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Innovating Epigenetics Solutions

Chromatin EasyShear Kit for Plant

Previous name: Chromatin Shearing Optimization Kit for Plant

Cat. No. C01020014

USER GUIDE

V3 04_2021

Please read this manual carefully
before starting your experiment

Summary

Introduction

The first critical step of a successful Chromatin Immunoprecipitation (ChIP) experiment is the preparation of sheared chromatin. We therefore suggest the use one of our optimized Shearing ChIP kits.

- Chromatin EasyShear Kit - Ultra Low SDS (Cat. No.C01020010)
- Chromatin EasyShear Kit – Low SDS (Cat. No. C01020013)
- Chromatin EasyShear Kit - High SDS (Cat. No.C01020012)
- Chromatin EasyShear Kit for Plant (Cat. No.C01020014)

Our Chromatin EasyShear kits are used in combination with the Bioruptor in order to ensure a highly reproducible chromatin shearing and make sure you obtain the right fragment size needed for your experiment. Establish optimal conditions required for shearing cross-linked chromatin (protein-DNA) is usually laborious; the protocol of the Chromatin EasyShear kits is fast, easy-to-use and optimized to get the best results possible.The ChIP assay offers great potential to improve knowledge about the regulation of gene expression. This technique is now used in a variety of life science disciplines including cellular differentiation, tumor suppressor gene silencing, and the effect of histone modifications on gene expression. Isolation and shearing of the chromatin after formaldehyde fixation is the first step in the ChIP method. The following two steps are: 1/ specific immunoprecipitation of chromatin fragments using antibody against the protein of interest and 2/ analysis of the immunoprecipitated DNA fragment. With the present kit, the first step of the ChIP method is guaranteed, providing you with the best results. Using standard protocols and kits leads to a significant decrease in potential variables that can occur from shearing to ChIP, which makes interpretation and analysis from experiment to experiment much more accessible.

Kit Materials

The content of the kit is sufficient to perform 12 chromatin extractions and chromatin shearing optimization reactions. Store the components at the indicated temperature upon receipt (Table 1).

Table 1: Components supplied with the kit

Description	Quantity	Storage
10x Crosslinking Buffer	36 ml	4°C
Crosslinking bags for plant tissue	15 pc	room temperature
Glycine	30 ml	4°C
4x Extraction buffer 1	90 ml	4°C
Extraction buffer 2	300 ml	4°C
Extraction buffer 3	60 ml	4°C
Sonication Buffer	7.2 ml	4°C
Elution Buffer 1	1.2 ml	4°C, incubate at room temperature before use
Elution Buffer 2	48 µl	4°C
DNA precipitant	120 µl	4°C
DNA co-precipitant	60 µl	-20 °C
TE buffer	240 µl	4°C

Required Material Not Provided

- Formaldehyde (high-quality, ex: Sigmaaldrich)
- Miracloth (Calbiochem)
- Funnels (adapted to 50 ml falcon tubes)
- Vacuum pump
- Desiccator
- Liquid nitrogen
- dH2O
- Ceramic mortar and pestle
- 50 ml falcon tubes
- 1.5 ml standard tubes
- 1.5 ml Safe-Lock tubes
- 1.5 ml Bioruptor tubes (Depending on the Bioruptor® model used)
- Bioruptor®
- Thermomixer
- Rotating wheel
- Phenol/Chloroform/Isoamylalcohol {25:24:1}
- Chloroform/Isoamylalcohol {24:1}
- ß-Mercaptoethanol
- Protease inhibitors (recommended: Protease Inhibitor Cocktail for plant cell and tissue extracts P 9599 from Sigma)
- Pincer, Scalpel & blades, spatula etc...
- Towel papers

Kit Method Overview & Time Table

Step		Time needed
STEP 1	Crosslinking of plant tissue	1 to 2 hours
STEP 2	Chromatin extraction from plant tissue and chromatin shearing	2 to 5 hours
STEP 3	Chromatin shearing analysis	1 day

Remarks Before Starting

Please read the complete manual very carefully before starting. It contains many details which are very important and essential for the proper conduct of experiments. We highly recommend using the detailed protocol for your first assays. You can use the short protocol after being experienced with this kit.

1. Starting material

This kit has been tested and validated for both monocots and dicots plants. It was optimized for many model plant species belonging to many plant families and several types of tissues (see table 2).

Depending on the plant tissue and/or species, the sampling method can be different. In the majority of cases, we recommend cutting samples into small pieces in order to facilitate the fixation with formaldehyde. A cold support such as a glass plate on ice can be used to cut the samples:

Arabidopsis seedlings (two weeks old) can be crosslinked intact. There is no need to cut them before crosslinking.

Arabidopsis adult rosette leaves can be sampled from 25 days old seedlings (18 leaves stage) and cut to small pieces of approximately 5 mm.

For maize cut the inner stem of 2-3 weeks old seedlings into “cylinders” of approximately 2 to 3 mm thick using a sterile blade.

For maize husk material, about 3 months old plants are used. The husks are sampled when the first silks begin to emerge from female inflorescence. The outer lignified layers are discarded. The rest of the hush is cut into pieces of about 2 to 3 mm thick using a sterile blade.

For rice cut the seedlings into fragments of about 3 to 4 mm using a blade or sterile scissors then homogenise all organs (leaves, roots ...) with a pincer.

For tomato, cut young leaves into “discs” of about 4 to 5 mm using a blade or sterile scissors.

Tomato anthesis are sampled during the meiotic phase (buds between 3 to 6 mm). There is no need to cut anthesis.

For in vitro poplars, cut the aerial part of the plant (leaves and stem) into pieces of about 2 to 4 mm and homogenize pieces from different organs.

For poplar plants grown in greenhouse, cut the stem at 1 to 2 cm above the culture substrate using loppers. Remove the apical part of the plant which has a thin and soft stem and keep the rest. Remove the leaves and make a longitudinal "wound" along the stem using a sterile blade, this will facilitate the removal of the bark. Before removing the bark, cut the stem into "cylinders" of about 10 cm using loppers and keep them on ice. The bark can be removed immediately before the crosslinking.

For poplars grown in field, cut young branches that have a stem diameter greater than 1 cm using loppers. Prepare samples exactly as described for poplar plants grown in greenhouse.

If you work on the same or similar plant material, the sampling method can be similar to this description. If you work on a completely different tissue, the sampling procedure should be optimized.

The optimal weight of plant tissue for ChIP experiments depends on many factors:

- Plant species and/or families: ex herbaceous vs woody plants.
- Plant organs: different organs (leaves, roots, flowers etc...) may contain different cell types that yield more/less chromatin.
- Age and/or size of the cells: the younger cells are usually smaller with a thinner cell wall, therefore, for the same weight of plant material, the younger tissue contains more cells and by consequence more nuclei/chromatin that are more easily available compared to older cells.
- Type of the cells: ex somatic cells vs sex organs
- The genome size: plants with a bigger genome will yield more chromatin with fewer number of cells

This protocol has been optimized for chromatin extraction from 0.1 to 2 g fresh weight (fw) of different plant materials (see examples in table 2). It is possible to start with more than 2 g of plant material; however, the volumes of extraction and sonication buffers should be optimised. One ChIP reaction is performed using 40 µl of the extracted chromatin. Consequently, many ChIP reactions can be processed from 1 chromatin extraction depending on the final volume of chromatin.

Table 2: Optimal conditions for chromatin extraction depending on the plant tissues

Plant material	Optimal weight*	Crosslinking duration	Sonication (cycles)**	Centrifuge speed ***
Arabidopsis seedlings Arabidopsis thaliana ecotype columbia (col-0)	1 g	15 min	8	1000 x g
Arabidopsis adult rosette leaves Arabidopsis thaliana ecotype columbia (col-0)	1 g	15 min	8	1000 x g
Tomato leaves Solanum lycopersicum cv. Micro-Tom	1 g	10	6-8	2100 x g
Tomato anthesis Solanum lycopersicum	65 mg	10	6-8	4000 xg
Maize inner stem Zea mays cv. B73	1.5 g	10	8-10	2900 x g
Maize husk ** Zea mays cv. B73	1.5 g	10	8-10	2900 x g
Rice seedlings (2-3 leaves stage) Oriza sativa ssp Japonica cv. Nipponbare	2 g	15 min	8-10	2900 x g
In vitro poplar plants (leaves + stem) Populus trichocarpa clone Nisqually Populus tremula x alba clone INRA 717-1B4	> 0.1 g	15 min	8	2900 x g
Poplar (stem-differentiating xylem) Populus trichocarpa clone Nisqually Populus tremula x alba clone INRA 717-1B4	2 g	15 min	10	2900 x g

* The weight indicated in the table is optimized for 1 chromatin extraction. This weight was tested and validated in ChIP using this kit.

*** The number of cycles (1 cycle = 30 s ON / 30 s OFF) indicated in the table is valid only when a Bioruptor® Pico is used for sonication (see Remarks before starting, section 3)*

**** The indicated values correspond to the centrifuge speed during the chromatin extraction step after adding the Extraction buffer 1, 2 and 3.*

***** For all plant materials cited in table 2, the concentration of formaldehyde in the crosslinking buffer is 1%, except for maize husk material for which the concentration of formaldehyde is 3 %.*

2. Crosslinking of plant tissue

Crosslinking is one of the major steps in ChIP experiments. It allows the fixation of proteins (histones, transcription factors etc...) to DNA by the fixation agent (ex: formaldehyde). Ideally, crosslinking is performed in vivo using fresh tissue in order to obtain the most accurate biological information relative to the research questions. This kit was therefore optimized on fresh/living plant material. We note that it may be possible to crosslink frozen samples. For this, the crosslinking/sampling conditions have to be optimized.

Inefficient crosslinking causes DNA loss and/or reduced antigen availability in chromatin. However Over-crosslinking can result in inefficient shearing and in non-specific binding events. The optimal cross-linking time for maximal specificity and efficiency of ChIP should be determined empirically. A crosslinking time course (ex: 5-10-15 min) can be performed.

For the majority of plant tissues, crosslinking is performed under vacuum which permits a more efficient fixation. In fact, the cell wall may inhibit the penetration of formaldehyde.

The Crosslinking buffer provided in this kit was optimized to yield more chromatin after nuclei preparation in combination with the chromatin extraction protocol of this kit.Crosslinking of plant material is a delicate step, especially the washes following the fixation (see STEP 1. Crosslinking of plant tissue, 7). When removing the remaining crosslinking buffer after each wash, it is very difficult to avoid losing plant material as small tissue fragments can be lost. To render this step easy to perform, we provide in the kit crosslinking bags for plant tissue with an easy closure system. Crosslinking bags will avoid any loss of material and will significantly facilitate the crosslinking, the washes and sample handling after fixation.

Please note that provided crosslinking bags are not adapted to powder (ground plant material).

3. Shearing optimization and sheared chromatin analysis

Before starting the immunoprecipitation, the chromatin should be sheared to fragments ranging between 200 and 600 bp approximately (see figure 1 below). The shearing conditions mentioned in the protocol were optimized using the Bioruptor® Pico (#B01060001) in combination with a water cooling system (#B02010003; 115V or #B02010002; 230V). We recommend using 1.5 ml Bioruptor® Microtubes with Caps (#C30010016) for use with the Bioruptor® Pico. If you use a different sonicator, the sonication conditions must be optimized accordingly. The shearing conditions mentioned in the protocol are adequate for chromatin extracted from plant tissues we tested using this kit. If you work with a different plant tissue(s) the sonication conditions should be optimized for each tissue systematically before processing a large number of samples. If you work with similar plant material, these conditions will help you to approach the optimal sonication conditions for your tissue. However, we highly recommend optimizing the shearing conditions before doing any new experiment. In fact, the shearing efficiency depends on numerous factors other than the sonication device and the plant material such as the sample volume, the chromatin concentration and/or density, the fixation time, etc...

An initial time-course experiment of 6-8-10-12 sonication cycles 30'' ON/30'' OFF is recommended when starting a new ChIP project using the Bioruptor Pico. In some experimental conditions, even a shorter sonication time of 2-3 cycles might result in satisfactory chromatin shearing. As best practice, choose the shortest sonication time resulting in a satisfactory ChIP efficiency (highest recovery/lowest background). Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments, especially when non-histone proteins are to be evaluated by ChIP. A guide for successful chromatin preparation using the Bioruptor® Pico is available on the Diagenode website.

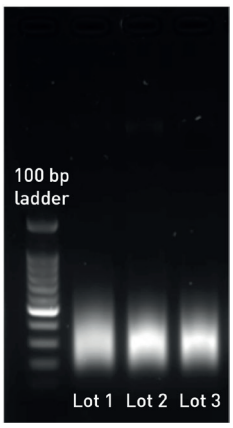


Figure 1

The figure shows gel electrephoresis of sheared chromatin of 3 independent nuclei preparations from maize inner stem. The sonication was performed using Bioruptor Pico (8 cycles: 30s ON / 30s OFF),

4. Magnetic beads

This kit includes DiaMag protein A-coated magnetic beads (cat. No. C03010020). Make sure the beads do not dry during the procedure as this will result in reduced performance. Keep the beads in a homogenous suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility. Do not freeze the beads. The amount of beads needed per IP depends on the amount of antibody used for the IP. The protocol below uses 20 µl of beads. The binding capacity of this amount is approximately 5 µg of antibody. With most of Diagenode's high quality ChIP-seq grade antibodies the recommended amount to use is 1 to 2 µg per IP reaction. However, if you plan to use more than 5 µg of antibody per IP we recommend increasing the amount of beads accordingly.

5. DNA purification after ChIP

The DNA purification after ChIP reaction is performed using the IPure kit v2 (#C03010015) included in this kit in this protocol (STEP4. Elution, de-crosslinking and DNA isolation). The IPure kit v2 gives high quality DNA for PCR and library preparation for next generation sequencing. It was also tested and validated for all plant tissues / or species indicated in table 2.

It's highly recommended to quantify the eluted DNA using a Qubit for example. This will give a first idea about the success of ChIP as the recovered DNA quantity should be different between IgG or no antibody control (low), Input (high) and sample of interest (usually in between).

6. Quantitative PCR analysis and data interpretation

For qPCR analysis, depending on the concentration/quantity of immunoprecipitated DNA, use 3 µl of purified DNA and Input samples as template. The quantity of immunoprecipitated DNA for one PCR reaction is generally comprised between 100 and 500 pg (indicative values). However it still possible to perform PCR using 30 pg or up to 3 ng of DNA in some cases. You can follow the manufacturer's recommendations relative to template DNA concentration depending on your qPCR system and/or reagents.

The efficiency of chromatin immunoprecipitation of particular genomic loci can be expressed as the recovery of that locus calculated as the percentage of the input (% Input: the relative amount of immunoprecipitated DNA compared to input DNA). The enrichment of immunoprecipitated DNA on target fragments can also be calculated in comparison with the negative control (IgG or No Antibody control). In this case, it's considered as Fold Enrichment. The enrichment can also be normalized to a reference gene (Normalized Enrichment). See below formulas for each type of calculation. These equations assume that the PCR is 100 % efficient (amplification efficiency = 2). For accurate results the real amplification efficiency, if known, should be used.

> ***NOTE**: It is important to verify the Melting Peaks of each primer for each sample before any calculation. For some samples, especially IgG negative control, when DNA quantity is low, the primers may have tendency to make dimers resulting in a false peak (and as a consequence a false Ct value). In order to avoid this problem, use enough DNA for PCR. The Melting Peaks of IgG and samples should be superposed with those of the Input (which generally has a higher DNA concentration).*

% Input = (100/Df) x [2^(Ct Input - Ct Sample)]

Df: dilution factor of the Input i.e. if Input is 1% (see STEP 3. Magnetic Immunoprecipitation, 33) the Df is 100. If Input is 10 %, the Df is 10 etc...

Ct: Ct values are the threshold cycles from the exponential phase of the qPCR

Fold Enrichment = 2^(Ct Sample – Ct Nc)

Nc: negative control IgG or No Antibody control

Normalized Enrichment = 2^(Ct Sample – Ct Reference gene)

7. Negative and positive IP controls

The kit contains a negative (IgG, cat. No. C15410206) and a positive (H3K4me3, cat. No. C15410003) control antibody. We recommend including one IgG negative IP control in each series of ChIP reactions. We also recommend using the positive control ChIP-seq grade H3K4me3 antibody at least once. The kit contains qPCR primer pairs for amplification of a positive and negative control target for H3K4me3 in Arabidopsis thaliana (Arabidopsis FLC-ATG and Arabidopsis FLC-Intron1, respectively). Positive and negative control primer pairs for H3K4me3 in other plant species are also available but not included in the kit. They can be purchased separately (see table 1b).

8. Preparation of buffers

Check Elution buffer 1 for precipitation upon storage. If necessary, warm to 37°C until the precipitate has fully dissolved.

Crosslinking buffer and ChIP Dilution Buffer are provided as 10x concentrates. Extraction buffer 1 is provided as 4x concentrate. Before use prepare the necessary amount of 1x buffer using dH20.

Protease inhibitors, PMSF, and Ð-mercaptoethanol should be added freshly, where stated.

Short Protocol For Experienced Users

STEP 1. Crosslinking of plant tissue

1. Prepare 1x Crosslinking Buffer containing 1 % formaldehyde and store it on ice.

1x Crosslinking buffer	
10x Crosslinking buffer	3 ml
Formaldehyde	1 % (ex: 811 µl of Formaldehyde solution 37 %)
dH2O	Up to 30 ml
Total volume	**30 ml**

2. Harvest the plant material and wash it with dH2O. Dry it using towel papers and keep it on ice.

3. Weigh the plant material and put it in a crosslinking bag. Close and keep on ice.

4. Introduce the crosslinking bag to the crosslinking buffer in 50 ml Falcon tube.

5. Crosslink under vacuum in a desiccator pre-filled with ice during 10 to 15 min.

6. Replace 2.5 ml of the crosslinking buffer by 2.5 ml of Glycine. Mix the solution and apply vacuum for additional 5 min.

7. Discard the Crosslinking Buffer and wash 3 times with cold deionized water.

8. Dry plant material using paper towels and put it into a new 50 ml tube. Close the tube and snap-freeze in liquid nitrogen.

STEP 2. Chromatin extraction from plant tissue and chromatin shearing

9. Calculate the necessary amount of 4x Extraction buffer 1 (7.5 ml/sample), Extraction buffer 2 (25 ml/sample) and Extraction buffer 3 (5 ml/sample).

10. Prepare complete 1x Extraction buffer 1 by diluting the 4x Extraction buffer 1, Extraction buffer 2 and Extraction buffer 3 as indicated below and keep them on ice.

Complete Extraction buffer 1	
4x Extraction buffer 1	7.5 ml
dH2O	22.5 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 5 mM
Phenylmethylsulfonylfluoride (PMSF)	Add to a final concentration of 0.1 mM
Total volume	**30 ml**

Complete Extraction buffer 2	**(Volume for 1 sample)**
Extraction buffer 2	25 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 10 mM
Total volume	**25 ml**

Complete Extraction buffer 3	**(Volume for 1 sample)**
Extraction buffer 3	5 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 10 mM
Total volume	**5 ml**

11. Grind the plant tissue in liquid nitrogen to a fine powder. Transfer the powder to an empty pre-cooled 50 ml tube.

12. Take the tubes out of the liquid nitrogen, wait 15 s and add 30 ml of cold Complete Extraction Buffer 1.

13. Incubate the tubes at 4°C in a rotating wheel for at least 15 min and up to 1 h.

14. Filter the suspension twice through 1 layer of Miracloth (Calbiochem) and collect the flow-through in a new 50 ml tube on ice.

15. Centrifuge for 20 min at 4 °C at 1000 to 2900 x g (see table 2). Discard the supernatant and avoid losing any part of the pellet.

16. Wash the pellet containing the nuclei 5 times with 5 ml Complete Extraction Buffer 2. After each wash, centrifuge for 5 min at 4 °C at the same speed used in the previous step and discard the supernatant.

17. Wash once with 5 ml Extraction Buffer 3 as in step 15. Centrifuge for 5 min at 4 °C and discard the supernatant.

18. Centrifuge at 4 °C for 30s. Remove the rest of the extraction buffer 3 with a pipette.

19. Resuspend the pellet in 600 µl of Sonication Buffer (supplemented with protease inhibitors) by pipetting and transfer the suspension to a 1.5 ml Safe-Lock tube.

20. Incubate the 1.5 ml tube at 4°C in a rotating wheel for 15 min.

21. Transfer the lysate to a sonication tube (maximum 300 µl per tube).

22. Sonicate the chromatin with the Bioruptor® Pico for 6 to10 cycles (see table 2) with the settings 30 sec ON / 30 sec OFF at 4 °C.

23. Transfer the sonicated chromatin to a standard 1.5 ml tube and centrifuge for 5 min at 18.000 x g at 4 °C.

24. Transfer the supernatant to a new 1.5 ml tube.

> ***NOTE**: To identify correctly the fragment size distribution, sheared chromatin should be decrosslinked (see step 3)*

STEP 3. Magnetic Immunoprecipitation

25. Identify the number of ChIP reactions (nc) you will perform (don't consider the Input sample) and the number of antibodies (na) you will use including IgG.

26. Prepare the necessary volume (V) of 1x ChIP Dilution buffer as indicated.

V (µl) = (nc x 302) + [600 x (na + 1)]

1x ChIP dilution buffer	**(maximum volume for 1 sample)**
10x ChIP dilution buffer	150 µl
dH2O	1350 µl
Protease inhibitors	Add according to manufacturer's instructions
Total volume	**1.5 ml**

27. Calculate the necessary amount (ov1) of DiaMag protein A-coated magnetic beads (20 µl per ChIP reaction) and transfer it to a 1.5 ml tube. Place the tube in the DiaMag1.5-magnetic rack. Wait 1 min and remove the supernatant by pipetting.

28. Wash the beads 3 times with 200 µl of ice-cold 1x ChIP Dilution Buffer.

29. After the last wash, resuspend the beads in the original volume + 1 µl per ChIP reaction [ov1 + (nc x 1 µl)] by adding 1x ChIP Dilution Buffer and keep the tube on ice.

30. Prepare one Safe-Lock 1.5 ml tube per antibody including negative control IgG. Add the necessary amount (ov2) of washed beads (20 µl per ChIP reaction for each Antibody / IgG). For each tube, if the final volume is less than 100 µl, fill up with 1x ChIP Dilution Buffer.

31. Add x µl of antibody to the beads and incubate 4h to overnight on a rotating wheel at 4°C.

32. Wash the antibody coated beads 3 times with 200 µl 1x ChIP Dilution Buffer. After the last wash discard the supernatant and resuspend the beads in the original volume (ov2) + 1 µl per ChIP reaction using 1x ChIP Dilution Buffer and keep the tube on ice.

33. Per ChIP reaction: Dilute 40 µl of the sheared chromatin by adding 160 µl of cold 1x ChIP Dilution Buffer. Add protease inhibitors and mix thoroughly. Place 2 µl of the diluted chromatin aside (-20 °C) to be used as INPUT later.

34. Per ChIP reaction, add 20 µl of Antibody coated magnetic beads to the chromatin. Incubate 4h to overnight in a rotating wheel at 4 °C.

35. After the incubation briefly spin the tubes and place them in the DiaMag1.5-magnetic rack. Wait for one minute and remove the supernatant. Wash beads once with 200 µl of wash buffer 1, 2, 3 and 4.

STEP 4. Elution, de-crosslinking and DNA isolation using IPure Kit v2

36. Resuspend the beads pellet with 100 µl of Elution Buffer 1 and transfer it into a clean 200 µl strip tubes. Incubate for 30 min at room temperature on a rotating wheel.

37. Thaw your Input sample(s) from (step 3, 33) on ice.

38. After 30 min incubation, vortex, perform a short spin and put the strip on a DiaMag02 magnetic rack and wait for 1 min.

39. Collect the supernatant, transfer it into a new 200 µl strip tubes. Add 98 µl of Elution buffer 1 to Input sample(s) and transfer it into the 200 µl strip tubes. Add 4 µl of Elution Buffer 2 to all samples (ChIP + Input).

40. Vortex well until the sample is clear again. Incubate the strip 4 h to overnight at 65 °C.

41. Add 2 µl of carrier to each IP and input sample. Vortex briefly and perform a short spin.

42. Add 100 µl of 100% isopropanol to each IP and input sample. Vortex briefly and perform a short spin.

43. Resuspend the provided IPure Magnetic beads and transfer 10 µl to each IP and input sample.

44. Vortex and incubate 10 minutes at room temperature on a rotating wheel (40 rpm).

45. Prepare the Wash buffer 1 and 2 by adding 2 ml of Isopropanol (100 %) for each.

Wash buffer 1	
Wash buffer 1 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	**4 ml**

Wash buffer 2	
Wash buffer 2 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	**4 ml**

46. Perform a short spin of the strip. Place the strip on a magnet and discard the supernatant.

47. Add 100 µl of wash buffer 1 to the beads pellet. Vortex well and incubate for 30 seconds on a rotating wheel. Spin the strip, place it on a magnet and discard the supernatant.

48. Perform a second wash with 100 µl of wash buffer 2. Incubate for 30 s on a rotating wheel. Place on a magnet and discard the supernatant.

49. Spin the strip again and place it on the magnet. Discard any remaining wash buffer 2.

50. Resuspend the beads pellet in 25 to 100 µl of buffer C.

51. Incubate at room temperature for 15 minutes on a rotating wheel. Spin the strip and place it on a magnet.

52. Transfer the eluate into new tubes and discard the beads. The recovered DNA can be used immediately for PCR and/or library preparation or stored at -20 °C.

qPCR analysis and results

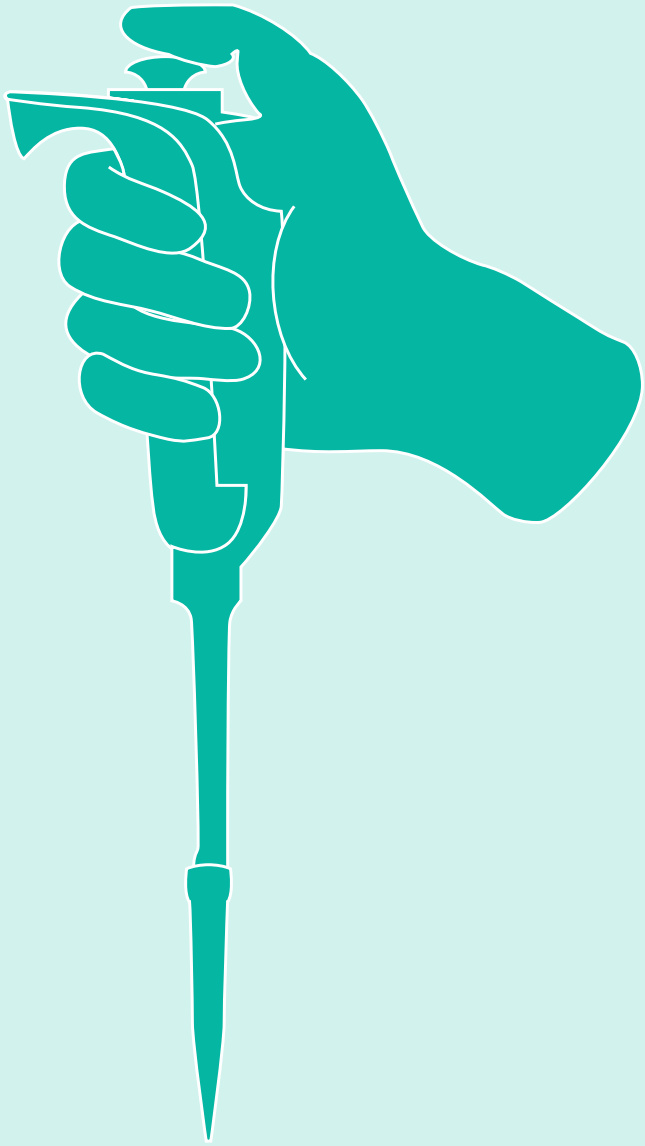
53. Prepare the qPCR master mix (total volume 10 µl = 7 µl PCR master mix + 3 µl DNA sample):

- 1 x SYBR Green PCR master mix (e.g.: iQ SYBR Green supermix)
- 0.25 µl of each primer pair (5 µM) (0.5 µl of the provided primer mix)
- Fill up to 10 µl with DNase-free water

54. Use 3 µl of purified DNA (100 to 500 pg) as template for qPCR.

55. Perform the qPCR using the following conditions: 3 to 10 min denaturation step at 95°C followed by 45 cycles of 30 s at 95 °C, 30 s at 60 °C and 30 s at 72 °C, followed by a Melting Curve step: 5 s at 95 °C, 60 s at 65 °C and 1 s at 97 °C.

DETAILED PROTOCOL



STEP 1

Crosslinking of plant tissue

***WARNING**: Steps 1.1, 1.4, 1.5, 1.6 and 1.7 should be carried out under the fume hood*

1.1 Prepare 1x Crosslinking Buffer containing 1 % formaldehyde and store it on ice.

1x Crosslinking buffer	
10x Crosslinking buffer	3 ml
Formaldehyde	1 % (ex: 811 µl of Formaldehyde solution 37 %)
dH2O	Up to 30 ml
Total volume	**30 ml**

1.2 Harvest the plant material (a description of the sampling procedure of each tested tissue and/or species is available in the section remarks before starting, Starting material). Wash the plant material with dH2O after harvesting. Dry it using towel papers and keep it on ice until the crosslinking step in order to protect tissues from dryness. Plant material should not be in direct contact with ice.

1.3 Weigh the necessary quantity of plant material mentioned in table 2 and starting material section. If the goal is to optimise the shearing efficiency or to compare many treatment conditions the weight should be exactly the same for all samples in order to have reliable results. Put the weighted sample in a crosslinking bag, make a node and pull simultaneously both ends of the thread. A second node can be made in order to close correctly the bag. Keep the crosslinking bag with the sample inside on ice until starting the crosslinking.

1.4 Introduce the closed crosslinking bag to the 50 ml falcon tube containing the crosslinking solution supplemented with formaldehyde. Make sure that the bag is submerged in buffer and not floating on top of it. A clean stainless steel paper clip can be fixed in the top of the crosslinking bag. This will somehow "lock" the crosslinking bag in the bottom of the tube and prevent it from floating on top of the solution. All samples should be introduced simultaneously to the 50 ml falcon tubes containing the crosslinking buffer supplemented with formaldehyde. Prolonged incubation of samples weighted first in crosslinking solution before applying the vacuum may induce variability.

1.5 Put the 50 ml falcon tubes containing samples in the desiccator (pre-filled with ice). During the crosslinking under vacuum, the tubes have to be opened. Otherwise, vacuum cannot be applied to the plant material. Close the desiccator lid and crosslink the plant tissue under vacuum (~ - 950 Millibars) during 10 to 15 min depending on plant tissue and/or specie (see table 2). If you use a different plant material and/or a different vacuum pressure, the crosslinking conditions should be optimized before doing any large scale fixation.

1.6 Turn off the vacuum pump and release the vacuum slowly. Replace 2.5 ml of the crosslinking buffer by 2.5 ml of Glycine. Mix the solution and apply vacuum for additional 5 min. Release the vacuum slowly.

1.7 Discard the Crosslinking Buffer following the legislation relative to dangerous chemical products of your country. Add 40 ml of cold deionized water (4 °C; not supplied), close the tube and mix. Discard the water with dangerous products as it may contain formaldehyde. Repeat the washing step twice (3 washes in total).

1.8 Take the crosslinking bag out of the 50 ml falcon tube. Open the bag or cut it with scissors, remove plant tissue and dry it thoroughly using paper towels. Insert the plant tissue into new 50 ml tubes, close the tubes and snap-freeze them in liquid nitrogen. At this point the tissue can be stored at - 80 °C for several months or directly used for chromatin extraction.

***NOTE**: For poplar plants grown in greenhouse and/or field, remove the bark from stem fragments. Place a clean box (ex: clean empty tips box) on ice in the desiccator and fill it with stem fragments. Add the necessary volume (v1 ml) of 1x Crosslinking Buffer supplemented with formaldehyde until all fragments are covered by the solution. A clean weight can be placed on top of the box to prevent stem fragments from floating. Crosslink the poplar tissue under vacuum (+/- 950 Millibars) during 15 min. If you use a different vacuum pressure, the crosslinking time should be optimized.*

*Turn off the vacuum pump and release the vacuum slowly. Remove a volume (v2) (v2 = 0.08 * v1 ml) from the crosslinking buffer and replace it with Glycine solution (ex: from 100 ml crosslinking solution remove 8 ml and add 8 ml of Glycine) to stop the crosslinking. Apply vacuum for additional 5 min. Release the vacuum slowly.*

Discard the Crosslinking Buffer and wash the stem fragments 3 times generously with cold deionized water. Place the fragments on towel paper.

Scrape gently the outermost layer of stem fragments corresponding to the stem-differentiating xylem (SDX) using a blade. The scraped SDX should be collected in a clean recipient containing liquid Nitrogen until the end of the sampling. Weigh the SDX and store in aliquots of 2 g each at -80°C up to several months.

STEP 2

Chromatin extraction from plant tissue and chromatin shearing

***WARNING**: Buffers containing ß-mercaptoethanol should be prepared, handled and discarded under the fume hood!*

2.1 Calculate the necessary amount of 4x Extraction buffer 1 (7.5 ml/sample), Extraction buffer 2 (25 ml/sample) and Extraction buffer 3 (5 ml/sample).

2.2 Prepare complete 1x Extraction buffer 1 by diluting the 4x Extraction buffer 1, Extraction buffer 2 and Extraction buffer 3 containing protease inhibitors, ß-mercaptoethanol and PMSF (only for Extraction buffer 1) as indicated below and keep them on ice.

Complete Extraction buffer 1	**(Volume for 1 sample)**
4x Extraction buffer 1	7.5 ml
dH_2O	22.5 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 5 mM
Phenylmethylsulfonylfluoride (PMSF)	Add to a final concentration of 0.1 mM
Total volume	**30 ml**

Complete Extraction buffer 2	**(Volume for 1 sample)**
4x Extraction buffer 2	25 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 10 mM
Total volume	**25 ml**

Complete Extraction buffer 3	(Volume for 1 sample)
4x Extraction buffer 3	5 ml
Protease inhibitors	Add according to manufacturer's instructions
ß-Mercaptoethanol	Add to a final concentration of 10 mM
Total volume	**25 ml**

2.3 Fill a ceramic mortar with liquid nitrogen and add the plant tissue. Grind the plant tissue thoroughly using a mortar and a pestle to a fine powder in liquid nitrogen. Avoid letting the tissue to thaw at any time. Transfer the powder to an empty pre-cooled 50 ml tube. As it is difficult to transfer all powder using pre-cooled spatula, you can use a small volume of liquid Nitrogen to transfer the rest of the powder. Make sure that all the liquid Nitrogen is evaporated completely before closing the tube. Close the tube and keep it in liquid Nitrogen. Repeat this procedure for the other samples.

2.4 When all samples are grinded, take the tubes out of the liquid nitrogen, wait 15 s and add 30 ml (20 ml if powder weight is lower than 0.5 g) of cold Complete Extraction Buffer 1. It is important to add the Extraction buffer 1 simultaneously to all samples to prevent variability. Vortex vigorously until the tissue is completely homogenized in the buffer.

2.5 Incubate the tubes at 4°C in a rotating wheel for at least 15 min and up to 1 h. This will improve the efficiency of the chromatin extraction. The Extraction buffer 1 will lysate the cells but not the nuclear membrane and will permit a better separation between cell lysates and nuclei.

2.6 Filter the suspension twice through 1 layer of Miracloth (Calbiochem) and collect the flow-through in a new 50 ml tube on ice. The pore size of Miracloth layer is about 22 µm. The nuclei having around 10 µm diameter will pass through the pores but not the bigger cell lysates.

2.7 Centrifuge for 20 min at 4 °C at 1000 to 2900 x g depending on plant material (see table 2). The nuclei being heavier than the remaining cell lysates will remain in the bottom of the falcon tube after centrifugation. Discard the supernatant and avoid losing any part of the pellet.

2.8 Wash the pellet containing the nuclei 5 times with 5 ml Complete Extraction Buffer 2. In each washing step carefully resuspend the pellet by gentle shaking (no vortexing or pipetting). The pellet may be difficult to resuspend. Thus alternate short shaking with incubation on ice for 15s. This will help to resuspend the nuclei. If the problem persists, you can use a tip for example to mechanically scatter the pellet. After each wash, centrifuge for 5 min at 4 °C at the same speed used in the previous step and discard the supernatant without disturbing the pellet.

2.9 Wash once with 5 ml Extraction Buffer 3 as in step 15. Centrifuge for 5 min at 4 °C and discard the supernatant.

2.10 Centrifuge at 4 °C for 30s. Remove very carefully the rest of the extraction buffer 3 with a pipette. Remove only the clear liquid without disturbing the pellet. If the liquid is not clear you can centrifuge again for 30s. It is important to remove all the remaining liquid before adding the sonication buffer. Keep the tubes with the pellets on ice. At this step, the nuclei pellet can be stored at - 80 °C.

2.11 Resuspend the crude nuclear pellet in 600 µl of Sonication Buffer (supplemented with protease inhibitors) by pipetting and transfer the suspension to a 1.5 ml Safe-Lock tube. If you started with less than 0.5 g of plant material you can resuspend the pellet in 300 µl. This volume will determine the chromatin concentration and will, by consequense, affect the shearing efficiency. It is an important factor to be taken into consideration when optimizing the shearing conditions.

2.12 Incubate the 1.5 ml tube at 4°C in a rotating wheel for 15 min. During this step, the sonication buffer will permit the lysis of the nuclear membrane before sonication making the chromatin more accesible for sonication.

2.13 Transfer the lysate to a sonication tube (tubes depend on the Bioruptor® model used). Be careful not to cause bubbles by pipetting. Add maximum 300 µl per tube. If the volume is bigger than 300 µl, split it in as many sonication tubes as necessary.

2.14 Sonicate the chromatin with the Bioruptor® Pico for 6 to10 cycles depending on the plant tissue (see table 2) with the settings 30 sec ON / 30 sec OFF at 4 °C. Optimization may be required depending on the tissue type, fixation time and Bioruptor® model used. We recommend performing a time course experiment in order to specify the optimal sonication conditions (see Remarks before starting).

2.15 Transfer the sonicated chromatin to a standard 1.5 ml tube and centrifuge for 5 min at 18.000 x g at 4 °C.

2.16 Transfer the supernatant to a new 1.5 ml tube. We recommend highly measuring the concentration of chromatin after sonication using a Qubit (DNA). Following the above procedure, you should obtain between 2 and 30 µg (indicative values). This will permit to identify the chromatin yield and to estimate the number of cells (N) corresponding to the obtained chromatin. Therefore, the number of cells that will be used for each ChIP experiment (n) can be calculated (see formulas below). A calculation table is available for downloading in Diagenode website (same location as the manual)

(N) = (0.978 x Q x V x 10^6) / Gs

(n) = (N/V) x 40

N: number of cells for 1 chromatin extraction

0.978: conversion factor from bp to pg (1 pg = 0,978 * 10^9 bp)

Q: concentration of sonicated chromatin (ng/µl)

V: volume of sonication buffer used to resuspend nuclei pellet

Gs: Genome size (Mbp)

n: number of cells for 1 ChIP reaction

40: volume of chromatin for 1 ChIP reaction

At this point the chromatin can be stored at - 80 °C or directly used for the immunoprecipitation. Avoid multiple freeze/thaw cycles.

***NOTE**: To identify correctly the fragment size distribution, sheared chromatin should be decrosslinked (see step 3)*

***NOTE**: Not sheared chromatin from step 20 can also be stored at -80°C and sheared after thawing*

STEP 3

Chromatin shearing analysis

3.1 Take an aliquot of 50 µl of sheared chromatin and spin the chromatin at 12,000 rpm for 10 min at 4°C. Transfer the supernatant to a new tube for chromatin analysis.

3.2 Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of ChIP-seq grade water).

3.3 Add 2 µl of diluted RNase cocktail.

3.4 Incubate 1h at 37°C.

3.5 Add 50 µl of elution buffer 1.

3.6 Add 4 µl of elution buffer 2, mix thoroughly.

3.7 Incubate samples at 65°C for 4h (or overnight).

3.8 Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Incubate the sample at RT for 10 min on a rotating wheel.

3.9 Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

3.10 Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the sample at RT for 10 min on a rotating wheel.

3.11 Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

3.12 Precipitate the DNA by adding 10 µl DNA precipitant, 5 µl of co-precipitant, and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.

3.13 Centrifuge for 25 min at 13,000rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.

3.14 Centrifuge for 10 min at 13,000 rpm at 4°C. Carefully remove the supernatant, leave tubes open for 30 min at RT to evaporate the remaining ethanol.

3.15 Re-suspended the pellet in 20 µl of TE buffer.

3.16 Run samples (20 µl of DNA + 4 µl of 6x loading dye) in a 1.5% agarose gel.

Troubleshooting

If you face an issue not cited below or if any of the proposed solutions don't help resolving an issue, please contact Diagenode

Issue	Possible solution(s)
Non-efficient shearing (abundant fragments with high size when analyzing the shearing efficiency)	• Re-sonicate chromatin 2 to 6 additional cycles (30s ON/30s OFF) on Bioruptor Pico and analyze the shearing efficiency. • Reduce concentration of chromatin (dilute in sonication buffer) • Reduce crosslinking time (over-crosslinking may reduce shearing efficiency). • A de-crosslinking step should be included when analyzing the shearing efficiency • Check all sonication parameters related to Bioruptor (recommended tubes, volumes ...). Please visit Diagenode website
Low chromatin yield	• Grind well the plant material to a fine powder • Increase quantity of starting material • Increase incubation time (step 13 and step 20) • Remove filtration step with Miracloth especially with very small quantity of starting material or rare samples (step 14) • Reduce the volume of sonication buffer used to take the nuclei pellet (ex: 200-300 µl instead of 600 µl) (step 19)

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