ChIP using plant samples – Arabidopsis

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Introduction

Eukaryotic chromatin is a complex of DNA and associated histone proteins which are involved in the higher order packaging of DNA into chromosomes. The chromatin state of a given DNA sequence influences transcriptional activity and replication timing and is regulated by potentially reversible covalent modifications of DNA and histones. Histone modifications at conserved lysine and arginine residues within the flexible N-terminal tails, such as phosphorylation, acetylation and methylation, specify a code which serves as an interaction platform with specific domains of chromatin-associated proteins. The immunoprecipitation (IP) of crosslinked chromatin with antibodies specific for certain histone modifications (chromatin immunoprecipitation. ChIP), followed by PCR to detect a potential enrichment or depletion of a DNA sequence of interest within IP fractions, constitutes an elegant and direct method to query specific chromatin states of individual genes and is already routinely used in many labs. In contrast to animal cells, however, plant cells have a rigid cell wall which poses limitations to the simple utilization of protocols established for animals. In this protocol, the method described in used to study histone modifications in the plant model organism Arabidopsis thaliana. This protocol is an adapted version of the original procedure published by Lawrence and co-workers (Lawrence et al., 2004).

Procedure

Arabidopsis seeds are stratified for 48 hours in 0.1% Phytablend at 4°C and then sown onto soil. 1.5 g of whole, 3-4 week old seedlings, are used per chromatin preparation. It is imperative to avoid contamination with soil as much as possible during harvest.

Day 1 : Chromatin crosslinking

1. Harvest 1.5 g seedlings and place them into a 50 ml tube.
2. Rinse seedlings twice with 40 ml double distilled water. Remove as much water as possible after second wash.
3. Add 37 ml 1% formaldehyde solution. Gently submerge seedlings at the bottom of the tube by stuffing the tube with nylon mesh. Screw on cap and poke cap with needle holes. Put in exsiccator and draw vacuum for 10 minutes.
4. Release vacuum slowly and shake exsiccator slightly to remove air bubbles. Seedlings should appear translucent.
5. Add 2.5 ml 2 M glycine to quench crosslinking. Draw vacuum for 5 minutes.
6. Again, release vacuum slowly and shake exsiccator slightly to remove air bubbles.
7. Remove nylon mesh, decant supernatant and wash seedlings twice with 40 ml of double distilled water. After second wash, remove as much water as possible and put seedlings between two layers of kitchen paper. Roll up paper layers carefully to remove as much liquid as possible.

At this step, plant material can be shock-frozen in liquid nitrogen and stored at -80°C

Chromatin preparation

1. Pre-cool mortar with liquid nitrogen. Add 2 small spoons of white quartz sand and plant material. Grind plant material to a fine powder.
2. Use cooled spoon to add powder to 30 ml of extraction buffer 1 stored on ice. Vortex to mix and keep at 4°C until solution is homogenous.
3. Rotate for 30 min at 4°C on a turning wheel or equivalent.

4. Filter extract through into a new, ice-cold 50 ml Falcon tube. Press to recover extract from solid material.

5. Repeat step 4.

6. Centrifuge extract using the rotor at 4000 rpm for 20 minutes at 4°C.

7. Gently pour off supernatant and resuspend pellet in 1 ml of extraction buffer 2 by pipetting up and down. Transfer solution to Eppendorf tube.

8. Spin in cooled benchtop centrifuge at 13000 rpm for 10 minutes.

9. Remove supernatant and resuspend pellet in 300 µl of extraction buffer 2 by pipetting up and down.

10. Spin in cooled benchtop centrifuge at 13000 rpm for 10 minutes. Add supernatant to fresh Eppendorf tube. Use pipette to carefully layer solution from step 9 onto it.

11. Spin in cooled benchtop centrifuge at 13000 rpm for 1 hour. In meantime, prepare 10 ml nuclei lysis buffer and 20 ml ChIP dilution buffer. Put buffers in cold room.

12. Remove supernatant and resuspend pellet in 300 to 500 µl of cold Nuclei Lysis Buffer. Resuspend by pipetting up and down and by vortexing. Keep solution cold between vortexing. Incubate for 20 minutes on ice.

13. Sonicate for 10 min at 4°C with sonicator: Setting “HIGH”, 10 sec “ON cycle”, 45 sec “OFF cycle”.

14. Spin in cooled benchtop centrifuge at 13000 rpm for 10 minutes. Add supernatant to new Eppendorf tube.

15. Repeat step 14. Remove 10 µl to run on an agarose gel.

16. Separate aliquots from steps 12 and 15 on 1.5% agarose gel. In the sonicated samples, DNA should be shifted and more intense compared to untreated samples and range between 200-2000 bp, centering around 500bp.

Following step 14, the chromatin samples can be “snap-frozen” in liquid nitrogen and further stored at -80°C. Repeated freezing/thawing cycles, however, should be avoided.

Pre-clearing and immuno precipitation (IP)

1. Transfer chromatin to 15ml Falcon tubes. Dilute 1/10 with fresh, ice-cold ChIP dilution buffer.

2. Prepare Protein A agarose beads pre-absorbed with sheared salmon sperm DNA by rinsing the required amount of beads 3 times with 1 ml ChIP Dilution Buffer in an Eppendorf tube. Spin in cooled benchtop centrifuge for 30 seconds at 13000 rpm between the washes to pellet the beads. After the last wash, resuspend the beads with ChIP dilution buffer to yield a 25% slurry (for pipetting reasons).

3. Pre-clear each chromatin sample by adding 140 µl of washed beads. Rotate for 1 hour at 4°C.

4. Spin Falcon tubes in a cooled centrifuge for 3 minutes at 3000 rpm to pellet the beads and transfer the supernatant to a new Falcon tube. Be careful not to carry over beads.

5. Store a 60 µl aliquot of pooled chromatin at -20°C. This will serve as input control later on.

6. Add 600 µl of chromatin solution per IP to an Eppendorf tube with an appropriate antibody. The optimal chromatin/antibody ratio has to be determined empirically for each antibody used. As a “rule of thumb”, we use about 10 µg of antibody per IP for polyclonal antibodies. For monoclonal antibodies, usually 1.5x to 4x higher concentration has to be used. Also set up 600 µl of chromatin solution without antibody or with an unrelated antibody as a mock IP. We use a non-specific rabbit serum (Sigma Cat.: R9133) for the mock IP.
7. Add 80 µl of washed Protein A agarose beads to the chromatin IPs (25% slurry; for the preparation see step 2). Rotate overnight at 4°C.

Day 2: Collection, washes and elution of immune complexes

1. Prepare fresh elution buffer and place it at 65°C.
2. Spin IPs in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard the supernatant.
3. Add 1 ml of low salt wash buffer per tube. Rotate for 5 minutes at 4°C.
4. Spin in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard supernatant.
5. Add 1 ml of high salt wash buffer per tube. Rotate for 5 minutes at 4°C.
6. Spin in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard supernatant.
7. Add 1 ml of LiCl wash buffer per tube. Rotate for 5 minutes at 4°C.
8. Spin in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard supernatant.
9. Add 1 ml of TE buffer per tube. Rotate for 5 minutes at 4°C.
10. Spin in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard supernatant.
11. Repeat TE wash. Spin in cooled benchtop centrifuge at 5000 rpm for 30 seconds to collect beads and discard supernatant.
12. Elute immune complexes by adding 250 µl of elution buffer. Vortex briefly to mix and incubate at 65°C for 15 minutes. Spin in benchtop centrifuge at 13000 rpm for 30 seconds and transfer supernatant to a fresh Eppendorf tube.
13. Repeat elution and finally combine the two elutes.

Reverse crosslinking

1. Add 20 µl of 5M NaCl to samples. Incubate overnight at 65°C.
2. Add 109 µl of TE buffer, 7.1 µl of 5M NaCl and 8.7 µl 20% SDS to the 60 µl input control aliquoted on Day 1. Incubate overnight at 65°C.

Day 3: DNA cleanup

1. Add 10 µl of 0.5M EDTA, 20 µl 1M Tris-HCl pH 6.5 and 1µl of 20 mg/ml proteinase K to the IP samples. Add 1.2 µl of 0.5M EDTA, 2.4 µl 1M Tris-HCl pH 6.5 and 1µl of 20 mg/ml proteinase K to the input control samples. Incubate for 1 to 3 hours at 45°C. The samples can be gently shaken through this incubation.
2. Purify the DNAs using a silica-gel membrane (e.g. PCR purification kits from Qiagen, Promega, etc.). Elute DNAs twice with 50 µl 10mM Tris-HCl pH 8.0 and pool elutes. Proceed to PCR reactions. An excellent guide to quantification of ChIP by real-time PCR is provided by Haring et al. (2007).

Materials & Reagents

Extraction buffer 1
0.4 M Sucrose
10 mM Tris-HCl, pH 8.0
10 mM MgCl₂
5 mM β-mercaptoethanol, with Protease Inhibitors
**Extraction buffer 2**
0.25 M Sucrose
10 mM Tris-HCl, pH 8.0
10 mM MgCl₂
1% Triton X-100
5 mM β-mercaptoethanol, with Protease Inhibitors

**Extraction buffer 3**
1.7 M Sucrose
10 mM Tris-HCl, pH 8.0
2 mM MgCl₂
0.15% Triton X-100
5 mM β-mercaptoethanol, with Protease Inhibitors

**Nuclei lysis buffer**
50 mM Tris-HCl, pH 8.0
10 mM EDTA
1% SDS, with Protease Inhibitors

**CHIP dilution buffer**
1.1% Triton X-100
1.2 mM EDTA
16.7 mM Tris-HCl, pH 8.0
167 mM NaCl

**Elution buffer**
1% SDS
0.1 M NaHCO₃

**Low salt wash buffer**
150 mM NaCl
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl, pH 8.0

**High salt wash buffer**
500 mM NaCl
0.1% SDS
1% Triton X-100
2 mM EDTA
20 mM Tris-HCl, pH 8.0

**LiCl wash buffer**
0.25 M LiCl
1% Nonidet P-40
1% sodium deoxycholate
1 mM EDTA
10 mM Tris-HCl, pH 8.0

**TE buffer**
10 mM Tris-HCl, pH 8.0
1 mM EDTA

**Protease inhibitor**
100 mM PMSF
1 tablet per 10ml of solution of complete mini protease inhibitor cocktail tablets
References:


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