Chapter 3

Genome-Wide Identification of Open Chromatin in Plants Using MH-Seq

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Abstract

Functional *cis*-regulatory elements (CREs) act as precise transcriptional switches for fine-tuning gene transcription. Identification of CREs is critical for understanding regulatory mechanisms of gene expression associated with various biological processes in eukaryotes. It is well known that CREs reside in open chromatin that exhibits hypersensitivity to enzyme cleavage and physical shearing. Currently, high-throughput methodologies, such as DNase-seq, ATAC-seq, and FAIRE-seq, have been widely applied in mapping open chromatin in various eukaryotic genomes. More recently, differential MNase (micrococcal nuclease) treatment has been successfully employed to map open chromatin in addition to profiling nucleosome landscape in both mammalian and plant species. We have developed a MNase hypersensitivity sequencing (MH-seq) technique in plants. The MH-seq procedure includes plant nuclei fixation and purification, differential treatments of purified nuclei with MNase, specific recovery of MNase-trimmed small DNA fragments within 20~100 bp in length, and MH-seq library construction followed by Illumina sequencing and data analysis. MH-seq has been successfully applied for global identification of open chromatin in both *Arabidopsis thaliana* and maize. It has been proven to be an attractive alternative for profiling open chromatin. Thus, MH-seq is expected to be valuable in probing chromatin accessibility on a genome-wide scale for other plants with sequenced genomes. Moreover, MHS data allow to implement footprinting assays to unveil binding sites of transcription factors.

Key words Chromatin accessibility, Open chromatin, MNase-hypersensitive sites, MH-seq, Plants

1 Introduction

Precise spatiotemporal physical interactions between *cis*-regulatory elements (CREs) and transcription factors (TFs) form the hub of complex transcriptional regulatory networks, which play key roles in fine-tuning gene transcription during normal growth and development and in response to internal and external stimuli in plants [1]. Active CREs reside in open chromatin accessible for recruiting *trans*-factors. Open chromatin is loosely packed and is usually nucleosome-depleted or in low nucleosome occupancy in various eukaryotic genomes [2–4]. Therefore, identification of open

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chromatin is a key step toward deciphering regulatory genomic loci, which lead to deep understanding of transcriptional regulatory mechanisms underlying various biological processes in eukaryotes. The currently available methods for genome-wide mapping of chromatin accessibility can be classified into indirect and direct assays. Enzyme- or chemical-based nucleosome mapping, like MNase-seq [5, 6], MPE-seq [7], and copper ion-mediated Fenton reaction coupled with sequencing [8], can be used to indirectly profile accessible chromatin across the genome. Direct assays include epitope-dependent ChIP-seq assay [9], including TFs, Pol II, silencer, or insulator ChIP-seq [10–13], and a number of antibody-free methods, including DNase-seq [14–16], FAIRE-seq [17], MH-seq [18], ATAC-seq [19, 20], and NOMe-seq [21], have been successfully applied to comprehensively measure chromatin accessibility across various eukaryotic genomes, ranging from yeast and humans to plants.

MNase was first isolated from bacterium *Staphylococcus aureus*, exhibiting endo- and exonuclease activity to naked DNA [22]. At chromatin level, MNase preferentially cleaves linker DNA between neighboring nucleosomes relative to DNA sequences tightly wrapped around nucleosomes. Recovery of MNase-trimmed mononucleosomal DNA fragments (approximately 150 bp) combined with high-throughput sequencing, referred as to MNase-seq, is commonly used for genome-wide mapping of nucleosomes landscape [23–25]. MNase accessibility (MACC) or differential MNase treatment can simultaneously measure nucleosome and accessible chromatin regions in bulk cells in *Drosophila* [5] or a single human cell [26]. Similar to DNase hypersensitive sites (DHSs), chromatin-accessible regions are more sensitive to MNase treatment as compared to regions associated with nucleosomes. A methodology with light MNase cleavage, termed as MNase hypersensitivity sequencing (MH-seq), was successfully developed to specifically identify open chromatin, MNase hypersensitive sites (MHSs), across maize [18] and *Arabidopsis* [27] genomes. In this chapter, we describe a detailed and robust MH-seq methodology for global mapping of open chromatin in plants. The MH-seq procedure consists of nuclei fixation and purification, differential cleavage of purified nuclei with MNase, specific recovery of MNase-trimmed DNA fragments ranging from 20 to 100 bp, MH-seq library preparation coupled with Illumina sequencing, and data analysis for identification of MHSs across the genome. This protocol has been successfully employed in both *Arabidopsis* [27] and maize [18] for mapping functional genomic loci or CREs and can also be adapted for other plant species with a sequenced genome.

2 Materials

2.1 Plants

Plant species with a sequenced genome are suited for MH-seq-based identification of open chromatin. Plants grown to a certain developmental stage under artificially controlled environment or in the field can be used for nuclei preparation for MH-seq experiments (*see* **Note 1**).

2.2 Isolation and Purification of Nuclei

1. Beaker.
2. Scissor.
3. Vacuum pump.
4. Mortar and pestles.
5. 50-mL Corning conical tubes.
6. Centrifuge with swing bucket rotor with cooling system.
7. Miracloth.
8. Funnel.
9. Pre-chilled spatula.
10. Fixation buffer: 20 mM HEPES, 1 mM EDTA, 100 mM NaCl, 1 mM PMSF, and 1% formaldehyde.
11. 2 M glycine.
12. Nuclei isolation buffer (NIB): 10 mM Tris–HCl (pH 8.0), 80-mM KCl, 10 mM EDTA, 1 mM spermidine, 1 mM spermine, 0.5 M sucrose, pH 9.5. Store at 4 °C. Add fresh β-mercaptoethanol to achieve a final concentration of 0.15% before use.
13. Nuclei washing buffer (NWB): NIB containing 0.5% of freshly prepared Triton X 100.
14. MNase digestion buffer (MNB): Mix 1 mL of 50% sucrose, 250 μL of 1 M Tris–HCl (pH 7.5), 20 μL of 1 M $MgCl_2$, 5 μL of 1 M $CaCl_2$; add ddH_2O to make up to 5 mL.

2.3 MNase Digestion and DNA Extraction

1. Micrococcal nuclease (MNase) (NEB, cat # M0247S), RNase A, and proteinase K.
2. Pipettes with various scales.
3. 1.5 mL cap-locked Eppendorf tubes.
4. 37 °C, 65 °C, and 55 °C water bath.
5. Stop solution: 0.5 M EDTA.
6. 5 M NaCl.
7. 20% SDS.
8. 1× TE buffer.
9. Tris-saturated pure phenol solution (pH 8.0) and chloroform.
10. Glycogen, 5 mg/mL stock solution.

11. Sodium acetate (NaOAc, 3 M, pH 5.2).
12. Ethanol (100% and 70%).
13. EB buffer: 10 mM Tris–HCl, pH 8.0.

2.4 Recovery of Small-Sized DNA Fragments

1. Certified low range ultra agarose.
2. 1× TBE buffer: 90 mM Tris-borate, 2 mM EDTA, pH 8.3.
3. GeneRed (TIANGEN, cat # RT211).
4. 100 bp ladder.
5. Electrophoresis apparatus and Gel Doc XR+.
6. Gel knife.
7. QIAquick Gel extraction kit (QIAGEN, cat # 28704).

2.5 MH-seq Library Preparation

1. Thermocycler.
2. 0.2-mL PCR strip tubes.
3. Centrifuge with 24 × 2 mL rotor at RT.
4. Pipettes with various scales.
5. Magnetic stand (Alpaqua, cat # A001219).
6. 0.5 mL/1.5 mL/2 mL cap-locked Eppendorf tubes.
7. Gel knife.
8. Electrophoresis apparatus and Gel Doc XR+.
9. Thermo Labquake™ rotator.
10. Spin-X filter (Sigma, 0.45 μm, cat # CLS8162).
11. 21 gauge needle.
12. NEBNext Ultra II DNA Library Prep Kit for Illumina (NEB, cat # E7645S) including the following components:
    NEBNext Ultra II End Prep Enzyme Mix;
    NEBNext Ultra II End Prep Reaction Buffer;
    NEBNext Ultra II Ligation Master Mix;
    NEBNext Ligation Enhancer;
    NEBNext Ultra II Q5 Master Mix.
13. AMPure XP Beads (Bechman Coulter, A63881).
14. 80% freshly prepared ethanol.
15. 1× TBE: 90 mM Tris-borate, 2 mM EDTA, pH 8.3.
16. EB buffer: 10 mM Tris–HCl, pH 8.0.
17. 50 bp DNA ladder.
18. NEBNext Multiplex Oligos for Illumina (96 Index Primers, NEB, cat # E6609) including the following components:
    NEBNext Adaptor for Illumina;
    USER Enzyme;
    NEBNext Index/Universal Primer Mix Plate.

3 Methods

3.1 Preparation of Purified Nuclei for MNase Digestion

1. Cut the collected fresh tissues (~2 g) into a length of 1–2 cm pieces, and then immerse them in excess fixation buffer under a vacuum for 10 min at room temperature (RT) (*see* **Note 2**).
2. Quench the excessive formaldehyde by adding 2 M glycine to a final concentration of 0.125 M under a vacuum for another 5 min.
3. Discard the liquid, wash the materials three times using sterilized water followed by absorbing residual water with absorbent papers, freeze the materials in liquid nitrogen for 5 min followed by transferring to −80 °C for a long-term storage, or directly use in the next step.
4. Grind the cross-linked tissues into fine powder in liquid nitrogen, and then transfer the ground powder into a 50 mL ice-cold Corning centrifuge tube. Store the powder at −80 °C if not used immediately (*see* **Note 3**).
5. Add an equal volume of ice-cold NIB containing 0.15% fresh β-mercaptoethanol to 5 mL of powder, and completely mix with a chilled spatula.
6. Gently shake the tube on ice for 6 min to make the powder and liquid mixed intensively, and then filter the slurry solution through four-layer Miracloth into a new 50 mL Corning tube.
7. Centrifuge the filtered solution at 1000 × *g* for 10 min at 4 °C, and discard the supernatant as much as possible.
8. Add 10 mL of cold NWB and resuspend the pellets using a soft nylon paintbrush, and then centrifuge at 1000 × *g* for 10 min at 4 °C to pellet the nuclei (*see* **Note 4**).
9. Decant the supernatant, and then repeat **step 8** 2–3 times until the nuclei become white or yellowish.
10. Resuspend the pellet with 5 mL of MNB, and centrifuge at 1000 × *g* for 10 min at 4 °C; purified nuclei can be obtained after removing supernatant.

3.2 MNase Digestion and Purification of Digested DNA Fragments

1. Gently resuspend the purified nuclei in 1.2 mL of cold MNB using a paintbrush.
2. Equally aliquot the suspension into six 1.5 mL prechilled Eppendorf tubes with 200 μl per aliquot; all samples need to be placed on ice.
3. Add various amount of MNase with a specific enzyme unit to each tube; mix well by gently inverting the tubes several times (*see* **Note 5**).
4. Incubate the tubes in a 37 °C water bath for 10 min, and gently mix every 3 min.

5. Stop the reaction by adding 16 μl of 0.5 M EDTA (pH 8.0), mix well by inverting the tubes, and place samples on ice.
6. Equally divide each sample into two parts, and store one cross-linked part at 4 °C overnight; add 16 μL of 5 M NaCl, 8 μL of 20% SDS, and 160 μL of 1 × TE to the second part of each sample, and incubate the mixture at 65 °C overnight for reverse cross-linking.
7. Add 20 μg of RNase A to each tube, and incubate for 30 min in a 37 °C water bath; then add 100 μg of proteinase K, and incubate for 2 h in a 55 °C water bath. Make the total volume in each tube approximately up to 400 μL by adding 1 × TE.
8. Extract DNA using an equal volume of phenol (400 μL), phenol:chloroform mixture (1:1), chloroform, respectively. Vibrate violently, and transfer upper liquid after centrifugation at 15,000 × *g* for 10 min at RT for each time.
9. Pipet the upper supernatant into a new 1.5 mL Eppendorf tube after the last round of extraction; add 20 μg glycogen, 1/10 volume of 3 M NaOAc (pH 5.2), and 2 × volumes of ice-cold 100% ethanol; gently invert for several times; and store at −20 °C for at least 1 h to precipitate DNA.
10. Centrifuge at 15,000 × *g* for 15 min at 4 °C and remove supernatant.
11. Wash the DNA pellet with 500 μL of 70% ethanol, and centrifuge at 15,000 × *g* for another 5 min; decant all residual liquid.
12. Air-dry the DNA for 5 min at RT, and then dissolve the DNA in 20 μL EB.

3.3 Recovery of MNase-Cleaved Small-Sized DNA Fragments

1. Prepare 2% of agarose gel containing GeneRed with 1 × TBE (*see* **Note 6**).
2. Load 10 μL of each reverse cross-linked DNA to each well, use the corresponding cross-linked DNA as a control, and run agarose gel electrophoresis at 100 V for 1 h.
3. Recover DNA fragments <100 bp from MNase-trimmed nuclei under an appropriate MNase unit (*see* Fig. 1).
4. Purify the DNA using a QIAGEN gel extraction kit (QIAGEN, part # 28704), and elute DNA with 30 μL EB.

3.4 MH-seq Library Preparation According to the Manual for the Kit

3.4.1 End Repair and Addition of "A" Base

1. Use 5–20 ng fragmented DNA dissolved in EB (or 1 × TE) as starting material. Add EB to a final volume of 50 μL.
2. Make 60 μL of mix in a PCR tube. Gently pipette up and down for at least 10 times to mix thoroughly (*see* **Note 7**).

   NEBNext Ultra II End Prep Enzyme Mix: 3 μL

   NEBNext Ultra II End Prep Reaction Buffer: 7 μL

   Fragmented DNA: 50 μL

   The total volume: 60 μL

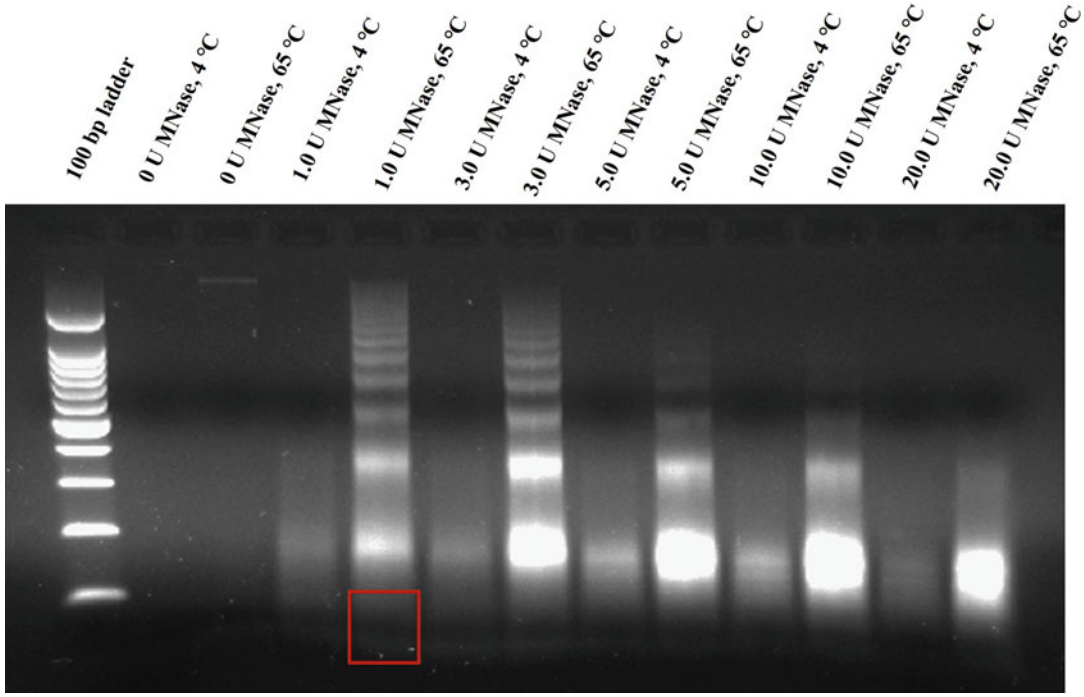

Fig. 1 Agarose gel image showing separation of MNase-trimmed nucleosomal DNA in 2% of agarose gel. Nuclei were purified from formaldehyde-fixed rice seedling tissues and cleaved by various amounts of MNase as indicated on the top. DNA was purified from MNase-trimmed nuclei with (MNase-trimmed fixed nuclei incubated at 65 °C overnight) or without (MNase-trimmed fixed nuclei kept at 4 °C overnight) reverse-cross-linking. The DNA fragments with size less than 100 bp indicated by red box were recovered from fixed nuclei with 1.0 U MNase treatment for MH-seq library preparation. The optimal MNase digestion should be determined by the sequencing data

3. Place the tube containing the mixture in a thermocycler, with the heated lid set to 80 °C. The running program is as follows: 30 min at 20 °C; 30 min at 65 °C; hold at 4 °C.

3.4.2 Adaptor Ligation

1. Add 33.5 μL of the following components to the 60 μL of mixture obtained in the last step. Gently mix well using pipettes (*see* **Note 8**).

   NEBNext Ultra II Ligation Master Mix: 30 μL

   NEBNext Ligation Enhancer: 1 μL

   NEBNext Adaptor for Illumina: 2.5 μL

   The total volume: 93.5 μL

2. Incubate at 20 °C for 15 min in a thermocycler with the heated lid off.
3. Add 3 μL of USER™ Enzyme to the adaptor–ligation mixture; the total volume is 96.5 μL. Gently mix well using pipettes.
4. Incubate at 37 °C for 15 min in a thermocycler with the heated lid set to 50 °C.

3.4.3 Cleanup of Adaptor-Ligated DNA

1. Add 87 μL (0.9×) resuspended AMPure XP beads, which are prewarmed at RT for at least 30 min before use, to 96.5 μL mixture, mix well, and incubate at RT for 10 min.
2. Put the tube on a magnetic stand for 5 min to completely separate the beads from the supernatant. Gently move the tube to converge beads if necessary.
3. Carefully discard the supernatant, wash beads using 200 μL of freshly prepared 80% ethanol for two times, and incubate at RT for 30 s each time. All operations are performed on the magnetic stand.
4. Remove the supernatant as much as possible, air-dry the beads on the magnetic stand until all visible liquid has been evaporated, but do not overdry the beads (*see* **Note 9**).
5. Remove the tube from the magnetic stand. Elute DNA with 15–17 μL EB, pipet up and down to mix well, and incubate at RT for 10 min.
6. Place the tube back on a magnetic stand for 5 min at RT, transfer the supernatant into a new tube after the liquid becomes clear, do not contain the beads in the final solution, and extend the time on the magnetic stand to thoroughly converge beads if necessary.

3.4.4 Enrichment of Adaptor-Ligated DNA by PCR

1. Any adaptor-ligated DNA fragments can be amplified by PCR. PCR cocktail:

   Adaptor-ligated DNA fragments: 15 μL

   NEBNext Ultra II Q5 Master Mix: 25 μL

   Index/universal primer: 10 μL

   The total volume: 50 μL
2. Run PCR program with the following parameters: 98 °C × 30 s; 11 cycles of 98 °C × 10 s, 65 °C × 75 s; 65 °C × 5 min; then hold on at 4 °C (*see* **Note 10**).

3.4.5 Cleanup of PCR Products

1. Prewarm the AMPure XP beads at RT for at least 30 min.
2. Add 45 μL (0.9×) resuspended beads to the PCR products. Mix well and incubate at RT for 10 min, and then place the tube on a magnetic stand for 5 min to converge beads.
3. Carefully decant the supernatant without disturbing the beads.
4. Wash the beads for two times using 200 μL freshly prepared 80% ethanol, incubate at RT for 30 s each time, and remove all the residual liquid.
5. Air-dry the beads on the magnetic stand until all visible liquid has been evaporated, but do not overdry the beads.

6. Remove the tube from the magnetic stand. Elute DNA with 15–17 μL EB, mix well, and incubate at RT for 10 min.
7. Place the tube back on the magnetic stand for 5 min, and transfer the supernatant into a new liquid. The final liquid can be stored at −20 °C for later use (*see* **Note 11**).

3.4.6 Purification of Bead-Purified PCR Products Using Polyacrylamide Gel Electrophoresis (PAGE) (See ***Note 12***)

1. Prepare 15% TBE polyacrylamide gel (PAGE); make sure it is fully solidified before use.
2. Premix the 16 μL of PCR products with 4 μL 5 × loading buffer. Load the mix into one well of PAGE gel; add 1 μL of 50 bp DNA marker to another single well as control.
3. Run the PAGE gel at 100 V for about 3 h in 1 × TBE buffer.
4. Stain the gel in a petri dish (150 × 15 mm) containing 5 mL of sterile water or 1 × TE plus 10 μL of GeneRed (TIANGEN). Observe the gel under the UV light.
5. Cut the DNA band with size ranging from 150 bp to 250 bp with a clean gel knife (*see* Fig. 2); place the gel pieces into a 0.5 mL Eppendorf tube with bottom punched by a 21 gauge needle.

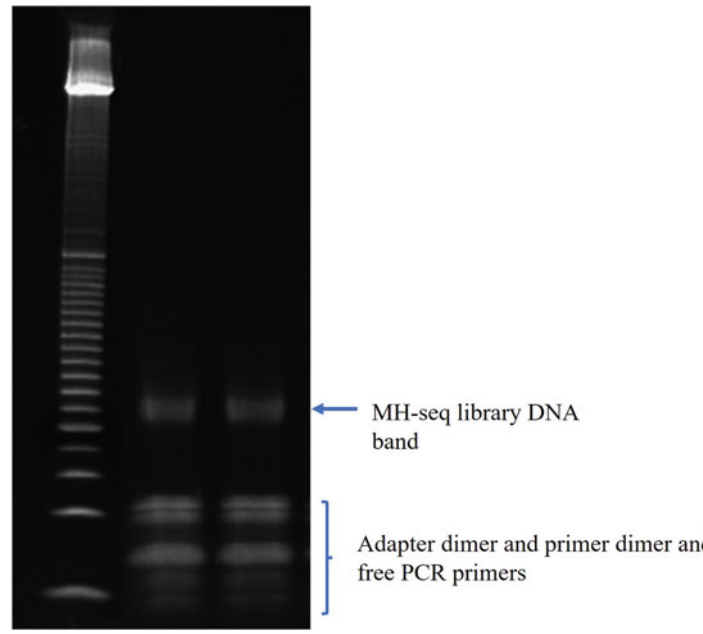

Fig. 2 PAGE image showing the final PCR enrichment of MH-seq library. Approximately 25–30 μl of the PCR product with loading dye were loaded in each lane. The DNA band of interest with sizes ranging from 150 to 220 bp is indicated by the blue arrow. The single bracket indicates the contamination of adapter and PCR primer dimer and free PCR primers that need to be removed after purification

6. Put the 0.5 mL tube into a 2 mL Eppendorf collection tube with round bottom, and centrifuge the tube at 15,000 × *g* for 5 min at RT to crash the gel through the hole.
7. Add 400 μL of 1 × TE buffer into the 2 mL tube, and elute the DNA by gently rotating the tube using Thermo Labquake™ rotator at RT for 2 h.
8. Transfer the crashed gel mixture to the column of the Spin-X filter (Sigma, 0.45 μm) using a cut pipet tip. Spin at 15,000 × *g* for 3–5 min, and transfer the filter liquid into a new 1.5 mL Eppendorf tube.
9. Repeat the same procedures as **steps 8–11** in Subheading 3.2 to precipitate the DNA.
10. Air-dry the DNA for 5 min at RT, and then add 15–17 μL of EB to dissolve DNA for 5–10 min.
11. Rerun 1–2 μL of recovered DNA on 15% TBE PAGE gel to verify the quality of the MH-seq library (*see* Fig. 3).
12. Quality control and estimate the concentration of MH-seq library by using BioAnalyzer before performing Illumina sequencing (*see* Fig. 4).

3.4.7 Bioinformatics Analysis of MH-seq Datasets

The raw sequencing reads are trimmed using Cutadapt [28] and are mapped to the *Arabidopsis* genome (TAIR 10) and maize genome (B73_RefGen_v4) by Bowtie 2 [29], respectively. MACS2 [30] with default parameters is used to call peaks for identification of MHSs across the whole genome. IGV [31] is used for displaying MHS peaks, and the profiles of open chromatin could be plotted by R. More details for MH-seq data analysis can be found in previous publications [27].

3.4.8 MHS-Related Footprinting Assay

FIMO from meme suite [32] is used to search motifs on a genome-wide scale, which are located within binding sites of transcription factors (TFs). TF data are derived from the current database of plant transcription factors and newly generated transcription factor-related ChIP-seq/DAP-seq datasets. All TF-binding motifs identified within DHSs or MHSs are used for downstream footprinting assays. The motif center is used as a reference point; the average DNase or MNase cut frequencies with 5 bp windows are computed around the reference point. The conspicuous dip corresponding to the motif center represents the presence of footprinting (*see* Fig. 5), which is caused by the protection of TF protein binding, therefore resulting in less DNase or MNase cuts occurred relative to immediately flanking regions.

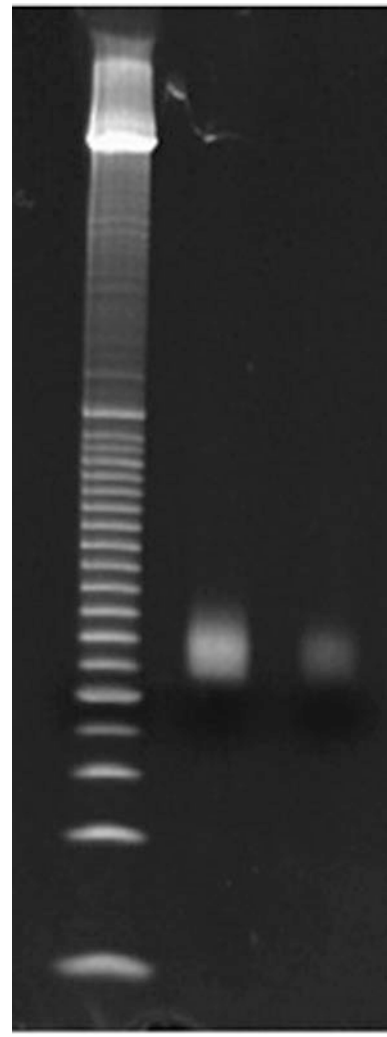

Fig. 3 Confirmation of purified MH-seq library using 1-μl re-running 15% PAGE. Only a single DNA band with the expected sizes, ranging from 150 to 200 bp, is observed in the gel

4 Notes

1. This protocol has been successfully applied in model plant species, *A. thaliana* and maize. It should be applicable to all plant varieties with sequenced genomes.
2. Formaldehyde can create covalent bonds between DNA and proteins to stabilize their interactions in vivo. The final concentration of formaldehyde and incubation time need to be adjusted according to species and tissue types. Excessive cross-linking will impact solubility of protein–DNA complexes; it is better to control the cross-linking time under vacuum within 10 min.

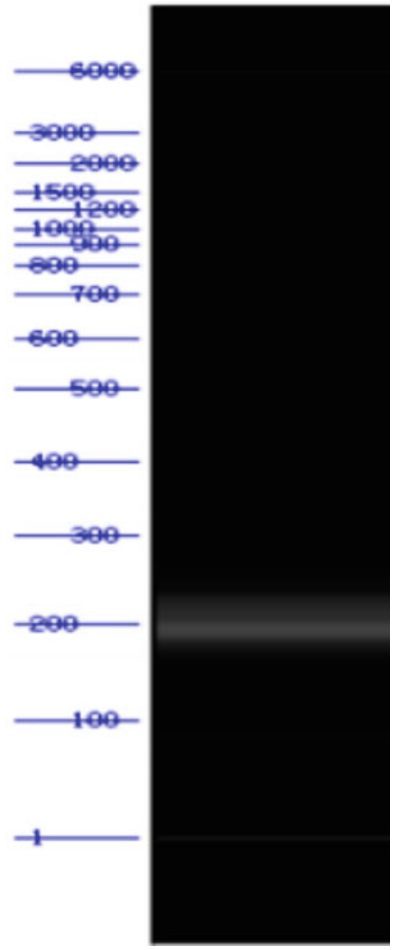

Fig. 4 A BioAnalyzer gel image showing the quality of MH-seq library. An ideal MH-seq library with sizes ranging from 170 to 200 bp, reflecting inserted DNA fragments with sizes ranging from 50 to 100 bp

3. Young tissues from plant seedlings can result in a higher yield of nuclei. Fresh plant tissues should be ground into powder as fine as possible in liquid nitrogen. Keep the powder frozen before adding ice-cold NIB.
4. Triton X-100 (0.5% final concentration) is used to remove chloroplast and mitochondria contamination by breaking the membrane. The concentration of Triton X-100 needs to be adjusted to achieve the best results. The nuclei should become yellowish or white after washing for 3 times; otherwise, the concentration of Triton X-100 needs to be increased (about 0.8–1%) to limit the wash time within three.
5. The extent of MNase digestion can be determined by digestion time and enzyme concentration. The concentration of MNase used in experiments would depend on the number of nuclei

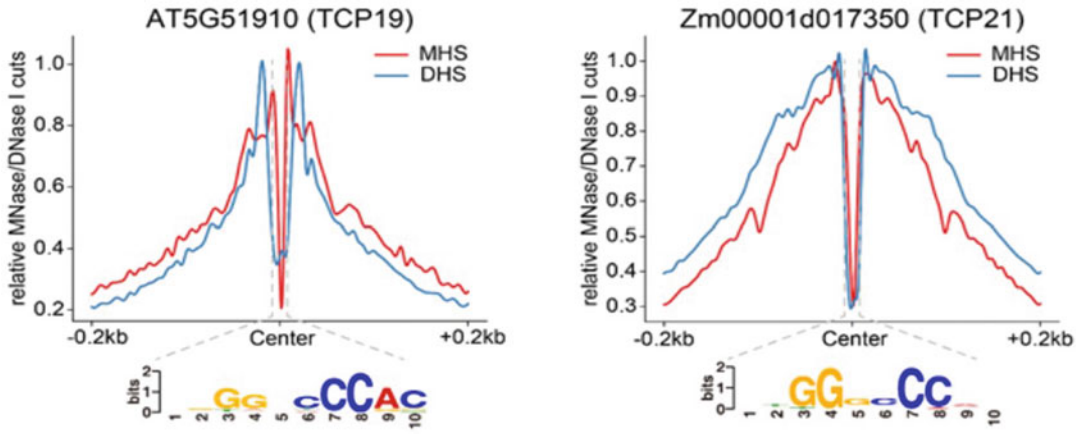

Fig. 5 Curve plot showing the presence of MHS/DHS-related footprint corresponding to the motif of transcription factors, TCP19 and TCP21. The binding motif is predicted using 50 bp DNA sequences in the MHS/DHS peak center. MNase/DNase cutting sites are calculated using MHS data with a bin window as 5 bp

and varies among different tissues or species. To make the operation easier and time saving, we recommend to test a series of enzyme concentrations with a fixed digestion time, by which at least one sample can be recovered for MH-seq.

6. Make the agarose powder fully dissolved in 1 × TBE solution; the final gel with a thickness of 0.5 cm is recommended. The resolution of the DNA band may be affected if the gel is too thick.
7. Gently pipet up and down for at least 10 times to mix well when preparing reaction solution during library construction; try to avoid foam and bubbles.
8. The adaptor needs to be diluted (by mix of 10 mM Tris–HCl, 10 mM NaCl, pH 8.0) if the sample input is <10 ng; the dilution ratio is typically 1:10 or 1:25 when the input is between 5 ng and 100 ng or less than 5 ng, respectively. Excess adaptor should be removed before PCR reaction.
9. AMPure XP Beads should be prewarmed at RT for at least 30 min before use. The orientation of tubes placing on a magnetic stand can be switched to adequately converge the beads. Overdried beads will result in lower recovery of DNA; thus it is necessary to elute DNA timely when the beads are still dark grown but all external liquid evaporates.
10. This step is for enrichment of adaptor-ligated DNA. The number of PCR cycles is determined based on the amount of starting DNA; it should be high enough to generate sufficient DNA fragments, but avoid over-amplification; the cycle number is always set between 3 and 15 cycles.

11. For more details for library preparation for Illumina, please refer to the manual of NEBNext Ultra II DNA Library Prep Kit for Illumina.
12. This step is optional. It can be skipped if the final MH-seq library lacks adaptor–dimer contamination.

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