

Jul 02, 2019 Version 1

Plant Chromatin Immunoprecipitation V.1

DOI

dx.doi.org/10.17504/protocols.io.zmff43n



Laura Poza-Viejo¹, Ivan del Olmo¹, Pedro Crevillén¹

¹Centro de Biotecnología y Genómica de Plantas, Universidad Politécnica de Madrid (UPM) - Instituto Nacional de Investigación y Tecnología Agraria y Alimentaria (INIA), 28223 Pozuelo de Alarcón (Madrid), Spain



Pedro Crevillén

OPEN  ACCESS



DOI: dx.doi.org/10.17504/protocols.io.zmff43n

Protocol Citation: Laura Poza-Viejo, Ivan del Olmo, Pedro Crevillén 2019. Plant Chromatin Immunoprecipitation. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.zmff43n>

Manuscript citation:

High ambient temperature leads to reduced FT expression and delayed flowering in Brassica rapa via a mechanism associated with H2A.Z dynamics. Del Olmo I, Poza-Viejo L, Piñeiro M, Jarillo JA, Crevillén P. *Plant J*. 2019 doi: 10.1111/tpj.14446

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Protocol status: Working

We use this protocol routinely in our group for ChIP experiments with Arabidopsis and Brassica crop samples and it is working.

Created: March 29, 2019

Last Modified: July 02, 2019

Protocol Integer ID: 21895

Keywords: Chromatin Immunoprecipitation, ChIP, plants, Arabidopsis, Brassica

Abstract

Chromatin Immunoprecipitation (ChIP) is a crucial technique to study chromatin regulation, epigenetic phenomena and transcription factor DNA-binding in vivo. This technique is widely used in model plant systems like *Arabidopsis* but reliability in other plant systems is challenging. We adapted our well-established *Arabidopsis* ChIP protocol to be used with leaves and inflorescences of Brassica crops species (*Brassica rapa* and *Brassica napus*). This protocol was originally based on Gendrel et al. *Nature Methods* volume 2, pages 213–218 (2005), but has several modifications including chromatin sonication using a BioRuptor (Diagenode), immunoprecipitation using magnetic Dynabeads (Thermofisher) and DNA elution recovery performed using Chelex 100 (Bio Rad). The protocol is quick and very reproducible and has been validated against a number of histone modifications and protein tag antibodies.

Guidelines

Notes: (please read step-by-step protocol)

¹ For Dual crosslinking: to detect histone modifications this is enough, but for protein binding we perform a dual crosslinking with DSG 2 mM. Dissolve 65 mg of DSG in 200 µl of Dimethyl Sulfoxide (DMSO), and then add to 100 ml of PBS 1X to obtain a 2 mM solution. Vacuum first with PBS+DSG for 30 min, remove and wash with PBS. Then, vacuum again with PBS+FAA1% and continue with the standard protocol.

² Vacuum time depends on the strength of the pump and the type of material. We have done up to 20 minutes in some cases. It is recommended a trial experiment to set up the conditions with your device.

³ Volume can be increased to 300 µl for two IPs.

⁴ Before and after sonication, take a 5 µl aliquot to check efficiency on gel. Check efficiency of sonication running an agarose gel with samples before and after sonication (you want most of the signal around 600-300 bp in the gel). If needed, you can do more cycles of sonication, but do not over sonicate samples because this is detrimental for the immunoprecipitation.

⁵ Sonication time needs to be adjusted experimentally. You may require more or less depending on many factors (type of plastic tube, sample material, etc.). For some protein ChIP, high sonication may be harmful for weak DNA-protein interactions so you can try sonicating at LOW setting.

⁶ We start our ChIP experiments in the late afternoon, so the IP is always between 12-16 h. Shorter or longer incubations times can be used depending on the antibody.

⁷ In this protocol, DNA is not purified so the INPUT DNA could have some colour and carry SDS from lysis buffer, so it needs to be diluted prior PCR amplification. In some extreme cases, after Chelex treatment DNA can be purified and concentrated by standard molecular biology techniques or column-based methods. However, this will only reduce yield and could introduce some bias.

Materials

MATERIALS

These are some specific materials used in this protocol:

- o **Miracloth**, Merk Millipore, REF: 475855-1R.
- o **Dynabeads Protein G**, Invitrogen by Thermo Fisher Scientific, REF: 10003D.
- o **Dynabeads Protein A**, Invitrogen by Thermo Fisher Scientific, REF: 10001D.
- o **cOmplete Tablets EDTA-free** protease inhibitor, Roche, REF: 04 693 132 001.
- o **DSG (Disuccinimidyl glutarate)**, Santa Cruz Biotechnology, REF: sc-285455A.
- o **Formaldehyde 37-38%** w/w stabilized with metanol (USP, BP, Ph. Eur.) pharma grade, PanReac AppliChem, REF: 141328.
- o **Proteinase K**, Sigma, REF P2308
- o **Chelex® 100** Chelating Resin molecular biology grade, Bio-Rad, REF: 1421253.
- o **DMSO (Dimethyl Sulfoxide)** (Reag. Ph. Eur.) for analysis ACS, PanReac AppliChem, REF: 131954.
- o **Sodium deoxycholate monohydrate (deoxycholic acid)**, Sigma, REF: D-5670.
- o **2-Mercaptoethanol (βME)**, Sigma, REF: M3148.
- o **Triton X-100**, Sigma, REF: T8787.
- o **Nonidet P 40** Substitute, SIGMA, REF:74385.
- o **DynaMag-2 magnet magnetic rack**, Thermo Fisher Scientific, REF: 12321D.
- o **Rotary vane vacuum pump**, Thermo Fisher Scientific, REF: 12911141.

SOLUTIONS

Note that you maybe need to prepare different solution volumes depending of the number of samples. For histone acetylation ChIPs, add sodium butyrate (NaBu) 5mM in all buffers. For best results Extraction buffers are prepared the same day from stock solutions. Triton X-100 10% and Sucrose 2M stock solutions should be recently prepared and not autoclaved. Rest of the buffers should be autoclaved and different from general laboratory stock to avoid cross DNA contaminations.

<i>Final concentration</i>	
PBS 10x	for 1 L:
1.3 M NaCl	74 g
30 mM Na ₂ HPO ₄	4.25 g
30 mM NaH ₂ PO ₄	4.14 g
adjust pH to 7	

Extraction Buffer 1	for 1 L:
0.4 M sucrose	20 ml 2 M sucrose
10m M Tris-HCl pH 8	1 ml 1 M Tris-HCl pH 8
10m M MgCl	1 ml 1 M MgCl
*5m M β ME	35 μ l 14.3 M β ME
*Protease Inhibitor cOmplete (stock 50X, use 20 μ l for 1 ml of buffer)	
Extraction Buffer 2	for 10 ml:
0.25 M sucrose	1.25 ml 2 M sucrose
10 m M Tris-HCl pH 8	100 μ l 1 M Tris-HCl pH 8
10 m M MgCl ₂	100 μ l 1 M MgCl ₂
1% Triton X-100	1 ml 10% Triton X-100
*5 mM β ME	3.5 μ l 14.3 M β ME
*Protease Inhibitor cOmplete (stock 50X, use 20 μ l for 1 ml of buffer)	
Extraction Buffer 3	for 10 ml:
1.7 M sucrose	8.5 ml 2 M sucrose
10 mM Tris-HCl pH 8	100 μ l 1 M Tris-HCl pH 8
0.15% Triton X-100	150 μ l 10% Triton X-100
2 mM MgCl ₂	20 μ l 1 M MgCl ₂
*5 mM β ME	3.5 μ l 14.3 M β ME
*Protease Inhibitor cOmplete (stock 50X, use 20 μ l for 1 ml of buffer)	
Nuclei Lysis Buffer	for 5 ml:



50mM Tris-HCl pH 8	0.25 ml 1 M Tris-HCl pH 8
10 mM EDTA	100 µl 0.5 M EDTA
1% SDS	0.5 ml 10% SDS
*Protease Inhibitor cOmplete (stock 50X, use 20µl for 1 ml of buffer)	100 µl
*add just before using the buffer	
ChIP Dilution Buffer	for 10 ml:
1.1% Triton X-100	1.1 ml 10% Triton X-100
1.2 mM EDTA	24 µl 0.5 M EDTA
16.7 mM Tris-HCl pH 8	167 µl 1 M Tris-HCl pH 8
167 mM NaCl	334 µl 5 M NaCl
WASHING SOLUTIONS	Prepare, autoclave and store at 4°C.
Low Salt	for 50 ml:
150 mM NaCl	1.5 ml 5M NaCl
0.1% SDS	0.5 ml 10% SDS
1% Triton X-100	5 ml 10% Triton X-100
2 mM EDTA	200 µl 0.5M EDTA
20 mM Tris-HCl pH 8	1 ml 1 M Tris-HCl pH 8
H ₂ O	41.8ml
High salt	For 50 ml:
500 mM NaCl	5 ml 5 M NaCl

0.1% SDS	0.5 ml 10% SDS
1% Triton X-100	5 ml 10% Triton X-100
2 mM EDTA	200 µl 0.5M EDTA
20 mM Tris-HCl pH 8	1 ml 1 M Tris-HCl pH 8
H ₂ O	38.3 ml
LiCl wash	For 50 ml:
0.25 M LiCl	3.125 ml 4 M LiCl
1% NP-40	5 ml 10% NP-40
1% deoxycholic acid	0.5 g deoxycholic acid
1 mM EDTA	100 µl 0.5M EDTA
10 mM Tris-HCl pH 8	0.5 ml 1 M Tris-HCl pH 8
H ₂ O	41.275 ml

Safety warnings

- ⚠ Sample crosslinking must be performed in fume hood. Exposure to formaldehyde (FAA), a flammable gas with a strong smell, can cause leukemia and cancers.

Crosslinking

- 1 Harvest 1-2 g of Arabidopsis or Brassica seedlings/leaf tissue/inflorescence in a 50ml conical centrifuge tube.
- 2 Add Phosphate-buffered saline buffer (PBS) with 1% formaldehyde (FAA)¹. To detect histone modifications this is enough, but for protein binding we perform a dual crosslinking with DSG (disuccinimidyl glutarate) 2 mM.
- 3 Crosslink in a desiccator attached to a vacuum pump for 5 + 5 min (for Arabidopsis seedlings) or 5 min + 10 min + 10 min (for Brassica leaves and inflorescences)².
- 4 Stop the crosslinking by adding Glycine 2 M to a final concentration of 0.125M. (2.5 ml of 2M Glycine in 37ml of water), vacuum for 5 min.
- 5 Remove the tissue from the vacuum and rinse off the formaldehyde with MilliQ water twice. Remove as much water as possible from the seedlings by placing on a paper towel.
- 6 Freeze in liquid nitrogen and store crosslinked tissue at -80°C

Chromatin Extraction and Sonication

- 7 Grind with mortar in liquid nitrogen to a fine powder.
- 8 Pass to a 50ml centrifuge tube and add the powder to 10 ml of Extraction Buffer 1. After adding buffer, ensure you mix well so all the tissue has dissolved into the buffer.
- 9 Filter the solution through a layer of Miracloth into a 50ml centrifuge tube. Repeat step 9 until the solution is clear of big tissue particles.
- 10 Centrifuge at 5000 xg for 10 min at 4°C.
- 11 Gently remove supernatant and resuspend the pellet in 1 ml of Extraction Buffer 2. Transfer the solution to 1.5ml centrifuge tube.
- 12 Centrifuge at 5000 xg for 10 min at 4°C.

- 13 Remove supernatant and resuspend pellet in 300 µl of Extraction Buffer 3.
- 14 In a clean eppendorf, add 600 µl of Extraction Buffer 3. Take the 300 µl solution (resuspended pellet) from step 13 and carefully lay it on top of the clean 600 µl of Extraction Buffer 3.
- 15 Centrifuge at 16000 xg for 60min at 4 °C. At this point, it is advisable to prepare Nuclei Lysis Buffer and ChIP Dilution Buffer, to turn on the Bioruptor and to prepare the Dynabeads (go to step 20).
- 16 Remove the supernatant and resuspend the chromatin pellet in 200 µl of Nuclei Lysis Buffer³.
- 17 Resuspend the pellet by pipetting up and down (keep solution cold). Take a 5 µl aliquot of each sample to check sonication efficiency⁴.
- 18 Sonicate the chromatin solution for 20 min (30sec on/off intervals) HIGH setting⁵ (LOW setting protein ChIPs) in the Bioruptor (sonicator water bath must be pre-cooled). Take a 5µl aliquot of each sample to check sonication efficiency⁴.
- 19 Spin chromatin solution at 2500 xg for 5 min at 4 °C to pellet debris.

Immunoprecipitation

- 20 Prepare the Dynabeads magnetic beads (Protein A for rabbit antibody or Protein G for mouse antibody). For each immunoprecipitation wash 15 µl Dynabeads in 500 µl ChIP dilution buffer twice. Then, add 1-5 µg of antibody and incubate the Dynabeads and the antibody in 50 µl ChIP Dilution Buffer rotating at 4°C for 1 h.
- 21 Wash prepared antibody coated Dynabeads with 500 µl of ChIP dilution buffer. Let Dynabeads attach to the magnet. Then, discard buffer. Resuspend Dynabeads in 50µl of ChIP Dilution Buffer and keep aside on ice.
- 22 For the immunoprecipitation, measure chromatin solution from step 19, usually ~150 µl. Take INPUT: 10% of the volume (15 µl) of chromatin and store it at -20°C.
- 23 Dilute chromatin solution 10 times adding 1.5 ml of ChIP Dilution Buffer. The point here is to dilute the 1% SDS to 0.1% SDS with ChIP dilution buffer. This is enough for two immunoprecipitations or one immunoprecipitation and no-antibody control sample. For three immunoprecipitations is better to start from 300 µl of Nuclei Lysis Buffer (step 16).



- 24 Add antibody coated Dynabeads into the 750 μ l of chromatin solution in a DNA low binding microtube. Incubate the immunoprecipitation rotating at 4°C overnight⁶.

DNA recovery

- 25 The next morning perform the washes using 1 ml of each buffer (see below). Wash at 4°C for 5 min with gentle rotation and let beads attach to magnets in between washes. Use the sequence of buffers listed below:
- a) Low Salt Wash Buffer: 2 washes.
 - b) High Salt Wash Buffer: 1 wash (optional).
 - c) LiCl Wash Buffer: 1 wash (optional).
 - d) TE Buffer: 2 washes.
- 26 Take the INPUTs frozen the day before and elute immune complexes and INPUTs by adding 200 μ l 10% Chelex 100 resin (10g in 100ml water) and boil the samples at 98°C for 10min with agitation in a thermomixer.
- 27 Cool in ice and incubate with 2 μ l Proteinase K (10 mg/ml) at 37°C for 20min.
- 28 Boil samples again at 98°C for 10 min with agitation in thermomixer to inactivate Proteinase K.
- 29 Cool in ice and spin the tubes in microcentrifuge at 16000 xg for 5 min at 4°C. Transfer ~160 μ l of the supernatant to a new DNA low binding microtube. Do not take any Chelex particle. Store at 4°C or at 20-°C for long term storage.
- 30 For real-time quantitative PCR analysis, ChIP DNA can be used directly but INPUT DNA must be diluted at least 1:10 in water⁷. Usually 1-2 μ l of the DNA solution is enough for PCR amplification.