diagenode

A Hologic Company

Auto Plant ChIP-seq kit

Cat. No. **C01010151** (24 rxns)

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Content

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 Plant ChIP-seq kit

The Auto 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 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 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 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

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.

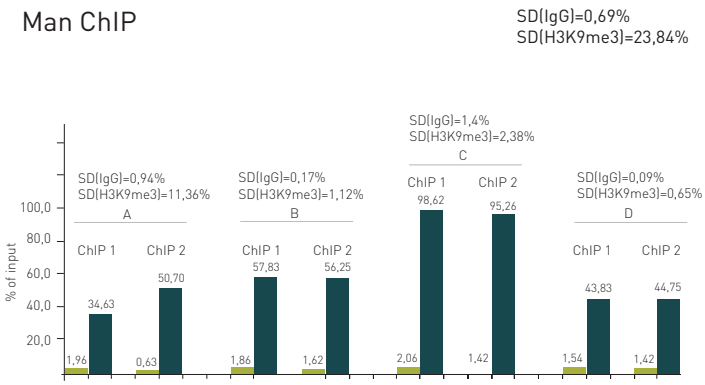


Figure 1. Manual ChIP

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.

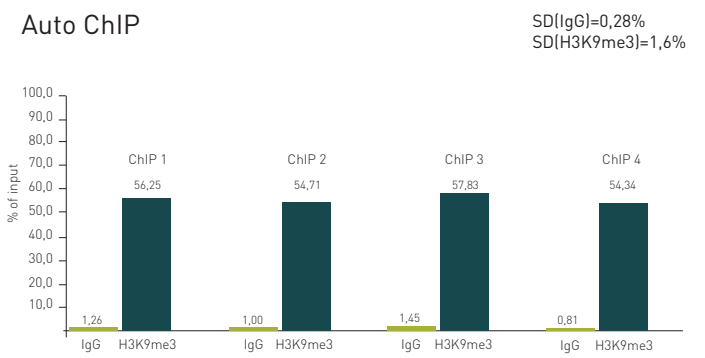


Figure 2. Automated ChIP

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

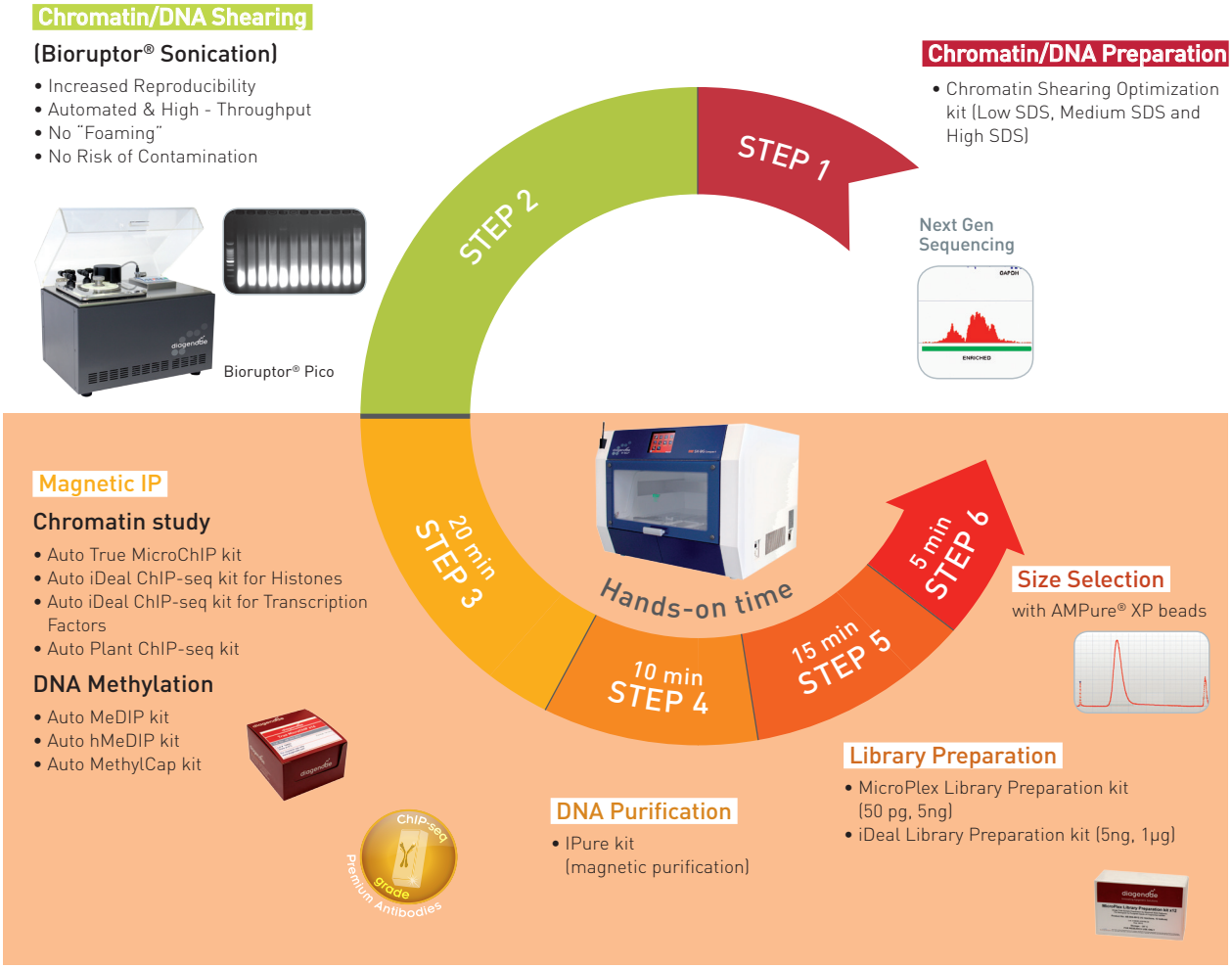


Figure 3. Diagenode provides a full suite of automated solutions for ChIP experiments

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.

Kit materials

The content of the kit is sufficient to perform 24 ChIP assays, 6 chromatin extractions and 6 chromatin shearing optimization reactions. Store the components at the indicated temperature upon receipt (Table 1).

Table 1. Kit content

Description	Quantity (x24)	Storage
10x Crosslinking Buffer	13 ml	4°C
Extraction Buffer 1	130 ml	4°C
Extraction Buffer 2	160 ml	4°C
Extraction Buffer 3	35 ml	4°C
10x ChIP Dilution Buffer	13 ml	4°C
Sonication Buffer	2.2 ml	4°C
Protein-A coated magnetic beads	530 µl	4°C - DO NOT FREEZE!
Wash Buffer 1	50 ml	4°C
Wash Buffer 2	50 ml	4°C
Wash Buffer 3	50 ml	4°C
Wash Buffer 4	26 ml	4°C
Elution Buffer 1	16 ml	4°C - incubate at RT before use
Elution Buffer 2	640 µl	4°C
DNA Precipitant	1.6 ml	4°C
DNA Co-precipitant	200 µl	-20°C
Glycine	14 ml	4°C
Rabbit IgG	10 µg (1 µg/µl)	-20°C
ChIP-seq grade premium antibody H3K4me3	10 µg (1 µg/µl)	-20°C
FLC-ATG Primer pair (positive control)	5 µM each/50 µl	-20°C
FLC-Intron1 Primer pair (negative control)	5 µM each/50 µl	-20°C

Table 2. Kits and Modules available separately

Description	Reference	Quantity
Auto IPure kit v2 x100	C02010012	100 rnx

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 materials not provided

- Formaldehyde (high-quality methanol-free)
- Miracloth
- Funnels
- Vacuum pump
- Desiccator
- Liquid nitrogen
- dH_2O
- Ceramic mortar and pestle
- 50 ml tubes
- 1.5 ml Safelock tubes
- 1.5 ml Bioruptor® tubes (Depending on the Bioruptor® model used)
- Bioruptor®
- Thermomixer
- Rotating wheel
- Magnetic rack
- Phenol/Chloroform/Isoamylalcohol (25:24:1)
- Chloroform/Isoamylalcohol (24:1)
- ß-Mercaptoethanol
- Protease Inhibitor Cocktail for plants (e.g. Protease inhibitor cocktail for plant cell and tissue extracts, Sigma Aldrich #P9599)

Remarks before starting

1. Starting material

This protocol has been optimized for chromatin extraction from 1 g fresh weight (fw) of *Arabidopsis thaliana* seedlings. One ChIP reaction is performed with 40 µl of the yielded chromatin equalling approximately 0.2 g (fw) of seedlings. It is possible to use more material per IP, however we recommend performing separate IPs and pooling the IP'd DNA before purification.

2. Shearing optimization and sheared chromatin analysis

Before starting the ChIP, the chromatin should be sheared to fragments ranging between 200 and 600 bp. Our kits and protocols are optimized for chromatin shearing using the Bioruptor® (Bioruptor® Pico (#B01060001); Bioruptor® Plus (#B01020001); Bioruptor® Standard (#B01010001)) in combination with a water cooling system (#B02010003; 115V or #B02010002; 230V). If you use a different sonicator, the sonication conditions must be optimized accordingly. Tubes and corresponding volumes depend on the Bioruptor® system used, refer to www.diagenode.com. We recommend using 1.5 ml Bioruptor® Microtubes with Caps (#C30010016) for the Bioruptor® Pico and 1.5 ml TPX microtubes (#C30010010) for the Bioruptor® Standard and Plus. The shearing conditions mentioned in the protocol are adequate for chromatin from different plant tissues. However we recommend optimizing the sonication conditions for each tissue before processing a large number of samples. Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation. A protocol to assess the shearing efficiency can be found in the "Additional Protocols" section.

3. Magnetic beads

This kit includes DiaMag protein A-coated magnetic beads (#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.

4. Negative and positive IP controls

The kit contains a negative (IgG, #C15410206) and a positive (**H3K4me3**, #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 also contains qPCR primer pairs for amplification of a positive and negative control target for **H3K4me3** in Arabidopsis thaliana (**FLC-ATG** and **FLC-Intron1**, respectively).

5. 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. Before use prepare the necessary amount of 1x Buffer using dH20. Proteinase Inhibitor Cocktail and ß-mercaptoethanol should be added freshly, where stated.

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

IP-STAR® COMPACT

Protocol

STEP 1. Crosslinking of plant tissue

Caution: Steps 1-7 should be carried out under the fume hood!

1. Prepare 1x **Crosslinking Buffer** (20 ml per 1 g fresh weight plant tissue) by diluting 2 ml of **10x Crosslinking buffer** with 18 ml dH20. Add formaldehyde (high-quality methanol-free solution) to a final concentration of 1 % and store it at 4 °C.
2. Harvest 1 g of plant material into a 50 ml tube and keep harvested tissue cooled on ice.
3. Add 20 ml of 1x Crosslinking Buffer supplemented with 1% formaldehyde (4°C) to each 50 ml tube containing 1 g of plant material. Make sure that the tissue is submerged in buffer and not floating on top of the buffer.
4. Crosslink the plant tissue in the (opened) 50 ml tube by placing the tube in a desiccator and applying vacuum for 15 min (on ice). If the tissue is floating on top of the buffer slowly release the vacuum after 5 min, submerge the tissue and reapply the vacuum for the remaining 10 min. Release the vacuum slowly.
5. Stop the crosslinking by adding 2 ml of **Glycine** solution (provided) to each 50 ml tube containing 1 g of plant tissue. Mix the solution and apply vacuum for additional 5 min. Release the vacuum slowly.
6. Discard the Crosslinking Buffer and wash the plant tissue twice with 40 ml of deionized water (4 °C; not supplied). For washing add the water to the 50 ml tube, invert the closed tube twice and discard the water. Alternatively you can use a funnel and Miracloth to wash the plants with adequate amount of deionized water.
7. Remove the plant tissue from the 50 ml tube 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 or be directly used for the chromatin extraction.

STEP 2. Chromatin extraction from plant tissue and chromatin shearing

Caution: Steps 8-14 should be carried out under the fume hood!

Description	Reagent	Concentration / Dilution
Extraction Buffers 1-3	ß-Mercaptoethanol	Add to a final concentration of 5 mM
	Plant Protease Inhibitor Cocktail	Add according to manufacturer's instructions
Sonication Buffer	Plant Protease Inhibitor Cocktail	Add according to manufacturer's instructions

8. Prepare the necessary amounts of complete **Extraction Buffers 1-3** by adding ß-mercaptoethanol and Protease Inhibitor Cocktail and keep them cold.
9. 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 being careful not to let the tissue thaw at any time.
10. Transfer the powder to a pre-cooled 50 ml tube and add 20 ml of cold **Extraction Buffer 1**. (If you transfer the powder in liquid nitrogen, make sure it has evaporated completely before adding **Extraction Buffer 1**.) Vortex vigorously until the tissue is completely homogenized in the buffer.
11. Filter the suspension twice through Miracloth (Calbiochem) and collect the flow-through in a fresh 50 ml tube on ice.
12. Centrifuge for 20 min at 1000 g at 4 °C.
13. Wash the pellet containing the nuclei 5 times with 5 ml **Extraction Buffer 2**. In each washing step carefully resuspend the pellet by gentle shaking (no vortexing or pipetting). Centrifuge at for 10 min 1000 g at 4 °C after each wash.
14. Wash once with 5 ml **Extraction Buffer 3** as in point 13.Resuspend the crude nuclear pellet in 300 µl of **Sonication Buffer** by pipetting and transfer the suspension to a 1.5 ml sonication tube (tubes depend on the Bioruptor® model used). Since the **Sonication Buffer** contains SDS, be careful not to cause bubbles by pipetting. If the volume is bigger

than 300 µl, split it in two sonication tubes.

15. Sonicate the chromatin with the Bioruptor® for 8-10 cycles 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.
16. Transfer the sonicated chromatin to a standard 1.5 ml tube and centrifuge for 10 min at 12.000 g at 4 °C.
17. Transfer the supernatant to a new 1.5 ml tube, this is the extracted chromatin. Purify the DNA from 50 µl of the chromatin to check the shearing efficiency on an agarose gel (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.

STEP 3. Magnetic immunoprecipitation

The chromatin extraction from 1 g of plant material yields 300 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.

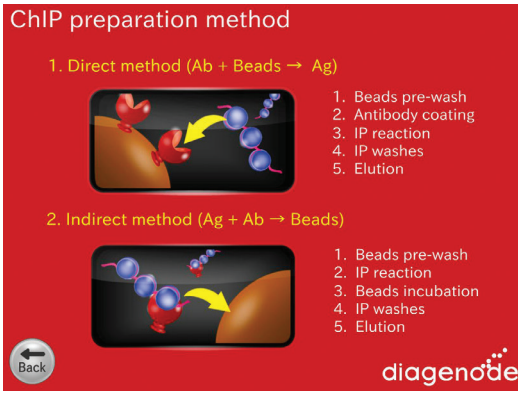
Determine the total number of IP's in the experiment. Please note that we recommend to include one negative control in each experiment (IP with the IgG negative control)

ChIP 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.

18. Switch ON the IP-Star® Compact.
19. Select "**Protocols**" icon and then "**ChIP**" category.

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

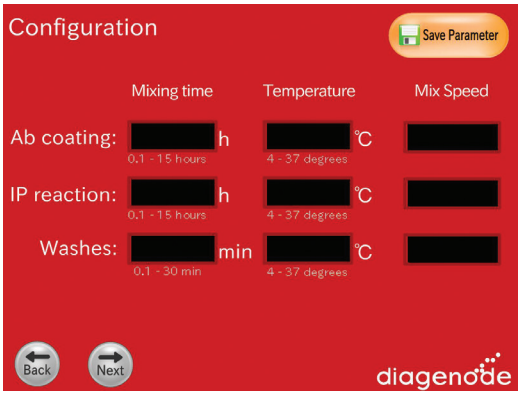


NOTE:
If you plan to run between 1 and 8 samples, chose "**ChIP_IPure_8_200_D**"
If you plan to run between 9 and 16 samples, chose "**ChIP_IPure_16_200_D**"

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

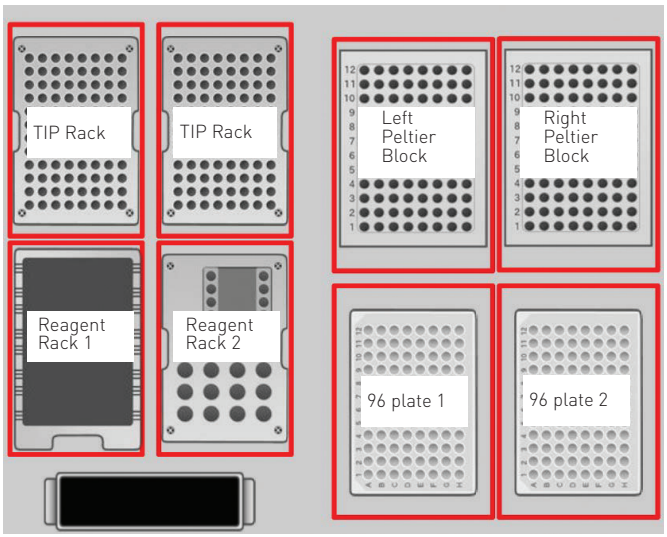
NOTE:
The **Peltier Block** is now cooling down to 4°C to keep your samples cold

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

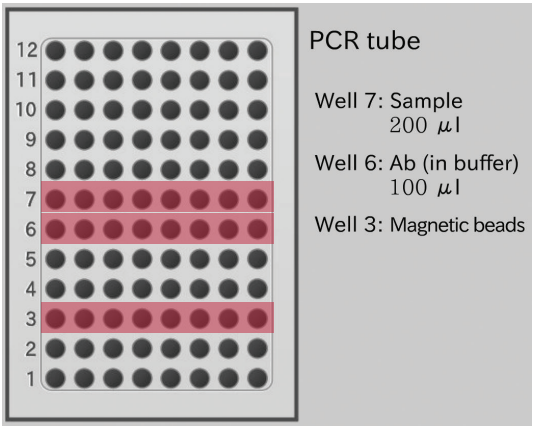


Setup the "Ab coating" step to 3 hours
Setup the "IP reaction" step to 8-10 hours (overnight)
Setup the "Washes" step to 5-10 min

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



24. Fill **TIP Rack 1** (and 2 if processing 16 samples protocol) with tips according to the screen.
25. Fill **Reagent Racks** 1 & 2 with reagent containers according to the screen.
26. Fill **Peltier block** with your sample, antibody and magnetic beads as mentioned her below



Direct ChIP

1. 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)

2. 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

3. Preparation Immunoprecipitation mix (Well 7)

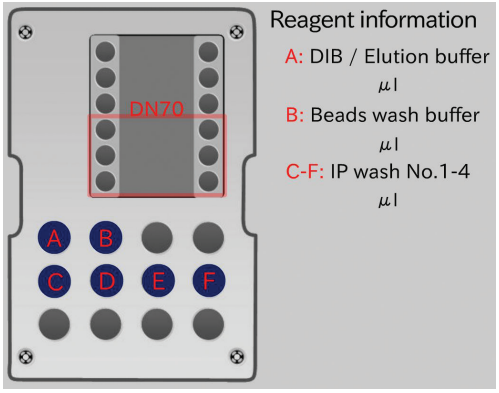
Sheared chromatin	40 µl
1x ChIP Dilution Buffer	160 µl
200x Plant Protease Inhibitor Cocktail	1 µl

Keep 2 µl of the Immunoprecipitation mix aside for the Input

This Auto 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.

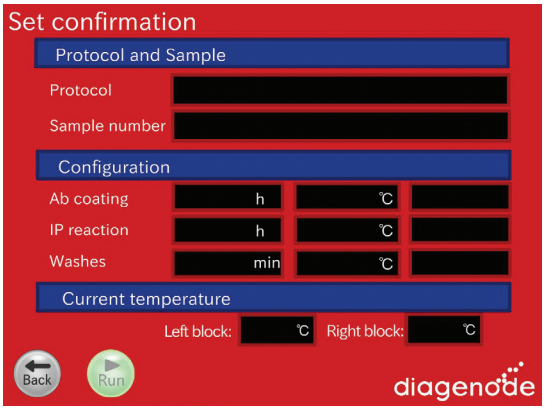
NOTE:
If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added to the chromatin sample.

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

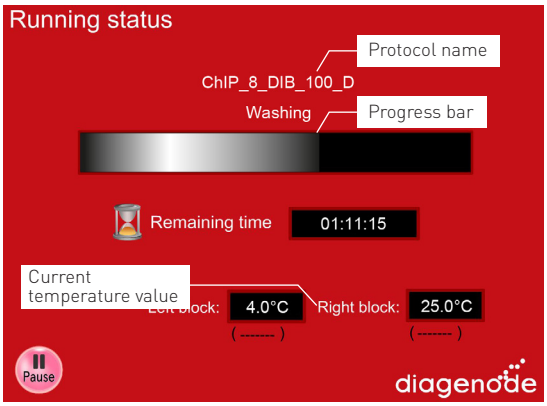


Beads Wash Buffer: 1x ChIP Dilution Buffer
Elution buffer: Elution Buffer 1
IP wash 1: Wash Buffer 1
IP wash 2: Wash Buffer 2
IP wash 3: Wash Buffer 3
IP wash 4: Wash Buffer 4

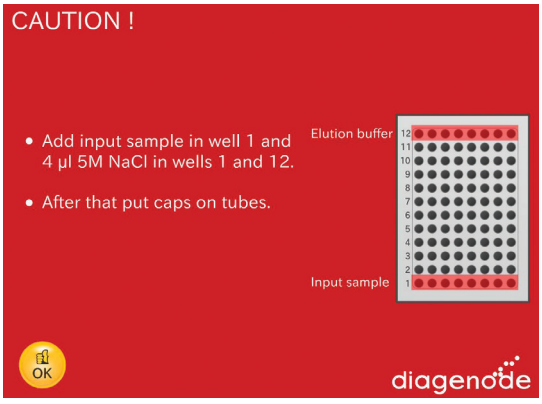
28. Check the selected parameters, close the door and press "Run" to start



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



30. 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 + 94 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

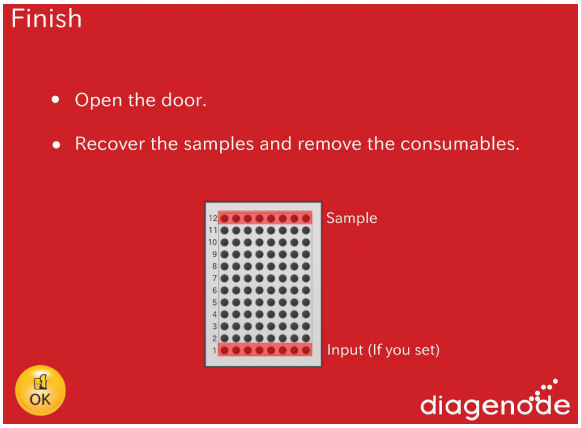
NOTE 1:

(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.

31. 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.



STEP 4. Elution, decross-linking and DNA isolation

After the reverse-crosslinking, DNA purification is performed using Phenol/Chloroform extraction. Alternatively, DNA purification can be performed using our simplified and validated Auto IPure kit v2 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).

Caution: steps 32-34 should be carried out under the fume hood!

DNA purification with Phenol Chloroform extraction:

32. Add an equal volume of phenol/chloroform/isoamyl alcohol {25:24:1} to each sample. Vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
33. Add 1 volume of chloroform/isoamyl alcohol {24:1}. Vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
34. Precipitate the DNA by adding 40 µl of DNA precipitant, 5 µl of DNA co-precipitant and 1 ml of cold 100 % ethanol to the sample.
35. Incubate for 30 min at -80 °C (or for 2 h at -20 °C). Centrifuge for 25 min at 12 000 g at 4 °C.
36. Carefully remove the supernatant and add 500 µl of ice-cold 70 % ethanol to the pellet. Centrifuge for 10 min at 12 000 g at 4 °C.
37. Carefully remove the supernatant and air dry the pellet at RT until all remaining ethanol has evaporated.
38. Resuspend the pellet in 20 µl of dH2O (DNase-free).
39. For PCR, dilute the DNA 1:10 and use 5 µl per reaction..

IP-STAR®

How to perform Automated ChIP on the IP-Star®

Protocol

STEP 1. Crosslinking of plant tissue

Caution: Steps 1-7 should be carried out under the fume hood!

1. Prepare 1x **Crosslinking Buffer** (20 ml per 1 g fresh weight plant tissue) by diluting 2 ml of **10x Crosslinking buffer** with 18 ml dH20. Add formaldehyde (high-quality methanol-free solution) to a final concentration of 1 % and store it at 4 °C.
2. Harvest 1 g of plant material into a 50 ml tube and keep harvested tissue cooled on ice.
3. Add 20 ml of 1x Crosslinking Buffer supplemented with 1% formaldehyde (4°C) to each 50 ml tube containing 1 g of plant material. Make sure that the tissue is submerged in buffer and not floating on top of the buffer.
4. Crosslink the plant tissue in the (opened) 50 ml tube by placing the tube in a desiccator and applying vacuum for 15 min (on ice). If the tissue is floating on top of the buffer slowly release the vacuum after 5 min, submerge the tissue and reapply the vacuum for the remaining 10 min. Release the vacuum slowly.
5. Stop the crosslinking by adding 2 ml of **Glycine** solution (provided) to each 50 ml tube containing 1 g of plant tissue. Mix the solution and apply vacuum for additional 5 min. Release the vacuum slowly.
6. Discard the Crosslinking Buffer and wash the plant tissue twice with 40 ml of deionized water (4 °C; not supplied). For washing add the water to the 50 ml tube, invert the closed tube twice and discard the water. Alternatively you can use a funnel and Miracloth to wash the plants with adequate amount of deionized water.
7. Remove the plant tissue from the 50 ml tube 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 or be directly used for the chromatin extraction.

STEP 2. Chromatin extraction from plant tissue and chromatin shearing

Caution: Steps 8-14 should be carried out under the fume hood!

Description	Reagent	Concentration / Dilution
Extraction Buffers 1-3	ß-Mercaptoethanol	Add to a final concentration of 5 mM
	Plant Protease Inhibitor Cocktail	Add according to manufacturer's instructions
Sonication Buffer	Plant Protease Inhibitor Cocktail	Add according to manufacturer's instructions

8. Prepare the necessary amounts of complete **Extraction Buffers 1-3** by adding ß-mercaptoethanol and Protease Inhibitor Cocktail and keep them cold.
9. 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 being careful not to let the tissue thaw at any time.
10. Transfer the powder to a pre-cooled 50 ml tube and add 20 ml of cold **Extraction Buffer 1**. (If you transfer the powder in liquid nitrogen, make sure it has evaporated completely before adding **Extraction Buffer 1**.) Vortex vigorously until the tissue is completely homogenized in the buffer.
11. Filter the suspension twice through Miracloth (Calbiochem) and collect the flow-through in a fresh 50 ml tube on ice.
12. Centrifuge for 20 min at 1000 g at 4 °C.
13. Wash the pellet containing the nuclei 5 times with 5 ml **Extraction Buffer 2**. In each washing step carefully resuspend the pellet by gentle shaking (no vortexing or pipetting). Centrifuge at for 10 min 1000 g at 4 °C after each wash.
14. Wash once with 5 ml **Extraction Buffer 3** as in point 13.Resuspend the crude nuclear pellet in 300 µl of **Sonication Buffer** by pipetting and transfer the suspension to a 1.5 ml sonication tube (tubes depend on the Bioruptor® model used). Since the **Sonication Buffer** contains SDS, be careful not to cause bubbles by pipetting. If the volume is bigger

than 300 µl, split it in two sonication tubes.

15. Sonicate the chromatin with the Bioruptor® for 8-10 cycles 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.
16. Transfer the sonicated chromatin to a standard 1.5 ml tube and centrifuge for 10 min at 12.000 g at 4 °C.
17. Transfer the supernatant to a new 1.5 ml tube, this is the extracted chromatin. Purify the DNA from 50 µl of the chromatin to check the shearing efficiency on an agarose gel (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.

STEP 3. Magnetic immunoprecipitation

The chromatin extraction from 1 g of plant material yields 300 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.

1. 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)

2. Preparation of Ab coating mix (Well 6)

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

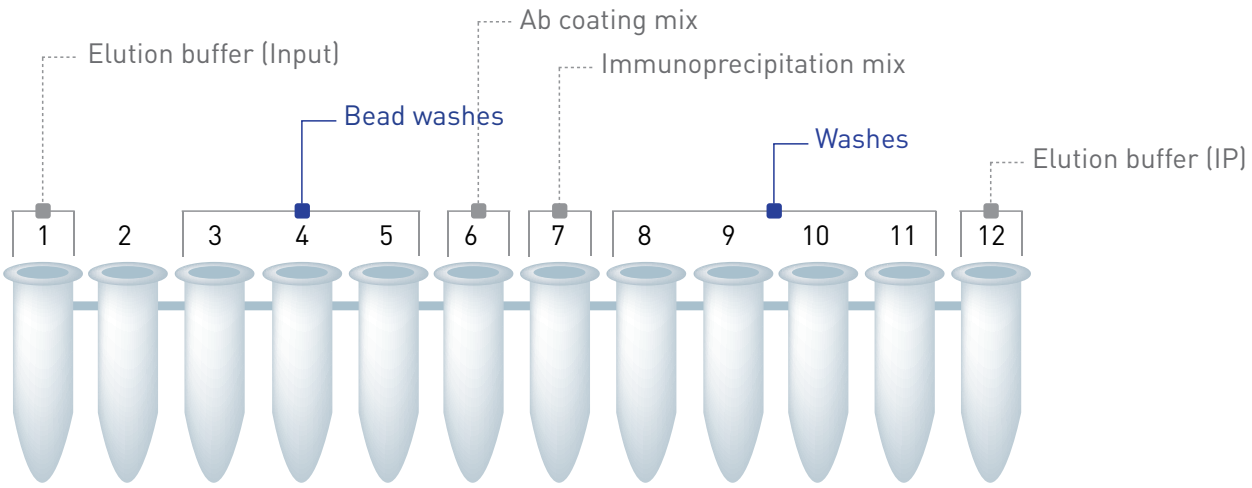
Use 1 µl of the rabbit IgG (1µg/µl) control antibody for the negative control IP. If a positive control IP is included in the experiment, use 1 µl of the H3K4me3 (1µg/µl) ChIP-seq grade control antibody

3. 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

4. Load the reagents



	IPURE	
Tube #	Description	200 µl protocol
1	Elution Buffer 1 + Elution Buffer 2	94 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	96 µl + 4 µl

* This Auto 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.

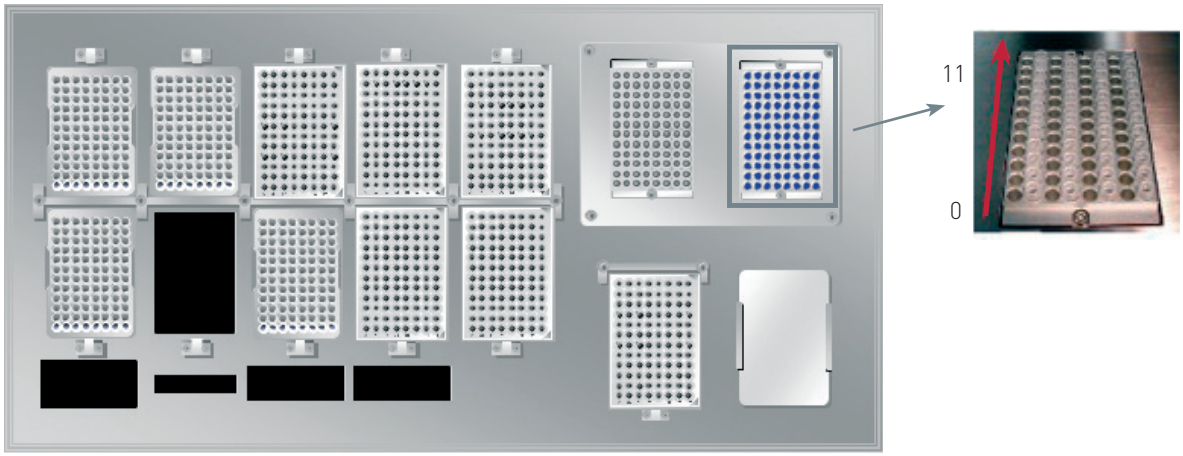
Running protocol

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.

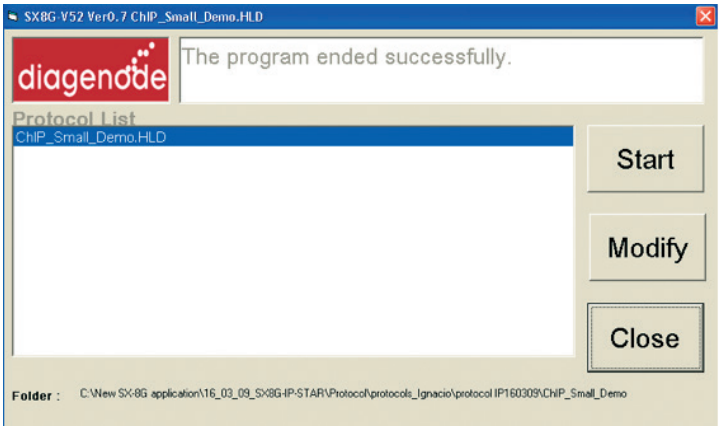
Table 3.

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:

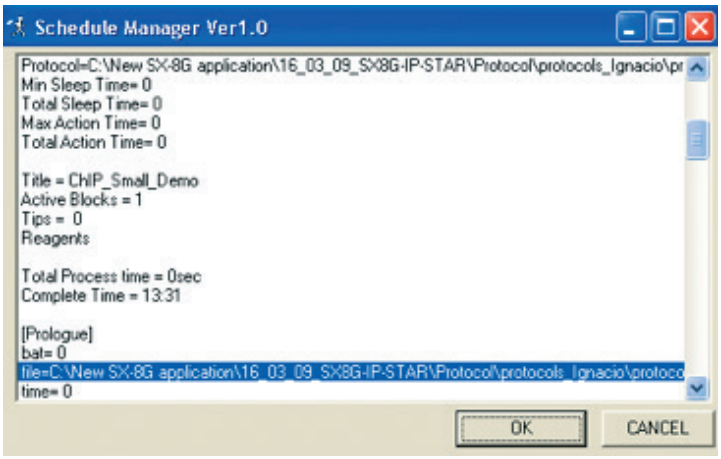
*[**EASYSTARTSCREEN**]*

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

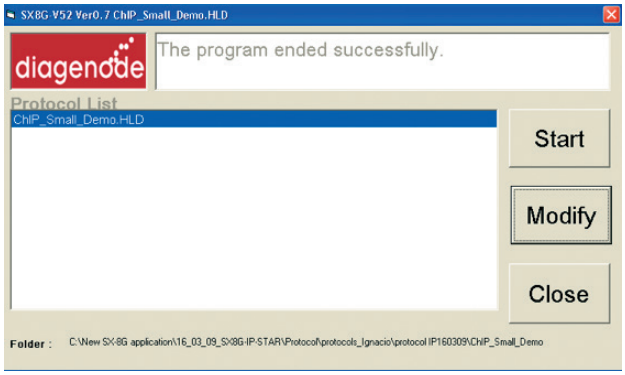
for loading ChIP Direct protocols

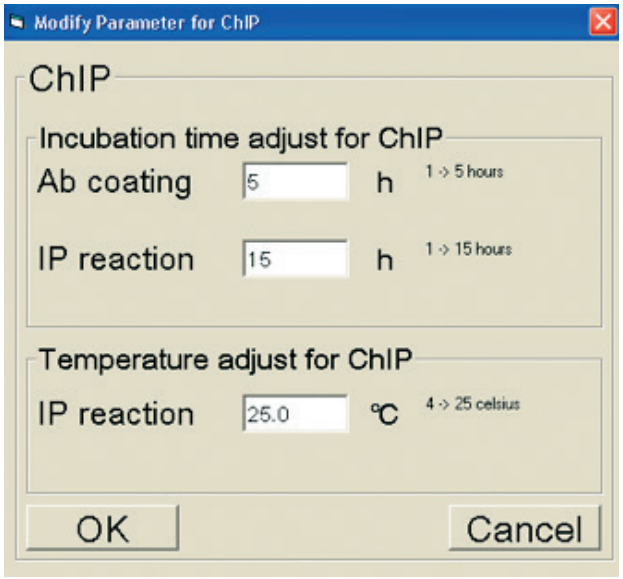
In red it is indicated the directory location of the ChIP protocols.

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

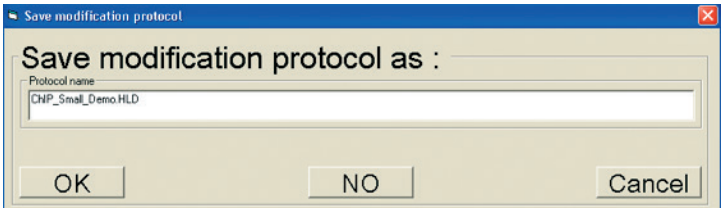


Alternatively, incubation time for antibody coating and temperature and incubation time for the IP reaction can be adjusted in an existing protocol by selecting the modify button. The modified protocol can also be saved as new protocol.

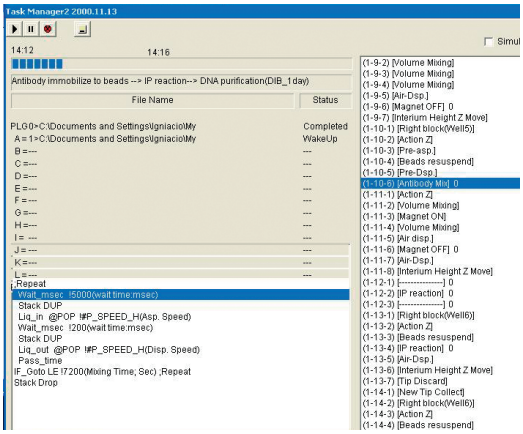




If running ChIP 16 protocol, setup half of the incubation time. It will incubate half of the time on each block but total time will be correct. (For instance, if you want 10h incubation, you have to setup 5h)



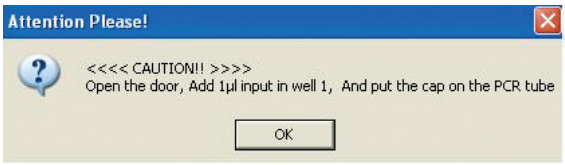
7. The program will run through the following steps: magnetic bead washes, IP and IP washes.



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 mmunoprecipitation 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.

STEP 4. Elution, decross-linking and DNA isolation

After the reverse-crosslinking, DNA purification is performed using Phenol/Chloroform extraction. Alternatively, DNA purification can be performed using our simplified and validated Auto IPure kit v2 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).

Caution: steps 13-15 should be carried out under the fume hood!

DNA purification with Phenol Chloroform extraction:

13. Add an equal volume of phenol/chloroform/isoamyl alcohol {25:24:1} to each sample. Vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
14. Add 1 volume of chloroform/isoamyl alcohol {24:1}. Vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
15. Precipitate the DNA by adding 40 µl of DNA precipitant, 5 µl of DNA co-precipitant and 1 ml of cold 100 % ethanol to the sample.
16. Incubate for 30 min at -80 °C (or for 2 h at -20 °C). Centrifuge for 25 min at 12 000 g at 4 °C.
17. Carefully remove the supernatant and add 500 µl of ice-cold 70 % ethanol to the pellet. Centrifuge for 10 min at 12 000 g at 4 °C.
18. Carefully remove the supernatant and air dry the pellet at RT until all remaining ethanol has evaporated.
19. Resuspend the pellet in 20 µl of dH2O (DNase-free).
20. For PCR, dilute the DNA 1:10 and use 5 µl per reaction..

qPCR analysis and results

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

1. Prepare the qPCR master mix (total volume 25 µl = 20 µl PCR master mix + 5 µl DNA sample):
   - 1 x SYBR Green PCR master mix (e.g.: iQ SYBR Green supermix)
   - 5 pmol of each primer (1 µl if you use the provided primer pair mixes)
   - Fill up to 20 µl with DNase-free water
2. As template use 5 µl of purified diluted IP'd DNA sample and purified diluted INPUT(s).
3. Perform the PCR using the following conditions: 3 to 10 minutes denaturation step at 95°C (please check carefully the manufacturer's recommendations about Taq polymerase activation time), followed by 45 cycles of 30 seconds at 95 °C, 30 seconds at 60 °C and 30 seconds at 72 °C, followed by an elongation step for 10 min at 72 °C. These conditions may require optimisation depending on the type of Master Mix or qPCR system used.

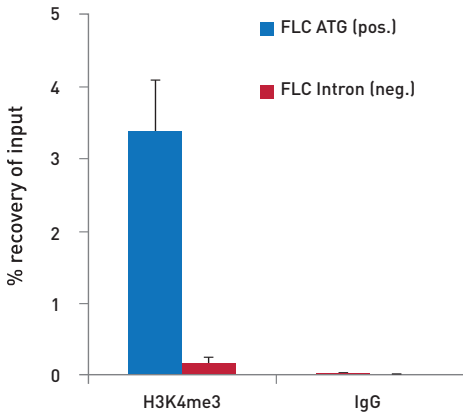


Figure 1: ChIP was performed on *Arabidopsis thaliana* (Col-0) seedlings 14 days after germination (dag) using our premium anti-H3K4me3 ChIP-seq grade antibody (#C15410003) and rabbit IgG (#C15410206). Data shown is derived from biological replicates. Sheared chromatin from 0.2 g (fw) of seedlings, 1 µg of H3K4me3 antibody and 1 µg of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control FLC-ATG and the negative control FLC-Intron1 primer sets from the kit. The recovery is expressed as % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

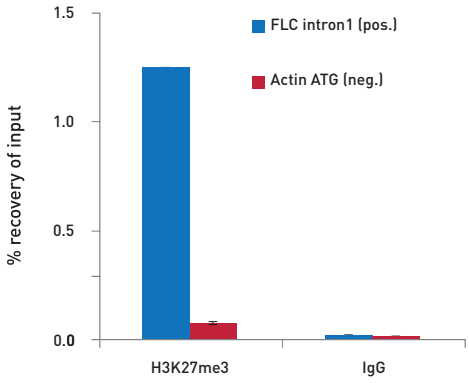


Figure 2: ChIP was performed on Arabidopsis thaliana (Col-0) seedlings 14 days after germination (dag) using our premium anti-H3K27me3 ChIP-seq grade antibody (#C15410195) and rabbit IgG (#C15410206). Data shown is derived from biological replicates. Sheared chromatin from 0.2 g (fw) of seedlings, 2 µg of H3K27me3 antibody and 2 µg of the negative IgG control were used per IP. Quantitative PCR was performed with primer pairs for FLC-Intron1 (positive control) and Actin ATG (negative control). The recovery is expressed as % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

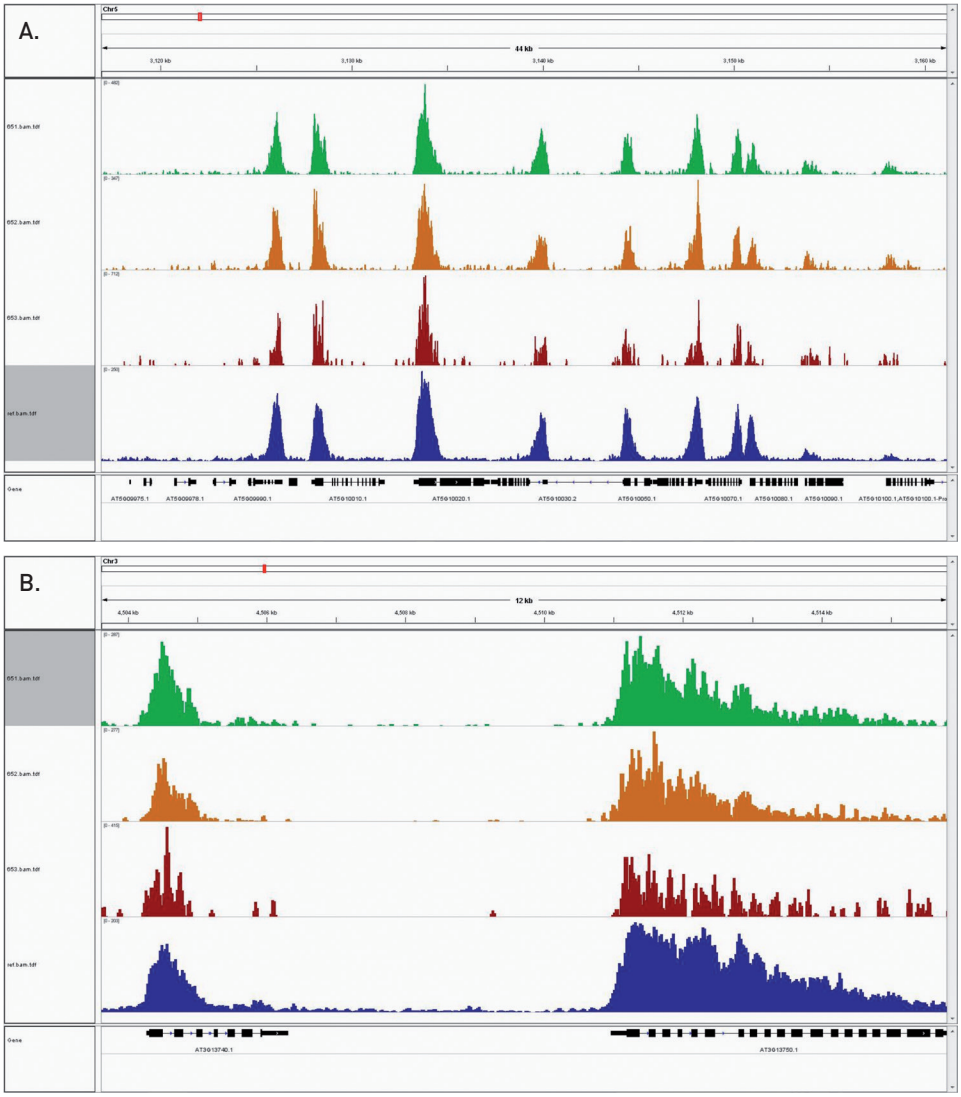


Figure 3: ChIP-seq was performed on *Arabidopsis thaliana* (Col-0) seedlings (grown for 14 days) using our premium anti-H3K4me3 ChIP-seq grade antibody (#C15410003). Libraries were prepared with our MicroPlex Library Preparation™ kit (#C05010010) from 1 ng (green), 500 pg (orange) and 100 pg (red) IP'd DNA and sequenced on an Illumina® HiSeq 2500. The enrichment in blue represents a public dataset (NCBI GEO Dataset GSM1193621) that we used as an external reference. A) Enrichments along a wide region of chromosome 5 are uniform regardless of the starting material amount. B) Detailed view of a region of chromosome 3 illustrating the similarity in the enrichment structures.

Aditional protocols

Sheared chromatin analysis

Reagents not supplied:

- RNase (e.g. Ambion, #AM 2286 A)
- Phenol/chloroform/isoamyl alcohol {25:24:1}
- Chloroform/isoamyl alcohol {24:1}
- 100 % Ethanol
- 70 % Ethanol
- Agarose and TAE buffer
- DNA ladder marker
- DNA loading dye

Caution: Steps 4-6 should be carried out under the fume hood!

1. Use the 50 µl aliquot of sheared chromatin from Step 2 point 18 to analyse the shearing efficiency.
2. Treat the chromatin with an RNase according to its manufacturer's instructions. (Usually using 1 µl RNase cocktail and incubation 1 h at 37 °C)
3. Add 350 µl of **Elution Buffer 1** and 16 µl **Elution Buffer 2** to the chromatin sample. Mix thoroughly and incubate the samples for 4 h (or overnight) at 65°C in a thermoshaker (1300 rpm).
4. Add an equal volume of phenol/chloroform/isoamyl alcohol {25:24:1} to the sample, vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
5. Add an equal volume of chloroform/isoamyl alcohol {24:1} to the sample, vortex and centrifuge for 3-5 min at 12 000 g at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
6. Precipitate the DNA by adding 1/10 volume of **DNA Precipitant**, 5 µl of **DNA Co-precipitant** and 1 ml of cold 100 % ethanol to the sample. Incubate for 30 min at -80 °C (or for 2 h at -20°C). Centrifuge for 25 min at 12,000 g at 4°C.
7. Carefully remove the supernatant and add 500 µl of ice-cold 70 % ethanol to the pellet. Centrifuge for 10 min at 12 000 g at 4°C.
8. Carefully remove the supernatant and air dry the pellet at RT until all remaining ethanol has evaporated.
9. Resuspend the pellet in 20 µl of dH2O which corresponds to the purified DNA from the sheared chromatin.
10. Add DNA loading dye (according to manufacturer's instructions) and analyse the sample(s) on a 1.5 % agarose gel along with DNA size marker to visualize the shearing efficiency.

Troubleshooting guide

Critical steps	Troubles, solutions and comments	
Cross-linking (formaldehyde fixation)	Optimize crosslinking time	Poor cross-linking causes DNA loss, elevated background, and/or reduced antigen availability in chromatin. Empirically determine optimal cross-linking time for maximal specificity and efficiency of ChIP. The optimal duration of cross-linking varies between cell type and protein of interest. Short cross-linking time (5-10 minutes) may improve shearing efficiency. Cross-linking duration should not exceed 15 minutes or shearing will be inefficient.
	Optimize formaldehyde concentration	Low formaldehyde concentrations like 1% (weight/volume) may improve shearing efficiency. For some proteins, however, especially those that do not directly bind DNA, this might reduce cross-linking efficiency and thus the yield of precipitated chromatin. Empirically determine the formaldehyde concentration as some antigen epitopes may be more sensitive to formaldehyde.
	Quality of formaldehyde	We recommend to use fresh, high-quality, methanol-free formaldehyde solution.
	Quenching of the cross-linking reaction	Use a final concentration of 125 mM glycine to stop the cross-linking reaction
Cell lysis	Make sure cells disrupt completely	Do not use too many cells per amount of lysis buffer (w/v) in order to reach complete cell disruption. Follow the instructions in the protocol.
	Maintain cold temperature during lysis	It is critical to perform the cell lysis at 4°C (cold room) or on ice. Keep the samples on ice at all times and use cold buffers.
	Prevent protein degradation	The amount of cells required per IP reaction is determined by cell type, protein of interest and the quality of antibodies used. Follow the advice given in the manual.
Number of cells required	Determine the number of cells per IP	The amount of cells required per IP reaction is determined by cell type, protein of interest and the quality of antibodies used. Follow the advice given in the manual.
Chromatin shearing	Optimization of shearing conditions	Shearing conditions need to be optimized empirically for each cell type, cell number and fixation time used. For optimization, a time course experiment is highly recommended and the chromatin shearing optimization modules can be used (C01020010; C01020011; C01020012)
	Analysis of sheared chromatin	Purify the DNA from the sheared chromatin as described in the protocol in order to analyze the shearing. We strongly advise to perform the reverse cross-linking step before the analysis. DNA from ≥100.000 cells can be visualized on a 1-1.5% agarose gel.
	Sample temperature	Maintain the temperature of the samples at 4°C before, during and after the shearing in order to maintain sample integrity.
	Optimize SDS concentration in shearing buffer	A high percentage of SDS favours better sonication but inhibits immunoselection. Optimize the SDS concentration (0.05% to 1%) depending on the cell type and cell number used
	Dilution of sheared chromatin prior to ChIP	For the immunoprecipitation step the SDS concentration needs to be reduced to 0.1% to 0.2%. (e.g. if the shearing buffer contains 1% SDS, dilute the chromatin 1:10 with the buffer used in the IP reaction)
	Bioruptor® models	Sonication conditions differ depending on the Bioruptor® model used. Refer to the Bioruptor® manuals and protocols available on www.diagenode.com for specific instructions.
Sonication with the Bioruptor®	Sonication conditions	We strongly advice to perform a time course experiment to assess shearing efficiency for the specific sample used. Sonicate chromatin samples for 1 to 3 runs of 5 to 10 cycles: [30 sec ON / 30 sec OFF] with high power setting. Precool the water bath to 4°C and make sure to maintain the temperature during sonication.
	Bioruptor® accessories	For optimal shearing results use tubes and chromatin volumes as advised in the Bioruptor® manuals and protocols available on www.diagenode.com.
	Chromatin shearing with Diagenode modules tips	You can also use the LowCell ChIP kit for shearing. 25 µl of complete Buffer B are added per 10 000 cells. After 5 minutes lysis on ice, 75 µl of HBSS are added and chromatin is sheared in 100 µl aliquots. Sheared chromatin have to be diluted 2 times with the complete ChIP Buffer tC1 from the True MicroChIP kit (Protocol STEP3 point 21) before adding antibodies.
	Water cooler	Temperature of the sonication bath can be controlled using the Diagenode water cooling system (B02010003; B02010002)
Magnetic beads	Keep beads in suspension	Beads should not dry out at any time.
	Storage	Store magnetic beads at 4°C. Do not freeze.
	Handling of magnetic beads	Use the DiaMag 0.2 or DiaMag 1.5 magnetic racks for the separation of beads and buffer, e.g. during the washing steps.
	Centrifugation methods	Use gentle centrifugation in order to pellet the beads (up to 1500 g)

Protease inhibitors	Storage	Store provided P.I. mix at -20°C and thaw directly before use.
	Add to buffers directly before use	Add protease inhibitor mix to buffers directly before use. Since some inhibitors are unstable in solution, use buffer within 24 h.
Protease inhibitors	Storage	Store provided P.I. mix at -20°C and thaw directly before use.
	Add to buffers directly before use	Add protease inhibitor mix to buffers directly before use. Since some inhibitors are unstable in solution, use buffer within 24 h.
Antibodies	Antibody binding capacity to protein A or protein G	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C and thawed before use.
	Antibody quality	Add protease inhibitor mix to buffers, just before use, in HBSS (Steps 1 and 2), Lysis Buffer tC1 (Step 2), ChIP Buffer tC1 (Step 3). Discard within 24 hours.
	Determine amount of antibody per IP	Add phosphatase inhibitors or others to Lysis Buffer tC1 and ChIP Buffer tC1, if necessary, depending on your research field and protein(s) of interest. Add NaBu for histone ChIPs.
	Antibody-antigen recognition may affect ChIP efficiency	Antibody-antigen recognition can be significantly affected by cross-linking resulting in loss of epitope accessibility and/or recognition.
Negative ChIP controls	IgG control	Use the non-immune IgG fraction from the same species the antibodies were produced in as a negative control.
	No-antibody control	Do not add antibody to the IP to serve as a negative control. Alternatively incubation with uncoated beads could also be used as a negative ChIP control.
	Usage of un-blocked and blocked antibodies	Use one antibody in ChIP and the same antibody that is blocked with specific peptide. To specifically block one antibody, pre-incubate the antibody with saturating amounts specific peptide for its epitope for about 30 minutes at room temperature before using it in the IP incubation mix.
	Determine number of negative controls needed	If multiple antibodies of the same species are to be used with the same chromatin preparation then a single negative ChIP control is sufficient for all of the antibodies used.
Immunoprecipita–tion conditions	Temperature	We advise to perform the IP reaction on a rotating wheel at 4°C, and for duration of 2 to 16 hours depending on the antibody used.
	Ultrasonic water bath	The IP reaction can be performed in an ultrasonic bath in order to accelerate antigen binding to antibodies, which is the rate limiting step in ChP.
qPCR	Primer design	Primer length: 18 to 24 nucleotides and primer Tm: 60°C (+/-3.0°C)/ % GC: 50% (+/-4%)
	Include negative and positive controls	Negative PCR controls: PCR with DNA from samples IP'd with non-immune antibodies (negative IgG). Alternatively, PCR using DNA from ChIP samples and primers specific for a DNA region to which your antigen of interest is not binding. Positive PCR control: PCR using input DNA.
	Troubleshoot high Ct values	Use more input chromatin in the case of high Ct values.
	Determine the ratio between Ct(NegCtrl) and Ct(Target)	The ratio between target IP and negative control IP depends on the antibody used.
	Minimize background	Keep the antibody binding beads in suspension during the experiment. Check by eye that equal pellets of beads are present in each tube. Washes are critical.
	Analyse PCR products with melting curve	The melting curve analysis tells you if only the desired PRC product has been amplified. If there are several products the Ct values may be misleading.
Sample storage	Samples can be frozen at several steps of the protocol	Snap freeze the samples in liquid nitrogen (e.g. fixed cell pellets or sheared chromatin). Pellets of formaldehyde fixed cells can be stored at -80°C for at least one year. Sheared chromatin can be stored at -80°C for months, depending on the protein of interest. Purified DNA from ChIP and input samples can be stored at -20°C for months. Thaw samples on ice before use and avoid multiple freeze/thaw cycles.

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NOTICE TO PURCHASER

LIMITED LICENSE

TRADEMARKS

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Ordering information

Products	Cat. No. (new)	Cat. No. (old)	Format
IP-Star® Compact	B03000002	UH-002-0001	1 unit
Auto True MicroChIP kit x16	C01010140	/	16 rxns
Auto True MicroChIP & MicroPlex Library Preparation™ Package	C01010141	/	16 ChIP rxns & 12 library prep rxns
MicroPlex Library Preparation™ kit x12	C05010010	AB-004-0012	12 rxns
MicroPlex Library Preparation™ kit x48	C05010011	/	48 rxns
Auto Histone ChIP-seq kit protein A x16	C01010020	AB-Auto02-A016	16 rxns
Auto Histone ChIP-seq kit protein A x100	C01010022	AB-Auto02-A100	100 rxns
Auto Histone ChIP-seq kit protein G x16	C01010021	AB-Auto02-G016	16 rxns
Auto Histone ChIP-seq kit protein G x100	C01010023	AB-Auto02-G100	100 rxns
Auto iDeal ChIP-seq kit for Histones x 24	C01010057	/	24 rxns
Auto Transcription ChIP kit protein A x16	C01010030	AB-Auto03-A016	16 rxns
Auto Transcription ChIP kit protein A x100	C01010032	AB-Auto03-A100	100 rxns
Auto Transcription ChIP kit protein G x16	C01010031	AB-Auto03-G016	16 rxns
Auto Transcription ChIP kit protein G x100	C01010033	AB-Auto03-G100	100 rxns
Auto iDeal ChIP-seq kit for Transcription Factors x24	C01010058	/	24 rxns
Auto Plant ChIP-seq kit x24	C01010151	/	24 rxns
Auto MeDIP kit x16	C02010011	AF-Auto01-0016	16 rxns
Auto MeDIP kit x100	C02010012	AF-Auto01-0100	100 rxns
iDeal Library Preparation Kit x24 (incl. Index Primer Set 1)	C05010020	/	24 rxns
Auto hMeDIP kit x16	C02010033	AF-Auto02-0016	16 rxns
Auto MethylCap x48	C02020011	AF-Auto01-0048	48 rxns
Auto IPure kit v2 x100	C03010010	AL-Auto01-0100	100 rxns

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