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Simultaneous single-molecule mapping of protein-DNA interactions and DNA methylation by MAPit

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Abstract

Sites of protein binding to DNA are inferred from footprints or spans of protection against a probing reagent. In most protocols, sites of accessibility to a probe are detected by mapping breaks in DNA strands. As discussed in this unit, such methods obscure molecular heterogeneity by averaging cuts at a given site over all DNA strands in sample population. DNA methyltransferase accessibility protocol for individual templates (MAPit), an alternative method described in this unit, localizes protein-DNA interactions by probing with cytosine-modifying DNA methyltransferases followed by bisulfite sequencing. Sequencing individual DNA products after amplification of bisulfite-converted sequences permits assignment of the methylation status of every enzyme target site along a single DNA strand. Use of the GC-methylating enzyme M.CviPI allows simultaneous mapping of chromatin accessibility and endogenous CpG methylation. MAPit is therefore the only footprinting method that can detect subpopulations of molecules with distinct patterns of protein binding or chromatin architecture, and correlate them directly with the occurrence of endogenous methylation. Additional advantages of MAPit methylation footprinting as well as considerations for experimental design and potential sources of error are discussed.

Keywords

Chromatin; Nucleosomes; DNA methylation; DNA methyltransferases; Footprinting; Single-molecule analysis

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Key References

Kladde et al., 1996. See above.
First demonstration of the utility of M.SssI for detection of nucleosome position and transcription factor binding.
Fatemi et al., 2005. See above.
Jessen et al., 2006. See above.
First demonstrations of the use of C-5 DNMTs in single-molecule footprinting.
Kilgore et al., 2007. See above.
First documented use of MAPit with M.CviPI, yielding simultaneous detection of chromatin accessibility and endogenous m^5CG at the single-molecule level.
Pardo et al., 2010. See above.
Development of MethylViewer program for rapid analysis of MAPit datasets.

Internet Resources

http://dna.leeds.ac.uk/methylviewer/
Site for download of MethylViewer program and detailed usage instructions.

INTRODUCTION

Crucial to a complete understanding of any biological function of DNA is the footprinting or mapping protein-DNA interactions at high resolution. Formerly, footprinting methods have relied on assaying accessibility of sites in DNA to probing reagents that result in DNA cleavage, such as nucleases (e.g., DNase I and micrococcal nuclease; *UNITS 12.4* and *21.1*, respectively) or chemicals (e.g. dimethylsulfate). Locations of protein binding to DNA are inferred by comparing sites of protection against damage in the absence and presence of the putative DNA-binding protein. Such conditions can be set up either in vitro or in vivo, e.g. wild-type cells *versus* the same cells with expression knock-down or bearing a null mutation in the gene of the factor of interest. While conventional footprinting methods have proven enormously informative, they are subject to several theoretical and practical limitations, as discussed in the Commentary. In particular, as only a single DNA break can be mapped per DNA molecule, conditions must approach limiting or single-hit kinetic levels of cleavage. By mapping cleavages at a given site over all molecules in a sample, the inherent complexity of protein-DNA interactions in biological systems is obscured by population averaging (Pondugula and Kladde, 2008).

These problems are overcome by probing protein-DNA interactions with DNA methyltransferases (DNMTs) that modify cytosine followed by bisulfite sequencing (Frommer et al., 1992; Clark et al., 1994) (*UNIT 7.9*), termed MAPit (Jessen et al., 2006; Kilgore et al., 2007; Pardo et al., 2009; Pondugula and Kladde, 2008). A key advantage of MAPit over other techniques is that it reports the methylation status (i.e. accessibility *versus* protection) of every C residue along one strand of individually-cloned and sequenced DNA molecules. This provides a single-molecule, non-averaged view of protein-DNA interactions that permits correlation between different footprints in a region within a sample population.

Methyl-5-C (m^5C), occurring predominantly at CpG sites (CG hereafter), is a common post-replicative DNA modification in vertebrates and many other organisms. A DNMT with different sequence specificity is needed to fully leverage MAPit. To this end, we cloned and characterized M.CviPI, an enzyme that methylates GC sites at C-5 (Xu et al., 1998a). Bisulfite sequencing of mammalian chromatin probed with M.CviPI therefore allows the methylation status of both CG and GC sites to be determined along a single DNA strand (Kilgore et al., 2007).

This unit describes a MAPit protocol using M.CviPI for simultaneous mapping of both endogenous cytosine methylation and protein-DNA interactions in cultured mammalian cells.

BASIC PROTOCOL PROBING MAMMALIAN NUCLEAR CHROMATIN WITH DNMTs

The three basic steps of MAPit are: (1) delivery of a suitable C-modifying DNMT to probe accessibility of DNA or chromatin; (2) bisulfite sequencing (*UNIT 7.9*), including bisulfite conversion of isolated and denatured DNA, PCR amplification of deaminated DNA, and sequencing cloned individual molecules from the PCR amplicon; and (3) assignment of the methylation status to each potential DNMT target sequence (Figure 1).

MAPit can be performed on organisms that lack detectable DNA methylation, such as the budding yeast *Saccharomyces cerevisiae* (Proffitt et al., 1984). Readers interested in MAPit probing of budding yeast chromatin are urged to consult any of several previously published detailed protocols (Jessen et al., 2004; Hoose and Kladde, 2006; Kilgore et al., 2007; Pardo et al., 2009). In principle, a DNMT probe can be used that modifies cytosine in any

sequence context on one of several positions. This is because bisulfite sequencing detects m^5C as well as methyl-N4-C ($m^{N4}C$) and hydroxymethyl-5-C (hm^5C) (Kriaucionis and Heintz, 2009; Tahiliani et al., 2009; Huang et al., 2010). Therefore, in organisms lacking detectable DNA methylation, the choice of DNMT need only consider the frequency and distribution of target sites on the analyzed strand of the locus of interest. However, C-modifying DNMTs with short recognition specificities, such as the C-5 DNMTs M.SssI (CG; Renbaum et al., 1990) and M.CviPI (GC; Xu et al., 1998a), are the most useful probes for MAPit analysis because they provide the highest footprinting resolution. An additional consideration of probe choice is that most genomic regions in vertebrate cells contain some m^5CG, limiting the usefulness of the CG-modification enzyme M.SssI (Fatemi et al., 2005; Gal-Yam et al., 2006). For this reason, we previously identified, cloned, overexpressed, and characterized the GC probe M.CviPI (Xu et al., 1998a), which is now commercially available.

Materials

Mammalian cell lines cultured under appropriate experimental conditions

Trypsin-EDTA solution (see recipe; store up to 6 months at −20°C)

Phosphate buffered saline (PBS; store indefinitely at 4°C)

Cell resuspension buffer (see recipe)

Cell lysis buffer (see recipe)

Methylation buffer (see recipe)

Methylation stop buffer (see recipe)

Enzyme dilution buffer (see recipe)

DNMT storage buffer (see recipe; store indefinitely at −20°C)

1 M dithiothreitol (DTT; store in single-use aliquots at −20°C)

100 mM phenylmethylsulfonyl fluoride (PMSF; dissolved in absolute ethanol; store up to six months at −20°C)

0.4% (w/v) trypan blue solution (store indefinitely at room temperature)

32 mM *S*-adenosyl-*L*-methionine (SAM) (store in single-use aliquots at −80°C)

80 U/μl M.CviPI fused to maltose binding protein (MBP, New England Biolabs) or fused to glutathione-*S*-transferase (GST, Zymo Research Corp.); aliquot and store at −20°C in non-frost-free freezer; see recipe for dilutions)

20 mg/ml proteinase K (store at −20°C in non-frost-free freezer)

5 U/μl HotStar Taq (Qiagen) (store at −20°C in non-frost-free freezer)

Phenol chloroform solution (see recipe; store indefinitely at 4°C)

10.0 M ammonium acetate, pH 8.0

Absolute ethanol

0.1× TE buffer (see recipe; store indefinitely at room temperature)

70% (v/v) ethanol (see recipe; store indefinitely at room temperature)

NOTE: Reagents should be prepared in sterile disposable labware. Use only distilled H_2O in all steps and solutions. Nuclei isolation and methylation buffers should be freshly prepared on the day of the experiment. DTT, PMSF and SAM should be added to solutions

immediately before use to avoid oxidation or hydrolysis. M.CviPI activity is very dependent on fresh addition of DTT.

Cell lines and growing conditions will vary according to the question being addressed and researcher discretion. Cells should be cultured using standard tissue culture techniques under desired experimental conditions until at least 1.5×10^6 cells per experimental sample (e.g. DNMT dose) are obtained. A refrigerated centrifuge and microcentrifuge or one in a cold room is recommended for isolation of nuclei.

Harvest cells

1 Add an appropriate volume of trypsin-EDTA solution pre-warmed to 37°C to remove cells from tissue culture plates or flasks. Incubate cells at room temperature until they detach from the growth surface.

The time needed for cell detachment varies from one cell line to another (~2–12 min), and can be determined by visualization with a light microscope. Cells can alternatively be harvested by adding ice-cold PBS directly to plates and scraping into 50 ml conical tubes on ice. Skip this step and step 2 if cells have been grown in suspension.

2 Add cell growth medium pre-warmed to 37°C (3× volume of trypsinization solution used in step 1) to terminate trypsinization.

Trypsin activity is inhibited by the protease inhibitor alpha-1-antitrypsin found in fetal bovine serum.

3 Centrifuge for 5 min at 1,000 × *g* at 4°C to pellet cells. Carefully aspirate supernatant.

4 Add 5 ml ice-cold PBS. Vortex gently and briefly to resuspend cell pellet and wash cells.

5 Centrifuge for 5 min at 1,000 × *g* at 4°C to pellet cells. Decant supernatant.

6 Resuspend cells with ice cold PBS to an approximate concentration of 10^6/ml and keep cells on ice.

7 Mix an aliquot of 20 μl cell suspension with 20 μl 0.4% (w/v) trypan blue solution. Pipette up-and-down several times to disperse cells and make the cell suspension homogeneous.

8 Count the number of live cells that exclude trypan blue either manually with a hemocytometer or using an automated cell counting device.

9 Aliquot 1.1×10^6 cells per experimental sample into pre-labeled 1.7 ml microcentrifuge tubes on ice.

Each DNMT probing reaction requires 10^6 cells. Starting with 1.1×10^6 cells per reaction (one reaction is one DNMT dose) allows for some loss during preparation of nuclei. We recommend setting up an untreated sample (0 U DNMT) and two concentrations of M.CviPI, therefore requiring 3.3×10^6 cells per experimental condition. In our experience, 30 and 100 U M.CviPI are good starting doses for either the M.CviPI-MBP or M.CviPI-GST reagents. Using two different concentrations of enzyme, while not essential, allows one to assess different degrees of chromatin accessibility and the extent of saturation of methylation by exogenously added M.CviPI at each GC site. The untreated sample (0 U DNMT) serves as a background control to monitor non-conversion of C in GC sites by bisulfite and/or sequencing errors. The untreated sample also

shows the level of endogenous CG methylation in the sample before probing, which should be taken into account when inferring whether GCG sites were likely methylated by endogenous DNMTs or exogenously-added by DNMT probe.

10 Centrifuge for 5 min at 1,000 × *g* at 4°C in a microcentrifuge to pellet cells.

11 Add 200 μl ice-cold cell resuspension buffer per 1.1×10^6 cells (i.e. add 600 μl, if 0, 30 and 100 U M.CviPI are used). Resuspend pellet by tapping tube gently.

NOTE: Isolating all nuclei for each experimental condition together in a single tube and aliquoting to separate tubes in step 17 ensures that the only variable will be the DNMT concentration.

12 Centrifuge for 5 min at 1,000 × *g* at 4°C to pellet cells. Decant supernatant.

Isolate mammalian nuclei

13 Resuspend cell pellet in 38.5 μl of ice cold cell lysis buffer per 1.1×10^6 cells (i.e. add 115.5 μl, if 0, 30 and 100 U M.CviPI are used). Incubate for 10 min at 4°C to lyse cells.

Inclusion of the non-ionic detergent Nonidet P-40 in cell lysis buffer allows for cell membrane lysis while maintaining nuclear integrity. Nonidet P-40 concentration and lysis time may need to be optimized for different cell types in order to obtain complete cell lysis without disrupting integrity of the nuclear envelope. To preserve nuclear structural integrity and native protein-DNA interactions, all steps for nuclei preparation should be done at 4°C. Nuclei should be handled carefully as they are prone to lysis. Avoid pipetting of nuclei until step 17; instead, resuspend by gentle tapping of the tube with a finger.

14 Prepare ice cold methylation buffer while cells are undergoing lysis by mixing on ice:

60.5 μl ice-cold cell resuspension buffer

0.55 μl freshly-thawed 32 mM SAM.

NOTE: These volumes are per each sample containing about 10^6 nuclei. Make enough extra solution to account for pipetting error. The methylation buffer contains 290 μM SAM, which will be diluted to a final concentration of 160 μM in the methylation reactions in step 20.

15 Add 61 μl ice-cold methylation buffer per 10^6 nuclei (i.e. add 183 μl, if 0, 30 and 100 U M.CviPI are used) to dilute Nonidet P-40 concentration. Mix by gently tapping the tube.

Dilution of Nonidet P-40 detergent to 0.08% (v/v) in this step helps maintain nuclear integrity.

16 Check the structural integrity of the nuclei. Stain an aliquot of 2 μl nuclei solution by adding 2 μl of 0.4% (w/v) trypan blue solution in a separate tube. Mix by gently tapping the tube, incubate for 3 min at room temperature, and examine nuclei by light microscopy.

Nuclei should stain blue as well as appear round and granular with no attached cytoplasmic debris. Nuclei may swell slightly during isolation and subsequent manipulations.

17 Aliquot 90 μl of nuclei resuspension containing 10^6 nuclei into 1.7 ml microcentrifuge tubes pre-labeled with each unit concentration of M.CviPI being used.

Probe nuclear chromatin structure by methylation with exogenous M.CviPI

18 On ice, freshly prepare M.CviPI dilutions for the methylation reaction.

For the 30 and 100 U M.CviPI samples, appropriate volumes of 3 U/μl and 10 U/μl M.CviPI solution are needed, respectively. Best results are achieved by setting up a dilution series to ensure that all samples are subjected to identical conditions in parallel (i.e., salt and 160 μM SAM), with the DNMT concentration being the only variable.

19 Pre-warm the nuclei dispensed in each tube by incubation for 5 min at 37°C. At the same time, pre-warm to 50°C a sufficient volume of 2× methylation stop buffer (100 μl per methylation reaction plus some extra to allow for pipetting error).

20 Staggering M.CviPI addition to each tube by 30 sec, add 10 μl of the corresponding M.CviPI dilution to each pre-warmed sample. Pipette up-and-down gently to mix and methylate for 15 min at 37°C.

Staggered addition of enzyme and respective staggered termination of methylation in step 21, ensure that the incubation time with the chromatin probing enzyme is held constant. The Nonidet P-40 detergent is diluted in this step to 0.07% (v/v), which is necessary for full activity of M.CviPI (and M.SssI). Parameters used during the chromatin probing reaction can be changed according to the requirements of the experiment. We recommend performing a pilot experiment under the conditions described here. Time and enzyme concentration can be adjusted accordingly (see Commentary).

21 Terminate each methylation reaction by adding 100 μl of 2× methylation stop buffer pre-warmed to 50°C, corresponding to the staggering scheme used in step 20. Vortex each sample immediately.

22 Add 1μl 20 mg/ml proteinase K to a final concentration of 100 μg/ml. Mix by inverting tubes and incubate overnight at 50°C.

Complete removal of protein from the DNA is necessary to achieve complete denaturation and hence bisulfite conversion (Warnecke et al., 2002). In our experience, incubation with proteinase K for at least 16 hr is required.

Purify mammalian genomic DNA

23 Extract proteins from the genomic DNA solution from step 22 by adding 200 μl (an equal volume) of phenol chloroform solution (*UNIT 2.3*). Vortex vigorously for 30 sec at room temperature to obtain a homogeneous suspension.

24 Separate the aqueous and organic phases by centrifugation for 5 min at 14,000 × *g* at room temperature in a microcentrifuge. Transfer the aqueous (top) phase to a new 1.7 ml microcentrifuge tube carefully avoiding transfer of denatured protein and SDS (white material located at the phase interface).

25 Add 1/4 volume of 10.0 M Ammonium acetate (i.e. 2.5 M final), and vortex briefly to mix.

26 Add 2.5 volumes of absolute ethanol, mix thoroughly by gentle inversion.

At this point, samples can be stored indefinitely at −20°C. Overnight incubation at −20°C increases recovery of low concentrations of nucleic acid.

27 Centrifuge for 5 min at 14,000 × *g* at room temperature in a microcentrifuge to pellet the nucleic acid.

28 Draw off supernatant carefully so as not to dislodge the nucleic acid pellet.

29 Add 0.4 ml 70% (v/v) ethanol. Vortex briefly to wash nucleic acid pellet.

30 Centrifuge for 5 min at 14,000 × *g* at room temperature in a microcentrifuge to pellet nucleic acid.

30 Carefully draw off supernatant without disturbing the pellet and air dry pellet for approximately 10 min.

31 Resuspend genomic DNA in 50 μl 0.1× TE.

Genomic DNA usually requires overnight incubation at 4°C to solubilize completely. Removal of RNA prior to bisulfite sequencing is not necessary. Samples can be stored at 4°C for many months or indefinitely at −20°C.

Bisulfite sequencing of mammalian DNA

Approximately 5–7 μg DNA are recovered from each reaction containing 10^6 nuclei. Bisulfite sequencing, including bisulfite conversion of purified DNA, PCR amplification of sequences of interest, cloning individual molecules from the PCR product, and sequencing cloned molecules, is performed as described in *UNIT 7.9*. Once clones of individual molecules have been sequenced the data are analyzed by MethylViewer (http://dna.leeds.ac.uk/methylviewer/) (Pardo et al., 2010). This computer program can concurrently score the methylation status of up to four user-defined sites either directly from *.ab1 sequencing files or from a FASTA file of sequences aligned with another program. For MAPit analysis of mammalian chromatin with M.CviPI, MethylViewer is used to concurrently score methylation at CG and GC sites along each sequenced molecule, and export publication-quality images. Other features, such as verification of bisulfite-conversion efficiency at non-CG and non-GC sequences can also be obtained. Occasional sequences with conversion efficiencies of <97% are typically omitted from further analyses, but this is up to the discretion of the investigator.

SUPPORT PROTOCOLS VERIFICATION OF METHYLATION OF DNA BY M.CviPI

When using a new enzyme preparation, one may wish to determine activity before investing time in sequencing and analysis of MAPit data. It is of course possible to methylate purified plasmids and test with various restriction enzymes. However, higher enzymatic activity is needed to methylate chromatin. We have found it most convenient to assay activity by methylation of nuclear DNA, using the actual experimental samples. To confirm that the DNMT used was active, we use one of two methods to screen for GC methylation, either quantitative methylation-sensitive restriction enzyme digestion (qMSRE) or methylation-specific PCR (MSP).

SUPPORT PROTOCOL I

For qMSRE, 20–250 ng of purified genomic DNA (not bisulfite treated) is subject to digestion with the methylation sensitive enzyme R.HaeIII. This enzyme can digest unmethylated GGCC sites but not GG-m^5CC sites. A parallel “mock” reaction containing all reaction components except R.HaeIII (replaced with glycerol) is included for each sample.

DNA from the R.HaeIII-digested or mock reaction is then amplified by real-time PCR with primers to a known open region containing a HaeIII site, such as the human *GAPDH* promoter (primers TACTAGCGGTTTTACGGGCG and TCGAACAGGAGGAGCAGAGAGCGA). Results are normalized to each sample's mock digestion control and quantified using the $\Delta\Delta C_T$ method to determine the levels of protection from R.HaeIII digestion achieved by each dose of DNMT.

SUPPORT PROTOCOL II

For MSP, 20 ng of bisulfite-treated DNA is amplified with two sets of primers that target human long interspersed nucleotide elements (LINE-1). One primer pair amplifies GC unmethylated or "U" LINE (primer sequences AGGTATTGTTTTATTTGGGAAGTGT and CCTTACAATTTAATCTCAAACTACTATA) and the second pair amplifies GC-methylated or "M" LINE (primers CATTGCTTTATTTGGGAAGCGC and CTTGCAATTTAATCTCAAACTGCTATG) DNA. The product of each PCR reaction is visualized on an agarose gel: the "M" product will be more abundant than the "U" product if the DNMT was active.

REAGENTS AND SOLUTIONS

Use deionized, distilled water in all recipes and protocol steps. For common stock solutions, see APPENDIX 2; for suppliers, see APPENDIX 4.

Trypsin-EDTA solution

0.25% (w/v) trypsin

2.21 mM ethylenediamine tetraacetic acid (EDTA)

Dissolved in Hank's-balanced salt solution (HBSS) without sodium bicarbonate, calcium and magnesium. Tested for porcine parvovirus as the same solution is used to harvest cells for passaging. Any good quality commercially-available solution will suffice.

Cell resuspension buffer

20 mM HEPES, pH 7.5

70 mM NaCl

0.25 mM EDTA, pH 8.0

0.5 mM EGTA, pH 8.0

0.5% (v/v) glycerol

10 mM DTT (always add fresh immediately before use, store at −20°C)

0.25 mM PMSF (always add fresh immediately before use, store at −20°C)

Cell lysis buffer—Cell resuspension buffer + 0.19% (v/v) Nonidet P-40

Nuclei methylation buffer—Cell resuspension buffer + 290 μM SAM (always add SAM immediately before use)

NOTE: The final concentration of SAM will be 160 μM in the methylation reaction.

2X Methylation stop buffer

100 mM NaCl

10 mM EDTA, pH 8.0

1% (w/v) SDS

DNMT storage buffer (M.CviPI)

15 mM Tris-HCl

200 mM NaCl

1 mM DTT

0.1 mM EDTA

200 μg/ml acetylated BSA (i.e. nuclease free)

50% (v/v) glycerol

pH 7.4 at 25°C

Enzyme dilution buffer (M.CviPI)—Dilute M.CviPI storage buffer by 8-fold (i.e. 1:7) with methylation buffer

M.CviPI dilutions—Immediately before use, on ice, prepare M.CviPI dilutions as follows:

1. Dilute 80 U/μl commercial stock of M.CviPI by 8-fold with ice-cold methylation buffer to make 10 U/1 μl dilution.
2. Make a 3.33-fold serial dilution of the 10 U/μl dilution with enzyme dilution buffer to make the 3 U/μl dilution.

About 8-fold less activity is needed if wild-type M.CviPI (i.e. non-fusion protein) is used. Adjust dilutions accordingly if a different stock concentration of commercial M.CviPI is used.

Phenol chloroform solution—Mix molecular biology grade phenol equilibrated to pH 8.0, chloroform, and isoamyl alcohol in the ratio of 25:24:1, respectively.

0.1× TE buffer

1 mM Tris-HCl, pH 8.0 (*APPENDIX 2*)

0.1 mM Na_2EDTA, pH 8.0

Autoclave and store indefinitely at room temperature.

70% (v/v) ethanol—Mix absolute ethanol and 0.1× TE buffer in the ratio of 37 ml:13 ml, respectively.

CAUTION: Flammable.

COMMENTARY

Background Information

Protein-DNA interactions play crucial roles in mediating all biological functions of DNA in every organism. Eukaryotes package their DNA into chromatin comprising a protein content of roughly half non-histone regulatory factors and half core histones. The fundamental repeating unit of eukaryotic chromosomes is the nucleosome core particle, composed of a histone octamer (central histone tetramer $(H3–H4)_2$ and H2A–H2B dimers) wrapped by a left-handed superhelix consisting of 1.65 turns or 147 bp of DNA (Luger et al., 1997).

Individual nucleosomes are repeated at a distance characteristic for each eukaryotic species, i.e. separated by a modal length of histone-free linker DNA in bulk chromatin. In contrast, at the single-molecule level, there can be considerable variation in linker length within a given region of chromatin. Nucleosomes are among the most stable protein-DNA interactions in eukaryotic chromosomes and act in concert with DNA-binding factors and other chromatin-associated factors to exert tight control of gene expression and other DNA functions (Kouzarides, 2007; Li et al., 2007).

In many eukaryotes, endogenous DNMTs post-replicatively modify the DNA component of chromatin at the 5 position of the cytosine base ring. C-5 methylation (m^5C) in vertebrates appears to occur exclusively at CG sites in most cell types, and plays essential roles in diverse aspects of vertebrate genome function (Bestor, 2000; Bird, 2002). These include repression or silencing of transcription, embryonic development, genomic imprinting of either the paternal of maternal alleles of some genes, inactivation of one of two X chromosomes in normal females of Eutherian mammals, and suppression of the mobility of parasitic genetic elements, e.g. retrotransposons (Robertson and Wolffe, 2000; Bestor and Bourc'his, 2004; Robertson, 2005; Goll and Bestor, 2005; McCabe et al., 2009). Aberrant DNA methylation is frequently associated with human aging and diseases, such as cancer (Robertson, 2001; Bird, 2002; Jaenisch and Bird, 2003; Robertson, 2005; Feinberg et al., 2006; Jones and Baylin, 2007). In other cases, m^5CG has been shown to activate transcription when it blocks binding of proteins to DNA that exert transcriptional repression (Nabilsi et al., 2009; Lai et al., 2010; Wu et al., 2010). At lower levels, m^5C is also present at non-CG sites, CHG and CHH (H is either A, C or T), in undifferentiated human embryonic stem cells (Kouidou et al., 2005; Grandjean et al., 2007; Latham et al., 2008; Lister et al., 2009; Hawkins et al., 2010; Laurent et al., 2010).

Chromatin structure is highly dynamic; nucleosomes are constantly being mobilized to different positions and/or are disassembled via the action of ATP-dependent chromatin remodelers, histone chaperones, or both (Längst and Becker, 2004; Saha et al., 2006; Clapier and Cairns, 2009). Nucleosome depletion at transcription start sites, for example, is often diagnostic of transcription initiation (Boeger et al., 2004; Korber et al., 2004; Mito et al., 2005; Jiang and Pugh, 2009). Although a hallmark of epigenetic m^5CG is its heritability from one cell to another, DNA methylation is also dynamic. First, methylation is not precisely maintained and thus modification of specific CG sites can fluctuate considerably. Second, cellular differentiation has recently been shown to involve oxidation of m^5C to hydroxymethyl C (hm^5C), which is subsequently removed by an as yet unknown mechanism (Tahiliani et al., 2009; Ito et al., 2010). In sum, dynamic changes in DNA methylation as well as occupancy by nucleosomes and non-histone regulatory factors lead to considerable epigenetic heterogeneity in chromatin.

Detection of the diverse epigenetic signatures present at a given region of interest by conventional footprinting methods poses several challenges (Pondugula and Kladde, 2008). Most of these stem from the nature of mapping DNA breaks introduced by nucleases or by genomic footprinting with chemical agents (e.g. dimethylsulfate). Limited digestion or chemical treatment of the footprinted sample is employed to achieve so-called single-hit kinetic levels of DNA cleavage, which are supposed to approximate a random Poisson distribution of cut sites. In practice, however, adherence to random Poisson statistics is hampered by biological complexity and non-randomness, especially when footprinted samples are of cellular origin. Second, even when single-hit digestion is achieved, only one cut site proximal to a radiolabeled DNA end, hybridizing primer, etc. can be mapped per single DNA molecule. Therefore, the position of nucleosomes or DNA-bound factors relative to one another on the same molecule, which requires mapping >1 cleavage site, cannot be determined. Third, a population of cut DNA molecules must be analyzed to

identify a footprint. Such population-ensemble methods average away differences between molecules and thus obscure molecular heterogeneity.

These problems are overcome by MAPit; single-molecule detection of protein-DNA interactions by exogenously-supplied C-methylating DNMTs (Jessen et al., 2006; Kilgore et al., 2007; Pardo et al., 2009; Pardo et al., 2010). MAPit builds on a large body of earlier studies by us and others demonstrating the usefulness of DNMTs as chromatin structural probes. To our knowledge, the earliest hints that chromatin structure might affect susceptibility to a DNMT were the preferential depletion of endogenous methyl-N6-adenine upon incubation of *Tetrahymena* nuclei with micrococcal nuclease (Pratt and Hattman, 1981; Pratt and Hattman, 1983). This suggested accessibility of linker DNA to the DNMT and its exclusion from nucleosome core DNA. Fehér et al. (1983) were the first to suggest that chromatin impeded access of specific sites in a yeast minichromosome to a C-5 DNMT expressed in vivo.. This observation was repeated almost a decade later, when *E. coli* M.Dam was used to differentiate between “open” and “closed” chromatin in budding yeast (Singh and Klar, 1992; Gottschling, 1992). We subsequently demonstrated that positioned nucleosomes and factors bound site-specifically to DNA impeded accessibility of M.Dam (Kladde and Simpson, 1994).

With the advent of bisulfite sequencing for detection of m^5C (Frommer et al., 1992; Clark et al., 1994), C-5 DNMTs became the logical choice for use as probes of protein-DNA interactions. M.SssI was used to probe chromatin structure first, because of its CG dinucleotide resolution and commercial availability (Kladde et al., 1996). In bisulfite sequencing, denatured DNA is subject to hydrolytic deamination of C to U with bisulfite ion, whereas m^5C is relatively non-reactive under optimal conditions (Hayatsu, 1976; Hayatsu et al., 2008; *UNIT 7.9*). Initially, we used C-5 DNMTs as in vivo probes of chromatin structure in yeast and for in vitro footprinting of yeast and mammalian factors (Kladde et al., 1996; Xu et al., 1998b; Dong et al., 1999; Duan et al., 1999; Vyhlidal et al., 2000; Samudio et al., 2001; Jessen et al., 2004). In these studies, PCR products obtained from DNMT-probed and bisulfite-converted samples were sequenced directly, generating a population-averaged view of chromatin accessibility. Subsequent work by us and others (Fatemi et al., 2005; Jessen et al., 2006) published within two months of each other, took the further steps to clone and sequence individual DNA molecules from PCR amplicons. This yielded the methylation status and hence accessibility state of the cytosine in each and every potential DNMT target site along single DNA strands; a powerful single-molecule view of chromatin accessibility. A later manuscript (Gal-Yam et al., 2006), introduced the name methylase-based single promoter analysis (M-SPA).

An important consideration for the utility of DNMT-base footprinting in vertebrate systems is to employ a DNMT with a sequence specificity that differs from the CG methylated by endogenous enzymes. To this end, we cloned the gene encoding the GC DNMT M.CviPI (Xu et al., 1998a). The first M.CviPI footprinting of single mammalian promoters proved its utility for chromatin-structure analysis (Kilgore et al., 2007; Pardo et al., 2010). A second advantage is that, unlike M.SssI, M.CviPI footprinting resolution is not limited by density of CG dinucleotides. Thus, MAPit need not be limited to studies of CpG islands, and may well be extended to studies of chromatin at regulatory elements besides promoters. Given the seminal advance afforded by M.CviPI – single-molecule, simultaneous mapping of m^5CG and chromatin accessibility at any genomic region – we respectfully request that users of the enzyme adopt the MAPit nomenclature.

As shown by the example in Figure 2, MAPit footprinting with M.CviPI has many advantages over other footprinting techniques. First, it is not at all subject to the constraints of single-hit kinetics, meaning that methylation of many CG and GC sites can be detected

per sequenced molecule. This makes MAPit the only method capable of correlating footprints, i.e. sequential or cooperative binding events, along individual DNA molecules (Jessen et al., 2006; Gal-Yam et al., 2006). In contrast, as only the first DNA cut can be mapped in nuclease-based footprinting, there is much potential for multiple cuts to "mask" signal at locations farther removed from the mapping primer, hybridization probe, etc. Second, MAPit data sets include molecules with no accessibility. Nuclease footprinting cannot score such molecules, as all signal generated from uncut molecules coalesces in the "parent" or "run-off" band. Lastly, the single-molecule view of footprints completely sidesteps population averaging and is thus able to detect distinct subclasses of molecules.

Critical Parameters and Troubleshooting

Exogenous DNMT, concentration, and treatment time—These are probably the most important variables to control when performing MAPit. Perhaps as expected, the wild-type M.CviPI polypeptide appears to be the most efficient probe for use in MAPit. This may be because DNMT fusion proteins have decreased affinity for DNA, catalytic activity, or both (Xu and Bestor, 1997). However, insolubility of wild-type M.CviPI led us to construct two commercially-available versions, M.CviPI fused to either maltose binding protein (MBP) or glutathione S-transferase (GST). While a high level of modification is desired for single-molecule footprinting, excessive DNMT activity (concentration and/or time of methylation) has the potential to physically compete for DNA-binding sites of proteins being footprinted. For example, we have observed that very high DNMT concentrations can invade the edges of nucleosomes in vitro. By no means is this a problem unique to probing with DNMTs, as all enzymes that act on DNA bind their substrate with measurable affinity. One advantage of DNMTs over nucleases is that multiple sites can be methylated per enzyme binding event. This is likely because DNMTs, like many proteins that associate with DNA, can slide or scan along DNA (Matsuo et al., 1994; Renbaum and Razin, 1992; Vilkaitis et al., 2005; Holz-Schietinger and Reich, 2010). That stated, we have not observed nor are we aware of any situations in which DNMTs have displaced either site-specific DNA-binding factors from DNA or histone octamers from nucleosomes.

It is recommended that pilot experiments be conducted to optimize footprinting results. In principle, enzyme concentration, time, or both can be varied. We have opted, however, to vary enzyme concentration in pilot studies in keeping with most footprinting protocols. Longer times of incubation may also lead to potential loss of DNMT activity, hydrolysis of SAM cofactor, and dissociation of factors of interest from DNA. It is important to realize that methylation by exogenous DNA probes is irreversible during the methylation probing step. Therefore, factors that subsequently bind to methylated sites cannot be footprinted. It is equally important to use a consistent number of nuclei (i.e. mass of chromatin) in each experiment. The conditions indicated in this basic protocol (number of nuclei, DNMT dosages, time, solutions, and temperature) have been standardized to provide an adequate level of modification in our hands.

Buffer composition—Buffers adopted in this protocol have been previously established as maintaining the structure of native chromatin (Richard-Foy and Hager, 1987). Buffers can be changed to suit specific needs, but care needs to be exercised to avoid reagents that affect DNMT activity. High salt concentrations, for example, inhibit DNMT activity, which are also undesirable as they disrupt protein-DNA interactions. It is critical when using M.CviPI to add DTT to a final concentration of 10 mM immediately prior to conducting the chromatin methylation reaction. SAM, the universal cofactor and methyl donor for methyltransferases (Hermann et al., 2004), hydrolyzes with repeated freeze-thaw cycles. It is therefore important to store SAM at −80°C as single-use aliquots and add freshly immediately prior to methylating chromatin.

DNA purification—Thorough degradation of DNA-bound proteins with proteinase K is required in order to obtain DNA of high purity. Incomplete proteinase K treatment can interfere with the efficiency of bisulfite conversion (Warnecke et al., 2002). To avoid denaturation, proteinase K should not be vortexed. Digest for at least 16 hr at 50°C. Removal of RNA has also been reported to be necessary for efficient bisulfite conversion; however, in our experience, using the bisulfite treatment protocol described in *UNIT 7.9*, this does not appear to pose a problem. Perhaps this is because the described "home brew" method uses a solution saturated with sodium metabisulfite and thus contains a higher concentration of reactive bisulfite ion than most other protocols. In addition, RNA is completely hydrolyzed under the alkaline and high temperature conditions used to denature DNA prior to deamination.

PCR amplification of sequences from bisulfite-converted DNA—Performing PCR with deaminated DNA as template presents several challenges. The main hurdle is that, although the genome remains the same size, it is reduced in complexity by bisulfite conversion, i.e. it has reduced GC content. Considerations for PCR with deaminated templates are discussed extensively in *UNIT 7.9*. It is worth mentioning that DNA strands are no longer complementary after bisulfite conversion, so strand-specific amplification is determined by primer design (see below). Ideally, one would design primer pairs for amplification of both strands of the locus of interest. Artifacts of DNA sequence can impair amplification or cloning of sequences corresponding to certain chromatin conformations at some loci, causing amplicon bias. Because each strand will produce a different sequence, they would not likely share amplicon biases. Comparison of data from both strands will therefore identify most biases, which must be known for quantitative interpretation of MAPit data. Alternatively, bisulfite sequencing of a set of mixtures (0:100, 25:75, 50:50, 75:25, 100:0) of placental DNA (primarily unmethylated):methylated DNA (genomic DNA methylated in vitro with M.SssI and/or M.CviPI) can be used to provide a direct test for amplification and cloning biases.

As it is single-stranded, deaminated DNA is prone to forming secondary structures that lead to spurious amplification. Performing hot-start PCR will avoid this amplification problem. In our hands HotStar Taq (Qiagen) has given good results with mammalian DNA templates. DNA polymerases can vary in tolerance to uracil containing templates, such as deaminated DNA. Long extension times of 2–4 min per kb can improve amplification yield, as can increasing the number of PCR cycles. Finally, for loci that are difficult to amplify, we employ PCR enhancers such as trimethylammonium chloride (TMAC; titrate concentration around 0.75 mM) or the Coral buffer supplied with HotStar Taq.

Primer design—Considerations for primer design are discussed in detail in *UNIT 7.9*. A main concern when working with native mammalian DNA or that which has been probed with the CG DNMT M.SssI is the presence of m^5CG. In such samples, PCR primers for amplification of bisulfite converted samples are designed to avoid CG sites, which may be potentially methylated. When using MAPit with the GC probe M.CviPI to footprint protein-DNA interactions, avoid CG and GC sites with primer binding sites as much as possible. When this is not feasible, degenerate bases should be incorporated into primers to avoid PCR bias towards molecules in which the primer binding sites are either methylated or unmethylated. Conventional guidelines for primer design, PCR conditions, and cycling parameters for PCR with bisulfite-converted DNA template are described in the PCR amplification step for bisulfite sequencing (*UNIT 7.9*).

Anticipated Results

MAPit analysis of a mammalian tumor suppressor gene promoter is shown in Figure 2 as an example of obtained results. The *SIM2* (*single-minded 2*) gene encodes a transcription factor that is highly expressed in breast tissue, where it has recently been reported to have tumor suppressor function (Metz et al., 2006; Kwak et al., 2007). We performed MAPit with wild-type M.CviPI on the immortalized human mammary epithelial cell line MCF10A. The zero M.CviPI control shows the level and distribution of endogenous m^5CG, as would be seen in any bisulfite sequencing experiment. For both m^5CG and G-m^5C, some background level is expected to result from incomplete deamination, base misincorporation during PCR, and sequencing error. The background can be estimated as equal to the percent unconverted cytosine outside methylation sites. Where endogenous m^5CG is not above background, as seen at *SIM2*, GCG methylation, which is otherwise ambiguous, can be ascribed to M.CviPI. This increases the resolution of MAPit.

The sequences reveal a nucleosome-free region of about 147 bp, located upstream of the TSS, and flanked by two protected areas that may accommodate at least one nucleosome on each side. Nucleosomes bound to DNA will generate protection footprints of ~150 bp. In a population of molecules, nucleosomal footprints can be shifted by several base pairs to either side due to different translational positions. Footprints comprising smaller sizes can be interpreted as DNA-bound factors, especially when located at known factor binding sites (Kladde et al., 1996; Xu et al., 1998b; Jessen et al., 2004; Hoose and Kladde, 2006). Higher-order chromatin structures and areas where nucleosomes are closely packed may generate larger footprints (Dechassa et al., 2010). Hypotheses derived from MAPit data can be tested, if necessary, by use of other techniques such as chromatin immunoprecipitation or expression knock-down.

Time Considerations

This protocol typically requires 4–5 days to complete, plus the time required to obtain DNA sequencing data. Probing isolated nuclei with exogenous DNMT can be performed in one day, including an overnight proteinase K digest, but may take more time depending on specific experimental goals and design. DNA purification takes 4 hr, plus an overnight elution step. Together, bisulfite conversion of purified DNA samples, PCR amplification, ligation and transformation take as many as 20 hr, which may be broken into separate days. After growing colonies overnight, analysis of cloning efficiency and preparation of 96-well sequencing plates takes under 6 hr. Plates are grown overnight, and preparation for transfer to a sequencing facility takes 1 hr the next day. Sequencing time depends on the sequencing facility.

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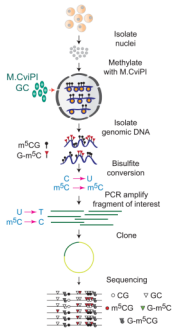

Figure 1.
MAPit overview for mapping m^5CG and chromatin accessibility in mammalian nuclei.

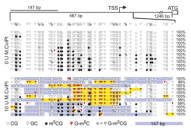

Figure 2.

MAPit analysis of the TSS region of human *SIM2* in MCF10A cells. Nuclei (10^6) were probed with 10 U of wild-type M.CviPI for 30 min at 37°C. *SIM2* is expressed in MCF-10A cells. Each horizontal line represents 524 bp of chromatin from a single cell. Circles represent CG sites and triangles represent GC sites. Black filled circles and red filled triangles, represent m^5CG and G-m^5C, respectively. GCG sites are represented by both gray triangle and circles. GCG site methylation cannot rigorously be discriminated as being placed by endogenous or exogenous DNMT, but this can often be inferred from context (see Anticipated Data for discussion). Blue highlighted areas represent 147 bp of contiguous M.CviPI DNA footprint. Note that about half of the alleles have relatively high levels of endogenous methylation (black filled circles). Based on molecules from cells not treated with M.CviPI, it can be inferred that gray GCG sites in these densely methylated MCF-10A alleles were likely methylated by endogenous DNMTs. The other half of the molecules is almost free of endogenous methylation but shows an accessible, nucleosome-length region high in M.CviPI methylation (red triangles) highlighted in red. No other technique can determine this bipartite pattern of chromosome structure. The high accessibility to M.CviPI is probably due to histone depletion near the TSS. In contrast, this putative histone-free region is flanked by protected spans of median length around 150 bp. Numbers at the right of each molecule depiction indicate the percentage of C conversion to T in non-CG and non-GC sequences. Nucleotides that failed to convert or reverted to a C during PCR amplification are indicated by vertical blue tick marks.