diagenode

A Hologic Company

Auto iDeal ChIP-seq kit for Histones

Cat. No. **C01010057** (24 rxns)
Cat. No. **C01010171** (100 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 iDeal ChIP-seq kit for Histones

The Auto iDeal ChIP-seq kit for Histones 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 iDeal ChIP-seq kit for Histones 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 iDeal ChIP-seq kit for Histones has been validated to perform ChIP-seq experiments using antibodies directed against histone 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 iDeal ChIP-seq kit for Histones 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.

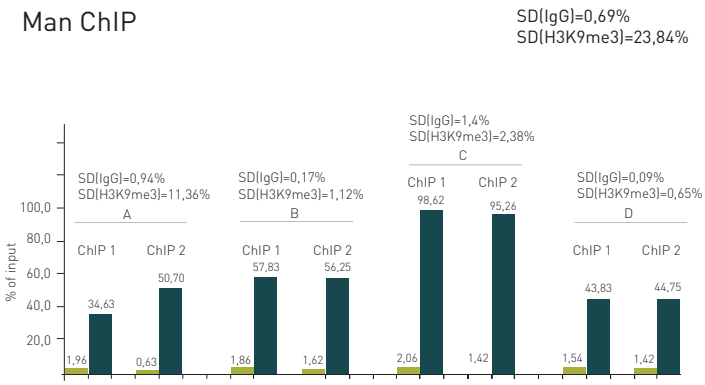


Figure 1. Manual ChIP

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.

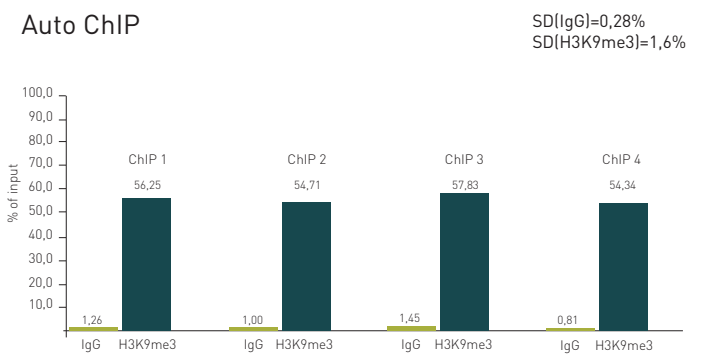


Figure 2. Automated ChIP

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

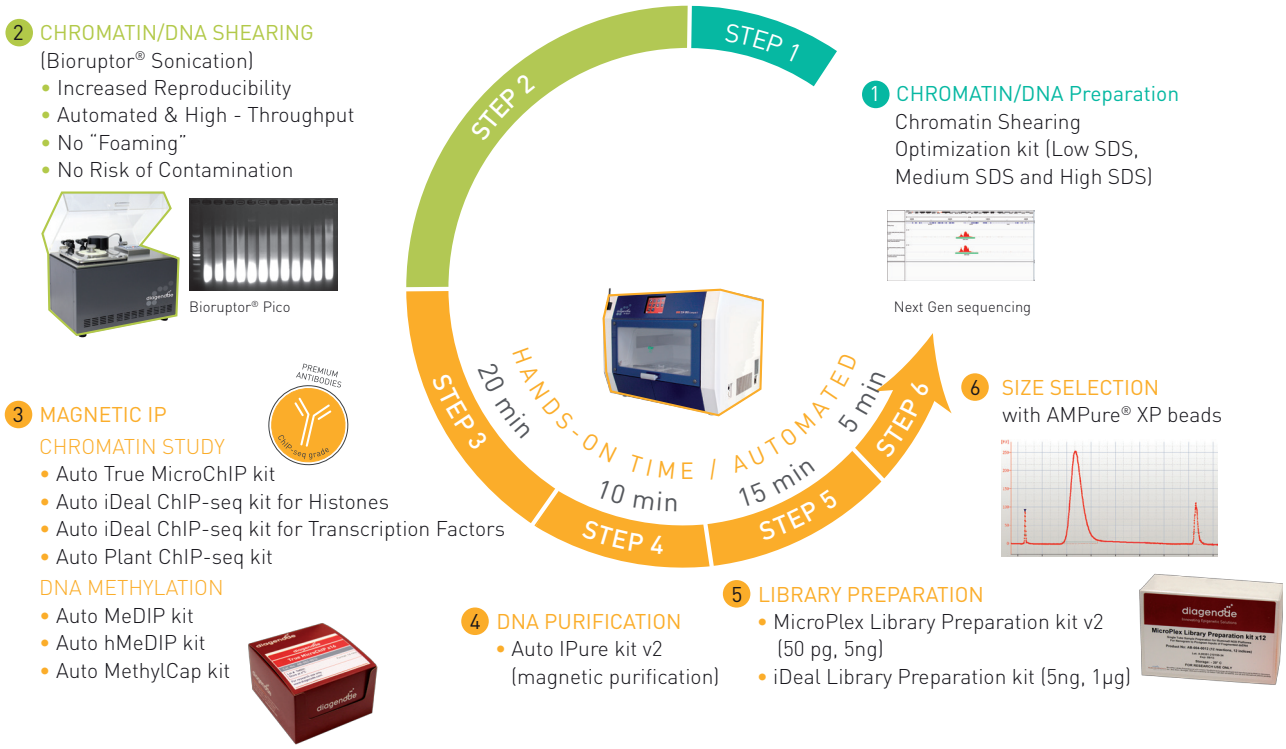


Figure 3. Diagenode provides a full suite of automated solutions for ChIP experiments

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 Auto iDeal ChIP-seq kit for Histones x100 contains reagents to perform 17 different chromatin preparations, 100 Chromatin Immunoprecipitations and DNA Purification by using the IP-Star's Automated System.

The Auto iDeal ChIP-seq kit for Histones x24 contains reagents to perform 4 different chromatin preparations, 24 Chromatin Immunoprecipitations and DNA Purification by using the IP-Star's Automated System. The kit content is described in Table 1. Upon receipt, store the components at the temperatures indicated in Table 1.

Table 1. Kit content

Description	Quantity (x24)	Quantity (x100)	Storage
Protease inhibitor cocktail	65 µl	365 µl	-20°C
5% BSA (DNA free)	175 µl	720 µl	-20°C
Rabbit IgG	15 µl (1µg/µl)	40 µg (1µg/µl)	-20°C
ChIP-seq grade antibody H3K4me3	10 µg (1µg/µl)	40 µg (1µg/µl)	-20°C
ChIP-seq grade GAPDH TSS primer pair	50 µl	500 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair	50 µl	500 µl	-20°C
Carrier	55 µl	320 µl	-20°C
Glycine	420 µl	1.9 ml	4°C
Shearing Buffer iS1	4 ml	34 ml	4°C
DiaMag protein A-coated magnetic beads	580 µl	2.2 ml	4°C - DO NOT FREEZE
Wash buffer iW1	10 ml	42 ml	4°C
Wash buffer iW2	10 ml	42 ml	4°C
Wash buffer iW3	10 ml	42 ml	4°C
Wash buffer iW4	10 ml	42 ml	4°C
ChIP-seq grade water	10 ml	40 ml	4°C
Elution Buffer iE2	580 µl	720 µl	4°C
Wash buffer 1 w/o iso-propanol	2 ml	8 ml	4°C
Wash buffer 2 w/o iso-propanol	2 ml	8 ml	4°C
Buffer C	4 ml	8 ml	4°C
IPure Beads v2	385 µl	1.6 ml	4°C - DO NOT FREEZE
Elution Buffer iE1	15 ml	16 ml	4°C
5x ChIP Buffer iC1	4 ml	11 ml	4°C
Lysis Buffer iL1	36 ml	190 ml	4°C
Lysis Buffer iL2	36 ml	190 ml	4°C

Table 2. Kits and Modules available separately

Description	Reference	Quantity
Chromatin shearing optimization kit-low SDS (for Histones)	C01020010	100 rxns
Auto IPure kit v2 x100	C02010012	100 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 materials not provided

Reagents

- Gloves to wear at all steps
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS) buffer
- 1 M Sodium butyrate (NaBu) (Cat. No. C12020010) (optional)
- 100% isopropanol
- RNase/DNase-free 1.5 ml tubes
- qPCR SYBR® Green Mastermix
- Reagents for library preparation, cluster generation (Illumina®) or ePCR (Ion TorrentTM PGMTM) and sequencing
- Quant-IT dsDNA HS assay kit (Invitrogen)

For library preparation, we highly recommend

- MicroPlex Library Preparation™ kit (Cat. No. C05010012, 12 reactions, 12 indices) (Cat. No. C05010011, 48 reactions, C05010014 indices)
- iDeal Library Preparation kit x24 (incl. Index Primer Set 1), (Cat. No. C05010020)

Equipment

- Diagenode DiaMag02 magnetic rack (Cat. No. B04000001)
- Diagenode Bioruptor® sonication device (Cat. No. Standard: B01010001, Plus: B01020001, and Pico: B01060001)
- Diagenode 1.5 ml TPX Microtubes (optimized for chromatin shearing with Bioruptor® Standard or Plus) (Cat.No. C30010010) or 1.5 ml Bioruptor® Microtubes with Caps (Cat No C30010016) optimized for chromatin shearing with Bioruptor® Pico
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- Cell counter
- Vortex
- Thermomixer
- Qubit system (Invitrogen)
- qPCR cycler
- For tissues:
    - Dounce homogenizer with loose and tight fitting pestles
    - Scalpel blades
    - Petri dishes

Remarks before starting

1a. Cell number (for cultured cells)

This protocol has been optimized for ChIP on 1,000,000 cells in 200 µl ChIP reaction. It is possible to use more cells. However, for optimal performance, we recommend performing separate ChIPs and pool the IP'd DNA before purification.

1b. Tissue amount (for fresh or frozen tissues)

This protocol has been optimized for ChIP from fresh or frozen mammalian tissues. The chromatin is prepared from 30-40 mg of tissue allowing up to 18 ChIP samples (about 1.5 - 2 mg of tissue per IP). However, the exact amount of tissue needed for ChIP-seq may vary depending on protein abundance, antibody affinity etc. and should be determined for each tissue type. We recommend performing a pilot experiment.

2. Shearing optimization and sheared chromatin analysis

Before starting the ChIP, the chromatin should be sheared into fragments of 100 to 600 bp. Our kits and protocols are optimized for chromatin shearing using the Bioruptor® (Standard, Plus and Pico). The maximum volume for shearing with the Bioruptor® is 300 µl per 1.5 ml Microtube (depending on the specific type). We recommend using TPX tubes (C30010010) for Bioruptor® Standard and Plus as shearing has been shown to be more efficient and reproducible using these tubes. For Bioruptor® Pico we recommend using 1.5 ml Microtubes with Caps (C30010016). The shearing conditions mentioned in the protocol are adequate for a variety of cell types. However, given that cell types are different, we recommend optimizing sonication conditions for each cell type before processing large quantities of cells or samples. It is important to 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. Make sure the beads do not dry during the procedure as this will result in reduced performance. Keep the beads homogenously in 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 (IgG and control Ab)

The kit contains a negative (IgG) and a positive (H3K4me3) 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 (GAPDH-TSS and Myoglobin exon 2, respectively).

5. Quantification

Determine the concentration of the IP'd DNA after the ChIP with a highly sensitive method such as the 'Quant-IT dsDNA HS assay kit' on the Qubit system from Invitrogen. PicoGreen is also suitable but UV spectrophotometric methods such as the NanoDrop are usually not sufficiently sensitive. In most cases it is sufficient to use approximately 10% of the IP'd material for quantification. The expected DNA yield will be dependent on different factors such as the cell type, the quality of the antibody used and the antibody target. The expected DNA yield obtained with the positive control H3K4me3 antibody on 1,000,000 HeLa cells is approximately 10 ng.

6. Quantitative PCR

Before sequencing the samples, we recommend analysing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. The kit contains a positive and negative control primer pair which can be used for the H3K4me3 positive control antibody in SYBR® Green qPCR assay using the protocol described in the manual. Use your own method of choice

for analysing the appropriate control targets for your antibodies of interest.

In order to have sufficient DNA left for sequencing, we recommend not using more than 10% of the total IP'd DNA for qPCR. You can dilute the DNA (1/10 or more) to perform sufficient PCR reactions. PCR reactions should be performed at least in duplicate although performing them in triplicate is recommended to be able to identify potential outliers.

7. Quantitative PCR data interpretation

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 (the relative amount of immunoprecipitated DNA compared to input DNA). If the amount used for the input was 1% of the amount used for ChIP, the recovery can be calculated as follows:

% recovery = $2^{(Ct_{input} - Ct_{sample})}$

Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and input, respectively. This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results the real amplification efficiency, if known, should be used.

For the positive control antibody (e.g. H3K4me3) the recovery of the positive control target (GAPDH TSS locus) is expected to be between 10 and 20% although this will depend on the cell type used. The recovery of the negative control target (Myoglobin exon 2 locus) should be below 1%.

Criteria to decide whether the sample is good enough for sequencing will be largely target dependant. Therefore, the following are only general guidelines:

- the recovery of the positive control target should be at least 5%
- the recovery of the negative control target should be below 1%
- the ratio of the positive versus the negative control target should be at least 5

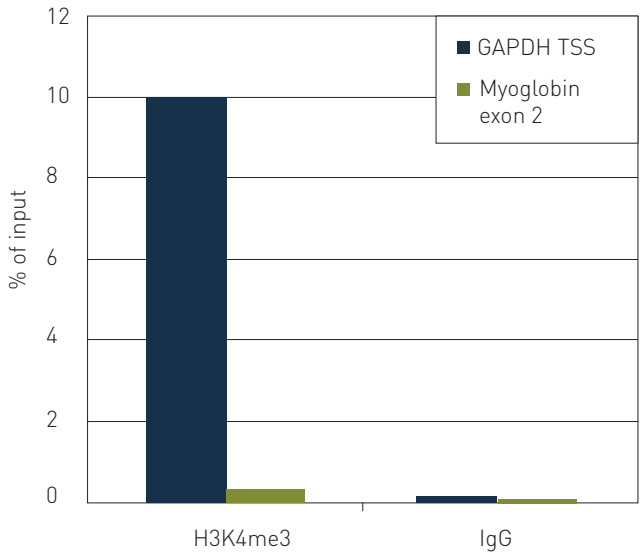


Figure 4

ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit for Transcription Factors. Sheared chromatin from 4 million cells, 0.5 µl of the positive control antibody and 1 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control H19 imprinting control region and the negative control Myoglobin exon 2 primer sets from the kit. The recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

IP-STAR® COMPACT

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

Protocol

STEP 1a. Cell collection and DNA-protein cross-linking (for cultured cells)

The protocol below is intended for adherent cells. Collect suspension cells by centrifugation and continue with the protocol starting from step 6.

1. Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.
2. Remove the medium and rinse the cells with pre-warmed PBS (10 ml for a 75 cm2 culture flask). Gently shake the flask for 2 min.
3. Remove the PBS and add sterile trypsin-EDTA to the culture flask to detach adherent cells from the bottom. Table 4 shows the required amount of trypsin for different numbers of cells. Gently shake the culture flask for 1-2 min or until the cells start to detach. The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment with trypsin may damage the cells. Regularly check if the cells start to detach.

Table 4			
# of cells	3x 10e6 cells	10e7 cells	5x 10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

4. Immediately add fresh culture medium to the cells when they are detached (Table 5). This will inactivate trypsin. Tansfer cell suspension to a 50 ml tube.

Table 5			
# of cells	3x 10e6 cells	10e7 cells	5x 10e7cells
Culture medium	2 ml	6 ml	30 ml

5. Rinse the flask by adding 10 ml of PBS. Add this volume to your 50 ml tubes containing cells from point 4.
6. Centrifuge for 5 min at 1600 rpm and 4°C and remove the supernatant.
7. Resuspend the cells in 20 ml of PBS and count them. Collect the cells by centrifugation for 5 min at 1600 rpm and 4°C.
8. Resuspend the cells in PBS to obtain a concentration of up to 10 million cells per 500 µl of PBS. If desired, the cell concentration can be decreased down to 1 million per 500 µl. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.
9. Add 13.5 µl of formaldehyde 37% to each tube containing 500 µl of cell suspension. Mix by gentle vortexing and incubate for 8 min at room temperature to allow fixation to take place.
10. Add 57 µl of **Glycine** to the cells to stop the fixation. Mix by gentle vortexing and incubate for 5 min at room temperature. Keep the cells on ice from this point onwards.
11. Collect the cells by centrifugation at 1600 rpm for 5 min and 4°C. Discard the supernatant without disturbing the cell pellet.
12. Wash the cells twice with 1 ml of cold PBS.
    → Proceed to Step 2a: *Cell lysis and chromatin shearing (for cultured cells)*

STEP 1b. Tissue disaggregation and DNA-protein cross-linking (for fresh and frozen tissue)

13. Weigh 30-40 mg of fresh or frozen tissue in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.
14. Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade.
15. Add 1 ml of ice-cold PBS with protease inhibitors cocktail and disaggregate the tissue using a dounce homogenizer (loose pestle) to get a homogeneous suspension.
16. Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 1300 rpm for 5 min at 4°C. Gently discard the supernatant and keep the pellet.

17. Resuspend the pellet in 1 ml of PBS containing 1% of formaldehyde at room temperature.
18. Rotate tube for 8-10 min at room temperature.
19. The fixation time might require an additional optimization. In general, histone marks require shorter fixation (8 min) than transcriptional factors (10-15 min). Please note that stronger fixation may lead to chromatin resistant to sonication.
20. Stop the cross-linking reaction by adding 100 µl of glycine. Continue to rotate at room temperature for 5 min.
21. Centrifuge samples at low speed (1300 rpm) at 4°C.
22. Wash the pellet with ice-cold PBS. Aspirate the supernatant and resuspend the pellet in 1 ml of ice-cold PBS plus protease inhibitors.
23. Centrifuge at low speed (1300 rpm) at 4°C and discard the supernatant.
24. Repeat the washing one more time.

→ Proceed to Step 2b: *Cell lysis and chromatin shearing (derived from tissue samples)*

STEP 2a. Cell lysis and chromatin shearing (for cultured cells)

25. Add 1 ml of ice-cold **Lysis buffer iL1** to the 1.5 ml tube containing 10 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 9 ml of **buffer iL1** and incubate for 10 min at 4°C with gentle mixing. If the starting amount of cells was less than 10 million, scale down accordingly (e.g. use a total of 5 ml **buffer iL1** for 5 million cells).
26. Pellet the cells by centrifugation at 1,600 rpm for 5 min and 4°C and discard the supernatant.
27. Add 1 ml of ice-cold **Lysis buffer iL2** and resuspend the cells by pipetting up and down several times. Add another 9 ml of **buffer iL2** and incubate for 10 min at 4°C with gentle mixing. Scale down accordingly when using less than 10 million cells.
28. Pellet the cells again by centrifugation for 5 min at 1,600 rpm (500 x g) and 4°C and discard supernatant.
29. Add 200x **protease inhibitor cocktail** to **Shearing buffer iS1**. Prepare 1 ml of complete shearing buffer per tube of 10 million cells. Keep on ice.
30. Add 1 ml of complete **Shearing buffer iS1** to 10 million cells. Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1 million cells per 100 µl **buffer iS1**. Split into aliquots of 100 to 300 µl and transfer the cell suspension to **1.5 ml TPX tubes** (Cat. No. C30010009). Incubate on ice for 10 min. Vortex and spin down the samples.
31. Split the samples into 300 µl aliquots in 1.5 ml sonication tubes and incubate on ice for 10 min. Please use only recommended tubes:
    - For Bioruptor® Standard or Plus, 1.5 ml TPX tubes (Cat. No. C30010009)
    - For Bioruptor® Pico, 1.5 ml Bioruptor® Microtubes with caps (Cat. No. C30010016)
32. Centrifuge at 13,000 rpm (16,000 x g) for 10 min and collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. If desired, the chromatin shearing effciciency can be analyzed at this step (see **"Additional protocols"**).

STEP 2b. Cell lysis and chromatin shearing (derived from tissue samples)

33. Add 10 ml of ice-cold **Lysis buffer iL1** to the pellet corresponding to 30-40 mg of tissue. Resuspend the pellet by pippeting up and down and incubate for 10 min at 4°C with gentle mixing.
34. Centrifuge for 5 min at 1,300 rpm at 4°C and discard the supernatant.
35. Add 10 ml of ice-cold **Lysis buffer iL2** to the pellet. Resuspend the pellet by pippeting up and down and incubate for 10 min at 4°C with gentle mixing.
36. Centrifuge for 5 min at 1,300 rpm at 4°C and discard the supernatant.
37. Resuspend the pellet in 1.8 ml of **shearing buffer iS1** containing protease inhibitors cocktail and homogenize using a dounce homogenizer (tight pestle).

38. Split the samples into 300 µl aliquots in 1.5 ml sonication tubes and incubate on ice for 10 min. Please use only recommended tubes:
    - For Bioruptor® Standard or Plus, 1.5 ml TPX tubes (Cat. No. C30010009)
    - For Bioruptor® Pico, 1.5 ml Bioruptor® Microtubes with caps (Cat. No. C30010016)

39. Shear chromatin by sonication using the Bioruptor®. An initial time course experiment is highly recommended.
    - For Bioruptor® Standard or Plus use High power setting for 10-30 cycles (30 sec ON/30 sec OFF). Stop the system after each run of 10 cycles, vortex and spin down sample.
    - For Bioruptor® Pico, sonicate samples for 5-15 cycles (30 sec ON/30 sec OFF). Vortexing is not required between runs.

40. Transfer samples to new 1.5 ml tubes and centrifuge at 13,000 rpm for 10 min.

41. Collect the supernatant which contains the sheared chromatin.

42. Take an aliquot of 100 µl for assessment of chromatin shearing (see "**Additional Protocol**"). The remaining chromatin can be stored at -80°C for up to 2 months for further use in the immunoprecipitation.

STEP 3. Magnetic immunoprecipitation

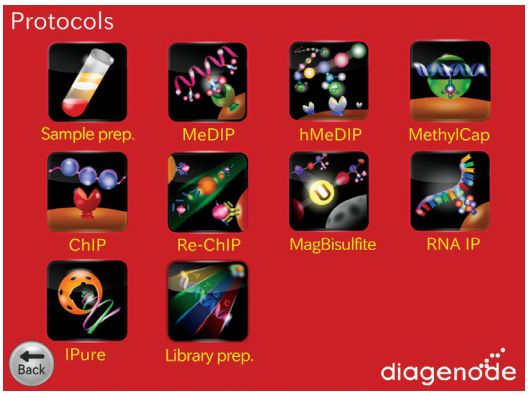
This protocol has been optimized for 1 million cells per ChIP. Although it is possible to use more cells, we recommend performing separate ChIP reactions and pool the samples before purification of the DNA. Determine the total number of IP's in the experiment. Please note that we recommend to include one negative control in each experiment (IP with the IgG negative control)

ChIP method

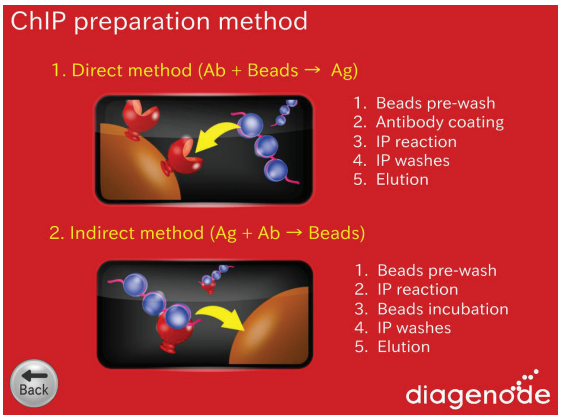
With this method the antibody is first coated on the surface of the magnetic beads and after that the bound antibodies are added to the sheared chromatin.

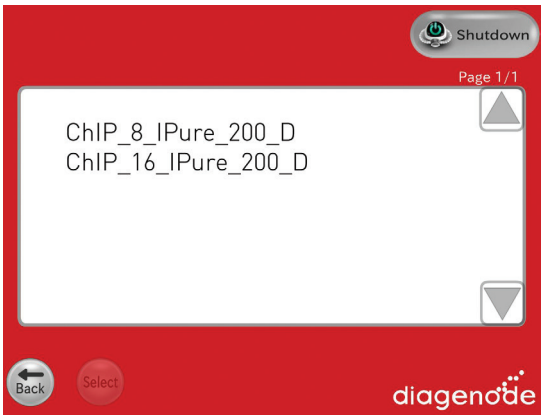
43. Switch ON the IP-Star® Compact.

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



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



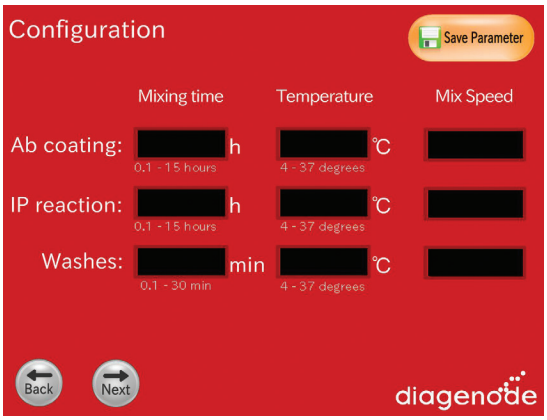


NOTE:
If you plan to run between 1 and 8 samples, chose "**ChIP_IPure_8_200_D**"
If you plan to run between 9 and 16 samples, chose "**ChIP_IPure_16_200_D**"

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

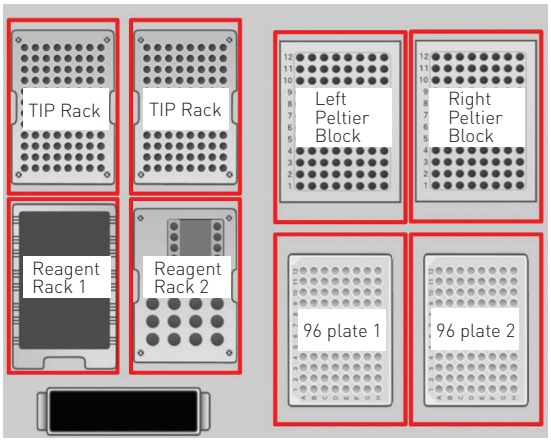
NOTE:
The **Peltier Block** is now cooling down to 4°C to keep your samples cold

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



Setup the "Ab coating" step to 3 hours
Setup the "IP reaction" step to 10-15 hours (overnight)
Setup the "Washes" step to 5-10 min

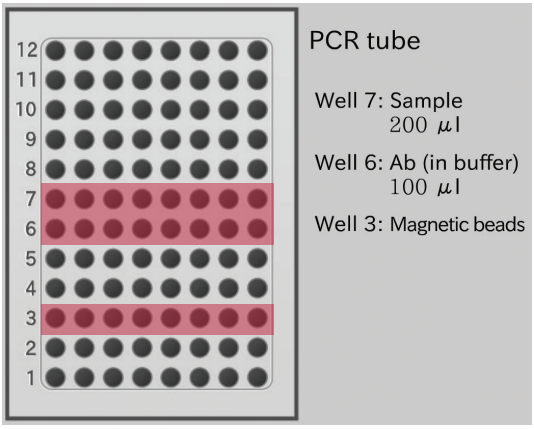
48. Setup all the plastics on the platform according to the screen layout.



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

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

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



Direct ChIP

1. Prepare 1x ChIP Buffer iC1

Mix for 1 IP (400µl per IP is needed)

5x ChIP Buffer iC1	80 µl
ChIP-seq grade Water	320 µl

- If ≤ 8 samples, prepare 400 µl excess (1 IP excess)
- If ≥ 9 samples, prepare 1200 µl excess (3 IP excess)

2. Preparation of Ab coating mix (Well 6)

Antibody	x µl
1x ChIP Buffer iC1	100 – x µl
200x Protease Inhibitor Cocktail	0.5 µl
5% BSA (DNA free)	2 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)

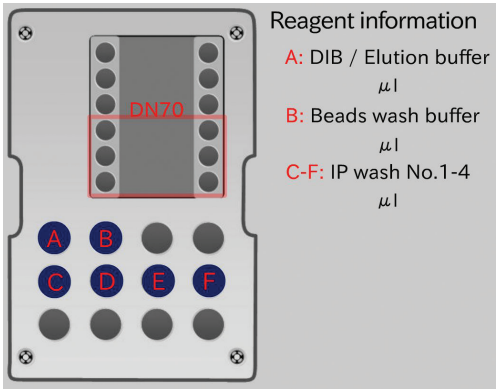
Sheared chromatin	100 µl
1x ChIP Buffer iC1	100 µl
200x Protease Inhibitor Cocktail	1 µl
5% BSA (DNA free)	4 µl

Keep 1 µl of the sheared chromatin aside for the Input

This Auto iDeal ChIP-seq kit for Histones 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.

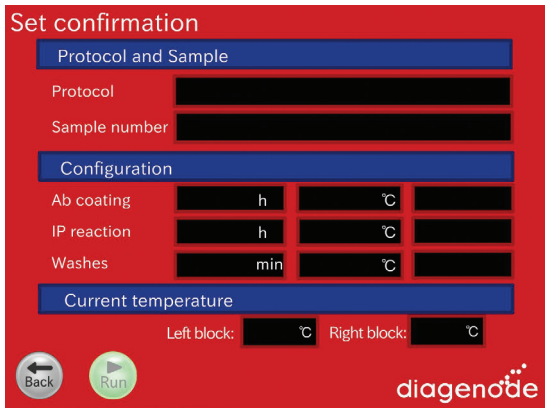
NOTE:
If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added to the chromatin sample.

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

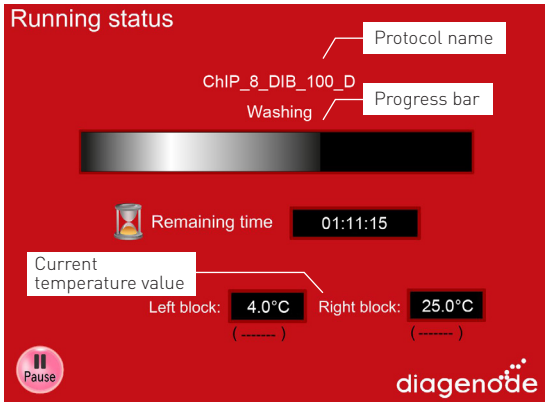


Beads Wash Buffer: 1x ChIP buffer iC1
Elution buffer: Elution Buffer iE1
IP wash 1: Wash Buffer iW1
IP wash 2: Wash Buffer iW2
IP wash 3: Wash Buffer iW3
IP wash 4: Wash Buffer iW4

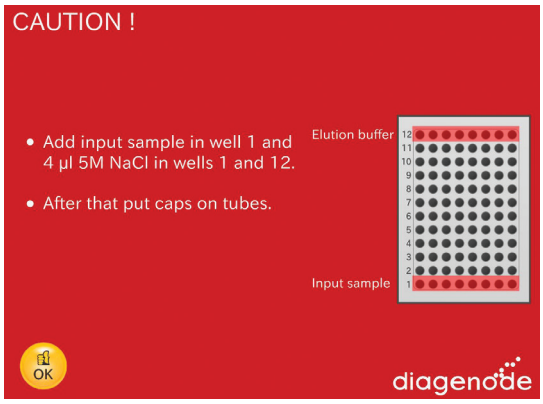
53. Check the selected parameters, close the door and press "Run" to start



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



55. 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.

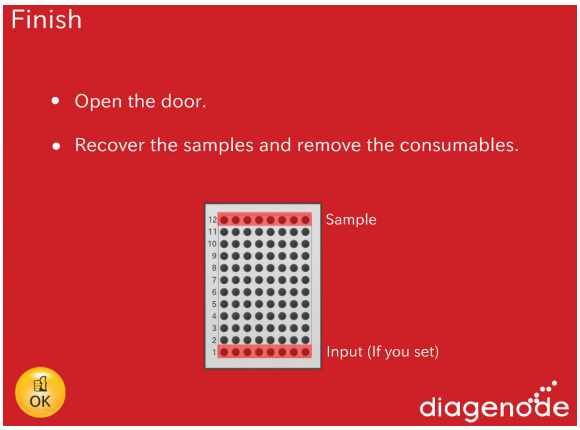


- Setup the Input in the 1st well
  INPUT= 1 µl sheared chromatin + 95 µl Elution Buffer iE1
- Add **4 µl of Elution Buffer iE2** (5M NaCl) in all the samples (well 12) and inputs (well 1)
- Close the tubes with the caps, close the door and press OK

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.

56. 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 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®.

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

58. 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**"

59. 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.

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

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

62. 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.

63. 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

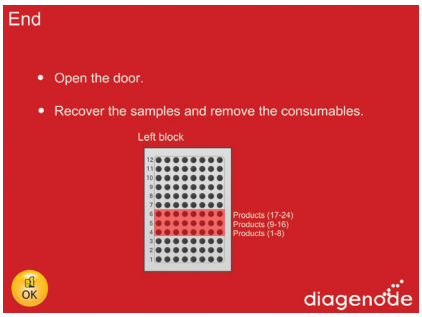
Wash buffer 2		
	24 rxns	100 rxns
Wash buffer 1w/o isopropanol	2 ml	8 ml
Isopropanol (100%)	2 ml	8 ml
Total volume	4 ml	16 ml

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

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

66. Press **Run** to start

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



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

69. 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®

Protocol

STEP 1a. Cell collection and DNA-protein cross-linking (for cultured cells)

The protocol below is intended for adherent cells. Collect suspension cells by centrifugation and continue with the protocol starting from step 6.

1. Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.
2. Remove the medium and rinse the cells with pre-warmed PBS (10 ml for a 75 cm2 culture flask). Gently shake the flask for 2 min.
3. Remove the PBS and add sterile trypsin-EDTA to the culture flask to detach adherent cells from the bottom. Table 4 shows the required amount of trypsin for different numbers of cells. Gently shake the culture flask for 1-2 min or until the cells start to detach. The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment with trypsin may damage the cells. Regularly check if the cells start to detach.

Table 4

# of cells	3x 10e6 cells	10e7 cells	5x 10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

4. Immediately add fresh culture medium to the cells when they are detached (Table 5). This will inactivate trypsin. Tansfer cell suspension to a 50 ml tube.

Table 5

# of cells	3x 10e6 cells	10e7 cells	5x 10e7cells
Culture medium	2 ml	6 ml	30 ml

5. Rinse the flask by adding 10 ml of PBS. Add this volume to your 50 ml tubes containing cells from point 4.
6. Centrifuge for 5 min at 1600 rpm and 4°C and remove the supernatant.
7. Resuspend the cells in 20 ml of PBS and count them. Collect the cells by centrifugation for 5 min at 1600 rpm and 4°C.
8. Resuspend the cells in PBS to obtain a concentration of up to 10 million cells per 500 µl of PBS. If desired, the cell concentration can be decreased down to 1 million per 500 µl. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.
9. Add 13.5 µl of formaldehyde 37% to each tube containing 500 µl of cell suspension. Mix by gentle vortexing and incubate for 8 min at room temperature to allow fixation to take place.
10. Add 57 µl of **Glycine** to the cells to stop the fixation. Mix by gentle vortexing and incubate for 5 min at room temperature. Keep the cells on ice from this point onwards.
11. Collect the cells by centrifugation at 1600 rpm for 5 min and 4°C. Discard the supernatant without disturbing the cell pellet.
12. Wash the cells twice with 1 ml of cold PBS.
→ Proceed to Step 2a: *Cell lysis and chromatin shearing (for cultured cells)*

STEP 1b. Tissue disaggregation and DNA-protein cross-linking (for fresh and frozen tissue)

13. Weigh 30-40 mg of fresh or frozen tissue in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.
14. Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade.
15. Add 1 ml of ice-cold PBS with protease inhibitors cocktail and disaggregate the tissue using a dounce homogenizer (loose pestle) to get a homogeneous suspension.
16. Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 1300 rpm for 5 min at 4°C. Gently discard the

supernatant and keep the pellet.

17. Resuspend the pellet in 1 ml of PBS containing 1% of formaldehyde at room temperature.
18. Rotate tube for 8-10 min at room temperature.
19. The fixation time might require an additional optimization. In general, histone marks require shorter fixation (8 min) than transcriptional factors (10-15 min). Please note that stronger fixation may lead to chromatin resistant to sonication.
20. Stop the cross-linking reaction by adding 100 µl of glycine. Continue to rotate at room temperature for 5 min.
21. Centrifuge samples at low speed (1300 rpm) at 4°C.
22. Wash the pellet with ice-cold PBS. Aspirate the supernatant and resuspend the pellet in 1 ml of ice-cold PBS plus protease inhibitors.
23. Centrifuge at low speed (1300 rpm) at 4°C and discard the supernatant.
24. Repeat the washing one more time.

→ Proceed to Step 2b: *Cell lysis and chromatin shearing (derived from tissue samples)*

STEP 2a. Cell lysis and chromatin shearing (for cultured cells)

25. Add 1 ml of ice-cold **Lysis buffer iL1** to the 1.5 ml tube containing 10 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 9 ml of **buffer iL1** and incubate for 10 min at 4°C with gentle mixing. If the starting amount of cells was less than 10 million, scale down accordingly (e.g. use a total of 5 ml **buffer iL1** for 5 million cells).
26. Pellet the cells by centrifugation at 1,600 rpm for 5 min and 4°C and discard the supernatant.
27. Add 1 ml of ice-cold **Lysis buffer iL2** and resuspend the cells by pipetting up and down several times. Add another 9 ml of **buffer iL2** and incubate for 10 min at 4°C with gentle mixing. Scale down accordingly when using less than 10 million cells.
28. Pellet the cells again by centrifugation for 5 min at 1,600 rpm (500 x g) and 4°C and discard supernatant.
29. Add 200x **protease inhibitor cocktail** to **Shearing buffer iS1**. Prepare 1 ml of complete shearing buffer per tube of 10 million cells. Keep on ice.
30. Add 1 ml of complete **Shearing buffer iS1** to 10 million cells. Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1 million cells per 100 µl **buffer iS1**. Split into aliquots of 100 to 300 µl and transfer the cell suspension to **1.5 ml TPX tubes** (Cat. No. C30010009). Incubate on ice for 10 min. Vortex and spin down the samples.
31. Split the samples into 300 µl aliquots in 1.5 ml sonication tubes and incubate on ice for 10 min. Please use only recommended tubes:
    - For Bioruptor® Standard or Plus, 1.5 ml TPX tubes (Cat. No. C30010009)
    - For Bioruptor® Pico, 1.5 ml Bioruptor® Microtubes with caps (Cat. No. C30010016)
32. Centrifuge at 13,000 rpm (16,000 x g) for 10 min and collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. If desired, the chromatin shearing effciciency can be analyzed at this step (see **"Additional protocols"**).

STEP 2b. Cell lysis and chromatin shearing (derived from tissue samples)

33. Add 10 ml of ice-cold **Lysis buffer iL1** to the pellet corresponding to 30-40 mg of tissue. Resuspend the pellet by pippeting up and down and incubate for 10 min at 4°C with gentle mixing.
34. Centrifuge for 5 min at 1,300 rpm at 4°C and discard the supernatant.
35. Add 10 ml of ice-cold **Lysis buffer iL2** to the pellet. Resuspend the pellet by pippeting up and down and incubate for 10 min at 4°C with gentle mixing.

36. Centrifuge for 5 min at 1,300 rpm at 4°C and discard the supernatant.

37. Resuspend the pellet in 1.8 ml of **shearing buffer iS1** containing protease inhibitors cocktail and homogenize using a dounce homogenizer (tight pestle).

38. Split the samples into 300 µl aliquots in 1.5 ml sonication tubes and incubate on ice for 10 min. Please use only recommended tubes:
- For Bioruptor® Standard or Plus, 1.5 ml TPX tubes (Cat. No. C30010009)
- For Bioruptor® Pico, 1.5 ml Bioruptor® Microtubes with caps (Cat. No. C30010016)

39. Shear chromatin by sonication using the Bioruptor®. An initial time course experiment is highly recommended.
- For Bioruptor® Standard or Plus use High power setting for 10-30 cycles (30 sec ON/30 sec OFF). Stop the system after each run of 10 cycles, vortex and spin down sample.
- For Bioruptor® Pico, sonicate samples for 5-15 cycles (30 sec ON/30 sec OFF). Vortexing is not required between runs.

40. Transfer samples to new 1.5 ml tubes and centrifuge at 13,000 rpm for 10 min.

41. Collect the supernatant which contains the sheared chromatin.

42. Take an aliquot of 100 µl for assessment of chromatin shearing (see "**Additional Protocol**"). The remaining chromatin can be stored at -80°C for up to 2 months for further use in the immunoprecipitation.

→ Proceed to Step 3: *Magnetic immunoprecipitation*

STEP 3. Magnetic immunoprecipitation

This protocol has been optimized for 1 million cells per ChIP. Although it is possible to use more cells, we recommend performing separate ChIP reactions and pool the samples before purification of the DNA.

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 Buffer iC1

Mix for 1 IP (400µl per IP is needed)

5x ChIP Buffer iC1	80 µl
ChIP-seq grade Water	320 µl

- If ≤ 8 samples, prepare 400 µl excess (1 IP excess)
- If ≥ 9 samples, prepare 1200 µl excess (3 IP excess)

2. Preparation of Ab coating mix (Well 6)

Antibody	x µl
1x ChIP Buffer iC1	100 – x µl
200x Protease Inhibitor Cocktail	0.5 µl
5% BSA (DNA free)	2 µl

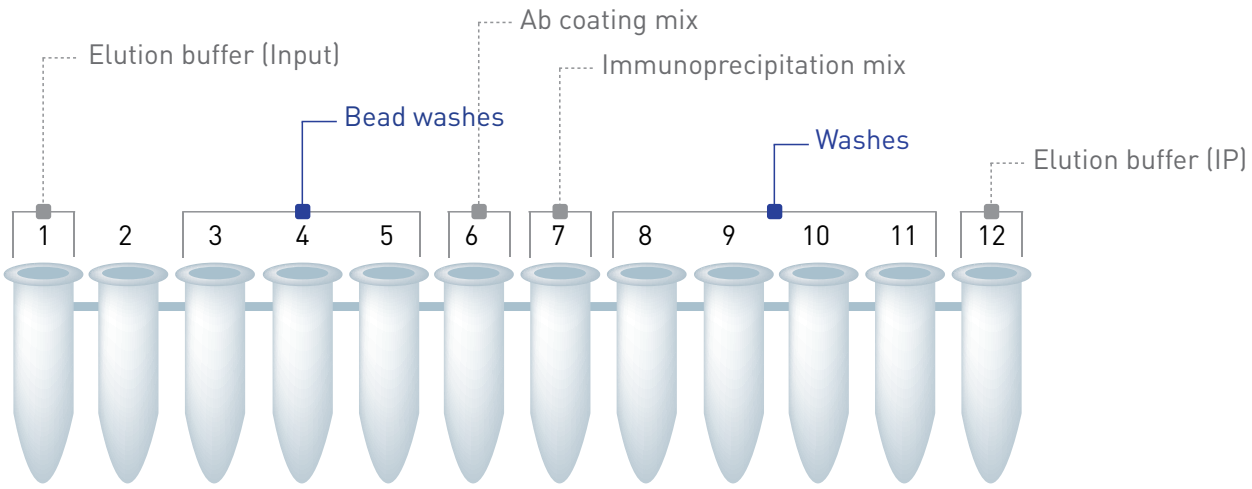
Use 1 µl of the rabbit IgG (1µg/µl) control antibody for the negative control IP. If a positive control IP is included in the experiment, use 1 µl of the H3K4me3 (1µg/µl) ChIP-seq grade control antibody

3. Preparation Immunoprecipitation mix (Well 7)

Sheared chromatin	100 µl
1x ChIP Buffer iC1	100 µl
200x Protease Inhibitor Cocktail	1 µl
5% BSA (DNA free)	4 µl

Keep 1 µl of the sheared chromatin aside for the Input

4. Load the reagents



	IPURE	
Tube #	Description	200 µl protocol
1	Elution Buffer iE1 + Elution Buffer iE2	95 µl + 4 µl
2	Empty	-
3	Magnetic beads*	10-20 µl
4	1x ChIP Buffer iC1	100 µl
5	1x ChIP Buffer iC1	100 µl
6	Ab coating mix	100 µl
7	Immunprecipitation mix	200 µl
8	Wash Buffer iW1	150 µl
9	Wash Buffer iW2	150 µl
10	Wash Buffer iW3	150 µl
11	Wash Buffer iW4	150 µl
12	Elution Buffer iE1 + Elution Buffer iE2	96 µl + 4 µl

* This Auto iDeal ChIP-seq kit for Histones 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

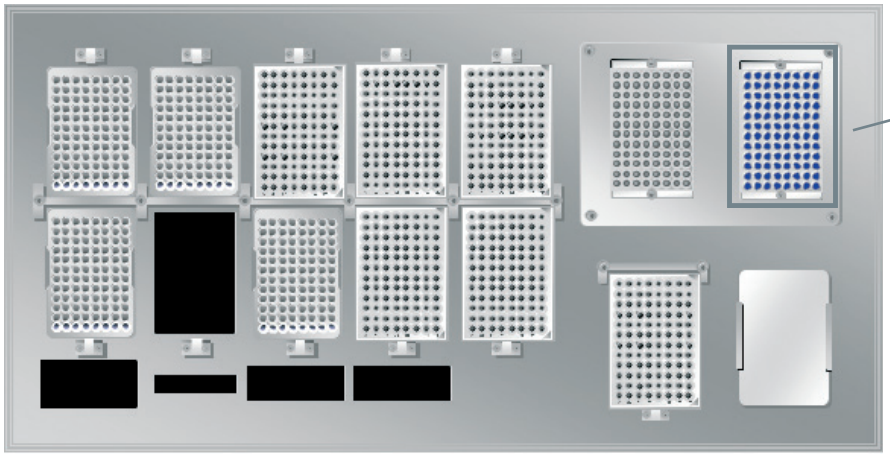
Be sure that the computer connected to the SX-8G IP-Star never switches to the standby modus. (standby modus has to be inactivated). Standby of the computer will lead to the abort of the protocol.

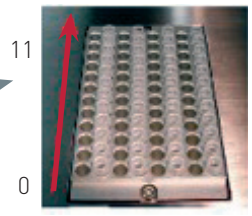
Table 3.

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

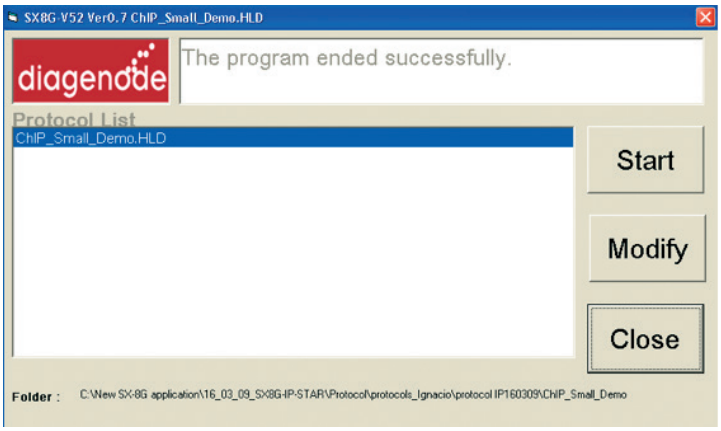
* Input required is sheared chromatin ready-to-ChIP

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:

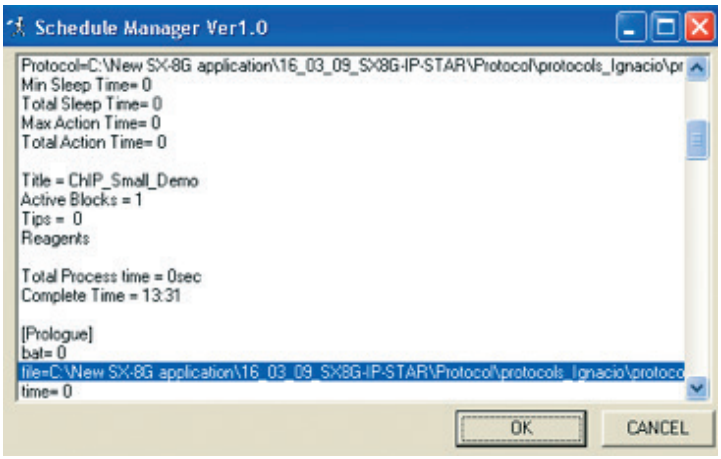
*[**EASYSTARTSCREEN**]*

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

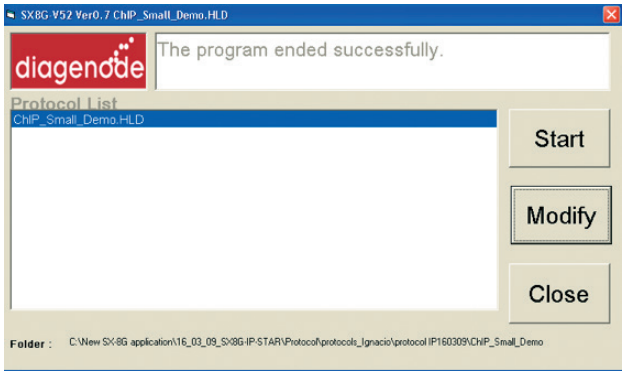
for loading ChIP Direct protocols

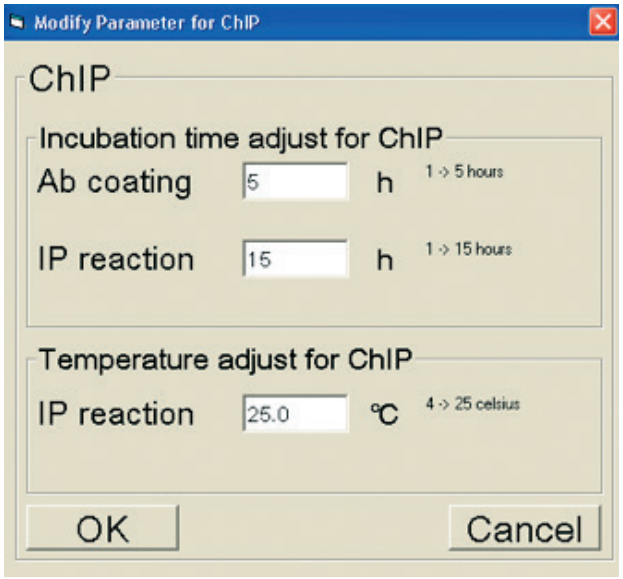
In red it is indicated the directory location of the ChIP protocols.

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

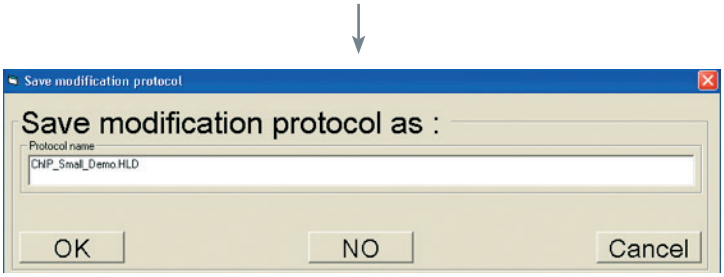


Alternatively, incubation time for antibody coating and temperature and incubation time for the IP reaction can be adjusted in an existing protocol by selecting the modify button. The modified protocol can also be saved as new protocol.

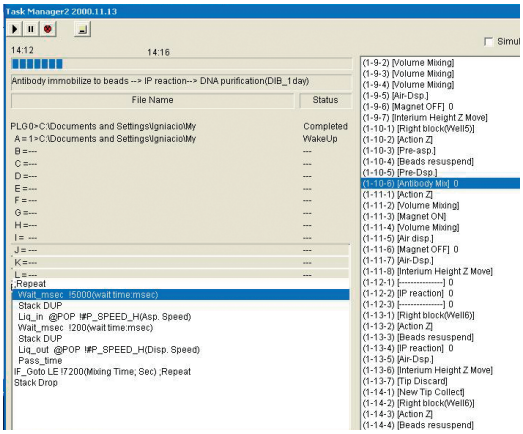




If running ChIP 16 protocol, setup half of the incubation time. It will incubate half of the time on each block but total time will be correct. (For instance, if you want 10h incubation, you have to setup 5h)



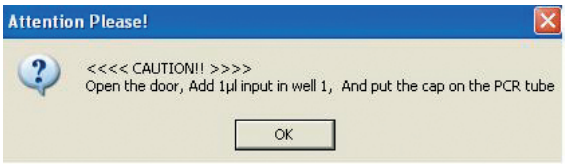
7. The program will run through the following steps: magnetic bead washes, IP and IP washes.



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.



1. Add 1 % Input to well 1: 1 µl of sheared chromatin

2. Close the tube strip with the corresponding caps

3. Press OK

4. 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 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. To run this protocol on the IP-Star, please follow the instructions from the manual **Auto IPure kit v2 (C03010010).**

STEP 5. Quantitative PCR analysis

Before sequencing the samples, we recommend analysing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. The kit contains a positive (GAPDH TSS) and negative (Myoglobin Exon 2) control primer pair which can be used for the positive control antibody provided in the kit (H3K4me3 ChIP-seq grade antibody) in SYBR® Green qPCR assay using the protocol described below. Use your own method of choice for analysing the appropriate control targets for your antibodies of interest.

43. Prepare the qPCR mix as follows (20 µl reaction volume using the provided **control primer pairs**):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer mix
- 4 µl of water
- 5 µl IP'd or input DNA

Use the following PCR program: 3 to 10 min denaturation step at 95°C (please check carefully supplier's recommendations about Taq polymerase activation time), followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72 °C. These conditions may require optimization depending on the type of Master Mix or qPCR system used.

ChIP-sequencing

The iDeal 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 SOLiD™ systems can also be used.

?

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

ChIP-seq data analysis recommendations

To find the captured regions of the genome after sequencing you must perform a) a reference alignment followed by b) a peak calling, then c) further data analysis (annotation, visualization etc.) to help you find what you are looking for. There are abundant software tools for each task that use different approaches to the same problem; choose your preferred one considering your dataset and scientific goals. The workflows for different sequencers basically differ only in the alignment step, since every sequencer has its own characteristic read set (short or long, fixed or variable length, nucleotide or colour code etc.).

a) The built-in aligners with default settings worked very well for our ChIP-seq experiments (e.g. **ELAND** for Illumina®, **TMAP** for PGM). If you cannot access them, open source tools are also available; we have positive experience with **BWA**: http://bio-bwa.sourceforge.net. If you use a multipurpose aligner, do not forget to use settings appropriate to your dataset; please consult with the manual of your software.

b) The purpose of the peak calling is to find the enriched regions in the alignment. Take extreme care when you choose and set up your peak caller, since the outcome can vary widely depending on the used software and its settings. We advise you to read the comparative literature and the software manuals to fully understand how a certain program works. One of the key features of your data is the expected length of the enrichment regions. Transcription factors tend to produce short and sharp peaks, while histone marks create broad islands of enrichment. A remarkable tool

for sharp peak detection is **MACS**, while **SICER** is dedicated to histone marks, and tools like **ZINBA** can be used for both with decent outcomes. **MACS 2** is reported to be better suited for histone marks than previous versions. The availability of the mentioned softwares:

- **MACS**: http://liulab.dfci.harvard.edu/MACS
- **MACS 2**: https://github.com/taoliu/MACS/tree/master/MACS2
- **SICER**: http://home.gwu.edu/~wpeng/Software.htm
- **ZINBA**: http://code.google.com/p/zinba

We are extensively using **MACS 1.4.1** for our experiments. While it is a prominent tool for shorter peaks, sometimes it has difficulties with broader regions, therefore we recommend you to set a wider local peak background and lower the pvalue cutoff if necessary for histone marks. In some cases turning off the local lambda calculation provides a better coverage of broad enrichment islands, though this can result in more false positive peaks detected. Please refer to the MACS manual (http://liulab.dfci.harvard.edu/MACS/README.html) if you are not sure how to tweak the parameters.

c) Having your peaks you can start decrypting the epigenetic code.

The visual inspection is a common first step, especially if the aim of your experiment was to see if certain genes have certain histone modifications/transcription factors attached, or you want to check some positive/negative control sites for enrichment. Choose the appropriate viewer software according to the output format of your peak caller and your preferences.

Annotation is always very useful, since you can identify biological features that are relevant to your peaks, or check if you have the peaks at the expected loci, like H3K4me3 enrichments in the promoter regions of active genes. You can expand the annotation with a gene ontology/pathway analysis of the peak associated genes, thus discovering how your transcription factor/histone modification is involved in the cell's or the whole organism's life.

Motif search is almost an obligatory analysis for the sequence specific transcription factors, but you may find common motifs among histone modification sites as well, so you can check for example if you indeed have promoter specific motifs in your theoretically promoter specific enrichments.

A lot of programs, including peak callers themselves output descriptive statistics of the peaks, measuring for example their enrichment ratios, significances, width, heights, reads in peaks. This characterization helps you better understand your data, which is essential for most applications; a typical example is the comparison of performance of different sample preparation protocols or different sequencer setups.

The final recommended analysis type is the comparative analysis. We encourage scientists to use replicates in their experiments; removing peaks that are not common could effectively reduce false positives. You can also use a validated reference set of peaks for comparisons, but that is rarely available. Additionally, if you have other biologically relevant data from your samples, it is wise to compare and integrate them. For example, an RNA-seq dataset is a great source of validation for histone marks that are supposed to regulate gene expression.

Recommended free tools for the peak analysis:

- **IGV** (visualization): http://www.broadinstitute.org/igv
- **UCSC Genome Browser** (visualization): http://genome.ucsc.edu
- **HOMER** (motif search, annotation, gene ontology, comparison, statistics): http://biowhat.ucsd.edu/homer
- **PinkThing** (annotation, conservation, comparison, gene ontology, statistics): http://pinkthing.cmbi.ru.nl
- **GREAT** (annotation, statistics): http://great.stanford.edu

When analysing ChIP-seq, please always keep an eye on sequencing quality and the performance of the software tools used for analysis. For example with a low quality sequencing with a lot of read errors you will have a hard time finding the peaks you are looking for, despite your excellent IP'd DNA. To control the quality use the **vendor supplied software** and metrics, like the ones available in the Illumina®® pipeline for GA II. Open source tools can also be used, e.g. the **FastQC** by Babraham Institute: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc.

Throughout this chapter we recommended some free tools, because they are accessible for everyone and we have

tested most of them. Please note that there are commercial softwares for the same purposes as well, most of them capable of performing several tasks, or even a complete ChIP-seq workflow. Here are a few examples that we know of (but we have not tested them):

- **CLC Genomics Workbench**: http://clcbio.com
- **Partek Genomics Suite**: http://www.partek.com/partekgs
- **NextGENe**: http://www.softgenetics.com/NextGENe.html
- **Avadis NGS**: http://www.avadis-ngs.com
- **Geneious**: http://www.geneious.com/web/geneious/geneious-pro
- **GenoMiner**: http://www.astridbio.com/genominer.html
- **GenoMatix**: http://www.genomatix.de

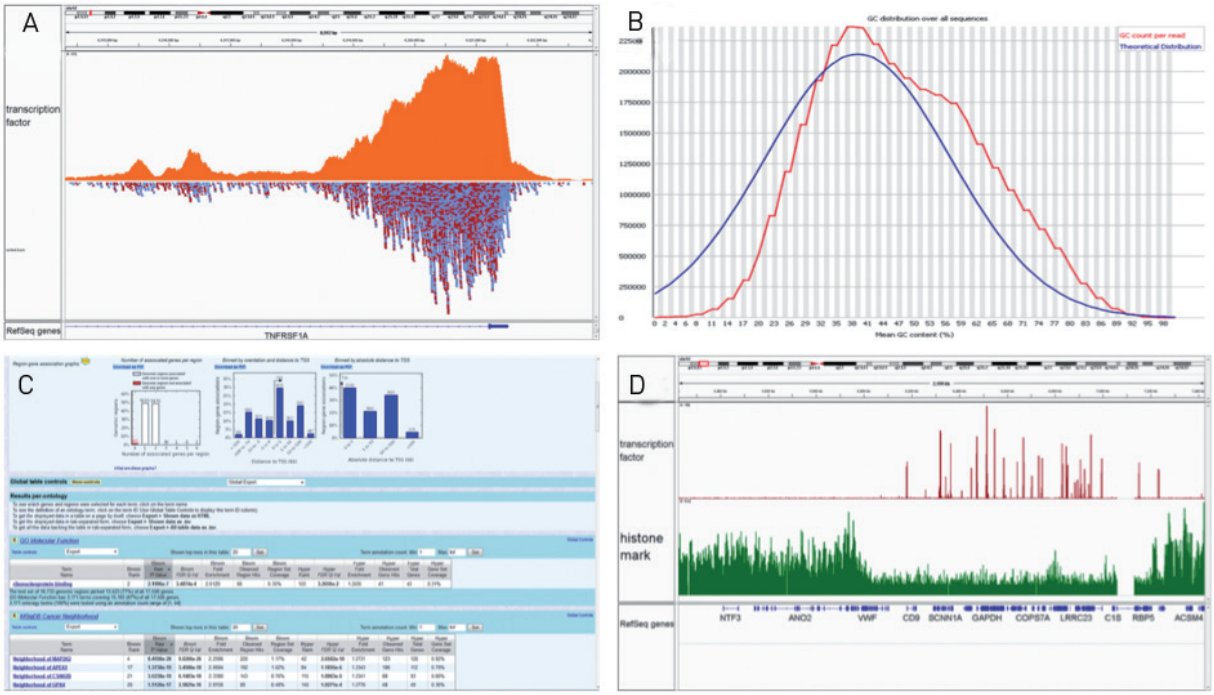


Figure 5: Various stages of bioinformatics data analysis

Representative images made during bioinformatics analysis of ChIP-seq data.

A: The reads are accumulating around the binding site to form a peak like structure in the coverage graph. Peak callers are used to detect these peaks.

B: A quality control software (like FastQC) anlyses numerous parameters that can help us assess the goodness of sequencing. Here we can monitor the GC content distribution.

C: Descriptive statistics and annotation output by GREAT.

D: Transcription factors tend to produce sharp peaks (upper red band), while broad enrichments are characteristic of many histone modifications (lower green band).

Aditional protocols

Sheared chromatin analysis

Reagents not supplied with the iDeal ChIP-seq kit

- 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)

1. Take an aliquot of 50 µl of sheared chromatin and spin the chromatin at 12,000 rpm for 10 min at 4°C. Transfer the supernatant to a new tube for chromatin analysis.
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 iE1**.
6. Add 4 µl of **elution buffer iE2**, 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.

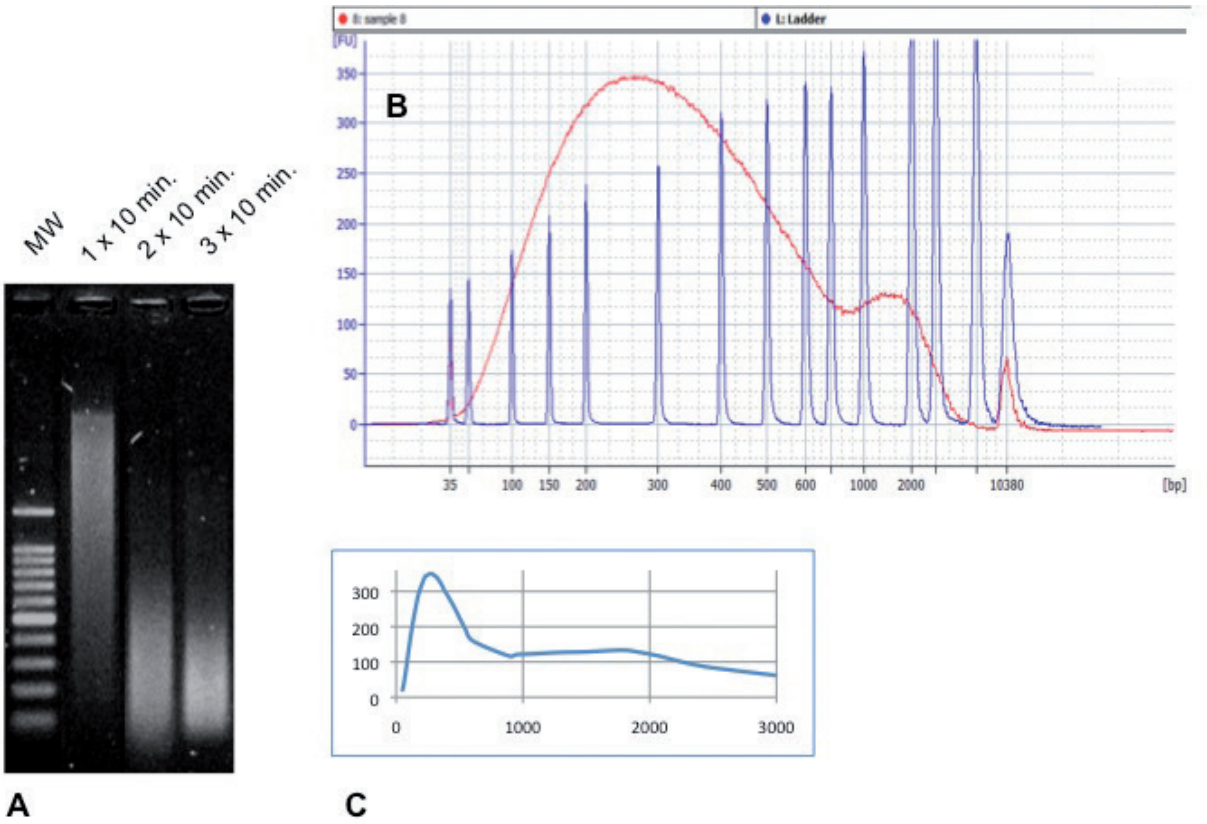


Figure 6: Superior chromatin shearing results with the Bioruptor® Plus using buffers and protocol of the Diagenode iDeal ChIP-seq kit

Hela cells were fixed with 1% formaldehyde (for 8 min at RT). Nucleus isolation of five million fresh or frozen (stored at -80°C) cells are performed using buffers of the Diagenode iDeal ChIP-seq kit (Cat. No. C01010050) and are then resuspended in 200µl of **Shearing Buffer iS1** prior to chromatin shearing.

Samples are sheared during 1, 2 or 3 rounds of 10 cycles of 30 sec ON / 30 sec OFF with the Bioruptor® Plus combined with the Bioruptor® Water cooler at HIGH power setting (position H). For optimal results, samples are vortexed before and after performing 10 sonication cycles, followed by a short centrifugation at 4°C. All samples were treated with RNase (see "**Additional Protocols**").

Panel A: 10 µl of DNA (equivalent to 300 ng) are analyzed on a 1.5% agarose gel.

Panel B and C: Sample 3 (3x 10 min) was analyzed on Bioanalyzer 2100 using DNA High Sensitivity chip. The default log scaled Bioanalyzer output can be seen in Panel A, while Panel C represents their linear transformation for better vizualisation. Out of range fragments were not shown in this graph.

In this example, the optimal shearing condition corresponds to 3 rounds of 10 cycles (30 sec ON / 30 sec OFF).

Troubleshooting guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak	Make sure you perform the fixation step for the correct period of time, at the right temperature and with the correct formaldehyde concentration. e.g: incubate for 8 minutes at room temperature with 1 % formaldehyde final concentration (weight/ volume). Also, use high quality, fresh formaldehyde.
	Cross-linking is too strong	
	Proteins have unique ways of interacting with the DNA. Some proteins are not directly bound to the DNA but interact with other DNA-associated proteins	Very short or very long cross-linking time can lead to DNA loss and/or elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity and efficiency of ChIP.
	Both cross-linking time and formaldehyde concentration are critical	Cross-linking can affect both efficiency of chromatin shearing and efficiency of specific antigen immunoprecipitation. Shorter cross-linking times (5 to 10 minutes) and/or lower formaldehyde concentrations (1%, weight/ volume) may improve shearing efficiency while, for some proteins especially those that do not directly bind DNA, this might reduce the efficiency of cross-linking and thus the yield of precipitated chromatin.
	The optimal duration of cross-linking varies between cell type and protein of interest	It is possible to optimize the fixation step by testing different incubation times: such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.
	Efficient fixation of a protein to chromatin in vivo is a crucial step for ChIP. The extent of cross-linking is probably the most important parameter.	Two major problems concerning the subsequent immunoprecipitation step should be taken into account: 1/ an excess of cross-linking can result in the loss of material or reduced antigen availability in chromatin, or both. 2/ the relative sensitivity of the antigen epitopes to formaldehyde. It is essential to perform the cross-linking step with care.
	It is essential to quench the formaldehyde	Use glycine to stop the fixation: quench formaldehyde with 125 mM glycine for 5 minutes at room temperature (add 57 µl of 1.25M glycine per 513.5 µl of sample, see STEP 2). Alternatively, wash the fixed cells properly and make sure you get rid of ALL the formaldehyde.
Cell lysis	Temperature is critical	Perform cell lysis at 4°C (cold room) or on ice. Keep the samples ice-cold at all times during the cell lysis and use ice-cold buffers see STEP 3.
	Protein degradation during lysis can occur	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Kit protocol validation	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells) and Kerationocytes have been used to validate the Magnetic ChIP protocol.
Cell number necessary per ChIP	The amount of cells required for a ChIP experiment is determined by cell type, protein of interest and antibodies used	You can use from 1,000,000 to 10,000,000 cells per IP.
Chromatin shearing	Optimal shearing conditions are important for ChIP efficiency	Shearing conditions for each cell type must be optimized from cell collection, fixation to shearing method (settings of the sonicator apparatus).
	Critical points for shearing optimization	1) Not to start with a too large amount of cells (1x 10e6 cells or less is ok) 2) Keep samples cold (4°C) 3) High % SDS favours better sonication but inhibits immunoselection (optimal range: 0,1 to 1%). Dilutions must be adapted accordingly prior to immunoselection; the final SDS concentration should not be higher than 0.15 to 0.20% (e.g. If the shearing buffer contains 0.75% SDS, the sheared chromatin is diluted 3.5 to 4.0 fold in the [P.I.-ChIP buffer 1x])
	Shear the samples of chromatin using the Bioruptor® from Diagenode (cat. No. UCD-200, UCD-300, UCD-400)	Maintain temperature of the samples close to 0°C. The chromatin shearing needs to be optimized for each cell type. A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.

Sheared chromatin analysis	Purify the DNA from the sheared chromatin as described in the kit protocol to analyse the shearing	Extract total DNA from an aliquot of sheared chromatin and run on 1% agarose gel (stain with EtBr). In order to analyse the sheared chromatin on gel, take DNA purified from the sheared chromatin input -prepared at STEP 3 . Some unsheared chromatin can be analysed on gel as well (purify it as done with the input sample (see "6. Additional protocols" section). Chromatin eqvivalent to 100,000 cells, one million cells or more can for sure be visualized on a gel.
	Do not load too much DNA on a gel	Loading of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation. The DNA amount to load depends on the size of the well and on the gel size.
	Agarose concentration	Do not use more than 1-1.5% agarose gel and run slowly (Volt/cm and time depend on the gel size).
	Running buffer concentration	1x TAE or TBE is preferred to 0.5x TAE, which can lead to smears on agarose gel.
Sheared chromatin amounts	How much sheared chromatin do I need to prepare?	Most of the sheared chromatin is to be used in the ChIP experiment, but remember that some of the sheared chromatin is needed as control as it corresponds to the input sample for the ChIP experiment and it can also be checked on agarose gel.
	Dilute the sheared chromatin in ChIP buffer for Immuno-selection incubation	The sheared chromatin is diluted in complete Buffer A prior to the immunoselection incubation (see STEP 3: Add 870 µl of complete Buffer A to the 130 µl of sheared chromatin). Dilute the sheared chromatin at least 7 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	Beads are in suspension	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead centrifugation	Don't spin the beads at high speed. Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm. (http://www.msu.edu/~venkata1/gforce.htm). It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Antibody binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C, and thawed before use.
Other enzyme inhibitors	Specific enzyme inhibitors are not included in the kit, such as phosphatase inhibitors	Add phosphatase inhibitors or others to Buffers A and B, if necessary, depending on your research field and protein(s) of interest to be ChIP'd Add NaBu for histone ChIPs.

<table>
<tr><td rowspan="6">Antibody in IP</td><td>How many negative controls are necessary?</td><td>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.</td></tr>
<tr><td>Why is my antibody not working in ChIP?</td><td>Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.</td></tr>
<tr><td>Which antibody should I use in ChIP?</td><td>Use ChIP-grade antibodies. If not available, it is recommended to use several antibodies directed against different epitopes of the same protein. Verify that the antibodies can work directly in IP on fresh cell extracts. Also, when testing new antibodies, include known ChIP-grade antibodies as positive control for your ChIP assay.</td></tr>
<tr><td>How do I choose an antibody for ChIP?</td><td>Be aware of the possible cross-reactivity of antibodies. Verify by Western blot analysis the antibody specificity. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.</td></tr>
<tr><td>Are my antibodies going to bind the protein A or protein G?</td><td>There is a significant difference in affinity of different types of immunoglobulins to protein A or G. Thererfore, in function of the antibody used for your ChIP, it is recommended to choose either protein A or protein G coated beads.
<table>
<tr><th colspan="2">Species immunglobulli isotype</th><th>Protein A</th><th>Protein G</th></tr>
<tr><td rowspan="8">Human</td><td>IgG1</td><td>+++</td><td>+++</td></tr>
<tr><td>IgG2</td><td>+++</td><td>+++</td></tr>
<tr><td>IgG3</td><td>-</td><td>+++</td></tr>
<tr><td>IgG4</td><td>+++</td><td>+++</td></tr>
<tr><td>IgGM</td><td colspan="2">Use anti human IgM</td></tr>
<tr><td>IgGF</td><td>-</td><td>+</td></tr>
<tr><td>IgGA</td><td>-</td><td>+</td></tr>
<tr><td rowspan="5">Mouse</td><td>IgG1</td><td>+</td><td>+++</td></tr>
<tr><td>IgG2a</td><td>+++</td><td>+++</td></tr>
<tr><td>IgG2b</td><td>++</td><td>++</td></tr>
<tr><td>IgG3</td><td>+</td><td>+</td></tr>
<tr><td>IgGM</td><td colspan="2">Use anti mouse IgM</td></tr>
<tr><td rowspan="4">Rat</td><td>IgG1</td><td>-</td><td>+</td></tr>
<tr><td>IgG2a</td><td>-</td><td>+++</td></tr>
<tr><td>IgG2b</td><td>-</td><td>++</td></tr>
<tr><td>IgG2c</td><td>+</td><td>++</td></tr>
<tr><td>Chicken</td><td>All isotypes</td><td>-</td><td>++</td></tr>
<tr><td>Cow</td><td>All isotypes</td><td>++</td><td>+++</td></tr>
<tr><td>Goat</td><td>All isotypes</td><td>-</td><td>++</td></tr>
<tr><td>Guinea pig</td><td>All isotypes</td><td>+++</td><td>++</td></tr>
<tr><td>Hamster</td><td>All isotypes</td><td>+</td><td>++</td></tr>
<tr><td>Horse</td><td>All isotypes</td><td>++</td><td>+++</td></tr>
<tr><td>Pig</td><td>All isotypes</td><td>+</td><td>++</td></tr>
<tr><td>Rabbit</td><td>All isotypes</td><td>+++</td><td>++</td></tr>
<tr><td>Sheep</td><td>All isotypes</td><td>-</td><td>++</td></tr>
</table></td></tr>
<tr><td>Freezing</td><td>Avoid multiple freeze/thawing.</td><td>Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)</td></tr>
</table>

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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 v2 x12 (12 indices)	C05010012	/	12 rxns
MicroPlex Library Preparation kit v2 x48 (48 indices)	C05010014	/	48 rxns
Auto iDeal ChIP-seq kit for Histones x 24	C01010057	/	24 rxns
Auto iDeal ChIP-seq kit for Histones x100	C01010171	/	100 rxns
Auto iDeal ChIP-seq kit for Transcription Factors x24	C01010058	/	24 rxns
Auto iDeal ChIP-seq kit for Transcription Factors x100	C01010172	/	100 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
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Auto IPure kit v2 x100	C03010010	AL-Auto01-0100	100 rxns

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