Chapter 16

Chromatin Immunoprecipitation of Meiotically Expressed Proteins from *Arabidopsis thaliana* Flowers

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Abstract

During meiosis recombination occurs between homologous chromosomes which can result in reciprocal exchanges of genetic information, called crossovers. Crossover rate is heterogeneous within the genome, with local regions having a significantly higher recombination rate relative to the genome average. These regions are termed hotspots and typically occur with widths of kilobases. Therefore, there is a need to profile recombination factors at a similar resolution during meiosis via techniques such as chromatin immunoprecipitation (ChIP). Here we describe a ChIP protocol, combined with high throughput sequencing (ChIP-seq) optimised for analysis of meiotically expressed proteins in *Arabidopsis thaliana* flowers. We provide methods to (1) isolate nuclei and prepare the chromatin for shearing, (2) immunoprecipitate DNA molecules cross-linked to a protein of interest, (3) to size-select and purify immunoprecipitated DNA molecules, and (4) to prepare DNA sequencing libraries suitable for high-throughput sequencing. Together, these methods allow the detection of binding sites for meiotic proteins in the Arabidopsis genome at high resolution, which will provide insights into relationships between meiotic chromosome organization, chromatin and recombination.

Key words Meiosis, Chromatin immunoprecipitation, Chromosome axis, Recombination, Arabidopsis

1 Introduction

Meiotic recombination initiates with the formation of DNA double strand breaks (DSBs) generated by SPO11 and accessory proteins [1, 2]. DSBs occur in the context of chromatin loops tethered to a meiotic chromosome axis, which promotes inter-homolog recombination and crossover formation [3, 4]. Meiotic recombination can result in allele reshuffling via crossovers and this contributes to genetic diversity [5]. Analysis of crossover frequency in plant genomes has revealed that recombination rate is heterogeneous, with narrow 1–2 kilobase (kb) hot spots exhibiting higher crossover frequency relative to the genome average [6–9]. However, the

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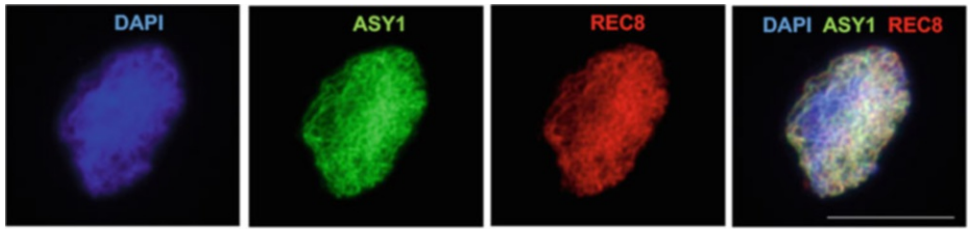

Fig. 1 Immunostaining of ASY1 and REC8 components of the chromosome axis in Arabidopsis. Immunostaining of ASY1 (green) and REC8 (red) on a pollen mother cell at leptotene stage. The chromatin is stained with DAPI (blue). Scale bar = 10 μM

genetic and epigenetic features that determine hotspot locations and activity remain to be fully understood.

Meiotic chromosomes are associated with a proteinaceous structure called the chromosome axis [10]. Several chromosome axis proteins have been identified in plants, including HORMA-domain containing proteins ASYNAPTIC 1 (ASY1)/HOMOLOGOUS PAIRING ABERRATION 2 (PAIR2) [11, 12], coiled-coil proteins ASYNAPTIC3 (ASY3)/PAIR3 [13, 14] and ASYNATPTIC4 (ASY4) [15, 16] and the cohesin subunits REC8/Absence of First Division 1 (AFD1) [17, 18] and STRUCTURAL MAINTENANCE OF CHROMOSOME 3 (SMC3) [19] (Fig. 1). Meiotic recombination occurs in association with the chromosome axis, and axis proteins are required to stabilize the interhomolog strand invasion protein DNA MEIOTIC RECOMBINASE 1 (DMC1) on chromatin and promote joint molecule formation between chromosomes [11–20]. In budding yeast [21] and *Caenorhabditis elegans* [22], meiotic axis proteins also play an important role in mediating crossover interference, which causes crossovers to be more widely spaced than expected at random, and their positioning along chromosomes.

The use of cytogenetic, molecular genetic, and biochemical approaches to study meiosis has helped to identify numerous proteins involved in the recombination pathways [23]. In addition, cytogenetic methods are useful to study meiotic progression, for example a recent study revealed that the chromosome axis remodels concomitantly with progression of meiosis [24]. However, these techniques have limited spatial resolution and are not suitable to determine the DNA binding sites of recombination factors and of chromosome axis components at the fine scale. One approach to study protein localization at the fine scale is chromatin immunoprecipitation sequencing (ChIP-seq) (Fig. 2) [25]. This technique detects the binding site of proteins with a resolution of ~200–400 bp [26]. The success of this experimental approach depends on the availability of an antibody with high affinity and specificity that can be used to "pull down" formaldehyde cross-linked protein–DNA complexes [26]. It is also possible to generate

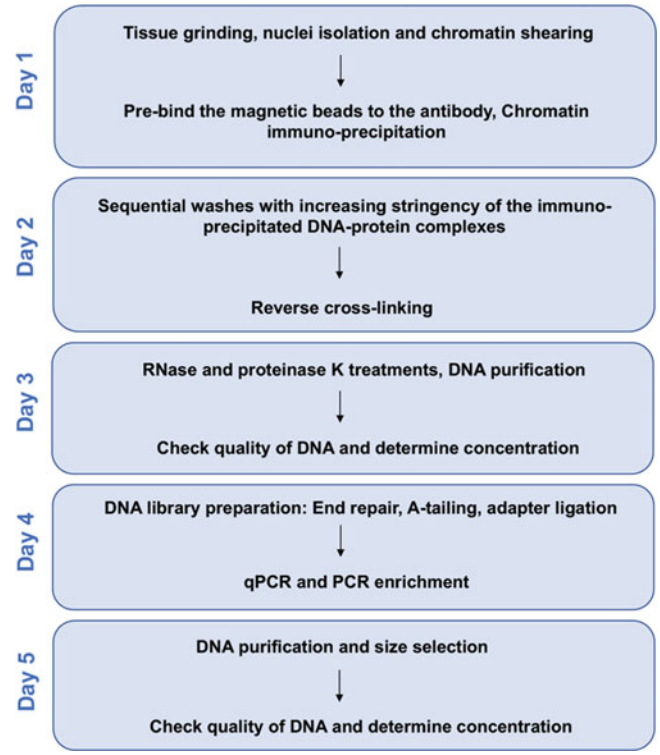

Fig. 2 Schematic representation of ChIP-seq procedure

tagged transgenic lines expressing a protein of interest translationally fused with an epitope. Several commercial antibodies are available to detect these epitopes with high specificity and which have been proven to work for ChIP-seq [25, 26]. However, it can be challenging to generate stable transgenic lines with a functional epitope-tagged protein that complements the mutant phenotypes. As an alternative, it is possible to raise an antibody against a recombinant protein of interest, or against synthesized peptides, in order to immunoprecipitate factors of interest.

Here we report detailed methodologies for ChIP-seq of meiotic proteins in Arabidopsis, which can be used to study the genome-wide profile of chromosome axis and recombination proteins. Arabidopsis flowers contain a larger number of male germ cells and comparatively few female germ cells [27]. We optimised the method to precipitate meiotic proteins from floral buds at stage 9 of flower development, which corresponds predominantly to male meiosis [28]. We describe a procedure for nuclei isolation, chromatin separation and shearing, chromatin immunoprecipitation, and DNA recovery. We also describe a method for DNA library preparation suitable for next-generation sequencing. Finally, we provide information on critical steps including chromatin preparation, PCR enrichment, and DNA size selection during the DNA library preparation.

2 Materials

2.1 Plant Growth and Tissue Collection

1. *Arabidopsis thaliana* plants are grown in controlled growth rooms under long day conditions (16 h light–8 h dark at 150 μmol light intensity) at 20 °C.

2.2 Nuclei Isolation and Chromatin Preparation

All the buffers are prepared fresh on the day of the ChIP experiment.

1. Nuclei isolation buffer: 1 M sucrose, 5 mM KCl, 5 mM $MgCl_2$, 60 mM Hepes pH 8.0 (pH adjusted with NaOH), 5 mM EDTA, 0.6% Triton X-100, 0.4 mM PMSF, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
2. EB2 buffer: 10 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 10 mM $MgCl_2$, 0.25 M sucrose, 1% Triton X-100, 1 mM EDTA, 5 mM 2-Mercaptoethanol, 0.1 mM PMSF, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
3. Nuclei lysis buffer: 50 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 10 mM EDTA, 1% SDS, 0.1 mM PMSF, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
4. ChIP dilution buffer: 20 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 167 mM NaCl, 1.1 mM EDTA, 1.1% Triton X-100, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).

2.3 Chromatin Immunoprecipitation

All the buffers are prepared fresh on the day of the ChIP experiment.

1. ChIP dilution buffer: 20 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 167 mM NaCl, 1.1 mM EDTA, 1.1% Triton X-100, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
2. Low-salt buffer: 150 mM NaCl, 20 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
3. High-salt buffer: 500 mM NaCl, 20 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 2 mM EDTA, 1% Triton X-100, 0.1% SDS, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution).
4. TE: 10 mM Tris–HCl pH 8.0 (pH adjusted with HCl), 1 mM EDTA.
5. Elution buffer: 100 mM $NaHCO_3$, 1% SDS.

3 Methods

3.1 Tissue Collection and Cross-Linking

1. Collect unopened Arabidopsis floral buds at stage 9 of flower development [26] from 5–6 week old plants in 2 ml SuperLock microcentrifuge tubes. It is recommended to keep the tube on ice during tissue collection. Once full, the tubes (a 2 ml Eppendorf tube full of floral buds weighs ~500 mg) are immediately frozen in liquid nitrogen and stored at −80 °C.
2. On the day of performing ChIP-seq: transfer 1 g of buds (corresponding to two 2 ml Eppendorf tubes of floral buds from **step 1**) from −80 °C to a mortar containing liquid nitrogen. Grind the buds to a fine powder in liquid nitrogen using a pestle. When all liquid nitrogen is evaporated, add more liquid nitrogen in the mortar and repeat the grinding step 2–3 more times until the buds are ground to a very fine powder.
3. Transfer the ground floral powder to an ice-cold mortar and add 25 ml of nuclei isolation buffer. Resuspend the powder in solution using a pestle.
4. Repeat **step 2** to grind an additional gram of buds in a mortar with liquid nitrogen. Add the ground power to the previous homogenate and resuspend the solution with a pestle.
5. Transfer the homogenate to a 50 ml conical tube and add 680 μl of 37% formaldehyde solution. The final ratio of formaldehyde should be 1% by volume.
6. Incubate the conical tube on a roller mixer at 12 rpm at room temperature for 25 min (*see* **Note 1**).
7. Stop the cross-linking reaction by adding 1.7 ml of 2 M glycine to a final concentration of 130 mM and incubate the solution on a tube roller mixer at 12 rpm and room temperature for 25 min.
8. Keep the solution on ice until all the samples are processed. Between 5 and 10 g of buds is usually needed to perform one ChIP experiment and **steps 2**–**7** are repeated until all the tissue material has been processed. The amount of starting material depends on the abundance of the protein on the chromatin. For example, axis proteins are abundant and 6 g of floral buds is usually enough for one experiment. In contrast, recombination proteins may be less abundant and 10 g of floral buds is recommended for one experiment.

3.2 Nuclei Isolation and Chromatin Preparation

During the procedure keep the samples on ice.

1. Filter the homogenate through one layer of miracloth into a 50 ml conical tube. Repeat the filtration once more with a fresh miracloth.

2. Centrifuge at 3000 × *g* for 20 min at 4 °C.
3. Discard the supernatant and resuspend the pellet in 1 ml of EB2 solution (*see* **Note 2**). Transfer the homogenate to a 1.5 ml Clear-view Snap-Cap low-retention microtube.
4. Centrifuge at 12,000 × *g* for 10 min at 4 °C.
5. Discard the supernatant (*see* **Note 3**). Resuspend the pellet in 600 µl of Nuclei Lysis Buffer and divide the sample into two new 1.5 ml Clear-view Snap-Cap low-retention microtube (300 µl into each).

3.3 Chromatin Shearing

During these procedures keep the samples on ice.

1. Sonicate the chromatin using a Bioruptor (High power, 30 s ON—30 s OFF) for 15 min (*see* **Note 4**).
2. Add 200 µl of ChIP dilution buffer to each tube and mix the solution by gently pipetting.
3. Sonicate the chromatin using a Bioruptor (High power, 30 s ON—1 min OFF) for 25 min.
4. Centrifuge the microtubes at 2400 × *g* for 10 min at 4 °C.
5. Pool the supernatants (~450 µl) from the microtubes for each sample, in order to homogenize the sonicated chromatin between tubes. For example, if 10 g of buds is ground for a wild-type sample, ten microtubes of chromatin are used for sonication and 10× 450 µl is recovered and pooled after sonication. 5% of the volume of sonicated chromatin is removed and stored at −20 °C. This sample corresponds to the input sample and will be used again from **step 2** in Subheading 3.5 onward.
6. Proceed immediately with the remaining sonicated chromatin to Chromatin immunoprecipitation (*see* Subheading 3.4.2), or store the chromatin at −20 °C. Chromatin can be stored for a few months at −20 °C without any loss in integrity.

3.4 Chromatin Immunoprecipitation

During all the procedures keep the samples on ice when possible.

3.4.1 Prebinding of the Antibody to Dynabeads Protein G

1. Transfer 50 µl of Dynabeads Protein G (*see* **Note 5**) to a 2 ml microcentrifuge tube and add 1.5 ml of ChIP dilution buffer. One tube is used per gram of buds. For example, prepare ten tubes of 50 µl of Dynabeads if 10 g of buds are used.
2. Rotate for 5 min at 12 rpm at 4 °C.
3. Place the microtubes in a magnetic separation rack and incubate for 2 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.

4. Carefully remove the supernatant without disturbing the beads and discard.
5. Add 1.5 ml of ChIP dilution buffer.
6. Remove the microtubes from the magnetic separation rack and gently flick the tubes to resuspend the beads in solution. Repeat **steps 2–6** twice.
7. Add 5–10 μg of antibody depending on the antibody specification (*see* **Note 5**).
8. Incubate the antibody solution on a rotator at 12 rpm between 6 h and overnight at 4 °C. The prebinding of the antibody to the beads can be prepared on the day before chromatin precipitation. In that case the incubation time corresponds to the time necessary to prepare the chromatin (~6–8 h). Alternatively, the prebinding of the antibody to the beads can be prepared the day before the preparation of chromatin and in that case the incubation can be carried out overnight. Overnight incubation has no negative impact on the downstream steps.
9. After incubation, place the microtubes in a magnetic separation rack and incubate for 2 min.
10. Carefully remove the supernatant without disturbing the beads.
11. Add 1 ml of ChIP dilution buffer.
12. Remove the microtubes from the magnets and gently flick the tubes to resuspend the beads in solution.
13. Rotate for 5 min at 12 rpm at 4 °C.
14. Place the microtubes in a magnetic separation rack and incubate for 2 min. Carefully remove the supernatant without disturbing the beads.
15. Add 1 ml of ChIP dilution buffer.
16. Remove the microtubes from the magnetic separation rack and gently flick the tubes to resuspend the beads in solution.
17. Repeat **steps 13–17** once. The resuspended beads correspond to the prebound antibody.

3.4.2 Immunoprecipitation of Chromatin with Prebound Antibody

1. Transfer ~420 μl of sonicated chromatin to the microtube containing the beads with prebound antibody.
2. Incubate the solution on a rotator at 12 rpm for 14–16 h at 4 °C.
3. Place the microtubes in a magnetic separation rack and incubate for 2 min.
4. Carefully remove the supernatant without disturbing the beads.
5. Add 1.5 ml of low-salt buffer to each tube.

6. Remove the microtubes from the magnetic separation rack and gently flick the tubes to resuspend the beads in solution.
7. Rotate for 5 min at 12 rpm at 4 °C.
8. Place the microtubes in a magnetic separation rack and incubate for 2 min.
9. Carefully remove the supernatant without disturbing the beads.
10. Repeat **steps 5–9** one more time.
11. Add 1.5 ml of high-salt buffer to each tube.
12. Remove the microtubes from the magnetic separation rack and gently flick the tubes to resuspend the beads in solution.
13. Rotate for 5 min at 12 rpm at 4 °C.
14. Place the microtubes in a magnetic separation rack and incubate for 2 min.
15. Carefully remove the supernatant without disturbing the beads.
16. Repeat **steps 11–15** once more (*see* **Note 6**).
17. Add 1 ml of TE buffer.
18. Remove the microtubes from the magnetic separation rack and gently resuspend the beads in solution by pipetting.
19. Transfer the homogenate to a fresh 1.5 ml Clear-view Snap-Cap low-retention microtube.
20. Place the microtubes in a magnetic separation rack and incubate for 2 min.
21. Carefully remove the supernatant without disturbing the beads.
22. Elute the chromatin from the beads by adding 200 μl of elution buffer.
23. Briefly vortex the tubes and then incubate at 65 °C for 10 min.
24. Place the microtubes in a magnetic separation rack and incubate for 2 min.
25. Transfer the eluted solution to a new 1.5 ml Clear-view Snap-Cap low-retention microtube without disturbing the beads. The solution contains the immunoprecipitated chromatin.
26. Add another 200 μl of elution buffer to the tubes with the beads.
27. Briefly vortex the tubes then incubate at 65 °C for 10 min.
28. Place the microtubes in a magnetic separation rack and incubate for 2 min.
29. Take the eluted solution and combine it with the previous eluted solution. In total ~400 μl of eluted solution is recovered for each tube.

3.5 Reverse Cross-Linking and DNA Purification

1. Add 20 µl of 5 M NaCl to the 400 µl of eluted immunoprecipitated chromatin solution. Mix the solution by gently pipetting and incubate the tubes at 65 °C overnight to reverse the cross-linking.
2. Thaw the input sample (from **step 5** in Subheading 3.3) and add elution buffer to a final volume of 400 µl, then add 20 µl of 5 M NaCl to a final concentration of 0.25 M. Mix the solution by gently pipetting and incubate the tubes at 65 °C overnight to reverse the cross-linking.

   For all the subsequent steps both input and ChIP samples are processed the same way.
3. Add 10 µl of 0.5 M EDTA, 20 µl of 1 M Tris–HCl pH 7.0 and 3 µl of 20 mg/ml RNase A. Gently mix the solution by pipetting. Incubate for 30 min at 37 °C.
4. Add 2 µl of 20 mg/ml proteinase K. Gently mix the solution by pipetting. Incubate for 2 h at 45 °C.
5. Add 450 µl of phenol–chloroform–isoamyl alcohol and vortex for 15 s.
6. Centrifuge at 14,000 × *g* for 25 min at room temperature.
7. Transfer the supernatant to a fresh 1.5 ml Clear-view Snap-Cap low-retention microtube.
8. Repeat **steps 6** and **7**.
9. Add 1/10 volume of 3 M sodium acetate pH 5.2 (typically 40 µl of 3 M sodium acetate is added to the solution), 2 µl of GlycoBlue and 2.5 volumes of 100% ethanol (typically 1 ml of ethanol is added to the solution).
10. Mix the tubes by inverting ten times and then incubate at −20 °C overnight.
11. Centrifuge the tubes at 14,000 × *g* for 20 min at 4 °C.
12. Discard the supernatant and add 500 µl of ice-cold 70% ethanol.
13. Centrifuge the tubes at 14,000 × *g* for 10 min at 4 °C.
14. Discard the supernatant and leave the DNA pellet to air dry for 15 min at room temperature.
15. Resuspend the DNA pellet from one tube in 20 µl of nuclease-free water by gently pipetting. Use this resuspended solution to resuspend the DNA pellet from a second tube corresponding to the same ChIP sample and repeat this for all the tubes of the same sample. For example, a total of ten Eppendorf tubes from 10 g of starting material.
16. Store the ChIP and input DNA samples at −20 °C.

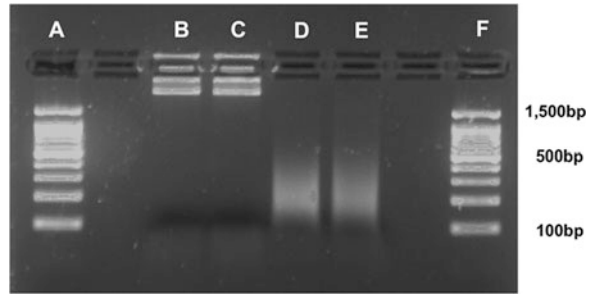

Fig. 3 Agarose gel electrophoresis of ChIP DNA before and after sonication. A 2% agarose gel with SYBR Gold nucleic acid stain showing, (**a**) 100 bp DNA ladder, (**b**) 15 ng of sample 1 DNA before sonication, (**c**) 15 ng of sample 2 DNA before sonication, (**d**) 15 ng of sample 1 DNA after sonication, (**e**) 15 ng of sample 2 DNA after sonication, and (**f**) 100 bp DNA ladder

3.6 DNA Quantification and Quality Control

Between 10 and 40 ng of DNA are routinely recovered from ChIP of meiocytes using this protocol. The amount of DNA recovered depends on the quality of the antibody, the amount of starting material and the abundance of protein on chromatin.

1. ChIP DNA is quantified using Qubit dsDNA HS assay kit.
2. Shearing of DNA is checked by loading 10–15 ng of input DNA on a 2% agarose gel and visualized after electrophoresis using SYBR gold nucleic acid gel stain (Fig. 3). DNA fragments ranging between 100 and 800 bp with enrichment at ~200 bp are suitable for DNA library construction.
3. The size of the immunoprecipitated DNA is checked using a High Sensitivity DNA kit with Agilent 2100 Bioanalyzer. This is particularly recommended when the amount of ChIP DNA recovered is relatively low (e.g., <20 ng).

3.7 DNA Library Preparation for Next-Generation Sequencing

3.7.1 End Repair

1. Add the following components in a 0.2 ml tube: 10 μl of 10× T4 DNA ligase reaction buffer, 4 μl of 10 mM dNTP mix, 5 μl of T4 DNA polymerase, 1 μl of DNA Polymerase I Large (Klenow) Fragment, 5 μl of T4 polynucleotide kinase, 10 ng of ChIP DNA or input DNA, and nuclease-free water to a final volume of 100 μl.
2. Gently mix the solution by pipetting, spin down (15,000 × *g*, 5 s) and incubate the solution at 20 °C for 30 min.
3. Spin down (15,000 × *g*, 5 s) the solution. Transfer the solution to a 1.5 ml Clear-view Snap-Cap low-retention microtube and add 1.8 volumes of Agencourt AMPure XP (180 μl). Gently mix the solution by pipetting ten times.
4. Incubate the tube for 10 min at room temperature.

5. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
6. Carefully remove the supernatant without disturbing the beads.
7. Add 400 μl of 75% ethanol and incubate the tube for 1 min at room temperature.
8. Carefully remove the supernatant without disturbing the beads and repeat **steps 7** and **8**.
9. Air-dry the beads for 10 min at room temperature.
10. Remove the microtube from the magnetic separation rack. Add 32 μl of nuclease-free water and resuspend the beads by gently pipetting ten times. Incubate the tube for 2 min at room temperature.
11. Place the microtubes in a magnetic separation rack and incubate for 5 min.
12. Carefully remove the supernatant (32 μl) without disturbing the beads and transfer it to a 0.2 ml Eppendorf tube.

3.7.2 dA-Tailing

1. Add the following components: 5 μl of NEBuffer 2, 10 μl of 1 mM dATP, and 3 μl of Klenow Fragment ($3' \rightarrow 5'$ exo-).
2. Gently mix by pipetting, spin down (15,000 × *g*, 5 s) the solution and incubate the solution at 37 °C for 30 min.
3. Spin down (15,000 × *g*, 5 s) the solution. Transfer the solution to a 1.5 ml Clear-view Snap-Cap low-retention microtube and add 1.8 volumes of Agencourt AMPure XP (90 μl). Gently mix the solution by pipetting ten times.
4. Incubate the tube for 10 min at room temperature.
5. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
6. Carefully remove the supernatant without disturbing the beads.
7. Add 400 μl of 75% ethanol and incubate the tube for 1 min at room temperature.
8. Carefully remove the supernatant without disturbing the beads and repeat **steps 7** and **8**.
9. Air-dry the beads for 10 min at room temperature.
10. Remove the microtube from the magnetic separation rack. Add 25 μl of nuclease-free water and resuspend the beads by gently pipetting ten times. Incubate the tube for 2 min at room temperature.

11. Place the microtubes in a magnetic separation rack and incubate for 5 min.
12. Carefully remove the supernatant (25 µl) without disturbing the beads and transfer it to a fresh 0.2 ml Eppendorf tube.

3.7.3 Ligation of Illumina Adapters

1. Add the following components: 4.5 µl of diluted Illumina adapters (1 µl of Illumina adapter diluted in 249 µl of nuclease-free water), 12.5 µl of 4× QS buffer, 5 µl of Quick-Stick ligase, and 3 µl of nuclease-free water.
2. Gently mix the solution by pipetting, spin down (15,000 × *g*, 5 s) and incubate the solution at 20 °C for 30 min.
3. Spin down (15,000 × *g*, 5 s) the solution. Transfer the solution to a 1.5 ml Clear-view Snap-Cap low-retention microtube and add 1 volume of Agencourt AMPure XP (50 µl). Gently mix the solution by pipetting ten times.
4. Incubate the tube for 10 min at room temperature.
5. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
6. Carefully remove the supernatant without disturbing the beads.
7. Add 400 µl of 75% ethanol and incubate the tube for 1 min at room temperature.
8. Carefully remove the supernatant without disturbing the beads and repeat **steps 7** and **8**.
9. Air-dry the beads for 10 min at room temperature.
10. Remove the microtubes from the magnetic separation rack. Add 20 µl of nuclease-free water and resuspend the beads by gently pipetting ten times. Incubate the tube for 2 min at room temperature.
11. Place the microtubes in a magnetic separation rack and incubate for 5 min.
12. Carefully remove the supernatant (20 µl) without disturbing the beads and transfer it to a fresh 0.2 ml tube.

3.7.4 PCR Enrichment

1. Prepare a real time quantitative PCR (qPCR) reaction as described in Table 1. Oligonucleotides with sequences matching the Illumina adapters are used to amplify DNA templates that will subsequently form clusters on a flowcell of the Next-Generation sequencing platform. Real-time qPCR allows the quantification of DNA molecules in the sample in order to determine the number of PCR cycles to use during the enrichment step (*see* **Note 7**, Fig. 4).

Table 1
Real time qPCR reaction composition

Components	Volume (μl)
DNA template	1
2× KAPA SYBR mix	5.5
Primer set (Illumina)	1
Nuclease-free water	2.5
Total	10

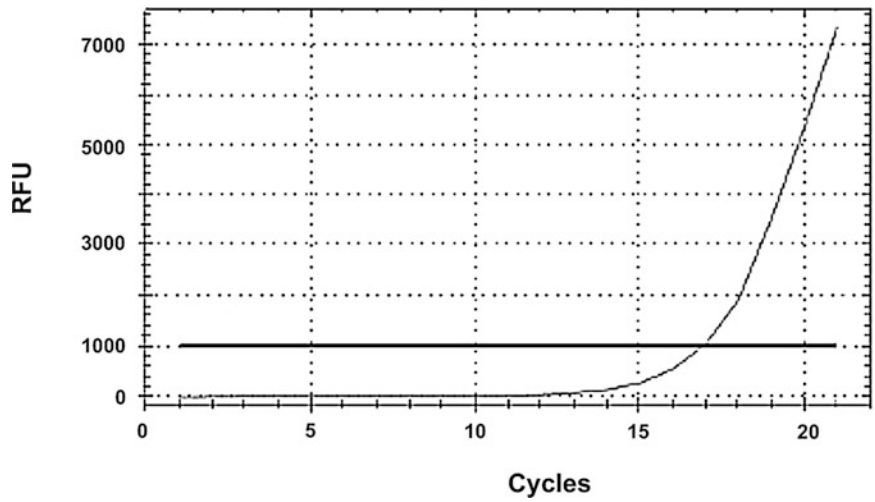

Fig. 4 Real-time qPCR amplification chart of a ChIP-seq DNA library. It is recommended to perform a real time qPCR of the ligated DNA library sample to determine the number of PCR cycles to use during the enrichment step. The number of cycles should be below 20 and should correspond to the number of cycles required to observe an increase of relative fluorescence units (RFU) minus 1 cycle. For example, on the chart the number of DNA molecules significantly increases after 17 cycles (arrow), as seen by the curve exponentially increasing and crossing the horizontal green line marking 1000 RFU. Hence, a number of 16 cycles should be used for the subsequent PCR enrichment step

2. Run real time qPCR in a thermal cycler with the parameters:
   1 cycle: 95 °C for 3 min;
   20 cycles: 95 °C for 30 s, 63 °C for 30 s, 72 °C for 30 s;
   Dissociation curve: from 65 to 95 °C with an increment of 0.5 °C after 5 s each time.
3. Prepare a PCR reaction as described in Table 2.
4. Run PCR for DNA enrichment in a thermal cycler with the parameters:
   1 cycle: 95 °C for 3 min;
   X cycles (number of cycles is determined by the real time qPCR): 95 °C for 30 s, 60 °C for 30 s, 72 °C for 30 s;
   1 cycle: 72 °C for 5 min.

Table 2
PCR reaction composition

Components	Volume (μl)
DNA template	19
2× KAPA SYBR mix	20
Primer set (Illumina)	1
Total	40

5. Spin down (15,000 × *g*, 5 s) the solution and add 10 μl of nuclease-free water to the PCR product.
6. Transfer the solution to a 1.5 ml Clear-view Snap-Cap low-retention microtube.

3.7.5 DNA Purification and Size Selection

1. Remove fragments that are >500 bp by adding 0.6 volume Agencourt AMPure XP (30 μl). Gently mix the solution by pipetting ten times.
2. Incubate the tube for 10 min at room temperature.
3. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
4. Transfer the supernatant to a new 1.5 ml Clear-view Snap-Cap low-retention microtube.
5. Add 1.4 volumes of Agencourt AMPure XP (70 μl for a 50 μl PCR reaction). Gently mix the solution by pipetting ten times.
6. Incubate the tube for 10 min at room temperature.
7. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
8. Carefully remove the supernatant without disturbing the beads.
9. Add 200 μl of 75% ethanol and incubate the tube for 1 min at room temperature.
10. Carefully remove all the solution without disturbing the beads and repeat **steps 9** and **10**.
11. Air-dry the beads for 10 min at room temperature.
12. Remove the microtubes from the magnetic separation rack. Add 35 μl of nuclease-free water and resuspend the beads by gently pipetting ten times. Incubate the tube for 2 min at room temperature.
13. Place the microtubes in a magnetic separation rack and incubate for 5 min.

14. Carefully remove the solution (35 μl) without disturbing the beads and transfer it to a new 1.5 ml Clear-view Snap-Cap low-retention microtube.
15. Add 1 volume of Agencourt AMPure XP (35 μl). This step will remove DNA fragments that are <250 bp, which frequently consist of adapter dimers (*see* **Note 8**). Gently mix the solution by pipetting ten times.
16. Incubate the tube for 10 min at room temperature.
17. Place the microtubes in a magnetic separation rack and incubate for 5 min. All the beads should be concentrated to the side of the tube adjacent to the magnet.
18. Carefully remove the supernatant without disturbing the beads.
19. Add 200 μl of 75% ethanol and incubate the tube for 1 min at room temperature.
20. Carefully remove all of the solution without disturbing the beads.
21. Repeat **steps 19** and **20**.
22. Air-dry the beads for 10 min at room temperature.
23. Remove the microtubes from the magnetic separation rack. Add 10 μl of nuclease-free water and resuspend the beads by gently pipetting ten times. Incubate the tube for 2 min at room temperature.
24. Place the microtubes in a magnetic separation rack and incubate for 5 min.
25. Transfer 10 μl of solution to a new tube. This corresponds to the eluted DNA library.
26. Check the quality and quantify the concentration of the DNA library sample as described in **step 3** in Subheading 3.6 (*see* **Note 8**, Fig. 5).

4 Notes

1. The cross-linking step should be optimized for local laboratory conditions. Both under– (causing poor protein–DNA cross-linking) and over–cross-linking (reducing the accessibility of epitope) may reduce the yield of immunoprecipitated DNA. Therefore, it is necessary to add a sufficient volume of formaldehyde solution per gram of ground tissue. Increasing the mass of ground buds in 25 ml of 1% formaldehyde solution may decrease the yield of cross-linking reaction. Small scale ChIP experiments with different cross-linking conditions can be performed to compare the amount of DNA being precipitated in order to optimize the cross-linking step.

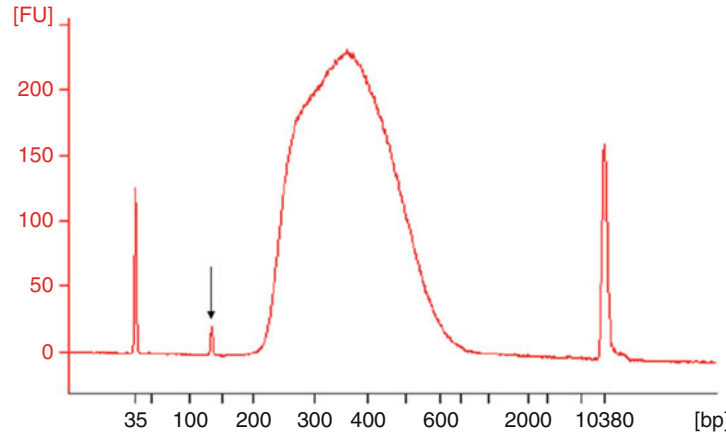

Fig. 5 Bioanalyzer electropherogram of a ChIP-seq DNA library from sonicated chromatin. The two peaks at 35 bp and 10.380 bp represents the lower and upper marker peaks from the Agilent High sensitivity DNA kits. These markers are important to calculate the size and concentration of the peak sample. The DNA library has a size ranging between 200 and 600 bp with enrichment for DNA molecules between 300 and 400 bp. Adapter dimers are marked with a black arrow and have a length between 120 and 140 bp. It is advisable to minimize the presence of adapter dimers before proceeding to next-generation sequencing

2. During nuclei isolation and chromatin preparation (Subheading 3.2) it is important to gently resuspend the pellet of nuclei in solution. For example, using a paintbrush and slowly resuspending the pellet by brushing the surface of the pellet to ensure good recovery of intact nuclei. Resuspension by pipetting is not recommended as it will create foam and risk breaking the nuclei.
3. After centrifugation, **step 4** in Subheading 3.2, make sure that the pellet of nuclei is mostly white and has little green residue, which indicates the presence of chloroplasts. Otherwise include a second wash with EB2 buffer. Alternatively, wipe off the green residue with a thin layer of Kimwipes tissue rolled around a spatula.
4. DNA sonication conditions should be optimised for local laboratory equipment. During sonication, ultrasonic waves generate heat and so the water bath should be kept at 4 °C to preserve the integrity of cross-linked protein-DNA complexes.
5. The choice of antibody depends on the epitope. It is recommended to test the antibody by western blot, immunoprecipitation, and immunofluorescence. If the antibody detects the epitope without significant background noise in these techniques it is a good indication that the antibody may also work for chromatin immunoprecipitation. Both Protein A and Protein G Dynabeads can be used for ChIP-seq. However, they have

different binding strengths to different immunoglobulins (IgG) and the choice of Dynabeads depends on the IgG class antibodies used for immunoprecipitating DNA.

6. More stringent wash conditions can be used to remove non-specific binding to the antibodies. For example, the second wash with High salt buffer (**step 16** in Subheading 3.4.2) can be replaced by a wash with Lithium chloride buffer (250 mM LiCl, 10 mM Tris–HCl pH 8.0, 1 mM EDTA, 1% Nonidet P-40, 1% sodium deoxycholate, 1 μM pepstatin A, EDTA-free Protease Inhibitor Cocktail (one tablet dissolved into 50 ml of solution)).
7. It is important to minimize the number of PCR cycles (<20) in **step 4** in Subheading 3.7.4. An increase in the number of PCR cycles cause an increased number of PCR-derived duplicate reads, which can cause bias when mapping reads and in the downstream bioinformatics analysis.
8. The final step of DNA size selection of the library (**step 15** in Subheading 3.7.5) is important to remove any adapter dimers. Adapter dimers cluster on the flow cell and may be sequenced more efficiently than the ligated ChIP DNA molecules, causing a reduction in the number of genome-mappable reads. Checking the final product of a DNA library on a bioanalyzer will indicate how much adapter dimer is present in the DNA library sample (Fig. 5). If the sample has a high molarity of adapter dimers, an additional step of size selection is recommended to remove them.

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