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Plant Chromatin Immunoprecipitation V.2

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We use this protocol in our group and it is working.

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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 is based on Gendrel et al. *Nature Methods* volume 2, pages 213–218 (2005), but has several modifications including chromatin sonication using a BioRuptor, immunoprecipitation using magnetic Dynabeads, and DNA elution is performed using Chelex 100 for ChIP-PCR or magnetic SPRI beads for ChIP-seq. The protocol is quick and very reproducible and has been validated against a number of histone modifications and protein tag antibodies. In this new version V2 we describe how to purify and recover DNA after immunoprecipitation if DNA is used for next-generation sequencing library preparation in ChIP-seq experiments.

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 **Rotary vane vacuum pump,** Thermo Fisher Scientific, REF: 12911141.
o **DynaMag-2 magnet magnetic rack,** Thermo Fisher Scientific, REF: 12321D
o **Bioruptor Plus** sonication bath, Diagenode REF: UCD-300.
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 **DNA Purification SPRI Magnetic Beads**, AMB, REF: G950-ABM.
o **Qubit Fluorometer** and **dsDNA HS Assay Kit**, Thermo Fisher Scientific, REF: Q32854.

SOLUTIONS

Note that you maybe need to prepare different solution volumes depending of the number of samples. For histone acetylation ChIPs, add sodium butirate (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.

	PBS 10x	for 1 L:
	1.3 M NaCl	74 g
	30 mM Na2HPO4	4.25 g
	30 mM NaH2PO4	4.14 g

	adjust pH to 7	
	Extraction Buffer 1	for 1 L:
	0.4 M sucrose	20 ml 2 M sucrose
	10 mM TRis-HCl pH8	1 ml 1 M Tris-HCl pH 8
	10 mM MgCl2	1 ml 1 M MgCl
	*5 mM βME	35 µl 14.3 M βME
	*Protease Inhibitor cOmplete (stock 50X, use 20µl 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 µl 1 M Tris-HCl pH 8
	10 mM MgCl2	100 µl 1 M MgCl2
	1% Triton X-100	1 ml 10% Triton X-100
	*5 mM βME	3.5 µl 14.3 M βME
	*Protease Inhibitor cOmplete (stock 50X, use 20µl 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 µl 1 M Tris-HCl pH 8
	0.15% Triton X-100	150 µl 10% Triton X-100
	2 mM MgCl2	20 µl 1 M MgCl2

	*5 mM βME	3.5 µl 14.3 M βME
	*Protease Inhibitor cOmplete (stock 50X, use 20µl 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
	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_20	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_20	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_20	41.275 ml
	Elution Buffer	For 10 ml:
	1% SDS	1 ml 10%
	0.1M NaHCO3	0.084 g

Troubleshooting

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.

Harvest and 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 vaccum pump for 5 + 5 min (for Arabidopsis seedlings and inflorescences) or 5 min + 10 min + 10 min (for Brassica leaves and inflorescences)[2].

STEP CASE

Alternative crosslinking method 32 steps

In the case of very hard tissues, like Brassica inflorescences or fruits, better results are obtained by crosslinking during chromatin extraction. In that case follow this modifications (steps 2 to 8):

- Frozen fresh plant material without fixing.
- Grind with mortar in liquid nitrogen to a fine powder.
- Pass to a 50 ml centrifuge tube and add the powder to 10 ml of "modified Extracction Buffer 1" (supplemented with 1% FAA and using HEPES 10 mM pH 8 buffer).
- Incubate for 10 min at room temperature; stop crosslinking by adding Glycine to final concentration of 0.125 M.
- Continue the protocol at step 9.

4 Stop the crosslinking by adding Glycine 2 M to a final concentration of 0.125 M. (2.5 ml of 2 M Glycine in 37 ml 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

7 Grind with mortar in liquid nitrogen to a fine powder.

8 Pass to a 50 ml 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 1.5 ml 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 Buffer[3].

17 Resuspend the pellet by pipetting up and down (keep solution cold). Take a 5 µl aliquot of each sample to check sonication efficiency[4].

Immunoprecipitation

18 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.

19 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.

20 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.

21 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).

22 Add antibody coated Dynabeads into the 750 µl of chromatin solution in a DNA low binding microtube. Incubate the immunoprecipitation rotating at 4°C overnight[6].

23 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)

STEP CASE

DNA recovery for ChIP-PCR or ChIP-seq 12 steps

If DNA is going to be used for standard ChIP-PCR quantification perform DNA purification with Chelex 100 (steps 24 to 28). This quick DNA purification yields greater amounts of DNA and improved real-time PCR quantification. However it is not suitable for next-generation library sequencing preparation.

If DNA is required for ChIP-seq perform classic reverse crosslinking teratment and purify DNA using SPRI beads (steps 29 to 35). This DNA can also be used for real-time PCR quantification.

DNA recovery for ChIP-PCR

24 Take the INPUTs frozen the day before and elute immune complexes and INPUTs by adding 200 µl 10% Chelex 100 resin (10 g in 100 ml water) and boil the samples at 98°C for 10 min with agitation in a thermomixer.

25 Cool in ice and incubate with 2 µl Proteinase K (10 mg/ml) at 37°C for 20 min.

26 Boil samples again at 98°C for 10 min with agitation in thermomixer to inactivate Proteinase K.

27 Cool in ice and spin the tubes in microcentrifuge at 16000 xg for 5 min at 4°C. Transfer ~160 µl 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.

28 For real-time quantitative PCR analysis, ChIP DNA can be used directly but INPUT DNA must be diluted at least 1:10 in water[7]. Usually 1-2 µl of the DNA solution is enough for

PCR amplification.

DNA recovery for ChIP-seq

29 Prepare the Elution buffer. Elute immune complexes by adding 50 µl of fresh Elution Buffer to the pelleted beads. Vortex briefly to mix and incubate at 65 °C for 15 min with gentle agitation. Let beads attach to magnet and carefully transfer 40 µl of the supernatant fraction (eluate) to another tube and repeat elution. Combine the two eluates (Final volume = 80 µl).

30 Take the INPUTs frozen the day before. Add 3.2 µl 5 M NaCl to each ChIP eluate and to the INPUTs and reverse crosslink incubating overnight at 65 °C.

31 Next day, add 180 µl (2.2x vol.) of SPRI beads. Carefully mix by pipetting up and down (at least ten times) and incubate for 5 min at room temperature. Separate supernatant from beads by placing the tubes in the magnet for 5 min. Discard supernatant but leave tubes on magnet.

32 Wash SPRI beads with 100 µl of 70% ethanol incubating or 30 sec, without disturbing the beads, then discard supernatant. Repeat wahses four times.

33 Move tubes off the magnet and air dry the SPRI beads for 4 min at room-temperature.

34 Elute DNA from SPRI beads in 30 µl of Tris-HCl 10 mM pH 8. Mix by pipetting at least ten times and incubate for 2 min.

35 Place tube on magnet and incubate for 5 min, and finally transfer supernatant to a new tube (aprox. 25 µl). DNA concentratin can be determined using a Qubit fluorometer. Usually 1-10 µl are used for ChIP-seq library preparation.