***A. paniculata* Histone ChIP-seq protocol**

(by Ling et al.)

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**Required reagents and equipment**

**[1] Required reagents**

1. Sucrose, 2.5 M Glycine, 10 % SDS, 4 M NaCl, 1 M MgCl2, 1 M KCl, 1 M LiCl, Formaldehyde (37 %), 80 % Ethanol, 20 % Triton X-100, RNase, 40 µM cell filter; (All these reagents are purchased from Sangon)

2. Phenylmethanesulfonyl fluoride (PMSF, 200 mM) (Sangon CatNo. MFCD00007424);

3. Protease Inhibitor Cocktail EDTA Free (PI, 50X) (Roche, CatNo.11873580001);

4. 1 M Ultrapure Tris-HCl (pH 8.0) (Invitrogen CatNo.15568025);

5. 0.5 M Ultrapure EDTA (pH 8.0) (Invitrogen CatNo.15575020);

6. Maximum recovery tube (1.5 mL) (Axygen, CatNo.MCT-150-L-C);

7. Real-time tubes (Axygen);

8. Zymo DNA Clean & Concentrator-5 Kit (Zymo, CatNo. D4003);

9. Q5 High-Fidelity DNA Polymerase (NEB, CatNo.M0491S);

10. Eva Green (20X) (Biotium, CatNo.31000);

11. AmPure XP beads (BECKMAN COULTER, CatNo.A63881);

12. Qubit dsDNA HS Assay Kit (Thermo, CatNo.Q32854);

13. Antibody strains Invitrogen Protein A/G Dynabeads, CatNo.10003D and 10001D

14. Corresponding Histone antibodies (ChIP Grade)

15. Proteinase K (NEB CatNo. P8107S);

16. Tn5 Kit (Vazyme, CatNo.TD501)

17. Sequencing Primers (N7XX and N5XX): (Vazyme, CatNo. TD202)

**[2] Equipments**

1. Covaris series (e.g. Covaris M220) or Bioruptor;

2. Magnetic stand for 1.5mL; Magnetic stand for 0.2mL;

3. PCR instruments/real-time instruments;

4. Metal bath;

5. Qubit Fluorimeter;

6. 1.5/15 mL Rotator;

7. Centrifuge (can use 4°C for 15 mL as well as 1.5 mL tube).

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**Step by Step Protocol**

**Step 1. Sample Crosslinking**

(1) Cut plant material into 2-5 mm strips (0.1-0.2 g per sample), transfer to a 50 mL tube and add 36 mL of pre-cooled 10 mM Tris-HCl Buffer (pH 8.0);

(2) In hood, add 1 mL of 37 % formaldehyde (final concentration 1 %), Vacuum infiltrate (85 kPa) for 5 min for fixation; Repeat the infiltration for 3-4 times.

(3) Add 2 mL of 2.5 M glycine and vacuum infiltrate (85 kPa) for 5 min to quench the formaldehyde.

(4) Filter out all the fixation buffer and rinse the cross-linked leaves with 40 mL ddH2O twice.

(5) Remove excess water by gently pressing cross-linked leaves with tissue paper, freeze the samples in liquid nitrogen and store at -80 °C, or proceed directly to the next step.

**Step 2. Nuclei Extraction**

(1) Prepare the following buffer and place on ice:

**Nuclei Extraction Buffer I** (approximately 15 mL per sample): 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.25 M Sucrose, 10 mM KCl, 40 mM NaCl, 0.1% SDS, 1 % Triton-x100, 1 mM PMSF, 1 x PI;

**Nuclei Extraction Buffer II** (approximately 2 mL per sample): 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.1% SDS, 1 % Triton-x100, 1 mM PMSF, 1 x PI;

**Nuclei Lysis Buffer** (approximately 500 µL per sample): 50 mM Tris-HCl, pH 8.0, 5 mM EDTA, 0.5% SDS, 1 mM PMSF, 1 x PI;

**Note:** PMSF and PI should be freshly added.

(2) Grind the plant samples in liquid nitrogen to a powder, transfer about 0.05-0.1 g of the material to 10 mL of Nuclei Extraction Buffer I in a 15 mL centrifuge tube.

(3) Stir by spoon (or shaker) to help the powder to resuspend.

(4) Filter the solution by a 40 µm filter and transfer the filtrate to a new 15 mL tube.

(5) Centrifuge at 6000 g for 5 min at 4 °C.

(6) Add 1 mL of Nuclei Extraction Buffer I to resuspend the precipitate, transfer to a 1.5 mL maximum recovery tube, centrifuge at 6000 g for 5 min at 4 °C; Repeat this step 3-5 times until supernatant becomes almost colourless.

(7) Add 1 mL of Nuclei Extraction Buffer II, resuspend the precipitate, and transfer to a 1.5 mL maximum recovery tube and centrifuge at 6000 g for 5 min at 4 °C. Repeat this step one more times.

(8) Remove the Nuclei Extraction Buffer II and the nuclei pellet can be stored at -80 °C or proceed directly to the next step.

**Step 3. Chromatin Sonication**

(1) Add 300 µL of Nuclei Lysis Buffer to suspend the nuclei pellet from the last step.

(2) Save about 15 µL as a control without sonication.

(3) Sonicate the chromatin to 500-1000 bp size (with major peak located 750 bp) using the Covias or Bioruptor according to the manual instructions.

(4) Centrifuge the sonicated chromatin at 12000 g for 5 min at 4 °C,

(5) Transfer **supernatant** to a new 1.5 ml tube and keep on ice.

**Step 4. Chromatin Immunoprecipitation (ChIP)**

(1) Prepare the following Buffer:

**Binding Buffer**: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 100 mM NaCl, 0.1 % SDS, 1 % Triton-X100, 1 mM PMSF, 1 x PI; (ready to use, 20 mL)

Note: PMSF and PI should be freshly added.

(2) Aliquot 25 µL of the corresponding Dynabead Protein A (or Protein G) beads, into a new 1.5 mL maximum recovery tube.

(3) Place the tube on a magnetic stand, wait until solution becomes clear and discard solution. Then, add 1 mL of Binding buffer to resuspend the beads. Repeat this step 2 times.

(4) Resuspend the magnetic beads by adding 750 µL of Binding buffer, Aliquot 150 µL to a new 1.5 mL tube, and keep the remaining 600 µL on ice.

(5) Transfer the supernatant (approximately 300 µL) to the tube containing 150 µL of washed beads, add 450 µL of Binding buffer to make up to approximately 900 µL, and spin at 4°C for 30 – 60 min to remove the non-specifically bound chromatin on the Dynabeads.

(6) Place the tube on a magnetic stand, wait until solution becomes clear (DO NOT discard the supernatant.) and save about 50 µL this pre-cleared chromatin to a new tube as **INPUT** control which can be store in -20 °C.

(7) Transfer the remaining supernatant (the pre-cleared chromatin) into the 600 µL Binding buffer containing the washed Dynabeads from Step (5) (approximately 1.5 mL).

(8) Add 2 µg of histone antibody (ChIP grade, in this study the anti-H3K27me3 antibodies from Diagenode [Cat.no. C15410195] was used).

(9) Rotate the tube overnight with a rotator (18-60 rpm) at 4 °C cold room. Or, overnight with 80 rpm shaking.

**Step 5 On-beads Tagmentation**

**Note: In this step, we used Tn5 for ChIP-seq library construction instead of the traditional ChIP-seq library construction protocol following end-repairing, dA-tailing and Y-shaped adaptor ligation.**

(1) (Second Day) Prepare the following Buffer:

**Low Salt buffe**r: 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 150 mM NaCl, 0.1 % SDS, 1 % Triton-X100, 10 mM PMSF, 1 X PI.

**High Salt buffer 1:** 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 300 mM NaCl, 0.1 % SDS, 1 % Triton-X100, 10 mM PMSF, 1 X PI.

**High Salt buffer 2:** 10 mM Tris-HCl pH 8.0, 1 mM EDTA, 250 mM LiCl, 0.1 % SDS, 1 % Triton-X100, 10 mM PMSF, 1 X PI.

**Tris Wash buffer:** 10 mM Tris-HCl pH 8.0.

(2) Place the tube on a magnetic stand. Wait until solution becomes clear and discard solution. Add 1 mL Low Salt buffer to resuspend the magnetic beads; Rotate the tube at 4 °C for 3 min. Repeat this step.

(3) Place the tube on a magnetic stand. Wait until solution becomes clear and discard solution. Add 1 mL High Salt buffer 1 to resuspend the magnetic beads; Rotate the tube at 4 °C for 3 min.

(4) Repeat step (2)-(3) with (i) High Salt Wash 2 and (ii) Tris Wash buffer

(5) Add 150 µL Tris Wash buffer to resuspend the Dynabeads and transfer to a 0.2 mL PCR tube. Keep on ice;

(6) Prepare Tn5 reaction mixture according to the instruction of Vazyme TD501 kit, 50 µL per tube.

(7) Place the PCR tube on a magnetic stand, discard the Tris buffer.

(8) Add 50 µL of Tn5 reaction mixture, resuspend the beads and place in a 37 °C chamber and rotate for 30 min.

(9) Place the tubes on a magnetic stand, discard the Tris buffer.

(10) Washed the beads by 150 µL(i) Low Salt buffer, (ii) Hight Salt buffer (iii) Tris buffer, respectively.

(11) Resuspend the Dynabeads by 150 µL Tris buffer, keep on ice;

**Step 6 DNA recovery and amplification**

(A) ChIPed DNA reverse crosslinking

(1) Prepare the reverse crosslinking buffer (**RC buffer**): 10 mM Tris-HCl (pH 8.0),2 mM EDTA (pH 8.0), 0.2% SDS, 300 mM NaCl;

(2) Place the tube from the previous step on a magnetic stand and discard the Tris buffer;

(3) Add 70 µL of RC buffer to resuspend the Dynabeads and transfer to a new 0.2 mL PCR tube.

(4) Add 1 µL RNase and incubate at 37 °C for 30 min.

(5) Add 1 µL Proteinase K; incubate at 55 °C for 1 h, then hold at 63 °C for 8 h.

(6) Place PCR tubes on a magnetic stand and transfer the supernatant (~ 70 µL) to a new 0.2 mL PCR tube.

(7) Add another 30 µL of RC buffer to resuspend the beads

(8) Place the tube on a magnetic stand, transfer the supernatant, and combine it into step (6) (in total 100 µL).

(9) Purify ChIPed DNA according to the Zymo kit instructions (Cat No. D4003); DNA is eluted with 50 µL ddH2O.

(B) **INPUT** DNA reverse crosslinking

(1) Transfer 10 µL INPUT DNA into a 0.2 mL PCR tube;

(2) Add 60 µL RC buffer and 1 µL RNase; Incubate the tube at 37 °C for 30 min.

(3) Add 1 µL Proteinase K; incubate at 55 °C for 1 h, then hold at 63 °C for 8 h.

(4) Purify the INPUT DNA according to the Zymo kit instructions (Cat No. D4003); DNA is eluted with 20 µL ddH2O.

(5) Measure the eluted DNA concentration by Qubit.

(6) Use 50 ng DNA for Tn5 tagmentation according to the manual instruction.

(7) Purify tagged DNA according to the Zymo kit instructions. DNA is eluted with 50 µL ddH2O.

(8) Measure the eluted DNA concentration by Qubit.

(C) PCR amplification

 (1) Prepare the PCR reaction mixture as follows:

|  |  |  |
| --- | --- | --- |
| Component | Stock Concentration  | Volume (μL) |
| 5 X Q5 buffer (NEB) | 5 X | 10 |
| dNTP | 10 mM  | 1.5 |
| Q5 enzyme (NEB.cat.no. M0491S) | - | 0.5  |
| ChIPed/INPUT DNA | - | 50 μL ChIPed DNA/1-5 ng INPUT DNA  |
| Primer 1, N5XX index | 10 µM | 1.5 |
| Primer 2, N7XX index | 10 µM | 1.5 |
| ddH2O | - | Up to 65  |

(2) Perform initial PCR:

(a) Pre-PCR: incubate at 72 oC for 5 min.

(b) Initial denaturation: 98 oC for 15 sec.

(c) Pre-amplification for 5 cycles: 98 oC for 15 s, 63 oC for 20 s, 72 oC for 20 s.

(d) Hold at 4 oC.

(e) Place the PCR tube on ice.

Note: For INPUT DNA, 5 cycles initial amplification is sufficient to obtain enough DNA ( > 30 ng) for sequencing.

(3) Take 19 μL of the initial PCR mix (ChIPed DNA), add 1 μL Eva Green dye (20X) (Biotium) and then quantify on a Bio-Rad real-time PCR system to estimate the number of cycles (X) required for amplification.

(4) Perform final PCR of the remaining 46 μL reaction:

(a) Hold at 98 oC for 30 s.

(b) Amplification for X+3 cycles: 98 oC for 15 s, 63 oC for 20 s, 72 oC for 20 s.

(c) Hold at 4 oC.

(5) Library cleanup is performed using Beckman AMPure XP beads (cat.no. A63880) according to the manual instructions.

(6) Elute the library DNA in 50 μL TSE buffer (10 mM Tris-HCl pH 8.0, 0.1 mM EDTA pH 8.0).

(7) Measure the library concentration using Qubit.

(8) Run 50 ng of library DNA on 1 % agarose gel to check the library size.

(9) Store at -20 oC or send for sequencing.