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## Chromatin Immunoprecipitation protocol for rice seedlings

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We use this protocol and it's working

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## Abstract

Chromatin Immunoprecipitation followed by sequencing (ChIP-seq) is a powerful technique for identifying transcription factor (TF) binding sites across the genome. By combining the immunoprecipitation of DNA-bound TFs with high-throughput sequencing, it enables precise mapping of target regions. However, applying ChIP in plants remains challenging due to the low abundance of many TFs, which often results in weak signals and the requirement of large amounts of starting material. Obtaining sufficient tissue is particularly difficult for specific developmental stages or cell types. Additionally, the rigid cell wall and compact chromatin structure in plants complicate chromatin extraction and immunoprecipitation efficiency. Arabidopsis protocols are more optimized and better documented, thanks to its status as a model organism. In contrast, crop species such as rice lack robust protocols. Protoplast-based ChIP methods have been developed to mitigate these challenges, but they compromise the *in vivo* context of chromatin interactions. To address these limitations, we present an optimized ChIP protocol for rice seedlings, providing a reliable approach for studying TF binding sites in this key crop species.

**Keywords:** ChIP-Seq, rice, transcription factor binding sites (TFBS)



## Materials

### Equipment

Centrifuge

Mortar and pestle

Miracloth (Millipore)

Sonicator (Bioruptor® Plus diagenode)

Vacuum chamber

### Reagents

37% formaldehyde

2 M glycine

Ultrapure water

Liquid nitrogen

β-mercaptoethanol

0.2 M phenylmethylsulfonyl fluoride (PMSF)

Protease inhibitor cocktail tablets

Triton X-100 (diluted to 20%)

Extraction buffer 1: 0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 1x protease inhibitor cocktail tablet

Extraction buffer 2: 0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1x protease inhibitor cocktail tablet

Extraction Buffer 3: 1.7 M sucrose, 10 mM Tris-HCl pH 8, 2 mM MgCl<sub>2</sub>, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1x protease inhibitor cocktail tablet 1)

Glycogen carrier (10 mg/ml; Roche)

Phenol-chloroform-isoamyl alcohol (25/24/1) pH 8

Proteinase K (20 mg/ml)

RNAse (20 mg/ml)

NaAc pH 5.2 3 M

EtOH 96%

Anti-FLAG® M2 magnetic beads (Sigma, USA)

Low Salt Buffer: 150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8

High Salt Buffer 200 mM NaCl, 0.1 % SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8

LiCl wash buffer 50 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8

TE buffer: 10 mM Tris-HCl pH 8, 1 mM EDTA)

## Troubleshooting

## Before start

*All the centrifuge and spin steps are performed at 4°C. Samples are always kept on ice or 4°C cold room and all buffers used are precooled unless otherwise indicated. All buffers should be prepared fresh.*

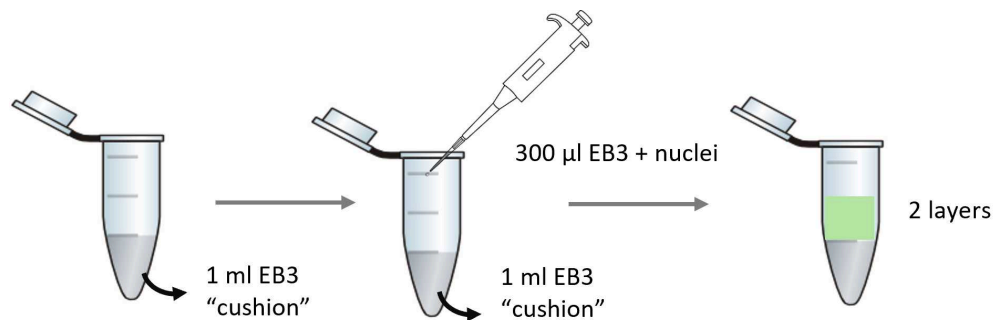
## Tissue Cross-Linking

- 1 Harvest 3 g of young rice leaves (14 days seedlings young leaves ~ 70 plants), cut them into pieces of approximately 1–2 cm length, and put the leaves immediately, as loose as possible, in a vacuum flask with a stirrer.
- 2 Submerge leaves in 200 ml of 1% p-formaldehyde solution. Vacuum infiltrate at room temperature for 15 min (meaning vacuum up to 50 mbar, a short release of vacuum, and then repeating the cycle), allowing penetration of fixative into leaf tissues. Stir for an additional 1 min without vacuum.
- 3 Add Glycine 2 M (13 ml of 2M Glycine in 200 ml of 1% formaldehyde solution) to the flask and mix vigorously to stop cross-linking. Vacuum infiltrate again for 5 min, releasing vacuum every 30 seconds.
- 4 Rinse the tissue two or three times with water to remove all the formaldehyde. After the rinses, remove as much water as possible by blotting between paper towels if necessary.

## Isolation of nuclei and chromatin fragmentation

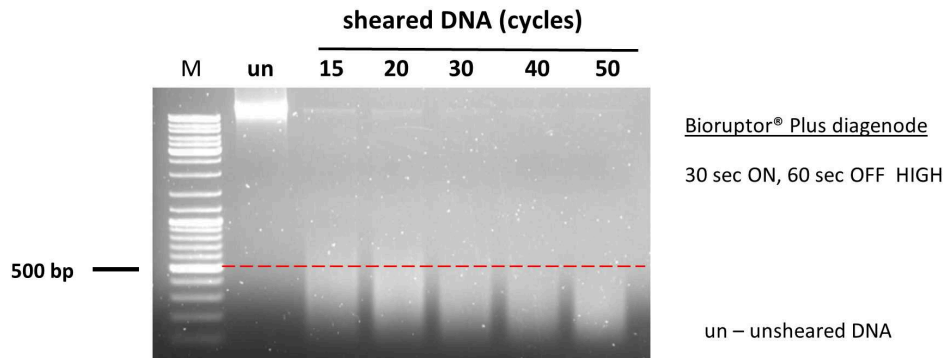
- 5 Grind 3 g of the cross-linked tissue in liquid nitrogen into fine powder. Insert into 50 ml tubes and resuspend the ground material in extraction buffer 1 (0.4 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 5 mM β-mercaptoethanol, 0.1 mM PMSF, and 1x protease inhibitor cocktail tablet).  
**Note: Do not add the entire buffer at once. Tap the tube on the bench to get the N<sub>2</sub> out of the mix.**
- 6 Incubate for 10 min on ice and vortex once in a while.
- 7 Filter the solution through four layers of Miracloth placed in a funnel into a fresh 50 ml Falcon tube placed on ice. Repeat once if necessary to remove additional debris.
- 8 Centrifuge the filtered solution for 20 min at 3000× g at 4°C.
- 9 Gently, remove supernatant and resuspend the pellet in 1 ml of extraction buffer 2 (0.25 M sucrose, 10 mM Tris-HCl pH 8, 10 mM MgCl<sub>2</sub>, 1% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1x protease inhibitor cocktail tablet).

- 10 Transfer the solution to a 1.5 ml Eppendorf tube and proceed to centrifugation at 12,000× g for 10 min at 4°C.
- 11 Gently, remove the supernatant and resuspend the pellet in 300 µl of extraction buffer 3 (1.7 M sucrose, 10 mM Tris-HCl pH 8, 2 mM MgCl<sub>2</sub>, 0.15% Triton X-100, 5 mM β-mercaptoethanol, 0.1 mM PMSF, 1x protease inhibitor cocktail tablet) and layered on top of 1 ml of the same extraction buffer.



- 12 Centrifuge for 1 h at 12,000 × g at 4°C.
- 13 Remove supernatant and resuspend the chromatin pellet in 300 µl Nuclei Lysis Buffer (50 mM Tris-HCl pH 8, 10 mM EDTA, 1% SDS, 1x protease inhibitor cocktail tablet).  
**Note: Keep 5 µl aliquot on ice and add 5 µl of H<sub>2</sub>O for the agarose gel analysis. This will represent 'unsheared' chromatin control.**
- 14 Sonicate the chromatin solution. A time course sonication experiment should be performed to assess the number of cycles that provide suitable DNA fragment sizes.

Example:



For the time course sonication experiment start with 300 µl of sample and select the cycles you wish to test. After each cycle collect 10 µl aliquot of the sheared DNA for agarose gel analysis.

- 15 Pellet cell debris by centrifuging at 12,000 x g for 10 minutes at 4°C.
- 16 Transfer the clear supernatant to a new 1.5 ml vial (approximately 300 µl).

## 16.1 Analysis of sonication efficiency

### 1. Reverse Crosslinking

-5 µl unsheared DNA sample aliquot + 5 µl water collected on step 13 AND 10 µl sheared DNA sample obtained on step 14.

-Add 0.67 µl NaCl 4 M.

-Incubate overnight at 65°C.

### 2. RNase and Proteinase K treatment

-For each sample add:

2.5 µl EDTA 0.5 M

5 µl Tris-HCl pH 6.5 1 M

2 µl RNase (20 mg/ml)

82 µl H<sub>2</sub>O

-Incubate 30 min at 42°C .

-Add 0.5 µl Proteinase K (20 mg/ml).

-Incubate 90 min at 42°C.

### 3. Phenol-chloroform isoamyl pH 8

-Add 100 µl H<sub>2</sub>O.

-Add 200 µl Phenol-chloroform-isoamyl alcohol (25/24/1) pH 8.

-Spin for 10 min at full speed.

-Keep the supernatant.

### 4. DNA precipitation

- Add to supernatant:
  - 20 µl NaAc pH 5.2 3 M
  - 2 µl glycogen 500 µl EtOH 96%
- Incubate at – 80°C for 15 minutes.
- Spin 20 min at full speed.
- Remove the supernatant and wash with EtOH 70% (500µl).
- Spin 10 min at full speed.
- Remove the supernatant and dry the pellet.
- Resuspend the pellet in 7 µl H<sub>2</sub>O.
- Estimate the sonication efficiency and chromatin concentration by agarose gel electrophoresis (1 % TAE gel).

- 17 Remove 50 µl to a clean tube and set aside at –20 °C to serve as the 'input DNA control'.

## Chromatin immunoprecipitation

- 18 Dilute the sample 10x with ChIP dilution buffer (1.1 mM Tris-HCl pH 8, 1.2 mM EDTA, 167 mM NaCl, and 1.1% Triton X-100).
- 19 Divide the samples into two tubes and add 50 µl Anti-FLAG<sup>®</sup> M2 magnetic beads (Sigma, USA) to the chromatin solution and incubate at 4 °C overnight with rotation.
- 20 After recovering the beads, wash twice with Low Salt Buffer (150 mM NaCl, 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8) for 5 min at 4 °C .
- 21 Wash the beads with High Salt Buffer (200 mM NaCl, 0.1 %SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-HCl pH 8) twice for 5 min at 4 °C .
- 22 Wash the beads with LiCl wash buffer (50 mM LiCl, 1% NP-40, 1% sodium deoxycholate, 1 mM EDTA, 10 mM Tris pH 8) twice for 5 min at 4 °C .
- 23 Wash the beads with TE buffer (10 mM Tris-HCl pH 8, 1 mM EDTA) twice for 5 min at 4 °C .
- 24 After the washing steps, carefully remove as much TE buffer as possible from the beads.
- 25 To elute the DNA complexes add 250 µL elution buffer (1% SDS, 10 mM EDTA, 0.1 M NaHCO<sub>3</sub>) to the beads, vortex briefly, and incubate at 65°C for 15 min with gentle agitation.





- 26 Combine the two supernatants into a single tube.
- 27 Reverse the cross-linking to the samples (including the input control samples set aside in Step 17+500 µl of elution buffer) by incubating at 65°C for 6 hours with the addition of 20 µL of 5 M NaCl.
- 28 Add to the samples 10 µl of 0.5 M EDTA, 20 µl 1 M Tris-HCl (pH 6.5), and 2 µl of 10 mg/ml proteinase K, and incubate at 45°C.
- 29 Extract the DNA with phenol-chloroform-isoamyl alcohol (25/24/1) pH 8 and recover by precipitation with ethanol in the presence of 0.3 M NaAc (pH 5.2) and 2 µl glycogen carrier (10 mg/ml) as explained before. Wash the DNA pellets with 70% ethanol and resuspend each pellet in 20 µl of sterile distilled water.

## Protocol references

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