DNA methyltransferase-based single-molecule (MAPit) assay for mapping protein-DNA interactions \textit{in vitro} (PROT45)

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\textbf{Introduction}

Analysis of protein-DNA interactions in the context of chromatin is pivotal for understanding the complex structure and functions of eukaryotic genomes. Packaging DNA with histones into nucleosomes impedes the binding of most \textit{trans}-acting factors requiring access to their specific target sites. Alteration of nucleosome structure and organization is therefore a central feature of gene regulation. Factors gain access to their target sites when the intrinsically dynamic nucleosomes preferentially expose DNA sequences at the nucleosome termini by a process termed site exposure (Polach and Widom, 1995, 1996). In addition, nucleosome-mediated repression is relieved by multi-protein chromatin remodeling complexes that disrupt nucleosomal structure in an ATP-dependent manner. Nucleosomes are reorganized either by remodeler-directed repositioning, disassembly and/or exchange of H2A-H2B dimers for histone variants (Längst and Becker, 2004; Saha et al., 2006).

Since the emergence of the basic nucleosome model of chromosome structure, various methods have been developed for mapping and characterization of histone and non-histone protein-DNA interactions. Most often, enzymatic (e.g., micrococcal nuclease, DNase I, DNase II, restriction endonucleases, exonuclease III) or chemical (e.g., dimethyl sulfate, methidiumpropyl-EDTA, psoralens, ultraviolet light) footprinting techniques, which rely on DNA cleavage, are used to localize nucleosome positions and/or factor binding sites (Simpson, 1998). In general, these footprinting techniques are based on the premise that protein-DNA complexes are more resistant to nuclease digestion than free DNA. Large nucleosomal or small regulatory factor footprints are inferred by comparison of the patterns of DNA cleavage in protein-containing and -free samples.
Although significant progress has been made in elucidating basic chromatin structure and dynamics, several problems associated with classical footprinting techniques have severely restricted the scope of questions that can be addressed. First, these methods fail to detect heterogeneity between individual chromatin molecules in a sample as they report the average behavior of all molecules in the population. Second, techniques based on DNA cleavage cannot detect multiple footprints arising from coordinated or sequential binding of factors along an individual DNA molecule within a population of molecules. This is because, considering a single molecule, only the first cleavage site proximal to the probe can be mapped. Related to this, quantitative assessment of factor occupancy is subject to the constraints of Poisson single-hit kinetic conditions; random cleavage of $\leq 1$ per molecule in at least 90% of the molecules in the population. However, biological processes are complex and often involve non-random situations leading to a multiple-hit regime where the probe modifies the same DNA molecule more than once. Lastly, nucleases exhibit strong cleavage preferences (Flick et al., 1986) that frequently limit accurate assignment of nucleosome or factor positions.

Efficient single-molecule techniques that do not damage DNA are therefore necessary to provide a detailed view of chromatin structure. DNA methyltransferases (DMTases) offer an attractive alternative for investigating chromatin architecture and deciphering dynamic chromatin-mediated processes. We recently developed a single-molecule assay termed MAPit (Methyltransferase Accessibility Protocol for individual templates) for analyzing a wide range of protein-DNA interactions by DNA methylation protection rather than nuclease-based strand scission (Jessen et al., 2006). Independently, a similar approach was developed, referred to as methyltransferase-based single-promoter analysis (MSPA) (Fatemi et al., 2005). We previously employed MAPit in living yeast cells to provide the first evidence for a stochastic and heterogeneous nature of chromatin transitions occurring during transactivation of the phosphate-responsive PHO5 promoter (Jessen et al., 2006). Here, we describe the application of this method to map nucleosome positions in biochemically reconstituted chromatin preparations either directly or after remodeling with the prototypical yeast ISW2 complex purified from budding yeast. This method should provide a powerful new approach for exploring mechanistic aspects of chromatin remodeling.

**Procedure**

To map nucleosome positions using MAPit, nucleosomes are first reconstituted and probed directly or subjected to remodeling in the presence of a purified ATP-dependent chromatin remodeling complex. After terminating remodeling reactions, DMTases are added to methylate accessible DNA sites. Subsequently, samples are analyzed by bisulfite genome sequencing (BGS), which reveals methylation patterns of individually cloned molecules at single-nucleotide resolution (Clark et al., 1994; Frommer et al., 1992) (Fig. 1).

**Preparation of Nucleosome Substrate**

1. Recombinant core histones (*Xenopus laevis*) are expressed, purified and refolded into the octamer using standard methods (Dyer et al., 2004).
2. Double-stranded DNA fragments (note 1) are prepared with one Cy5-labeled and one unlabeled primer in a preparative PCR reaction.
3. The large-scale PCR reactions are then concentrated (we use Millipore ultracel YM-50 filters) and purified by phenol-chloroform extraction.
4. After ethanol precipitation, the final concentration of DNA is adjusted to 1 mg/ml for reconstitutions.
5. Mononucleosomes are reconstituted on a micro-scale using rapid salt dilution method. Typically, we titrate the DNA-to-octamer in molar ratios of 0.3:1.0, 0.5:1, 0.75:1.0 and 1.0:1.0 in a 10 µl reaction containing 2 M NaCl and 10 µg of octamer and use the sample with the least amount of free DNA for further analysis. Higher molar ratios can also be employed to achieve maximal reconstitution of free DNA.
6. After incubating for 25 min at 37°C, the salt concentration is serially diluted to 1.5 M, 1 M, 0.7 M and 0.3 M by the step-wise addition of 25 mM Tris-HCl, pH 8.0 at 10 min intervals.
7. The efficiency of each nucleosome reconstitution is verified by polyacrylamide gel electrophoresis (PAGE) at 4°C on a 4% native gel buffered with 0.2X TBE.
8. An image of the gel is documented using an appropriate imaging apparatus.

**Nucleosome Remodeling**

1. Yeast ISW2 complex (or other desired activities) is purified as previously described (Tsukiyama et al., 1999).
2. Remodeling reactions can be performed as described in Zofall et al. (2004). A typical remodeling reaction of 75 µl contains (note 2):

| Reconstituted nucleosome | ~ 6 µl (1-18 pmol) |
| ISW2 binding buffer      | 1X final            |
| ISW2 complex             | 3-84 nM final       |
| 10 mM ATP                | 0.6-0.8 mM final    |

Reactions are incubated at 30°C for 30 min and stopped by adding ATP-γ-S (5 mM final).
3. Reactions(4-6 µl) are analyzed by electrophoresis on a 5% non-denaturing PAGE buffered with 0.2X TBE at 4°C.
4. Document the gel using an instrument capable of fluorescent imaging (note 3).

**Probing Mononucleosomes with DMTases**

Cytosine-5-specific DMTases that recognize specific dinucleotide sites (e.g., M.SssI with CG specificity (Renbaum et al., 1990) or M.CviPI with GC specificity (Xu et al., 1998) are particularly useful because of their high mapping resolution. C-5 methylation (m^5C) is required to provide a single-molecule view of DNA methylation via BGS.

**DMTase treatments**

1. Aliquots of each terminated reaction are subjected to methylation in a 30 µl reaction volume containing:

| Remodeled chromatin in 1X ISW2 binding buffer | 25 µl |
| S-adenosylmethionine                      | 160 µM (note 4) |
2. To perform DMTase titrations, serial dilutions of M.SssI DMTase (New England Biolabs) are prepared with the M.SssI storage buffer (manufacturer’s buffer in which the enzyme is stored) as the diluent (note 5).

3. Samples are pre-warmed to 30-37°C for 10 min.

4. An equal volume of each M.SssI dilution is added to the nucleosome mix prepared above (steps 1-2) at regular time intervals (i.e., staggered start) and incubated at 30-37°C for 5-30 min. In our titrations, a final DMTase concentration in the range of 0.01-0.64 U/µl was employed in various experiments. Dilutions of the enzyme can be avoided by adding the stock directly to the reactions but the samples must be brought up to similar volumes using the storage buffer.

5. Methylation is terminated by adding an equal volume of 2% (w/v) SDS (pre-heated to 70°C) and incubation at 70°C for 10 min (Notes 6 and 7).

Bisulfite Deamination of DNA

Several bisulfite conversion methods have been reported for processing cytosine-methylated DNA. Bisulfite treatment of DNA converts unmethylated cytosine bases to uracil while leaving 5-methylcytosine (m5C) chemically unaltered. Following PCR amplification of a region of interest, uracil arising from deamination is replaced by thymine and m5C is propagated as cytosine. The methylation status at every DMTase target site is then assessed by cloning of individual DNA molecules followed by sequencing. The following protocol was developed in our laboratory and can be used to achieve high efficiency of conversion of cytosine while minimizing DNA degradation, the two major concerns associated with other procedures. Commercially available bisulfite deamination kits such as the EZ DNA Methylation-Direct™ Kit (Zymo Research D5020) or EpiTect® Bisulfite Kit (Qiagen 59104) can also be used, but we have routinely achieved as good or better results with our protocol.

Kladde Lab Method

1. Degassed water (dg.dH2O) is prepared the day before bisulfite deamination or conversion of the DNA samples is to be performed. A 125-ml bottle is filled completely (above the lip taking advantage of surface tension) with dH2O that has been boiling for at least 20 min. Then, screw the air-tight cap on tightly and cool the water overnight on the bench top. Use this dg.dH2O for preparing solutions used for bisulfite deamination or conversion in subsequent steps.

2. Solutions of 3 N NaOH and 100 mM hydroquinone are freshly made immediately before use. Sample denaturation buffer (SDB) is mixed in the ratio of:

<table>
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<tr>
<th>Component</th>
<th>Volume</th>
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<tbody>
<tr>
<td>dg.dH2O</td>
<td>5.8 µl</td>
</tr>
<tr>
<td>3 N NaOH</td>
<td>3.0 µl</td>
</tr>
<tr>
<td>3 mg/ml glycogen (note 8)</td>
<td>0.7 µl</td>
</tr>
<tr>
<td>0.5 M EDTA, pH 8.0</td>
<td>0.5 µl</td>
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3. DNA (1-2 ng) (note 9) is aliquoted and brought up to 20 µl with dg.dH2O and 10 µl SDB are added to denature the DNA. The samples should be incubated at room temperature while subsequent reagents are prepared.

4. Sodium metabisulfite solution (SMBS, ~5 g sodium metabisulfite in 7 ml of dg.dH2O, plus 1 ml 3 N NaOH and 100 µl 100 mM hydroquinone) are prepared, adjusting to pH 5.0 with 3 N NaOH. Pre-warm the SMBS to 50°C (note 10).
5. Samples are denatured for 5 min at 98°C.
6. 200 µl SMBS is then added to each sample, followed by vigorous vortexing and incubation at 50°C for 6 h in the dark (note 11). It is not necessary to overlay the samples with mineral oil.
7. DNA is then desalted with the EZ bisulfite DNA Clean-Up Kit (Zymo Research, cat. D5026) according to the manufacturer’s directions and eluted with 52 µl 0.1X TE, pH 8.0, preheated to 95°C.
8. Desulfonation solution (DSS) is mixed in the ratio of:

| 3 N NaOH | 7.0 µl |
| 3 mg/ml glycogen (note 8) | 1.0 µl |

9. 8 µl DSS is added, and then the samples are vortexed and incubated in a 37°C water bath for 15 min to desulfonate the DNA.
10. 18 µl 10 M NH₄OAc and 200 µl 95% ethanol are then added, followed by incubation overnight at -20°C.
11. Samples are centrifuged at 16,000 g for 20 min at room temperature to pellet the DNA.
12. Pellets are washed once with 400 µl 70% ethanol (absolute ethanol diluted from 95% to 70% using 1X TE, pH 8.0 as diluent). Be careful as the pellets are easily dislodged from the tube.
13. Pellets are air dried and resuspended in 20 µl 0.1X TE, pH 8.0. The deaminated DNA can be stored indefinitely at -20°C.

**PCR Amplification of Deaminated DNA**

After bisulfite treatment, DNA strands are no longer complementary to each other. Hence, deaminated templates must be amplified with strand-specific primer pairs that are designed according to the original guidelines of Frommer et al. (1992). Primer pairs for exponential amplification of the bisulfite-converted top strand are designated a1 and a2, whereas those for the bottom strand are designated b1 and b2. Potential DMTase target sites should be avoided and all other cytosines should be changed to thymine (or guanines changed to adenine) in these primers. Degenerate bases should be incorporated in primers (A and G or C and T, depending on the specific primer) in regions where DMTase sites cannot be avoided (note 12). Also, restriction sites can be added to the 5’ ends of primers to enable subsequent directional cloning of PCR products.

1. 2-4 µl bisulfite-converted DNA is used as template in 50 µl PCR reactions as follows:

| Sigma JumpStart™ PCR buffer | 1X final |
| MgCl₂ | 2.5 mM final |
| dNTPs | 0.2 mM final |
| a1 (or b1) primer | 0.8 µM final |
| a2 (or b2) primer | 0.8 µM final |
| Sigma JumpStart™ Taq DNA polymerase | 1.25 U |
| dH₂O to volume |

2. The PCR amplification parameters are: 1 cycle of denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 30 sec, annealing of primers at 5°C below their
calculated $T_m$ for 30 sec and then extension at 72°C for a time interval equivalent to 60 sec per each kilobase of the PCR product size; ending with 1 final extension cycle at 72°C for 5 min (note 13).

3. PCR products are checked for yield and homogeneity by electrophoresis of 1-5 µl on a 1% agarose-TAE mini-gel containing 0.5 mg/ml ethidium bromide for 30 min at 100 V, and then visualized by UV transillumination. PCR products are purified with Promega Wizard® PCR preps DNA Purification System according to manufacturer’s directions. PCR products are eluted from the minicolumn with 35 µl sterile dH$_2$O and can be stored indefinitely at -20°C.

Cloning and sequencing

1. High-quality PCR products constituting single bands are cloned using standard recombinant DNA procedures (note 14).
2. Chemically-competent *E. coli* (we use DH10B™ cells) are transformed and plated on appropriate selection plates, using blue/white screening to enrich for insert-positive clones.
3. The efficiency of transformation with insert-containing plasmids can be verified by screening 5-10 clones by colony PCR or plasmid isolation followed by digestion with restriction enzymes (note 15).
4. Transformants bearing insert-positive plasmids are sequenced (note 16).

Data analysis and interpretation

1. Sequences obtained from individually cloned molecules are aligned and analyzed with Sequencher 4.2 or comparable alignment software.
2. Depending on which DNA strand was sequenced in the cloned molecule, cytosines are scored as methylated if they present a C (strand with a2 and b2 polarity; cf. Figure 1 of Frommer et al., 1992) or G (strand with a1 and b1 polarity). Conversely, unmethylated cytosines are scored if they sequence as T (a2 and b2 strands) or A (a1 and b1 strands).
3. It is also important to determine the total percentage of C (or G), excluding cytosines present in DMTase target sites. This fraction is indicative of cytosines that failed to convert to uracil during bisulfite treatment. The recommended rate of conversion for all unmethylated cytosines should be in the range of 99.5-99.7% and sequences of molecules with lower rates of conversion (< 98%) should be discarded. Note that cytosines obtained in scored molecules that are not present in the reference sequence can arise from single-nucleotide polymorphisms in the original sample, or, alternatively, from mutations that occur during PCR amplification and subsequent cloning steps. Such cytosines should be omitted from the calculation of conversion frequency.
4. Lastly, only sequences that are distinct in their methylation patterns should be kept for the final analysis. This is because one cannot distinguish if non-distinct molecules are sister molecules that arise during the PCR amplification, i.e., are derived from a common ancestor molecule. The diversity of template methylation patterns can be assessed more accurately if the fragments are labeled with molecular barcodes prior to PCR amplification using specialized techniques (Laird et al., 2004; McCloskey et al., 2007).
5. During data analysis, it should be kept in mind that, as is the case when probing with nucleases, DMTases are also occluded from histone-associated DNA as compared to
freely accessible linker DNA. Therefore, nucleosome footprints are generated when the bound histone complexes protect DNA against methylation. Spans of methylation protection of ~150 bp in size flanked by shorter methylated stretches of accessible DNA indicate individual nucleosome positions. However, nucleosomes tend to unwrap DNA transiently from the histone octamer by a rapid and spontaneous process termed site exposure, especially at the termini where the DNA enters-exits the nucleosome (Polach and Widom, 1995, 1996). Hence, a fraction of individual nucleosomes tend to display shorter or subnucleosomal size footprints with variable extents of methylation at termini that gradually decreases toward the pseudodyad centre. The observation of such subnucleosomal footprints increases in frequency with higher DMTase probe concentration. These products should not be omitted and taken into consideration to provide a detailed picture of chromatin mechanisms.

**Materials & Reagents**

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<th>Material</th>
<th>Composition</th>
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| **5X ISW2 Buffer** | 125 mM NaOH-HEPES, pH 7.8  
85 mM NaCl  
22.5 mM MgCl₂  
0.5 mg/ml bovine serum albumin (BSA) |
| **M.SssI storage buffer** | 10 mM Tris HCl, pH 7.5  
0.1 mM EDTA, pH 8.0  
1 mM DTT  
200 µg/ml BSA  
50% (v/v) glycerol |
| **3 N NaOH** | ~0.4 g in an appropriate volume of dg,dH₂O calculated using the equation: 8.333 X _ g of NaOH = _ ml |
| **100 mM hydroquinone** | ~0.04 g hydroquinone in an appropriate volume of dg,dH₂O calculated using the equation: 90.827 X _ g of HQ = _ ml |

**Author Notes**

1. We have prepared templates ranging from 147-219 bp containing the high-affinity nucleosome positioning sequence 601 that reconstitutes nucleosomes at a single translational position (Lowary and Widom, 1998). Longer templates of 219 bp were used because the ISW2 complex optimally binds nucleosomes with ≥ 70 bp of extranucleosomal DNA.
2. We always titrate the amount of remodeler added to the reactions to detect the range where subtle changes in nucleosome conformation, such as spontaneous site exposure, are detected. We often include a no ATP control as well.
3. Nucleosome remodeling can also be monitored by assaying for increased accessibility of a nucleosomal restriction site to restriction endonucleases.
4. SAM is a critical co-factor of DMTases that is rapidly hydrolyzed. Hence, it is important to freshly dilute SAM immediately prior to use and keep it on ice. We store 32 mM SAM stocks at - 80°C to minimize hydrolysis.
5. Serial dilutions of M.SssI made in the storage buffer will maintain uniform conditions in all the reactions with the only variable being the DMTase concentration. M.CviPI (New England Biolabs or Zymo Research) can also be used as probe.
6. Before proceeding to BGS, efficient methylation can be confirmed by assaying for inhibition of digestion of a methylation-sensitive restriction endonuclease whose site is located in an accessible region in the chromatin substrate.

7. For initial experiments, it is important to titrate the DMTase concentration or perform a time course to find the optimal amount of activity for mapping nucleosome positions. Owing to the inherent site exposure of nucleosomes, high DMTase levels or longer periods of methylation can result in excess methylation, and produce shorter nucleosome footprints. On the other hand, lower levels of DMTase or shorter methylation times can result in poor resolution of individual nucleosome footprints. As it is best to employ conditions that are well beyond single-hit kinetics for MAPit, sequence preferences for the DMTase do not present the same issues as encountered in conventional footprinting studies. However, a naked DNA control should be processed in parallel to establish that there are no sites that are refractory to methylation under the employed conditions.

8. Glycogen can be substituted with similar amounts of carrier DNA (salmon sperm or sonicated and denatured calf thymus DNA). Carrier DNA should not be added if non-specific products are obtained in PCR amplification of bisulfite-converted DNA. Consideration should also be given to the species of origin of the DNA fragment being studied (e.g., do not add carrier calf thymus DNA if the fragment is bovine or from a highly related species).

9. We have found that 1-2 ng purified DNA of fragments 147-219 bp in length is sufficient to achieve optimal conversion of unmethylated cytosine. Adding too much DNA increases the reformation of double-stranded DNA that inhibits conversion of unmethylated cytosine to uracil. In contrast, using too little DNA may cause problems with PCR product yield as some DNA degradation occurs during bisulfite conversion. Degradation occurs in the desulfonation step due to alkali-induced strand scission (i.e., β-elimination) at sites depurinated at the low pH used in bisulfite conversion.

10. We purchase 100 g sodium metabisulfite and aliquot approximately 5-g amounts into 5-g glass scintillation vials in a dH2O- and oxygen-free safety hood. To maintain high conversion efficiencies, the vials are then tightly capped and stored in the dark in a sealed bottle containing Drierite® dessicant to protect against oxidation. If a safety hood is not available, then quickly aliquot the reagent, cap the vials tightly and monitor the deamination efficiency over time of storage.

11. We have observed that 4-6 h of incubation is sufficient to achieve conversion efficiencies of at least 99% for unmethylated cytosine.

12. We do not score methylation status at degenerate sites in the primers as biases in methylation or non-methylation have been observed. This leads to high numbers of residues that are falsely positive or negative for DNA methylation.

13. To reduce stochastic differences that lead to potential amplification biases, we recommend setting up triplicate PCR reactions for each bisulfite-converted sample. Triplicate PCR reactions are pooled prior to PCR product purification and cloning.

14. We either digest the PCR products with the respective restriction enzymes for directional cloning or we use the TOPO™ TA-cloning™ kit (Invitrogen).

15. The frequencies of positive transformants vary from one insert to another. If efficiencies of ≥ 80% positives are obtained after screening a small number of colonies or plasmids, we typically do not screen all colonies for inserts. Instead, we routinely inoculate colonies from plates into liquid broth containing selective antibiotic in 96-well plates, grow the cells overnight at 37°C without shaking, bring to 8% (v/v) glycerol and store at - 80°C until plasmids are to be sequenced.
16. Clones can be sequenced using any available sequencing technology; however, the AT-richness of the templates can lead to problematic reads. Currently, we use TempliPhi™ φ29 DNA polymerase (GE Healthcare), which exponentially amplifies the cloned circular plasmid directly from bacterial cells by rolling-circle amplification. The resulting single-stranded template DNA is subsequently sequenced directly using BigDye (Applied Biosystems). This procedure is advantageous as purification of sequencing-grade plasmid DNA is avoided.

Figures
Figure 1. Overview of the \textit{in vitro} MAPit footprinting of reconstituted nucleosomes before and after remodeling. A protein-free DNA fragment (219 bp) containing a strong nucleosome-positioning sequence at the end (601; Lowary and Widom, 1998) was assembled with purified histone octamer (A) to reconstitute mononucleosomes (B). Reconstituted mononucleosomes were then treated with and without an ATP-dependent remodeling complex in the presence or absence of ATP (C), and subsequently analyzed by native PAGE (D). A gel containing free DNA (lane 1, species 1), reconstituted, end-positioned mononucleosomes (lane 2, species 2) and this same mononucleosome treated with purified ISW2 remodeler complex and ATP (Lane 3, species 3) is shown. In the presence of ATP, purified ISW2 complex catalyzes mobilization of the histone octamer from the end of this fragment to the center (Fitzgerald et al., 2004; Kagalwala et al., 2004; Zofall et al., 2004; Zofall et al., 2006), producing more slowly migrating nucleosomal species 3. After terminating the remodeling reactions, the samples were subjected to MAPit analysis (E), in which samples were: (i) challenged with M.SssI DMTase; (ii) deaminated by bisulfite ion and PCR amplified; and (iii) finally cloned and sequenced. Steps (ii) and (iii) comprise the conventional BGS procedure for mapping m$^{5}$C (Clark et al., 1994; Frommer et al., 1992). In (iii), each line represents a cloned DNA molecule and the positions of unmethylated (open circles) and methylated (filled circles) CG sites as determined by DNA sequencing are indicated. Histone-bound DNA (iii, middle and bottom panels) is relatively inaccessible to DMTases as compared to the free extranucleosomal DNA (iii, top panel), and the methylation patterns readily distinguish between the positions of nucleosomes pre- and post-remodeling. In addition, nucleosomes undergo a spontaneous process called site exposure in which sequences at their ends transiently dissociate from the histone octamer at a rate that is inversely proportional to thermodynamic stability (Polach and Widom, 1995, 1996). Therefore, as ISW2 mobilizes histone octamers from more thermodynamically stable to less stable positions (Whitehouse and Tsukiyama, 2006), increased spontaneous site exposure is observed after remodeling. Thus, MAPit reveals a variable extent of methylation at nucleosome termini that is gradually reduced towards the pseudodyad center on individual chromatin molecules. This yields some footprints shorter than the expected 147 bp length of nucleosomes. SHL0, superhelical location 0, i.e., nucleosome pseudodyad (center); Nuc, reconstituted mononucleosomes.

References


