCR reagents such as dNTPs, PCR Buffer and Taq polymerase)
- Gel extraction kit (Optional)
- Phenol/Chloroform/Isoamyl Alcohol (Optional)
- DNA vector for PCR fragment cloning and ligation reagents
- Competent *E. coli* cells for DNA cloning (Active Motif's RapidTrans™ TAM1 chemically competent cells, Catalog No. 11096)
- DNase-free sterile water

Notes: The polymerase chain reaction (PCR) process for amplifying nucleic acid is covered by U.S. Patent Nos. 4,683,195 and 4,683,202 assigned to Hoffmann-La Roche. Patents pending in other countries.

Use of methylation-specific PCR (MSP) is protected by U.S. Patent Nos. 5,786,146, 6,017,704, 6,200,756 & 6,265,171 and International patent WO97/46705. No license under these patents to use the MSP process is conveyed to the purchaser by purchasing this product.

Notes Before Starting

It is important to read through the entire manual before starting the protocol. The NOMe-Seq Assay is designed to map the location of nucleosomes to the chromatin structure. A comparison of samples treated with the GpC methyltransferase enzyme and those not treated will enable the analysis of endogenous methylation in addition to nucleosome positioning. The assay includes enough reagents to perform 10 enzymatic treatments and 10 negative control reactions. The assay is intended for locus-specific analysis.

To study the nucleosome positioning pattern of a specific region, it is necessary to design PCR primer mixes for bisulfite converted DNA. We recommend that primer design is performed prior to starting NOMe-Seq. Recommendations on bisulfite-specific primer design are listed below.

The approximate time frame to complete the NOMe-Seq Assay including chromatin preparation, enzymatic treatment of chromatin, bisulfite conversion of genomic DNA, PCR amplification of the gene locus of interest and DNA cloning is 5 days. The protocol does provide stopping points throughout the assay.

Please check the list of additional materials required but not provided prior to starting the protocol. Suggested protocols are provided for PCR amplification and DNA cloning, but we recommend you follow the manufacturer's instructions for your specific PCR and cloning reagents.

Bisulfite-Specific PCR Primer Design

We recommend designing PCR primers specific for bisulfite converted DNA using an *in silico* program such as the free program **Methylviewer**, **http://dna.leeds.ac.uk/methylviewer/**. MethylViewer provides an integrated solution for primer design and analysis of bisulfite sequencing[18].The GpC methyltransferase treatment that is performed on the chromatin sample prior to bisulfite conversion will methylate all the accessible cytosines in a GpC context. These cytosines will remain as cytosine following the bisulfite treatment. Additionally, any endogenous DNA methylation in a CpG context will also remain as cytosine following the bisulfite treatment.

The region of interest for analysis should contain a high GpC density in order to increase the resolution of the nucleosome footprint. Forward and reverse primer design should flank the GpC and CpG region of interest, but avoid containing GpC and CpG within the primer sequence itself.

The optimal amplicon length for Sanger sequencing analysis should be between 400-600 bp. This length will allow for enough resolution to identify regions of nucleosome or transcription factor occupancy while still enabling complete reads by single-pass sequencing.

Once bisulfite-specific PCR primers are designed, they should be tested to confirm their specificity for converted DNA. To do this, use non-converted genomic DNA as a PCR template with the primer pair to ensure there is no DNA amplification. The primers should only produce a PCR amplicon if the DNA has been bisulfite treated.

Buffer Preparation and Recommendations

Samples

The assay is intended for locus-specific analysis. The assay has been optimized for use on cultured cell lines, but has not been tested on tissue samples.

Our standard chromatin preparation protocols use cells grown in one 15 cm tissue culture plate (approximately 1.5 x 10^7 cells) and yield enough material to perform up to 20 reactions. Depending on your experiments, you may wish to work with different volumes of cells. The comments and table below are designed to help adapt our protocols for using different cell numbers.

- It is not recommended to use a sample with less than 500 µl or more than 2 ml of the Lysis Buffer for the cell lysis and dounce homogenization steps.
- If you intend to compare results from various samples, treat the samples equally. For example, grow induced and uninduced cells in the same size plate and to the same density, then use equal volumes and shearing conditions. This will help ensure that the chromatin preparations are equivalent.
- Each human diploid cell contains 6.6 picograms of DNA. This can be used to estimate DNA in a chromatin preparation if the number of cells in the starting material is known. We estimate DNA recovery of chromatin shearing to be about 60-70%, depending on the cell type.
- Chromatin sonication is performed on 750,000 cell equivalents in a final volume of 300 µl. If using plate sizes smaller than 15 cm plate, it may be necessary to pool multiple plates together to obtain enough chromatin material.
- Due to the sensitivity and stability of some of the bisulfite conversion reagents, we recommend performing the NOMe-Seq Assay with a minimum of 4 samples (2 GpG enzymatically treated and 2 negative control reactions) per experiment. This experimental design can be used for a total of 5 experiments, or 10 enzymatically treated reactions.

	1 well of a 6-well plate	60 mm dish	100 mm dish	15 cm plate
Number of Cells	0.9 x 10^6	2.4 x 10^6	6.6 x 10^6	15.0x 10^6
Fixation Solution	3 ml	5 ml	10 ml	20 ml
Glycine Stop-Fix	1.5 ml	2.5 ml	5 ml	10 ml
1X PBS	2 x 3 ml	2 x 5 ml	2 x 5 ml	2 x 10 ml
Cell Scraping Solution + PMSF	500 µl + 2.5 µl PMSF	1 ml + 5 µl PMSF	1 ml + 5 µl PMSF	5 ml + 30 µl PMSF
Lysis Buffer + PIC + PMSF	500 µl + 2.5 µl PIC + 2.5 µl PMSF	500 µl + 2.5 µl PIC + 2.5 µl PMSF	500 µl + 2.5 µl PIC + 2.5 µl PMSF	1 ml + 5 µl PIC + 5 µl PMSF

Recommendations are offered in the manual for an approximate time frame of the assay. Below are the buffer preparations required for each day of the protocol. Adjust buffer preparation as needed based on your specific experiment.

Day 1 Buffer Preparation

Fixation Solution

Add 0.54 ml 37% formaldehyde (not included in the kit) to 20 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.

1X PBS Solution

Dilute 10X PBS in dH_2O. Add 2.33 ml 10X PBS to 21 ml sterile water, mix and place on ice.

Glycine Stop-Fix Solution

Combine 1 ml 10X Glycine Buffer, 1 ml 10X PBS and 8 ml sterile water. Mix well and leave at room temperature.

Cell Scraping Solution

Add 0.6 ml 10X PBS to 5.4 ml dH_2O, mix and place on ice.

Lysis Buffer

Is provided ready to use.

1X NE Wash Buffer

Prepare a 1X solution by diluting 10X NE Wash Buffer 1:10 in dH_2O. For example, add 250 µl 10X NE Wash Buffer to 2.25 ml dH_2O. Mix thoroughly.

100 mM DTT

Prepare a fresh 100 mM working stock of DTT from the provided 1 M DTT each time reactions are set up. For every 750,000 cell pellet, dilute 10 µl 1M DTT into 90 µl dH_2O. Vortex to mix and place on ice. Avoid multiple freeze thaws of DTT as this will diminish its effectiveness. Discard any unused 100 mM DTT at the end of the day.

10X Reaction Buffer AM2

The reaction buffer is provided at a 10X concentration. It will be used as both a 1X and 10X solution at different stages in the protocol. Please read the manual instructions carefully to ensure use of the correct reaction buffer concentration.

To prepare a working solution of 1X Reaction Buffer, dilute 1:10 in dH_2O and supplement with a final concentration of 10 mM DTT. For example, for each 750,000 cell pellet add 40 µl 10X Reaction Buffer AM2 to 320 µl dH_2O plus 40 µl 100 mM DTT.

100X AdoMet

Is provided ready to use at a 100X concentration.

GpC Methyltransferase Enzyme

The enzyme is provided ready to use at a concentration of 4 U/µl. Enough enzyme is supplied to perform 10 enzymatic treatments.

Stop Solution AM2

Is provided ready to use. Stop Solution AM2 may precipitate, therefore it is necessary to completely resuspend any precipitates by incubating at 37°C for 2 minutes and mixing prior to use.

Day 2 Buffer Preparation

RNase A

Is provided ready to use at a concentration of 10 mg/ml.

Proteinase K

Is provided ready to use at a concentration of 10 mg/ml.

DNA Purification Binding Buffer (AM #103496)

Is provided ready to use.

DNA Purification Wash Buffer (AM #103497)

The DNA Purification Wash Buffer requires the addition of ethanol before use. The final concentration of ethanol should be 80%. Add 40 ml of fresh 100% ethanol to the DNA Purification Wash Buffer bottle. Invert repeatedly. The buffer can be stored at room temperature after the addition of ethanol. The ethanol only needs to be added before the first use, after that the Wash Buffer is ready for use.

3 M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

DNA Purification Elution Buffer (AM #103498)

Is provided ready to use.

Genomic DNA Purification Columns (AM #103945)

The kit includes 20 columns for 10 enzymatic treatments and 10 negative control reactions.

Conversion Buffer

Conversion Reagent is provided lyophilized. To make Conversion Buffer, resuspend the Conversion Reagent tube with 700 µl dH_2O, 350 µl Buffer A and 175 µl Denaturation Reagent. Mix at room temperature for 10 minutes with intermittent vortexing. The Conversion Buffer is a saturated solution and it is normal for it not to dissolve completely. The Conversion buffer is stable for one week at -20°C. Five vials of conversion reagent are provided. We suggest using each resuspended

conversion reagent vial for a minimum of 4 samples.

Hydroquinone

Hydroquinone is an alkalizing agent and prevents DNA from stand breakage because of depurination. Prepare a working stock of Hydroquinone by resuspending one tube with 100 µl dH_2O. Keep the mixture in the dark and discard after use. Five vials of hydroquinone are provided. We suggest using each resuspended hydroquinone vial for a minimum of 4 samples.

Day 3 Buffer Preparation

DNA Binding Buffer (AM #101232)

Is provided ready to use.

DNA Wash Buffer (AM #101233)

Is provided ready to use.

Desulfonation Buffer

For each reaction, combine 22 µl Buffer B with 88 µl dH_2O and 110 µl 100% isopropanol.

DNA Elution Buffer (AM #100762)

Is provided ready to use.

DNA Purification Columns (AM #101037)

The kit includes enough columns for 20 bisulfite conversion desulfonation reactions and 20 PCR amplification clean up reactions.

DNA Purification Binding Buffer (AM #103496)

Is provided ready to use.

3 M Sodium Acetate

It is important to check the sodium acetate before use to ensure that the salts have not precipitated out of solution. Once the sodium acetate is in solution it should be stored at room temperature.

DNA Purification Wash Buffer (AM #103497)

The DNA Purification Wash Buffer should be ready to use following ethanol addition from first use.

DNA Purification Elution Buffer (AM #103498)

Is provided ready to use.

Read the entire protocol before use.

Day 1	Approximate time frame for Day 1 is 6-8 hours. Stopping points are available after cell fixation and chromatin sonication if needed.

Step A: Cell Fixation

1. Prepare at least two 15 cm plates for each cell line to be tested. One plate will be used to determine the cell count, while the remaining plate(s) will be used for the fixation and extraction. Grow the cells to 70-80% confluency. Stimulate cells as desired to activate the pathway of interest.
2. When the plates are ready to harvest, use one 15 cm plate to perform a cell count. Trypsinize the plate and count cells using a hemocytometer. This cell count is representative of the total number of cells to be fixed on the second 15 cm plate. The remaining cells can be discarded after the count is completed.
3. Prepare fresh fixation solutions for the remaining 15 cm plate(s). The volumes listed below are enough to process one 15 cm plate. Volumes may be scaled as indicated on page 9.
   a. **Fixation Solution:** Add 0.54 ml 37% formaldehyde to 20 ml minimal cell culture medium and mix thoroughly. Leave at room temperature.
   b. **1X PBS Solution**: Add 2.33 ml 10X PBS to 21 ml dH_2O, mix and place on ice.
   c. **Glycine Stop-Fix Solution:** Combine 1 ml 10X Glycine Buffer, 1 ml 10X PBS and 8 ml dH_2O. Mix well and leave at room temperature.
   d. **Cell Scraping Solution:** Add 0.6 ml 10X PBS to 5.4 ml dH_2O, mix and place on ice.
4. Pour medium off the cells and add 20 ml Fixation Solution to each plate. Incubate on a shaking platform for 10 minutes at room temperature.

   Note: In standard protocols, chromatin is fixed for 10 minutes prior to shearing. While these are typical fixation conditions, some protein/chromatin combinations may work better with shorter fixation times (*e.g.* 5 minutes).

5. Pour Fixation Solution off and wash by adding 10 ml ice-cold 1X PBS to each plate. Rock the plate for 5 seconds, then pour off the PBS.
6. Stop the fixation reaction by adding 10 ml Glycine Stop-Fix Solution to each of the plates. Swirl to cover, and then rock at room temperature for 5 minutes.
7. Wash each plate by pouring off the Glycine Stop-Fix Solution, then adding 10 ml ice-cold 1X PBS. Rock the plate for 5 seconds, then pour off the PBS.
8. Just before use, add 30 µl 100 mM PMSF to Cell Scraping Solution. Add 5 ml of this ice-cold Cell Scraping Solution to each plate and scrape cells with a rubber policeman. Hold the plate at an angle and scrape cells down to collect them at the bottom edge of the plate. Use a 1 ml pipette to transfer the cells to a 15 ml conical tube on ice.

9. Pellet the cells from Step A.8 by centrifugation for 10 minutes at 2,500 rpm (720 RCF) at 4°C.
10. Remove the supernatant and discard. At this point the protocol can be continued or the pellet can be frozen. If freezing the pellet, add 1 µl 100 mM PMSF and 1 µl PIC and freeze at -80°C.

Step B: Preparation of Sheared Chromatin by Sonication

1. Thaw pellet (if necessary) on ice and resuspend cells in 1 ml ice-cold Lysis Buffer supplemented with 5 µl PIC + 5 µl 100 mM PMSF. Incubate on ice for 30 minutes.
2. **(Optional)** Transfer the cells to an ice-cold dounce homogenizer. Dounce on ice with 40 strokes to aid in nuclei release, pausing after every 10 strokes to ensure the chromatin does not overheat. If not performing the dounce homogenization, proceed to step #3.

   Monitor Cell Lysis: To ensure cell lysis, take 10 µl of the cell lysate from the dounce and look at it under a phase contrast microscope using a hemocytometer to verify that the nuclei have been released. It is often helpful to look at the cells before and after the lysis step as this makes it easier to identify the nuclei versus whole cells. Intact cells should have a dark central region (nucleus) surrounded by a halo of less dense cytoplasm. In lysed cells, the nuclei will appear as dots surrounded by asymmetric debris. If the cells are not lysed, then dounce on ice with an additional 10 strokes, or until the cells are lysed.
3. Transfer cells to a 1.5 ml microcentrifuge tube and centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
4. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 1 ml 1X NE Wash Buffer and centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge to pellet the nuclei.
5. Carefully remove the supernatant and discard. Resuspend the nuclei pellet in 1 ml 1X NE Wash Buffer and place on ice.
6. Using the information obtained in Step A.2 for the total number of cells fixed per plate, determine the volume of cell suspension needed to obtain 750,000 cells. This cell number is sufficient to perform two NOMe-Seq reactions (*e.g.* both a positive and negative control reaction can be performed on the same sample). For additional NOMe-Seq reactions, create multiple aliquots of 750,000 cells per tube. We do not recommend scaling cell numbers at this stage as it will affect downstream sonication and enzymatic reaction steps. If more sample is needed, please prepare multiple tubes.

   Total number of fixed cells (Step A.2) / 1 ml NE Wash Buffer = Concentration cells/ml

   750,000 cells needed / Concentration = Volume (ml) required per tube
7. Aliquot 750,000 cells per 1.5 ml microcentrifuge tube and centrifuge for 10 minutes at 5,000 rpm (2,400 RCF) in a 4°C microcentrifuge. Any unused nuclei should be discarded.
8. Prepare fresh dilutions of 100 mM DTT and 1X Reaction Buffer AM2 as described on page 10.
9. Carefully remove the supernatant and discard. Resuspend each nuclei pellet in 300 µl 1XReaction Buffer AM2 and place on ice.

10. Sonicate the nuclei pellet(s) to a chromatin size of greater than 1 kb using a microtip probe sonicator, such as Active Motif's EpiShear™ Sonicator with a 1/8" probe (Catalog No. 53051) in combination with an EpiShear Cooled Sonication Platform (Catalog No. 53073) to maintain probe height and temperature consistency between samples.
    Suggested settings: Sonicate on ice with a 25% amplitude, 30 seconds Sonicator "On" time and 30 seconds "Off" for a total time of 3 minutes.
11. Gently spin the tube to collect the liquid. Proceed directly to the GpC Methyltransferase reaction.

Step C: Methyltransferase Reaction

1. Set up the GpC Methyltransferase reaction in a 1.5 ml microcentrifuge tube as described below. We also recommend setting up a negative control reaction that does not include the GpC Methyltransferase enzyme for determination of endogenous methylation.

    Note: Be sure to use the provided 10X Reaction Buffer AM2 and not the diluted buffer. If the 100 mM DTT was prepared on the same day it can be used to set up the methyltransferase reaction. Otherwise, prepare fresh 100 mM DTT.

Reagents	Sample	Negative Control
dH_2O	32 µl	37 µl
10X Reaction Buffer AM2	5 µl	5 µl
100 mM DTT	5 µl	5 µl
100X AdoMet	3 µl	3 µl
Fragmented Chromatin (250,000 cells)	100 µl	100 µl
GpC Methyltransferase enzyme (4 U/µl)	5 µl	–
Total Volume	**150 µl**	**150 µl**

2. Incubate the reactions at 37°C for 4 hours, adding 1 µl of 100X AdoMet per hour to each reaction. Following each addition of AdoMet, mix tubes by inverting and return to 37°C.
3. During the final hour of the incubation, place Stop Solution AM2 at 37°C to pre-warm.
4. After the 4 hour incubation, stop the reaction by adding 50 µl of 37°C Stop Solution AM2 to each tube.
5. Reverse cross-links of the fixed chromatin by heating at 65°C overnight (~16 hrs).

    Note: Alternatively, reversal of cross-links can be accomplished by heating to 95°C for 15 minutes and then proceeding directly into Step D of the protocol.

Day 2	Approximate time frame for Day 2 is 7-8 hours. Stopping points are available after DNA purification or bisulfite conversion if needed.

Step D: Proteinase K Treatment and DNA Purification

1. Following reversal of the cross-links, return the tubes to room temperature and spin briefly to collect any liquid inside the caps.
2. Add 4 µl RNase A to each reaction and mix thoroughly. Incubate at room temperature for 30 minutes.
3. Add 6 µl Proteinase K to each reaction and mix thoroughly. Incubate at 50°C for 1 hour.
4. Briefly spin the tubes to collect the liquid to the bottom. Transfer each reaction to a new 1.5 ml microcentrifuge tube.
5. Add 1050 µl (5x sample volume) DNA Purification Binding Buffer (AM #103496) to each reaction. If needed, adjust the pH with 5 µl 3M Sodium Acetate as described below.

   Note: Check that the color of the DNA sample / DNA Purification Binding Buffer mixture is bright yellow, not light orange or violet. If the color is light orange or violet, this indicates that its pH is too high. Add more 3M Sodium Acetate 5 µl at a time, mixing after addition, until the color is bright yellow (see Figure 1). **This step is crucial to the successful binding and purification of your DNA sample.**

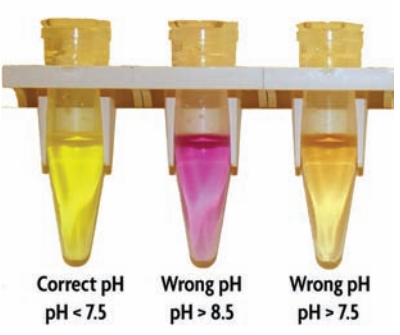


Figure 1: Solution color as a function of pH.
The DNA Purification Binding Buffer has a pH indicator dye, so that the pH of the solution can be easily determined. Only apply the sample to the column when the solution is bright yellow (left), indicating a pH under 7.5. DNA will not bind to the column if the pH is higher than 7.5.

Note: A full-color PDF of this manual can be downloaded from the Active Motif website, which will enable you to see this figure in color.

6. For each sample, place a Genomic DNA purification column (AM #103945) in the collection tube and add each pH adjusted sample to its own column in 750 µl increments. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute. Discard any flow through and repeat sample loading and centrifugation steps until no sample remains.
7. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
8. Prepare DNA Purification Wash Buffer (AM #103497) before the first use. Follow the instruction on page 11 for the addition of ethanol prior to using the solution. Add 750 µl DNA

Purification Wash Buffer to each column and cap the column.

9. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
10. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
11. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
12. Transfer the column to a clean microcentrifuge tube.
13. Add 30 µl of DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature.
14. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
15. Discard column and proceed to Step E below. Alternatively, purified DNA may be stored at -20C for future use.

Step E: Bisulfite Conversion

1. Prepare the Conversion Buffer and Hydroquinone reagents as described on pages 11-12.
2. In fresh 0.2 ml PCR tubes prepare the following reactions. The optimal range for bisulfite conversion is 500 ng - 2 µg per reaction. The maximum volume of DNA that can be used per reaction is 13 µl. Adjust the volume of DNA and water as needed to maintain a final volume of 140 µl per reaction.

Reagents	**Sample**	**Negative Control**
GpC Methyltransferase treated DNA	_____ µl	_____ µl
dH_2O	_____ µl	_____ µl
Hydroquinone	7 µl	7 µl
Conversion Buffer	120 µl	120 µl
Total Volume	**140 µl**	**140 µl**

3. Place the tubes in a thermocycler and set the program to start with an initial melt at 94°C for 3 minutes, then a 50°C conversion for 5 hours, followed by a hold at 4°C. To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.
4. Remove tubes and continue with Step F below. The protocol can be stopped here and DNA samples can be kept at 4°C in the dark for up to 5 days.

Day 3	Approximate time frame for Day 3 is 4 hours. Stopping points are available after DNA purification or PCR amplification if needed.

Step F: On-Column Desulfonation and DNA Purification

1. For each conversion reaction, aliquot 500 µl DNA Binding Buffer (AM #101232) into a 1.5 ml microcentrifuge tube. Add the entire conversion reaction mixture to the DNA Binding Buffer in the tube and vortex.
2. Remove the desired number of DNA purification columns (AM #101037) and place each one in a collection tube.
3. 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.
4. Add 200 µl DNA Wash Buffer (AM #101233) to each column and spin at 10,000 rpm for 30 seconds in a microcentrifuge.
5. Prepare Desulfonation Buffer as described on page 12.
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 (AM #101233) 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. Repeat Step 7 to perform a second wash.
9. Spin at 10,000 rpm for 1 minute in a microcentrifuge to remove any residual Wash Buffer from the column. Remove the column and place in a new 1.5 ml microcentrifuge tube.
10. Add 50 µl DNA Elution Buffer (AM #100762) directly to the filter of the column. Incubate 3 minutes at room temperature.
11. Spin at 10,000 rpm for 30 seconds in a microcentrifuge. The eluate will contain the converted DNA. DNA can be stored at -20°C until ready to use.

Step G: PCR Reactions to Amplify Genomic Region of Interest

1. Prior to performing sequencing, it is recommended to PCR amplify the genomic region of interest. This requires the use of bisulfite-specific PCR primer sets that surround the GpC methylated regions of interest. For Sanger sequencing, we suggest primer design such that the amplicon length falls between 400-600 bp. This will ensure a long enough sequencing read to confirm the location of nucleosomes, while still enabling reads by single-pass sequencing.
2. Please refer to page 8 for details on bisulfite specific primer design for PCR amplification.

3. Below is a suggested protocol for PCR amplification of bisulfite-treated DNA. Depending on the design of the locus-specific primers and PCR mix being used, some optimization may be required. We recommend using Bioline 2X MyTaq™ Master Mix for PCR reactions (Bioline Catalog No. BIO-25041). First prepare a Master Mix for the PCR reactions. The volumes listed in the chart are enough for 2 PCR reactions.

Reagents	**PCR Master Mix (2 PCR rxns)**
dH_2O	52.5 µl
2X MyTaq™ Mix	62.5 µl
Bisulfite-specific PCR primer mix (10 µM each)	5 µl
Total Volume	**120 µl**

4. In 0.2 ml PCR tubes, prepare the following PCR reactions.

Reagents	**Sample**	**Negative Control**
Bisulfite-treated DNA	2 µl	2 µl
PCR Master Mix	48 µl	48 µl
Total Volume	**50 µl**	**50 µl**

5. Mix tubes well and quick spin to collect liquid to the bottom of the PCR tube. Place tubes in a PCR cycler and program the thermocycler as shown below. Modify the annealing temperature as required for the locus-specific PCR primer mix. Optimal primer Tm should be between 50°C-60°C. To prevent evaporation of samples, use the heated lid or overlay reactions with mineral oil.

   95°C for 3 minutes

   (95°C for 30 sec., amplicon-dependent Tm for 30 sec., 72°C for 30 sec.) for 45 cycles

   72°C for 4 minutes

   Hold at 4°C

Step H: Purification of Amplified DNA

1. Following DNA amplification, purify the DNA. If the amplified PCR product is a single amplicon, the PCR amplified product can be purified using the included DNA purification reagents. However, if more than one PCR product is generated it is necessary to run the PCR reaction on an agarose gel to excise the proper amplicon using a gel extraction and purification kit.
2. For single amplicon DNA purification, use the DNA purification reagents provided:
. Add 250 µl (5x PCR sample volume) DNA Purification Binding Buffer (AM #103496) to each

reaction. If needed, adjust the pH of the solution with 5 µl 3M Sodium Acetate as described below.

Note: Check that the color of the DNA sample / DNA Purification Binding Buffer mixture is bright yellow, not light orange or violet. If the color is light orange or violet, this indicates that its pH is too high. Add more 3M Sodium Acetate 5 µl at a time, mixing after addition, until the color is bright yellow (see Figure 1). **This step is crucial to the successful binding and purification of your DNA sample.**

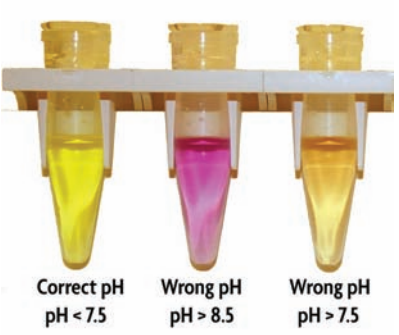


Figure 1: Solution color as a function of pH.
The DNA Purification Binding Buffer has a pH indicator dye, so that the pH of the solution can be easily determined. Only apply the sample to the column when the solution is bright yellow (left), indicating a pH under 7.5. DNA will not bind to the column if the pH is higher than 7.5.

Note: A full-color PDF of this manual can be downloaded from the Active Motif website, which will enable you to see this figure in color.

3. For each sample, place a DNA purification column (AM # 101037) in the collection tube and add each pH adjusted sample to its own column. Close the cap on each column, place them with the collection tubes in a microcentrifuge and spin them at 14,000 rpm for 1 minute. Discard any flow through.
4. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
5. Use the previously prepared DNA Purification Wash Buffer (AM #103497). Add 750 µl DNA Purification Wash Buffer to each column and cap the column.
6. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
7. Remove the column from the collection tube, then remove and discard the flow through from the collection tube. Return the column to the collection tube.
8. With the column cap open, spin at 14,000 rpm for 2 minutes in a microcentrifuge to remove any residual Wash Buffer from the column.
9. Transfer the column to a clean microcentrifuge tube.
10. Add 50 µl of DNA Purification Elution Buffer (AM #103498) to the center of the column matrix and incubate for 1 minute at room temperature.
11. Spin at 14,000 rpm for 1 minute in a microcentrifuge.
12. Discard column. Purified DNA may be stored at -20C for future use.

Day 4+	Time frame will depend on the manufacturer's instructions for the reagents used.

For locus-specific analysis of NOMe-Seq data, we recommend performing DNA cloning and Sanger sequencing using at least 10 colonies per gene region according to the instructions in Step I. Better coverage can also be obtained when a larger number of clones are sequenced and analyzed. Alternatively, to reduce the amount of labor and time required for analysis of a large collection of genes, it is possible to use Next-Gen sequencing on NOMe-Seq reactions. A protocol to prepare bisulfite converted, PCR amplified DNA samples for Next-Gen sequencing is available on the Active Motif website at www.activemotif.com/nome-seq.

Step I: DNA Cloning and Sequencing

1. The purified PCR product can be cloned into a DNA vector of choice according to the instructions of the manufacturer. If possible, plasmids containing the *LacZ* gene for blue/white screening are recommended.
2. Transform competent bacterial cells, such as Active Motifs RapidTrans TAMI (Catalog No. 11096), with 2 µl of ligation reaction. Follow the appropriate protocol for the competent cells used. The RapidTrans™ TAMI chemically competent cell protocol is provided below as an example.
    a. Remove the necessary number of chemically competent cell tubes from -80°C and place on ice to thaw.
    b. Add 1-5 µl of ligated DNA vector 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 each reaction.
    c. Incubate DNA and competent cells 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 reaction on pre-warmed LB agar plates with the appropriate antibiotic resistance for the cloning vector used. If using blue/white screening, ensure the selective plate contains X-gal.
    i. Allow plates to completely absorb any excess media
    j. Incubate inverted plates overnight at 37°C.
3. Select 10 colonies (or more if desired) for screening. Select white colonies if using blue/white screening as the expression of *LacZ* has been interrupted with the insertion of the ligation product.
4. Colonies can be screened by PCR to determine if they contain the proper insert prior to submitting samples for sequencing. PCR reactions should be set up using the forward and

reverse primers from the cloning vector for amplification of the insert. Prepare the PCR reactions as listed below. Prepare a PCR master mix containing everything except the template DNA and aliquot 30 µl to individual PCR tubes. Keep on ice.

Reagents	PCR Master Mix (10 PCR rxns)
dH_2O	243.2 µl
10X PCR Buffer	35 µl
dNTPs (2 mM each)	35 µl
Cloning vector PCR primer mix (10 µM each)	35 µl
Taq DNA polymerase (5 U/µl)	1.8 µl
Total Volume	**350 µl**

5. Pick the colonies for analysis and create a screening plate using LB agar plates with the appropriate antibiotic resistance by touching a pipet tip from the original colony selection onto the new screening plate and then place the tip directly into the appropriate PCR reaction. Pipet up and down to mix. Remove the pipet tip, and cap the PCR tubes. Place tubes in a thermocycler and amplify.

   95°C for 3 minutes

   (95°C for 30 sec., amplicon-dependent Tm for 30 sec., 72°C for 75 sec.) for 35 cycles

   72°C for 4 minutes

   Hold at 4°C

6. Following PCR amplification, run 2 µl of each PCR product on a 1% agarose gel to verify amplification. Confirm the amplicon is of the correct size. Proceed with plasmid DNA isolation and sequencing of correct colonies.
7. Isolate plasmid DNA from the colonies with correct insert sizes on the screening plate using any commercially available kit or published protocol.
8. DNA isolated from the positive clones can be submitted for Sanger sequencing using primers specific to the cloning vector of choice.

Section J: Data Analysis

Data analysis will require sequencing results to be evaluated separately for GpC and CpG methylation profiles. A comparison of the enzymatically treated sequencing results against the negative control reference sequence results will resolve if methylation that occurs in a GpCpG trinucleotide is the result of artificial or endogenous methylation.

The clarity of the methylation profile will depend on the number of GpC residues that cover a particular region. The more GpC residues present, the better the resolution.

1. First plot the GpC methylation from the sequencing data. This information provides the details about nucleosome or transcription factor positioning.
    a. A region of GpC sequence data containing unmethylated cytosines spanning a distance of 147 bp or larger can be identified as the location of a nucleosome.
    b. A region of GpC sequence data containing a range of unmethylated cytosines between 10-80 bp could be indicative of a protein binding site.
    c. Regions of methylated GpC dinucleotides indicate an area of the chromatin that is nucleosome depleted.
    d. Depending on the GpC density and quality of the sequencing read, sometime results are not as clearly defined as stated in Steps a-c. Additional criteria are provided to help with interpretation of the data.
    - 3 or more consecutively methylated GpC sites represent a nucleosome depleted region.
    - If one unmethylated GpC site exists within a stretch of methylated GpCs, the region can still be considered nucleosome depleted. The unmethylated site may represent a protein binding site.
    - If one methylated GpC site exists within a stretch of unmethylated GpCs, the region may still represent a nucleosome occupied sequence.
2. Second, plot the CpG methylation from the sequencing data. This information provides details about the endogenous DNA methylation present at the region of interest.
3. Manual methods will have to be used to overlay the two methylation profiles together in order to visualize the temporal relationship between nucleosome occupancy and DNA methylation.

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Appendix

Section K: Troubleshooting Guide

Problem/question	Recommendation
Poor yield of sheared chromatin	Nuclei not released. It is highly recommended to perform dounce homogenization. Use a dounce homogenizer with a small clearance pestle (Catalog No. 40401). Monitor cell lysis under a microscope. Generally, the more cells that are lysed, the higher the sheared chromatin yield.
	Decrease the fixation time. Over-fixed cells are often very resistant to lysis and shearing. Cross-linking for longer periods of time tends to cause cells to form into a giant cross-linked aggregate that is not sheared efficiently. Decrease the incubation time of the formaldehyde fixation step to 5 minutes.
	Sonication samples were emulsified. Avoid emulsification by turning up the power of the sonicator gradually. If a chromatin preparation becomes emulsified inadvertently, discontinue shearing and centrifuge the sample for 4 minutes at 8,000 rpm in a 4°C microcentrifuge to remove trapped air.
	Use fresh formaldehyde when preparing Fixation Solution.
	Buffers were not scaled proportionally to the size of the sample. Use the chart on page 9 to scale up or down chromatin preparation.
Low amount of GpC Methyltransferase activity	Confirm that all the reagents were added to the methyltransferase reaction including GpC enzyme and AdoMet and that reactions were performed at the temperatures indicated in the protocol. AdoMet should be spiked into the reaction every hour during the 4 hour incubation.
	Do not exceed the recommendations of 250,000 cell equivalents per reaction. The amount of enzyme is optimized for use in this range and cannot be scaled for use with different cell equivalent.
Poor bisulfite conversion	We recommend using a DNA starting volume of 13 µl. However, volumes up to 20 µl can be used without affecting the protocol.
	A 5 hour conversion incubation at 50°C is normally sufficient to efficiently convert DNA samples. However, DNA sequences that are GC-rich and/or contain extensive secondary structures may require a 9 hour conversion at 50°C. If noticing a loss of DNA after the conversion reaction, the conversion time can be reduced to as little as 3 hours.
	The Conversion Reagent is sensitive to air and moisture. We recommend performing bisulfite conversion on 4 samples per experiment to utilize a single tube of the supplied Conversion Reagent. Unused Conversion Reagent that is in solution should be discarded at the end of the day and not stored for future use.
	Hydroquinone solution is not stable long term. We recommend performing bisulfite conversion on 4 samples per experiment to utilize a single tube of the supplied Hydroquinone. Unused Hydroquinone that is in solution should be discarded at the end of the day and not stored for future use.
Storage of DNA	Once DNA is bisulfite converted samples may be stored at -20°C prior to PCR analysis. However, we recommend heating the frozen material to 37°C for 10 minutes before use in PCR, as heat-treatment releases any DNA bound to the tube during storage.

Problem/question	Recommendation
No amplification of DNA by PCR	Follow the recommendations for PCR primer design on page 8 to prepare bisulfite converted primers for your gene region of interest.
	PCR conditions may need to be optimized based on the annealing temperature of the gene-specific PCR primers, the Master Mix and PCR instrument used. We have validated the kit with Bioline 2X MyTaq™ Master Mix (Catalog No. BIO-25041).
	The pH of the Binding Buffer for the DNA purification step was too high. Your sample should be bright yellow when loading onto the DNA purification column. If the color is light orange or violet, add more 3 M Sodium Acetate 5 µl at a time, mixing after addition until the color is bright yellow. This step is crucial to the success of DNA binding and purification. For a full color image please see the manual for Active Motif's Chromatin IP DNA Purification Kit Catalog No. 58002 available online at www.activemotif.com.
	No ethanol in the DNA Purification Wash Buffer. Make sure that ethanol has been added to the DNA Purification Wash Buffer (AM # 103497) prior to first use.
	DNA will not be eluted from the silica matrix of the DNA purification column if the buffer used for elution contains high salt. Use the included DNA Purification Elution Buffer (AM # 103498) for DNA purification following reversal of chromatin cross-links and DNA purification of amplified locus-specific PCR products.

Section L. Related Products

ChIP-IT® Kits	Format	Catalog No.
ChIP-IT® High Sensitivity	16 rxns	53040
ChIP-IT® Express	25 rxns	53008
ChIP-IT® Express Enzymatic	25 rxns	53009
ChIP-IT® Express Shearing Kit	10 rxns	53032
ChIP-IT® Express Enzymatic Shearing Kit	10 rxns	53035
ChIP-IT® Express HT	96 rxns	53018
Re-ChIP-IT®	25 rxns	53016
RNA ChIP-IT®	25 rxns	53024
Chromatin IP DNA Purification Kit	50 rxns	58002
EpiShear™ Multi-Sample Sonicator	110 V	53062
EpiShear™ Probe Sonicator	110 V	53051
ChIP-IT® Protein G Magnetic Beads	25 rxns	53014
Siliconized Tubes, 1.7 ml	25 tubes	53036
ChIP-IT® qPCR Analysis Kit	10 rxns	53029
ChIP-IT® Control qPCR Kit – Human	5 rxns	53026
ChIP-IT® Control qPCR Kit – Mouse	5 rxns	53027
ChIP-IT® Control qPCR Kit – Rat	5 rxns	53028
ChIP-IT® Control Kit – Human	5 rxns	53010
ChIP-IT® Control Kit – Mouse	5 rxns	53011
ChIP-IT® Control Kit – Rat	5 rxns	53012
Ready-to-ChIP HeLa Chromatin	10 rxns	53015
Ready-to-ChIP Hep G2 Chromatin	10 rxns	53019
Ready-to-ChIP K-562 Chromatin	10 rxns	53020
Ready-to-ChIP NIH/3T3 Chromatin	10 rxns	53021
Bridging Antibody for Mouse IgG	500 µg	53017
Dounce Homogenizer	1 ml	40401
Dounce Homogenizer	15 ml	40415

ChIP-validated Antibodies

For an up-to-date list of over 125 ChIP-validated antibodies, please visit **www.activemotif.com/chipabs**.

DNA Methylation	Format	Catalog No.
hMeDIP	10 rxns	55010
MeDIP	10 rxns	55009
MethylDetector™	50 rxns	55001
MethylCollector™	25 rxns	55002
MethylCollector™ Ultra	30 rxns	55005
UnMethylCollector™	30 rxns	55004
Hydroxymethyl Collector™	25 rxns	55013
DNMT Activity / Inhibition Assay	96 rxns	55006
Methylated DNA Standard Kit	3 x 2.5 µg	55008
Fully Methylated Jurkat DNA	10 µg	55003
Jurkat genomic DNA	10 µg	55007

Modified Histones Array	Format	Catalog No.
MODified™ Histone Peptide Array	1 array	13001

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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