diagenode

A Hologic Company

Auto Universal Plant ChIP-seq kit

Cat. No. **C01010153** (24 rxns)

Content

MANUAL

Introduction

The Diagenode IP-Star® Automated System automates immunoprecipitation and increases reproducibility

Diagenode, the leading provider of complete solutions for epigenetics research, offers a variety of end-to-end systems to streamline DNA methylation and chromatin immunoprecipitation workflows. Central to this full offering is Diagenode's Automated Systems, simple yet robust automated bench-top instruments that standardize different epigenetic applications (i.e. ChIP, MeDIP or MethylCap). Diagenode designed these automation systems to make ChIP and DNA methylation studies accessible and reproducible, and ensure consistent data in every experiment.

Diagenode Automated Systems will produce consistent results from any operator regardless of the day, the experimental run, or the lab. Robust and reproducible results is a major goal of today's high resolution epigenomic studies.

Diagenode Automated Platforms replace the numerous manual, error-prone steps of complex epigenetic applications with a reliable, highly consistent and automated process that requires minimal operator intervention. We empower researchers to simplify the tedious protocols and the complexity of many epigenetic protocols. In addition, Diagenode Automated Systems minimize sample carryover, data variability, and costly errors. The platforms offer full workflow support for epigenetics research, utilizing our complete kits and laboratory-validated protocols to rapidly deliver high-quality and consistent data.

Auto Universal Plant ChIP-seq kit

The Auto Universal Plant ChIP-seq kit was developed to enhance the utility of the ChIP procedure, allowing one to perform many more ChIPs per day and per week. The entire procedure can be performed in a single day, since two overnight incubations have been eliminated. The IP has been optimized to specifically select and precipitate the chromatin with the use of our validated antibodies, buffers and protocols. Furthermore, the use of our automated system will drastically increase the consistency of your ChIP assay.

The Auto Universal Plant ChIP-seq kit allows quick and highly specific chromatin IP sample analysis. The Auto ChIP kit protocol has been improved to allow researchers to work with smaller volumes than other traditionally used methods. The kit ensures the use of small amounts of reagents per reaction (including antibodies and buffers) and also provides you with fewer buffers in comparison with other kits.

The Auto Universal Plant ChIP-seq kit has been validated to perform ChIP-seq experiments using antibodies directed against chromatin modifications. The combination of this high quality kit and the IP-Star® allows Chromatin IP to be performed in less than 10 hours. Starting with sheared chromatin, the Automated System provides purified immunoprecipitated DNA from your sample. The Auto Universal Plant ChIP-seq kit protocol has been validated using chromatin sheared by sonication using the Bioruptor.

Customer Feedback

Not only does the IP-Star® eliminate the problem of human variation associated with producing our samples, it also enables us to produce 1000-2000 ChIP-seq samples per year very reliably. The IP-Star® reduces our processing time down from one day of manual work to just one overnight run with only 30 minutes of hands-on work. The IP-Star® has made all our ChIPs consistent and the process completely reliable regardless of the operator or the time of day.

Dr. John Lambourne, Postdoctorate Researcher at the Innovation Centre, McGill University, Canada

IP-Star® and IP-Star® Compact Systems for automation of epigenetic applications

Diagenode has developed two automated platforms (IP-Star® and IP-Star® Compact) designed to increase your lab's productivity, efficiency and experimental reproducibility. The two automated platforms are capable of processing up to 16 samples per cycle. The automated systems processes sheared chromatin (or DNA) to deliver purified DNA ready for qPCR, amplification, microarray and sequencing analysis. Both, the IP-Star® and IP-star® Compact have an easy-to-use open software that provides you with flexibility. This allows you to create your personal protocol according to your specific needs.

Major benefits of Diagenode Automated Platforms

IP-Star® Compact

IP-Star®

- High resolution ChIP-seq and MeDIP-seq profiles
- Automated library preparation for Next Generation sequencing
- Reduces hands on time to just 30 minutes
- Reduces variability between operators and labs
- Ideal for low sample starting amounts
- Compatible with Diagenode Kits
- Reduces cross-contamination

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Improved reproducibility

Our IP-Star® will increase the immunoprecipitation reproducibility between IPs performed by the same as well as by different operators (see figure 1 and 2 below). Reagents (Antibodies, buffers,...) and sheared chromatin were identical for "ManChIP" and "AutoChIP". The IP-Star® Automated system removes variation that can be created by manual handling and allows you to optimize and standardize your assay within a lab. The IP-Star® is designed to improve the accuracy and the reproducibility of any immunoprecipitiation experiment.

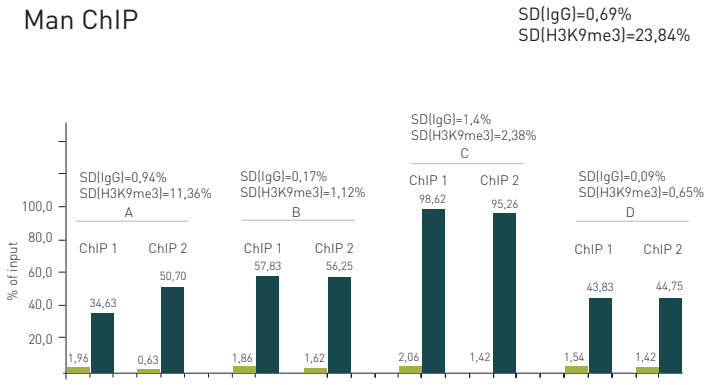

Figure 1. Manual ChIP

Four different operators have each performed two ChIP experiments using H3K9me3 antibody on the genomic region SAT2 (positive locus). 10,000 Hela cells have been used per IP. Reagents and sheared chromatin were identical per assay. The standard deviations between the ChIPs performed by the same operator and between the four different operators are displayed.

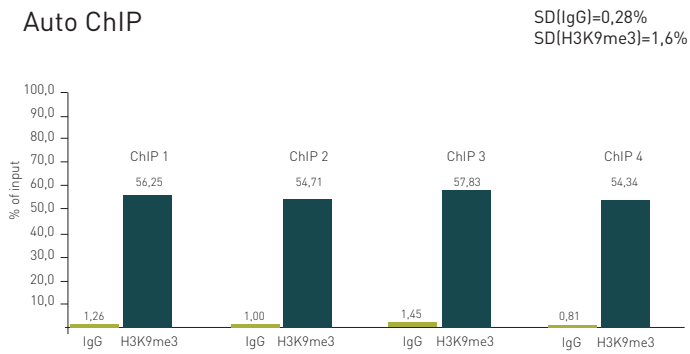

Figure 2. Automated ChIP

Four ChIP experiments using H3K9me3 antibody on the genomic region SAT2 (positive locus) have been performed by the IP-Star®. 10,000 Hela cells have been used per IP. Reagents and sheared chromatin were identical per assay. The standard deviations between the four ChIPs performed by the IP-Star® are displayed.

Kit method overview

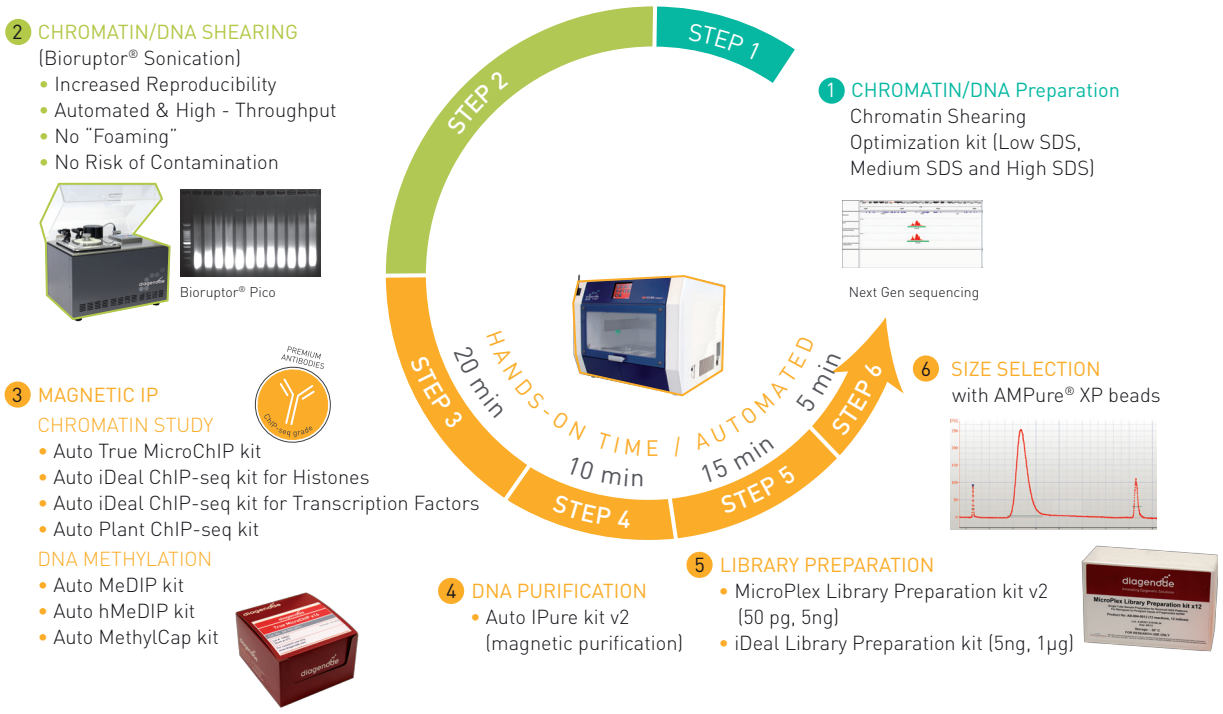

Figure 3. Diagenode provides a full suite of automated solutions for ChIP experiments

For Step 1, we offer products to isolate nuclei and chromatin. Step 2 describes reproducible sample shearing with the Bioruptor® product line. In Step 3 and Step 4, the Diagenode IP-Star Compact provides error-free, walk-away automation for all your immunoprecipitation and antibody capture needs.

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Kit materials

The Auto Universal Plant ChIP-seq Kit contains reagents to perform 8 different chromatin preparation (chromatin extractions and chromatin shearing optimization), 24 ChIP assays, and DNA Purification by using the IP-Star's Automated System. Store the components at the indicated temperature upon receipt (Table 1).

Table 1a: Components supplied with the Auto Universal Plant ChIP-seq kit

Description	Quantity	Storage
Rabbit IgG	8 µg (1µg/µl))	-20°C
ChIP-seq Grade Antibody H3K4me3	8 µg (1µg/µl))	-20°C
10x Crosslinking Buffer	24 ml	4°C
Glycine	20 ml	4°C
4x Extraction buffer 1	60 ml	4°C
Extraction buffer 2	200 ml	4°C
Extraction buffer 3	40 ml	4°C
10x ChIP Dilution Buffer	3.6 ml	4°C
Sonication Buffer	4.8 ml	4°C
DiaMag protein A-coated magnetic beads	480 µl	4°C do not freeze
Wash Buffer 1	4.8 ml	4°C
Wash Buffer 2	4.8 ml	4°C
Wash Buffer 3	4.8 ml	4°C
Wash Buffer 4	4.8 ml	4°C
Elution Buffer 1	4 ml	4°C incubate at RT before use
Elution Buffer 2	160 µl	4°C
Carrier	64 µl	-20°C
IPure Beads v2	320 µl	4°C
Wash buffer 1 w/o iso-propanol	2 ml	4°C
Wash buffer 2 w/o iso-propanol	2 ml	4°C
Buffer C	3.2 ml	4°C
Arabidopsis FLC-ATG Primer pair (positive control For Arabidopsis seedlings)	5 µM each/48 µl	-20°C
Arabidopsis FLC-Intron1 Primer pair (negative control for Arabidopsis seedlings)	5 µM each/48 µl	-20°C
Crosslinking bags for plant tissue	10	room temperature

Table 1b: Components available separately

Description	Cat. No.	Quantity	Storage
Maize B73 inner stem ZmB1-UTR primer pair	C17040004	50µl, 500µl	-20°C
Maize B73 inner stem ZmCopia primer pair	C17040005	50µl, 500µl	-20°C
Tomato leaves SlChr2-reg8 primer pair	C17040006	50µl, 500µl	-20°C
Tomato leaves SlChr4-NC1 primer pair	C17040007	50µl, 500µl	-20°C
Rice seedlings OsChr4-reg9 primer pair	C17040008	50µl, 500µl	-20°C
Rice seedlings OsMADS6 primer pair	C17040009	50µl, 500µl	-20°C
Poplar xylem PtrMYBTF1 primer pair	C17040010	50µl, 500µl	-20°C
Poplar xylem PtrCopia-orth primer pair	C17040011	50µl, 500µl	-20°C

Table 2. Kits and Modules available separately

Description	Reference	Quantity
Auto IPure kit v2 x100	C02010012	100 rnx
Chromatin Shearing Optimization Kit (Universal Plant ChIP-seq kit)	C01020014	12 rxns

Table 3. Plastics and consumables available separately

Description	Reference	Quantity
200 µl tube strips (12 tubes/strip) + cap strips	C30020001	80
200 µl tube strips (8 tubes/strip) + cap strips for SX-8G IP-Star® Compact	C30020002	120
96 well microplates for IP-Star®	C30080030	10
Tips (box)	C30040021	960
Tips (bulk)	C30040020	1000
2 ml microtube for SX-8G IP-Star® Compact	C30010014	100
Large reagent container for SX-8G IP-Star® Compact	C30020004	20
Medium reagent container for SX-8G IP-Star® Compact	C30020003	10

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)
- 200 µl strip tubes
- Bioruptor®
- Thermomixer
- Rotating wheel
- Magnetic rack (DiaMag1.5 - magnetic rack (Cat. No. B04000003)
- Magnetic rack (DiaMag02 - magnetic rack (Cat. No. B04000001)
- Phenol/Chloroform/Isoamylalcohol {25:24:1}
- Chloroform/Isoamylalcohol {24:1}
- ß-Mercaptoethanol
- Protease inhibitors (recommended: Protease Inhibitor Cocktailfor plant cell and tissue extracts P 9599 from Sigma)
- Pincer, Scalpel & blades, spatula etc...
- Towel papers

Remarks before starting

The Auto Universal Plant ChIP-seq kit is a new version of Diagenode Auto Plant ChIP-seq kit. This version replaces the previous one which is now discontinued. The Auto Universal Plant ChIP-seq kit is more efficient, more reliable, easier and gives better results than the previous one. Several modifications have been made to the protocol and the manual. Therefore, we invite you to read the current manual carefully.

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 ChIP 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 4).

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 4: Optimal conditions for ChIP experiments 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 ChIP, 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 (Cat. No. B01060001) in combination with a water cooling system (Cat. No. B02010003; 115V or Cat. No. B02010002; 230V). We recommend using 1.5 ml Bioruptor® Microtubes with Caps (Cat. No. 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.

A protocol to assess the shearing efficiency can be found in the "Additional Protocols" section. To identify the optimal shearing conditions before ChIP experiments we offer a shearing optimization kit (C01020014).

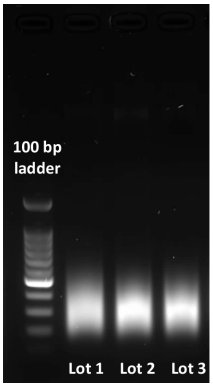

Figure 1

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 binding capacity of 20 µl of magnetic beads 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 Auto IPure kit v2 (Cat. No. C03010010) included in this kit in this protocol (STEP4. Elution, de-crosslinking and DNA isolation). The Auto 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 is 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 (diluted or undiluted) 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.

NB: 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) 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 1).

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 dH_2O.

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

IP-STAR® COMPACT

How to perform Automated ChIP on the IP-Star® Compact

MANUAL

Protocol

STEP 1. Crosslinking of plant tissue

Steps 1, 4, 5, 6 and 7 should be carried out under the fume hood!

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 (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.
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.
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.
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.
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.
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).
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.

NB: 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

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

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 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)**
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. Fill a ceramic mortar with liquid nitrogen and add the plant tissue. Grind the plant tissue thoroughly using mortar and pestle to a fine powder in liquid nitrogen. Avoid letting the tissue 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.
12. 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.
13. 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.
14. 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.
15. 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.
16. 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.
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 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.
19. 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.
20. 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.
21. 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.
22. 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, 4).
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. 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

25. Purify the DNA from 50 µl of chromatin to check the shearing efficiency on an agarose gel. **To identify correctly the fragment size distribution, sheared chromatin should be decrosslinked** (see Additional Protocols section).

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

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

STEP 3. Magnetic Immunoprecipitation

The chromatin extraction from 0.1-2 g of plant material yields 300-600 µl of sheared plant chromatin. This protocol has been optimized for 40 µl plant chromatin per IP reaction. Although it is possible to use more chromatin, we recommend performing separate IP reactions and pooling the samples together prior to DNA purification.

Determine the total number of IP's in the experiment. Please note that we recommend to include one negative control in each experiment (IP with the IgG negative control)

ChIP direct method

With this method the antibody is first coated on the surface of the magnetic beads and after that the bound antibodies are added to the sheared chromatin.Switch ON the IP-Star® Compact.

26. Select "**Protocols**" icon and then "**ChIP**" category.

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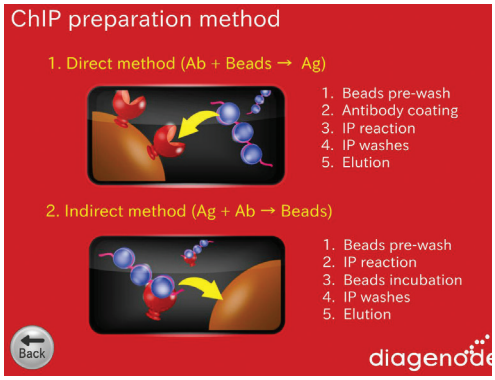
27. Select "**Direct method**" and then "**ChIP_IPure_200_D**" protocol in the list.



NOTE:

If you plan to run between 1 and 8 samples, chose "**ChIP_IPure_8_200_D**"

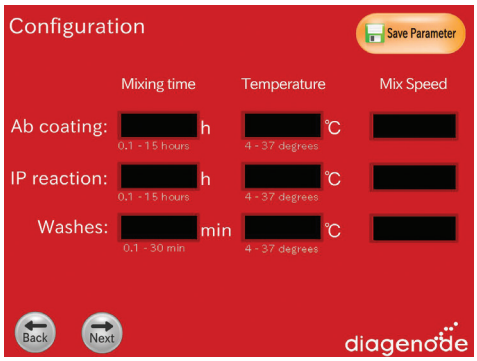
If you plan to run between 9 and 16 samples, chose "**ChIP_IPure_16_200_D**"

28. Setup the exact number of samples for your experiment. Each IP has to be counted as a sample. Input is not a sample.

Note:

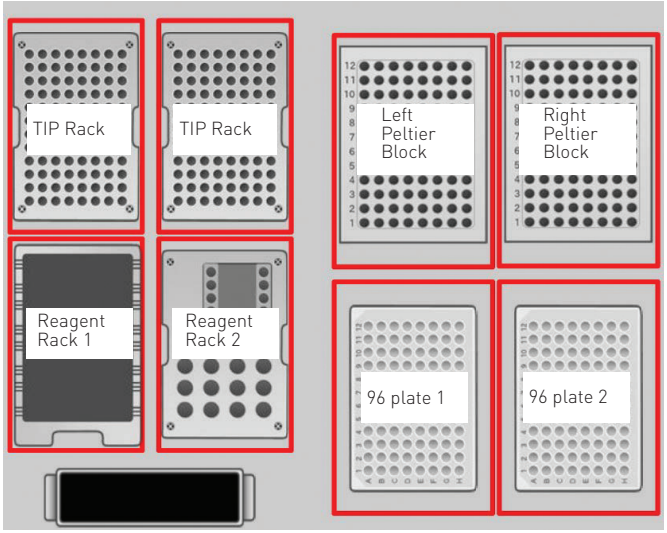
The **Peltier Block** is now cooling down to 4°C to keep your samples cold

29. Setup the parameters for your ChIP experiment and press "Next"



Setup the "Ab coating" step to 3 hours
Setup the "IP reaction" step to 8-10 hours (overnight)
Setup the "Washes" step to 5-10 min

30. Setup all the plastics on the platform according to the screen layout.

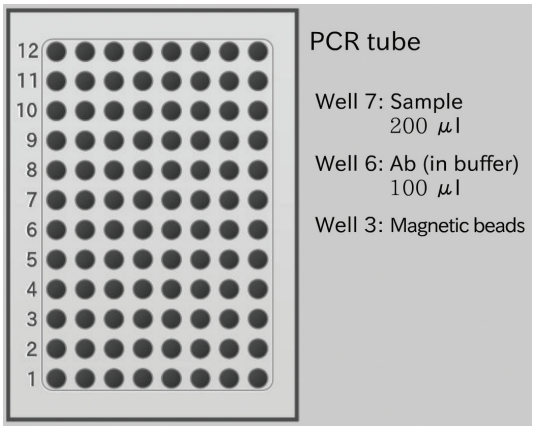


31. Fill **TIP Rack 1** (and 2 if processing 16 samples protocol) with tips according to the screen.

32. Fill **Reagent Racks** 1 & 2 with reagent containers according to the screen.

33. Fill **Peltier block** with your sample, antibody and magnetic beads as mentioned here below

Direct ChIP



34. Prepare 1x ChIP Dilution Buffer

Mix for 1 IP (460µl per IP is needed)

10x ChIP Dilution Buffer	46 µl
Water	414 µl

- If ≤ 8 samples, prepare 460 µl excess (1 IP excess)
- If ≥ 9 samples, prepare 1380 µl excess (3 IP excess)

35. Preparation of Ab coating mix (Well 6)

Antibody	x µl
1x ChIP Dilution Buffer	100 – x µl

Use 1 µl of the rabbit IgG (1µg/µl) control antibody for the negative control IP. If a positive control IP is included in the experiment, use 1 µl of the H3K4me3 (1µg/µl) ChIP-seq grade control antibody.

36. Preparation Immunoprecipitation mix (Well 7)

Sheared chromatin	40 µl
1x ChIP Dilution Buffer	160 µl
200x Plant Protease Inhibitor Cocktail	1 µl

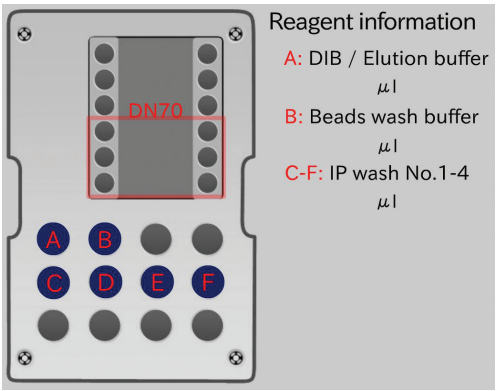
Keep 2 µl of the Immunoprecipitation mix aside for the Input

This Auto Universal Plant ChIP-seq kit has been optimized with Diagenode's high quality ChIP-grade antibodies and we use very low amounts of antibody per IP. The binding capacity of 10 µl of magnetic beads is ~3 µg of antibody. If you plan to use more than 3 µg of antibody per IP we recommend that the quantity of beads is adjusted accordingly. Please contact us for advice.

Note:

If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added to the chromatin sample.

37. Fill Reagent Racks 1 & 2 with reagent according to the screen instructions and Press "Next"



Beads Wash Buffer: 1x ChIP Dilution Buffer

Elution buffer: Elution Buffer 1

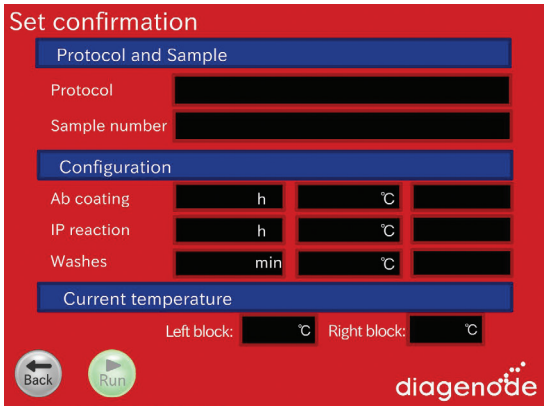
IP wash 1: Wash Buffer 1

IP wash 2: Wash Buffer 2

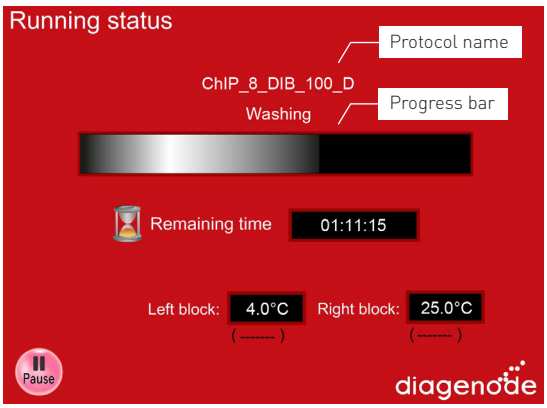
IP wash 3: Wash Buffer 3

IP wash 4: Wash Buffer 4

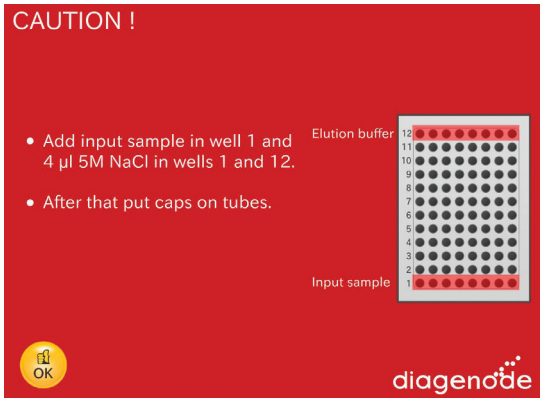
38. Check the selected parameters, close the door and press "Run" to start



39. ChIP is running. The "Remaining time" calculation will give you an estimation of the processing time of your experiment.



40. The next morning, after the overnight incubation. Recover the sample tubes and place them on the DiaMag02 magnetic rack (Cat. No. B04000001). Keep the supernatant and discard the beads.



- Setup the Input in the 1st well
  INPUT= 2 µl Immunoprecipitation mix + 98 µl Elution Buffer 1
- Add **4 µl of Elution Buffer 2** (5M NaCl) in all the samples (well 12) and inputs (well 1)
- Close the tubes with the caps, close the door and press OK

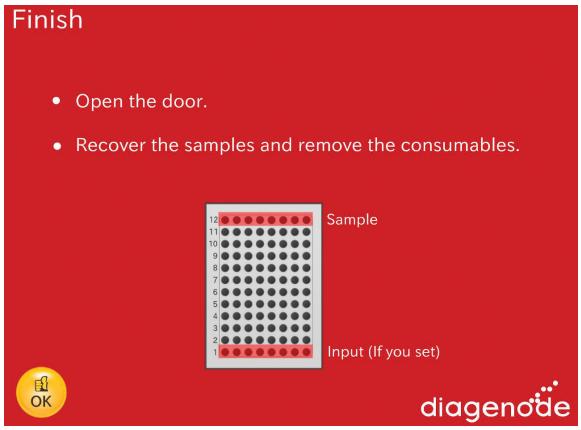
NOTE 1:

(optionnal) Proteinase K can be added for the reverse crosslinking. However, Diagenode does not provide Proteinase K.

NOTE 2:

(optionnal) RNase treatment by incubating the samples with RNase at 37°C during 30 minutes can be performed after the reverse crosslinking and it is recommended for ChIP-seq experiments. However, Diagenode does not provide RNase.

41. Recover the samples in the well 12 and inputs in well 1. Press "OK" and then "YES" to start a new run. Samples are now ready for purification.



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

After the reverse-crosslinking, DNA purification is performed using our simplified and validated Auto IPure reagents included in the Auto iDeal ChIP-seq kit for Histones and the related protocol on the IP-Star®.

42. Select "**Protocols**" icon and then "**IPure**" category.

43. Select **IPure** protocol for an elution in 50 µl and **IPure-seq** protocol for an elution in 25 µl.

Note:
If you plan to run between 1 and 8 samples, chose "**IPure_08 or IPure-seq_08**"
If you plan to run between 9 and 16 samples, chose "**IPure_16 or IPure-seq_16**"
If you plan to run between 17 and 24 samples, chose "**IPure_24 or IPure-seq_24**"

44. Setup the exact number of samples for your experiment. Each IP and input has to be counted as a sample.

Note:
The **Peltier Block** is now cooling down to 4°C to keep your samples cold.

45. Setup all the plastics on the platform according to the screen layout.

46. Add **2 µl of carrier** to each IP and input sample and place them on the Left block.

47. Resuspend and dispense 10 µl of **magnetic beads (IPure)** for each sample on the 96 well plate

Note:
Keep the magnetic beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
Make sure the beads are homogeneously in suspension at all the time during pipetting steps because the beads are precipitating rapidly.

48. Dilute **Wash Buffers 1:1 with isopropanol**

Wash buffer 1		
	24 rxns	100 rxns
Wash buffer 1w/o isopropanol	2 ml	8 ml
Isopropanol (100%)	2 ml	8 ml
Total volume	4 ml	16 ml

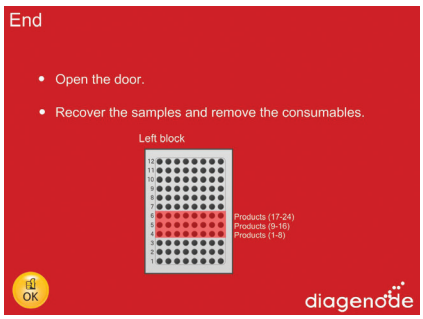
Wash buffer 2		
	24 rxns	100 rxns
Wash buffer 1w/o isopropanol	2 ml	8 ml
Isopropanol (100%)	2 ml	8 ml
Total volume	4 ml	16 ml

49. Dispense **Wash Buffers 1 & 2** with Isopropanol in the appropriate container in the IP-Star®

50. Dispense Buffer C in the appropriate container in the IP-Star®

51. Press **Run** to start

52. At the end of the run, recover your samples on the left block at 4°C



53. Press OK, remove the consumables and switch off the IP-Star®

54. Place the DNA on ice and proceed to any desired downstream applications, or store it at -20°C or -80°C until further use.

IP-STAR®

How to perform Automated ChIP on the IP-Star®

MANUAL

Protocol

STEP 1. Crosslinking of plant tissue

⚠ Steps 1, 4, 5, 6 and 7 should be carried out under the fume hood!

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 (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.
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.
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.
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.
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.
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).
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.

NB: 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

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

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 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)**
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. Fill a ceramic mortar with liquid nitrogen and add the plant tissue. Grind the plant tissue thoroughly using mortar and pestle to a fine powder in liquid nitrogen. Avoid letting the tissue 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.
12. 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.
13. 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.
14. 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.
15. 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.
16. 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.
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 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.
19. 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.
20. 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.
21. 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.
22. 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, 4).
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. 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

25. Purify the DNA from 50 µl of chromatin to check the shearing efficiency on an agarose gel. **To identify correctly the fragment size distribution, sheared chromatin should be decrosslinked** (see Additional Protocols section).

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

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

STEP 3. Magnetic Immunoprecipitation

The chromatin extraction from 0.1-2 g of plant material yields 300-600 µl of sheared plant chromatin. This protocol has been optimized for 40 µl plant chromatin per IP reaction. Although it is possible to use more chromatin, we recommend performing separate IP reactions and pooling the samples together prior to DNA purification.

Determine the total number of IP's in the experiment. Please note that we recommend to include one negative control in each experiment (IP with the IgG negative control)

ChIP direct method (Ab coating)

With this method the antibody is first coated on the surface of the magnetic beads and after that the bound antibodies are added to the sheared chromatin.

26. Prepare 1x ChIP Dilution Buffer

Mix for 1 IP (460µl per IP is needed)

10x ChIP Dilution Buffer	46 µl
Water	414 µl

- If ≤ 8 samples, prepare 460 µl excess (1 IP excess)
- If ≥ 9 samples, prepare 1380 µl excess (3 IP excess)

27. Preparation of Ab coating mix (Well 6)

Antibody	x µl
1x ChIP Dilution Buffer	100 – x µl

Use 1 µl of the rabbit IgG (1µg/µl) control antibody for the negative control IP. If a positive control IP is included in

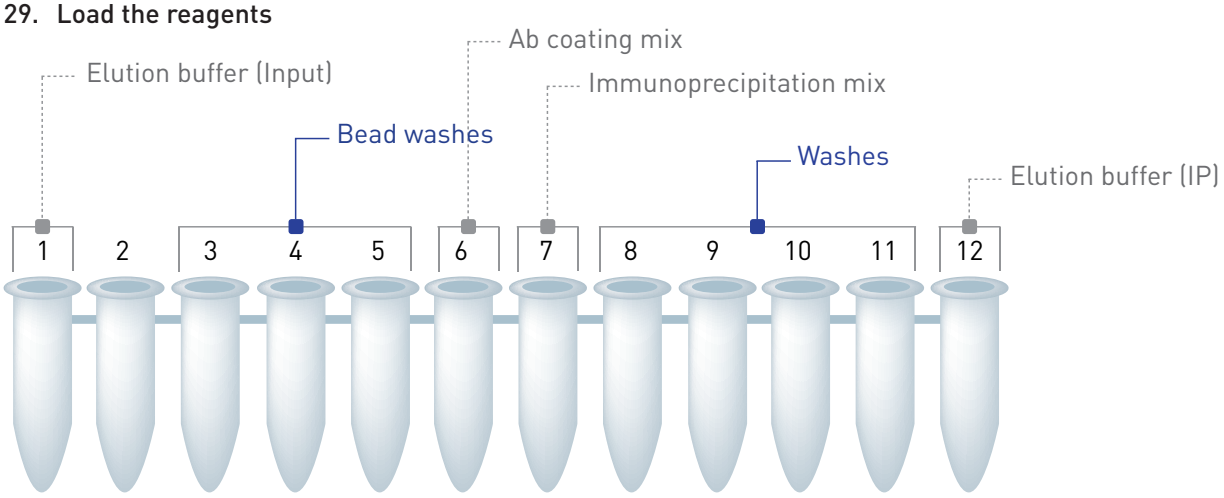
the experiment, use 1 µl of the H3K4me3 (1µg/µl) ChIP-seq grade control antibody.

28. Preparation Immunoprecipitation mix (Well 7)

Sheared chromatin	40 µl
1x ChIP Dilution Buffer	160 µl
200x Plant Protease Inhibitor Cocktail	1 µl

Keep 2 µl of the Immunoprecipitation mix aside for the Input

29. Load the reagents



	IPURE	
Tube #	Description	200 µl protocol
1	Elution Buffer 1 + Elution Buffer 2	98 µl + 4 µl
2	Empty	-
3	Magnetic beads*	10-20 µl
4	1x ChIP Dilution Buffer	100 µl
5	1x ChIP Dilution Buffer	100 µl
6	Ab coating mix	100 µl
7	Immunprecipitation mix	200 µl
8	Wash Buffer 1	150 µl
9	Wash Buffer 2	150 µl
10	Wash Buffer 3	150 µl
11	Wash Buffer 4	150 µl
12	Elution Buffer 1 + Elution Buffer 2	100 µl + 4 µl

* This Auto Universal Plant ChIP-seq kit has been optimized with Diagenode's high quality ChIP-grade antibodies and we use very low amounts of antibody per IP. The binding capacity of 10 µl of magnetic beads is ~3 µg of antibody. If you plan to use more than 3 µg of antibody per IP we recommend that the quantity of beads is adjusted accordingly. Please contact us for advice if required.

NOTE:

If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added to the Immunoprecipitation mix.

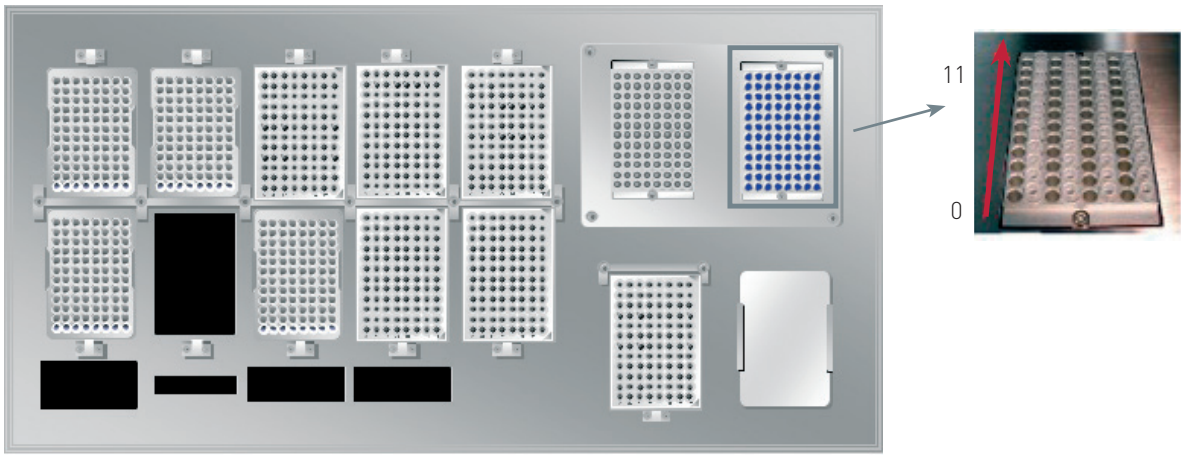
Running protocol

⚠ Be sure that the computer connected to the SX-8G IP-Star never switches to the standby modus. (standby modus has to be inactivated). Standby of the computer will lead to the abort of the protocol.

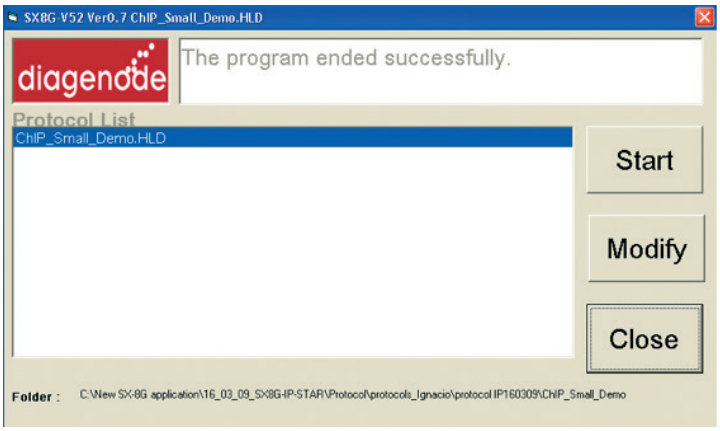
Table 3.

Protocol name	ChIP IPure 200 protocol
Reagent Preparation*	1h
Magnetic Bead Washes	30 min
Ab coating	3 hours
IP reaction	8-10 hours
Washes and elution	1h
Add reagents	15 min
DNA isolation or reverse cross-linking	4h (reverse cross-linking)
DNA recovery	ds DNA

* Input required is sheared chromatin ready-to-ChIP



1. Switch on the SX-8G IP Star. The power switch is on the right side of the instrument.
2. Switch on the computer.
3. Start SX-8G V52 software through the following icon → 
4. Place the prepared tube strip on the right cooling / heating block of the workstation
5. Close the workstation door and lock it using the following icon → 
6. Press the following icon→ 
   Select "**ChIP IPure 8 200**" protocol



IMPORTANT NOTE:

If the ChIP protocols do not appear in the screen,

1. Open the SX-8V52 directory
2. Open Easy start ini file. Write the directory location of the protocols

The Easy start ini file should contain the following information:

MANUAL

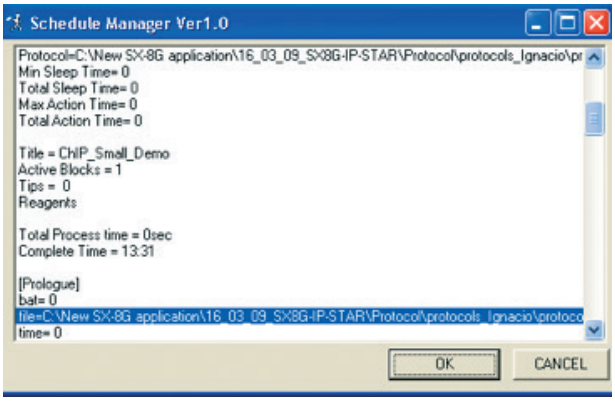
*[**EASYSTARTSCREEN**]*

*HoldFilePath = **C:\Documents and Settings\Desktop\New software protocols\ChIP\Ab Coating***

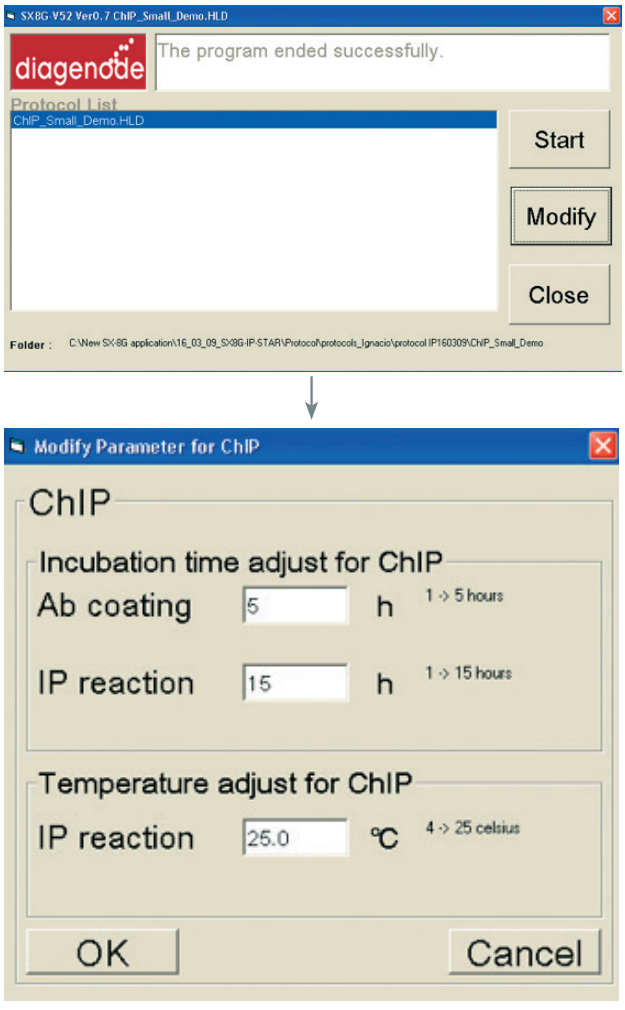
for loading ChIP Direct protocols

In red it is indicated the directory location of the ChIP protocols.

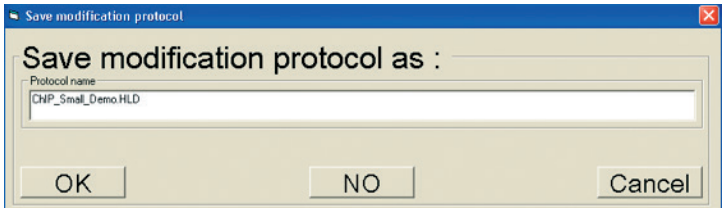
Before starting the protocol a start confirmation window will appear. Press OK and the protocol will run.



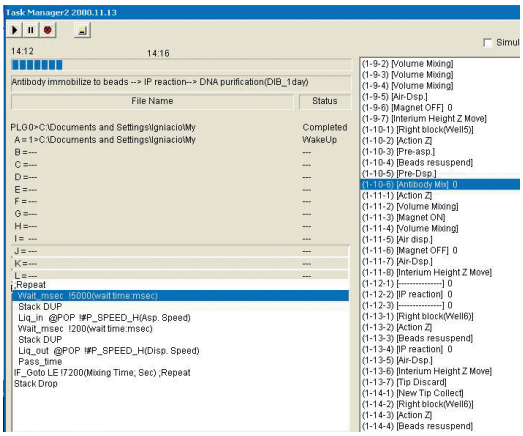
Alternatively, incubation time for antibody coating and temperature and incubation time for the IP reaction can be adjusted in an existing protocol by selecting the modify button. The modified protocol can also be saved as new protocol.



If running ChIP 16 protocol, setup half of the incubation time. It will incubate half of the time on each block but total time will be correct. (For instance, if you want 10h incubation, you have to setup 5h)



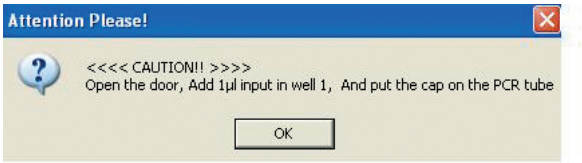
7. The program will run through the following steps: magnetic bead washes, IP and IP washes.



During protocol the next window will be displayed indicating the step that the protocol is processing.

8. Reverse crosslinking

After the IP washes the following window will be appear.



9. Add 1 % Input to well 1: 2 µl of immunoprecipitation mix

10. Close the tube strip with the corresponding caps

11. Press OK

12. Reverse crosslinking will be performed at 65°C for 4 hours or O.N

NOTE:

(Optional) RNase treatment by incubating the samples with RNase at 37°C during 30 minutes can be performed after the reverse crosslinking. Diagenode does not provide RNase.

MANUAL

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

After the reverse-crosslinking, DNA purification is performed using our simplified and validated Auto IPure reagents included in the Auto Universal Plant ChIP-seq kit and the related protocol on the IP-Star. To run this protocol on the IP-Star, please follow the instructions from the manual **Auto IPure kit v2 (C03010010).**

STEP 5. qPCR analysis and results

We recommend analysing the Immunoprecipitated DNA by qPCR using at least 1 positive and 1 negative control target.

30. 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

31. Use 3 µl of purified DNA (100 to 500 pg) as template for qPCR. For more details refer to the section: Remarks before starting, Quantitative PCR analysis and data interpretation.

32. Perform the qPCR using the following conditions: 3 to 10 min denaturation step at 95°C (please check carefully the supplier's recommendations concerning Taq polymerase activation time), followed by 45 cycles of 30 s at 95 °C, 30 s at 60 °C (temperature depending on primers) 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. These conditions may require optimisation depending on the type of Master Mix or qPCR system used.

For qPCR results and data interpretation, refer to the section: Remarks before starting, Quantitative PCR analysis and data interpretation.

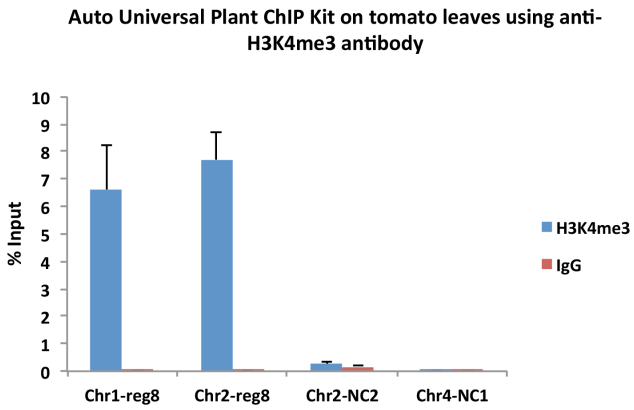


Figure 2

ChIP experiment using our premium anti-H3K4me3 ChIP-seq grade antibody (Cat. No. C15410003) and rabbit IgG (Cat. No. C15410206) on *Solanum lycopersicum cv Micro-Tom* young leaves has been performed by the IP-Star®. Error bars represent the standard deviation of at least 3 biological repetitions. 1 µg of H3K4me3 antibody and 1 µg of the negative IgG control were used per ChIP reaction. Quantitative PCR was performed with the positive controls Chr1-reg8 and Chr2-reg8 and the negative controls Chr2-NC2 and Chr4-NC1 primer sets. The recovery is expressed as % of input.

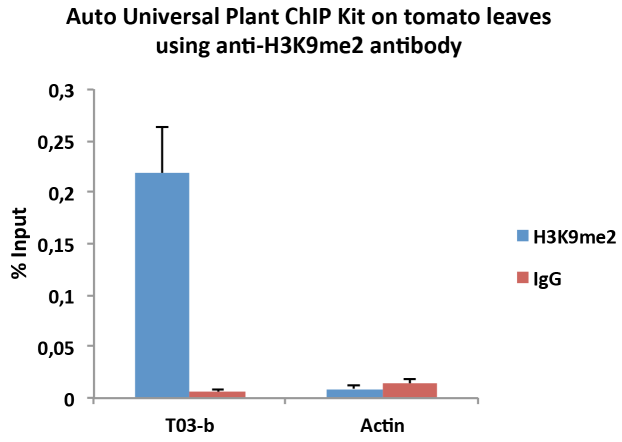


Figure 3

ChIP experiment using our premium anti-H3K9me2 ChIP-seq grade antibody (Cat. No. C15410060) and rabbit IgG (Cat. No. C15410206) on *Solanum lycopersicum cv Micro-Tom* young leaves using has been performed by the IP-Star®. Error bars represent the standard deviation of at least 3 biological repetitions. 1 µg of H3K4me3 antibody and 1 µg of the negative IgG control were used per ChIP reaction. Quantitative PCR was performed with the positive control T03-b and the negative control Actin primer sets. The recovery is expressed

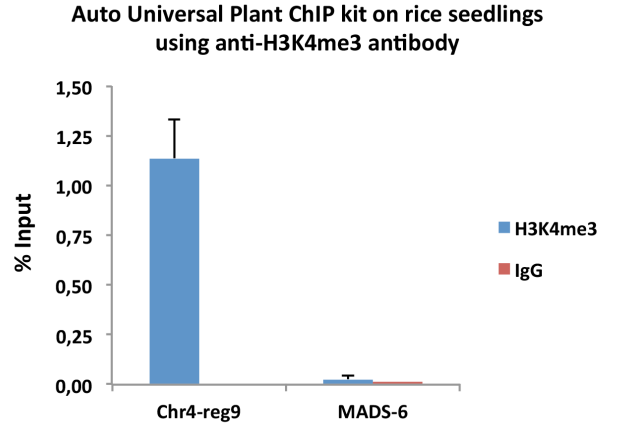


Figure 4

ChIP experiment using our premium anti-H3K4me3 ChIP-seq grade antibody (Cat. No.C15410003) and rabbit IgG (Cat. No.C15410206) on *Oriza sativa ssp Japonica cv. Nipponbare* 3 leaves stage seedlings has been performed by the IP-Star®. Error bars represent the standard deviation of at least 3 biological repetitions. 1 µg of H3K4me3 antibody and 1 µg of the negative IgG control were used per ChIP reaction. Quantitative PCR was performed with the positive control Chr4-reg9 and the negative control MADS6 primer sets. The recovery is expressed as % of input.

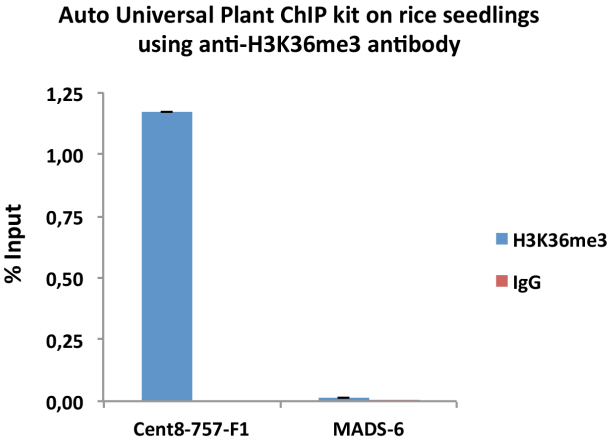


Figure 5

ChIP experiment using our premium anti-H3K36me3 ChIP-seq grade antibody (Cat. No. C15410192) and rabbit IgG (Cat. No. C15410206) on *Oriza sativa ssp Japonica cv. Nipponbare* 3 leaves stage seedlings has been performed by the IP-Star®. Error bars represent the standard deviation of at least 3 biological repetitions. 1 µg of H3K4me3 antibody and 1 µg of the negative IgG control were used per ChIP reaction. Quantitative PCR was performed with the positive control Chr4-reg9 and the negative control MADS6 primer sets. The recovery is expressed as % of input.

MANUAL

ChIP-sequencing

The Auto Universal Plant ChIP-seq protocol has been optimized for ChIP-seq on an Illumina® HiSeq Next-Gen sequencer. However, other sequencing systems such as the Illumina® MiSeq or the Life Technologies SOLiDTM systems can also be used.

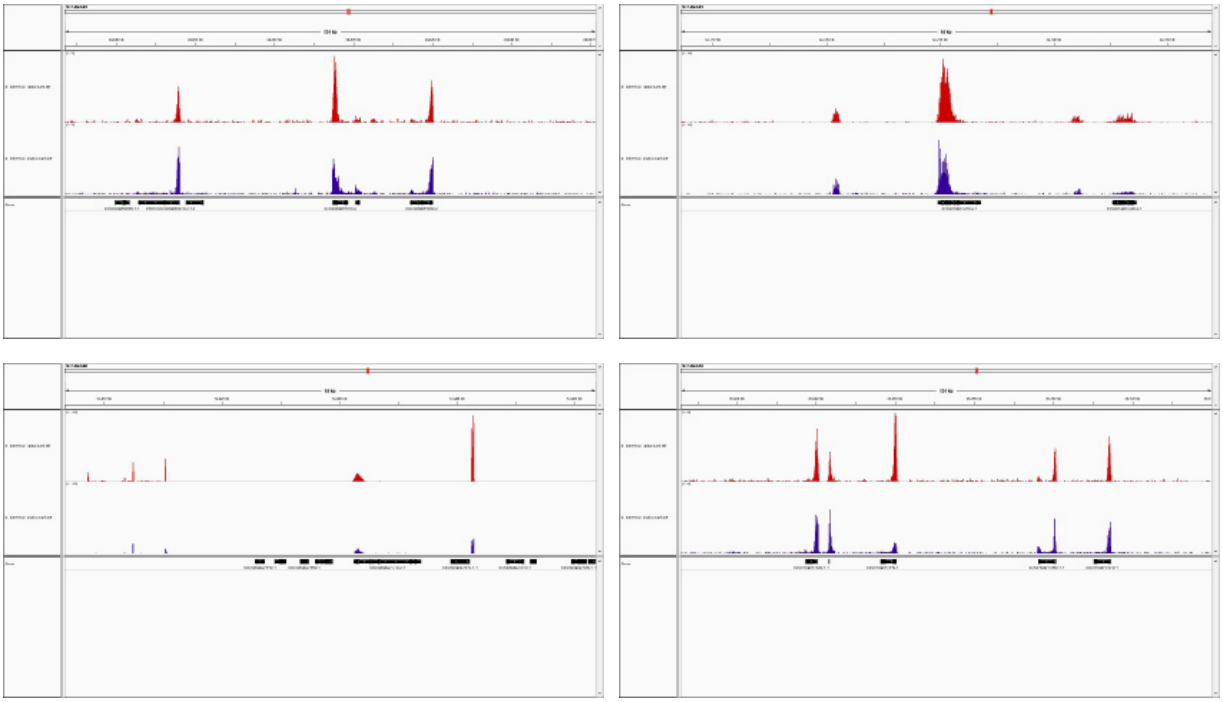


Figure 6.
ChIP-seq was performed on *Solanum lycopersicum cv MicroTom* leaves using our premium anti-H3K4me3 ChIP-seq grade antibody (Cat. No. C15410003). Libraries were prepared with our MicroPlex Library Preparation kit (Cat. No. C05010010) from 750 pg IP'd DNA and sequenced on an Illumina HiSeq 2500. Detailed view of 4 different regions of Solanum genome in red illustrates the enrichment profile. The enrichment in blue represents a dataset obtained from Nguyen et al. 2014 using similar material (tomato leaves) that we used as an external reference.

?

ASK THE EXPERTS

Please, do not hesitate to contact our customer support team if you have any questions about the design of your ChIP-seq experiment or the bioinformatics data analysis.

Contact for Europe, Asia, Oceania and Africa:
custsupport@diagenode.com
Contact for North and South America:
custsupport.na@diagenode.com

Additional Protocols

Sheared chromatin analysis

Reagents not supplied:

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol {25:24:1}
- Chloroform/isoamyl alcohol {24:1}
- 100 % Ethanol
- 70 % Ethanol
- DNA precipitant (Cat. No. C03030002)
- DNA co-precipitant (Cat. No. C03030001)
- Agarose and TAE buffer
- DNA ladder marker
- DNA loading dye

Caution: Steps 8-12 should be carried out under the fume hood!

1. Use 50 µl aliquot of sheared chromatin from Step 2 point 24 to analyse the shearing efficiency.
2. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of ChIP-seq grade water).
3. Add 2 µl of diluted RNase cocktail.
4. Incubate 1h at 37°C.
5. Add 50 µl of elution buffer 1.
6. Add 4 µl of elution buffer 2, mix thoroughly.
7. Incubate samples at 65°C for 4h (or overnight).
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.
9. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
10. Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the sample at RT for 10 min on a rotating wheel.
11. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
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.
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.
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.
15. Re-suspended the pellet in 20 µl of TE buffer.
16. Run samples (20 µl of DNA + 4 µl of 6x loading dye) in a 1.5% agarose gel.

Troubleshooting guide

If you face an issue not cited below or if any of the proposed solutions do not 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)
Low DNA yield after purification	• Reduce the elution volume (step 50) at the end of IPure purification • Apply remarks below (Low enrichment after Immunoprecipitation) • Pool many samples together to obtain necessary quantity
Low enrichment after Immunoprecipitation	• Avoid excessive shearing (Proteins can be damaged by very long sonication) • Increase the duration of incubation of beads with antibody (step 31) • Increase the duration of the Immunoprecipitation (ON instead of 4 h) (step 34) • Increase the quantity of chromatin (always respect the chromatin dilution ratio (1/5) to obtain an optimal SDS concentration for immunoprecipitation • Increase the quantity of magnetic beads (ex: 30 µl) • Increase the quantity of antibody • Use the recommended formula to calculate enrichment
High background	• Reduce the quantity of antibody (large excess of antibody might lead to lower specificity) • Decrease the duration of the Immunoprecipitation (step 34) • Decrease the quantity of chromatin for immunoprecipitation or dilute chromatin (if very concentrated)
No PCR amplification (including Input samples)	• Dilute the purified DNA After Purification (extremely concentrated samples may not be optimal for PCR

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