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Innovating Epigenetic Solutions

iDeal ChIP-seq kit for Histones

Cat. No. **C01010050** (10 rxns)

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Ordering information

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Content

MANUAL

Introduction

Association between proteins and DNA is a major mechanism in many vital cellular functions such as gene transcription and epigenetic silencing. It is crucial to understand these interactions and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation. Chromatin immunoprecipitation (ChIP) is a technique to analyze the association of proteins with specific genomic regions in intact cells. ChIP can be used to study changes in epigenetic signatures, chromatin remodelling and transcription regulator recruitment to specific genomic sites.

The different steps of the ChIP assay are cell fixation (cross-linking), chromatin shearing, immunoprecipitation, reverse cross-linking followed by DNA purification and analysis of the immunoprecipitated DNA.

In ChIP, living cells are first fixed with a reversible cross-linking agent to stabilize protein-DNA interactions. The most widely used reagent to fix cells is formaldehyde which generates covalent bonds between amino or imino groups of proteins and nucleic acids. Formaldehyde treatment crosslinks both DNA-protein as well as protein-protein complexes.

Following cross-linking, chromatin needs to be sheared very efficiently into homogeneous small fragments that can subsequently be used in immunoprecipitation (IP). The Bioruptor® shearing devices from Diagenode provide you with high quality sheared chromatin ready-to-ChIP. Currently, the Bioruptor® is the most widely used and the most cited chromatin fragmentation system as evidenced by more than 1000 publications. Shearing may also be accomplished with shearing kits from Diagenode which enable an easy and highly reproducible shearing process for any cell type. After fragmentation, the sheared chromatin is precipitated with a specific antibody (AB) directed against the protein of interest. The chromatin-AB complex is isolated using magnetic beads. Finally, the precipitated DNA fragments are released from the AB, and analyzed. Enrichment of specific sequences in the precipitated (IP'd) DNA indicates that these sequences were associated with the protein of interest in vivo. Analyzis of specific regions can be performed by quantitative polymerase chain reaction (qPCR). In recent years, ChIP combined with high-throughput Next-Generation sequencing (ChIP-seq) has become the gold standard for whole-genome mapping of protein-DNA interactions.

Although ChIP-seq is a powerful tool, the procedure requires tedious optimization of several reaction conditions that might lead to considerable time and lab expenditures. To reduce these tedious steps, Diagenode provides kits with optimized reagents and protocols for ChIP which enable successful ChIP-seq. The new iDeal ChIP-seq kit protocol has been thoroughly optimized by Diagenode for ChIP followed by high-throughput sequencing on Illumina® GAIIx and Ion Torrent™ PGM™ systems.

Diagenode also offers a wide range of additional tools and products for ChIP analysis including the Bioruptor® for chromatin shearing and nucleic acid fragmentation, the SX-8G IP-Star® for automated ChIP and DNA methylation analysis, magnetic racks for 0.2 or 1.5 ml tubes which enable you to work with magnetic beads under optimal conditions (4°C), and ChIP and ChIP-seq grade antibodies against many epigenetic targets. In addition, Diagenode provides individual reagents, such as magnetic beads, negative IP controls (mouse and rabbit IgG), protease inhibitors and deacetylase inhibitors (sodium butyrate). Diagenode also offers several qPCR primer pairs for the analysis of IP'd DNA. Peptides can additionally be purchased for use in blocking experiments (negative ChIP controls).

All our products have been extensively validated in ChIP on various targets. The combination of all our quality-controlled kits, reagents and equipment is a perfect starting point that will lead to your success.

Kit method overview & time table

Table 1 : iDeal ChIP protocol overview

Step		Time needed	Day
1a	Cell collection and DNA-protein cross-linking (for cultured cells)	1 to 2 hours	1
1b	Tissue Disaggregation and DNA-protein cross-linking (for fresh or frozen tissues)		
2a	Cell lysis and chromatin shearing (for cultured cells)	1 to 2 hours	1
2b	Cell lysis and chromatin shearing (derived from tissue sample)		
3	Magnetic immunoprecipitation	overnight	1-2
4	Elution, decross-linking and DNA purification	6 hours	2
5	Quantitative PCR and data analysis prior to Library preparation and Next-Generation Sequencing	2 to 3 hours	3

Kit materials

The content of the kit is sufficient to perform 10 ChIP assays from cell collection to IP'd DNA (Table 2). Store the components at the indicated temperature upon receipt.

Table 2: Components supplied with the iDeal ChIP-seq kit

Description	Quantity (x10)	Storage
Glycine	200 µl	4°C
Lysis buffer iL1	20 ml	4°C
Lysis buffer iL2	20 ml	4°C
Shearing buffer iS1	3.6 ml	4°C
Protease inhibitor cocktail	35 µl	-20°C
5x ChIP buffer iC1	950 µl	4°C
5% BSA (DNA free)	60 µl	-20°C
Protein A-coated magnetic beads	200 µl	4°C **Do NOT freeze !**
Wash buffer iW1	3.5 ml	4°C
Wash buffer iW2	3.5 ml	4°C
Wash buffer iW3	3.5 ml	4°C
Wash buffer iW4	3.5 ml	4°C
Elution buffer iE1 (warm to room temp. before use)	1.5 ml	4°C
Elution buffer iE2	64 µl	4°C
Rabbit IgG	8 µg (1µg/µl)	-20°C
ChIP-seq grade antibody H3K4me3	8 µg (1µg/µl)	-20°C
ChIP-seq grade GAPDH TSS primer pair (positive control) - human	45 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair (negative control) - human	45 µl	-20°C
ChIP-seq grade water	3 ml	Room temperature

Table 3: IPure kit content

Description	Quantity	Storage
Wash buffer 1 w/o iso-propanol (IPure)	700 µl	4°C
Wash buffer 2 w/o iso-propanol (IPure)	700 µl	4°C
Buffer C (IPure)	700 µl	4°C
Magnetic beads (IPure)	140 µl	4°C
Carrier (IPure)	28 µl	-20°C

Store DiaMag Protein A-coated magnetic beads and IPure Magnetic beads at 4°C. Do NOT freeze magnetic beads because they may become damaged. Keep the beads in liquid suspension during storage as drying will result in reduced performance.

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
- Trypsin-EDTA
- RNase/DNase-free 1.5 ml tubes
- qPCR SYBR® Green Mastermix
- Reagents for library preparation, cluster generation (Illumina®) or ePCR (Ion Torrent™ PGM™) and sequencing
- Quant-IT dsDNA HS assay kit (Invitrogen)

For library preparation, we highly recommend

- iDeal Library Preparation kit x24 (incl. Index Primer Set 1), (Cat. No. C05010020)
- MicroPlex Library Preparation™ kit
  (Cat. No. C05010010, 12 reactions, 12 indices) (Cat. No. C05010011, 48 reactions, 12 indices)

Equipment

- Optional: ChIPettor(tm) System for Histones (Cat.. No. C01010162)
- Diagenode DiaMag1.5 magnetic rack (Cat. No. B04000003)
- 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
- DiaMag Rotator (Rotating wheel) (Cat. No. B05000001)
- 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 300 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

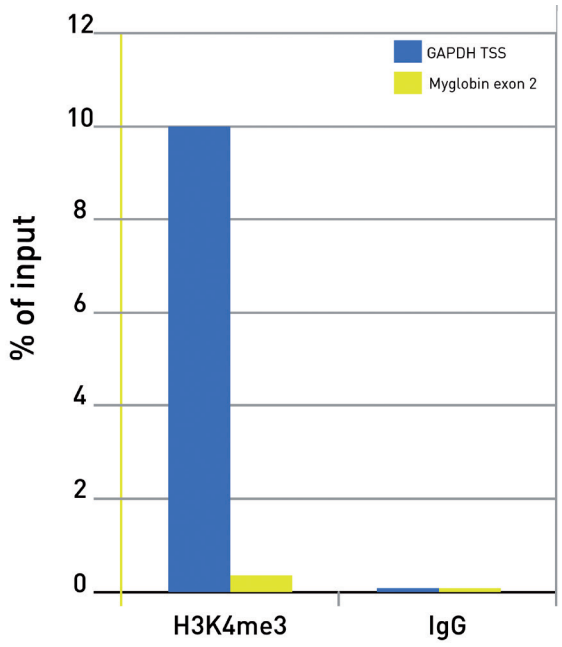


Figure 1: ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit. Sheared chromatin from 1 million cells, 1 µl of the positive control antibody and 2 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control GAPDH-TSS 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).

Short protocol for experienced users

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

1. Collect the cells by trypsinization and wash two times with PBS.
2. Count the cells and resuspend them in PBS to obtain up to 10 million cells in 500 µl of PBS. Aliquot 500 µl of cell suspension in 1.5 ml tubes.
3. Add 13.5 µl of formaldehyde 37%. Mix by gentle vortexing and incubate for 8 min at room temperature to allow fixation to take place.
4. Stop the fixation by adding 57 µl of **Glycine** solution. Mix by gentle vortexing and incubate for 5 min at room temperature. Work on ice from this point onwards.
5. Centrifuge at 1,600 rpm (500 x g) for 5 min at 4°C and gently aspirate the supernatant without disturbing the cell pellet.
6. Wash the cells twice with 1 ml PBS.

→ Proceed to Step 2a: *Cell lysis and chromatin shearing (for cultured cells)*

Step1b. Tissue disaggregation and DNA-protein cross-linking (for fresh or frozen tissues)

7. 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.
8. Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade.
9. 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.
10. Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 1,300 rpm for 5 min at 4°C. Gently discard the supernatant and keep the pellet.
11. Resuspend the pellet in 1 ml of PBS containing 1% of formaldehyde at room temperature.
12. Rotate tube for 8-10 min at room temperature. 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.
13. Stop the cross-linking reaction by adding 100 µl of glycine. Continue to rotate at room temperature for 5 min.
14. Centrifuge samples at low speed (1,300 rpm) at 4°C.
15. Wash the pellet with ice-cold PBS. Aspirate the supernatant and resuspend the pellet in 1 ml of ice-cold PBS plus protease inhibitors.
16. Centrifuge at low speed (1,300 rpm) at 4°C and discard the supernatant.
17. 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)

18. Add 10 ml of ice-cold **Lysis buffer iL1** to the cell pellet corresponding to 10 million cells. Resuspend the cells by pipetting up and down several times and incubate for 10 min at 4°C with gentle mixing. Scale down accordingly when using fewer cells.
19. Centrifuge for 5 min at 1,600 rpm (500 x g) and 4°C and discard the supernatant.

20. Add 10 ml of ice-cold **Lysis buffer iL2** to the cell pellet. Resuspend the cells by pipetting up and down several times and incubate for 10 min at 4°C with gentle mixing.
21. Centrifuge for 5 min at 1,600 rpm (500 x g) and 4°C and discard the supernatant.
22. Add 200x **protease inhibitor cocktail** to the **Shearing buffer iS1**. Keep on ice.
23. Add 1 ml of **Shearing buffer iS1** containing protease inhibitor to 10 million cells. Resuspend by pipetting up and down and incubate on ice for 10 min.
24. Shear chromatin by sonication using the Bioruptor(R). An initial time course experiment is recommended.
    - For Bioruptor® Standard or Plus use High power setting for 10-30 cycles (30 seconds ON, 30 seconds 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 seconds ON, 30 seconds OFF). Vortexing is not required between runs.
25. Centrifuge at 13,000 rpm (16,000 x g) for 10 min and collect the supernatant which contains the sheared chromatin.

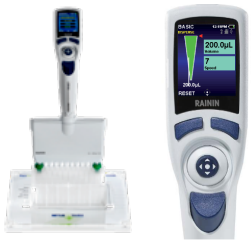
→ Proceed to Step 3: *Magnetic immunoprecipitation*

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

26. 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.
27. Centrifuge for 5 min at 1,300 rpm at 4°C and discard the supernatant.
28. 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.
29. Centrifuge for 5 min at 1300 rpm at 4°C and discard the supernatant.
30. Resuspend the pellet in 1.8 ml of **shearing buffer iS1** containing protease inhibitors cocktail and homogenize using a dounce homogenizer (tight pestle).
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. 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.
33. Transfer samples to new 1.5 ml tubes and centrifuge at 13,000 rpm for 10 min.
34. Collect the supernatant which contains the sheared chromatin.
35. 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



The immunoprecipitation step can also be performed using the semi-automated **ChIPettor™ System.** If doing so, please refer to the corresponding protocol delivered with this product. Alternatively, the protocol can also be downloaded from the website www.diagenode.com

36. Dilute the **5x ChIP buffer iC1** and with ChIP-seq grade water to obtain **1x ChIP buffer iC1**. Place on ice.
37. Take the required amount of **DiaMag Protein A-coated magnetic beads** (20 µl/IP) and wash four times with twice the volume of ice-cold **1x ChIP buffer iC1**.
38. Resuspend the beads after the last wash in the original volume **1x ChIP buffer iC1**.
39. Set aside 1 µl (1%) of the sheared chromatin to use as input sample and keep at 4°C.
40. Prepare the following ChIP reaction mix (1 IP) :
    6 µl of **5% BSA**
    1.5 µl of 200x **protease inhibitor cocktail**
    56 µl of **5x ChIP buffer iC1**
    100 µl of sheared chromatin
    20 µl of **DiaMag Protein A-coated magnetic beads**
    x µl **ChIP-seq grade antibody**
    add **ChIP-seq grade water** to a total volume of 300 µl
    If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added.
41. Incubate overnight at 4°C on a rotating wheel.
42. The next day, briefly spin the tubes, place them in the ice-cold magnetic rack and discard the supernatant.
43. Add 350 µl ice-cold **Wash buffer iW1** and incubate for 5 min at 4°C on a rotating wheel. Discard the wash buffer using the **Diagenode magnetic rack**.
44. Repeat step 21 and 22 once with **Wash buffer iW2, iW3 and iW4**, respectively.

STEP 4. Elution, decross-linking and DNA purification

45. After removing the last wash buffer, add 100 µl of **Elution bufferiE1** to the beads and incubate for 30 min on a rotating wheel at room temperature.
46. Briefly spin the tubes and place them into the Diagenode magnetic rack. Transfer the supernatant to a new tube and add 4 µl of **Elution buffer iE2**. Also add 99 µl **buffer iE1** and 4 µl of **buffer iE2** to the 1 µl input sample. Incubate for 4 hours or overnight at 65°C.
47. Purify the DNA using the IPure kit.
48. Add 2 µl of **carrier** to each IP and input sample.
49. Add 100 µl of 100% isopropanol to each IP and input sample. **ATTENTION**: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.
50. Resuspend the **IPure beads v2** and transfer 10 µl to each IP and input sample.
51. Incubate IP and input samples for 10 minutes at room temperature on a rotating wheel (40 rpm).
52. Prepare the **Wash buffer 1** containing 50% isopropanol:

Wash buffer 1	
Wash buffer 1 w/o isopropanol	700 µl
Isopropanol (100%)	700 µl
Total volume	1.4 ml

53. Briefly spin the tubes, place into the **DiaMag1.5 - magnetic rack**, wait 1 min and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 1**. Close the tubes, the beads and incubate for 5 min at room temperature on a rotating wheel (40 rpm).

54. Prepare the **Wash buffer 2** containing 50% isopropanol as follows:

Wash buffer 2	
Wash buffer 2 w/o isopropanol	700 µl
Isopropanol (100%)	700 µl
Total volume	1.4 ml

55. Briefly spin the tubes, place into the **DiaMag1.5 - magnetic rack**, wait 1 min and discard the buffer. Keep the captured beads and add 100 µl **Wash buffer 2** per tube. Close the tubes, resuspend the beads and incubate for 5 min at room temperature on a rotating wheel (40 rpm).

56. Briefly spin the tubes and place them into the DiaMag1.5, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 25 µl buffer C (alternatively, a higher volume can be used for the elution if necessary). Close the tubes and incubate for 15 minutes at room temperature on a rotating wheel (40 rpm). Resuspend the pelleted beads using the pipet and make sure that you drop them on the bottom of the tube.

57. Spin tubes and place them in the **DiaMag1.5 - magnetic rack**, wait 1 min and pool the supernatant with the corresponding IP or input sample (1.5 ml tube). Discard the beads.

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

STEP 5. Quantitative PCR analysis

59. 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

60. 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.

Detailed 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)

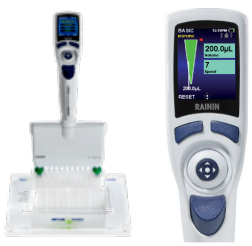
32. Centrifuge at 13,000 rpm (16,000 x g) for 10 min and collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. If desired, the chromatin shearing effciciency can be analyzed at this step (see **"Additional protocols"**).

→ Proceed to Step 3: *Magnetic immunoprecipitation*

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



The immunoprecipitation step can also be performed using the semi-automated **ChIPettor™ System.** If doing so, please refer to the corresponding protocol delivered with this product. Alternatively, the protocol can also be downloaded from the website www.diagenode.com

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.

43. 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). Take the required amount of **DiaMag Protein A-coated magnetic beads** (20 µl/IP). Dilute the **5x ChIP buffer iC1** with ChIP-seq grade water to obtain **1x ChIP buffer iC1**. The total amount of **1x ChIP buffer iC1** needed is 9 times the volume of beads required for the experiment. Place the diluted **ChIP buffer iC1** on ice.

44. Wash the beads 4 times with twice the volume of ice-cold **1x ChIP buffer iC1**. To wash the beads add **1x ChIP buffer iC1**, resuspend the beads by pipetting up and down several times and place the tubes in the **1.5 ml Diagenode magnetic rack** (Cat. No. B04000003). Wait for one min to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times. Alternatively, centrifuge the tubes for 5 min at 1,300 rpm, discard the supernatant and keep the bead pellet.

45. After the last wash, resuspend the beads in the original volume **1x ChIP buffer iC1**.

46. Prepare the ChIP reaction mix according to Table 5. If required, NaBu (20 mM final concentration) or other inhibitors can also be added. Use 2 µl of the **rabbit IgG control antibody** for the negative control IP. If a positive control IP is included in the experiment, use 1 µl of the **H3K4me3 ChIP-seq grade control antibody**. When preparing the reaction mix, place 1 µl of the sheared chromatin aside to be used as an input the next day.

Table 6. ChIP reaction mix

Number. of IP's	5% BSA (µl)	200x Protease inhibitor cocktail (µl)	5x buffer iC1 (µl)	Sheared chromatin (1e10 cells) (µl)	Magnetic beads (µl)	ChIP-seq grade water (µl)	Antibody (µl)
1	6	1.5	56	100	20	116.5-x	x
2	12	3	112	200	40	233-x	x
4	24	6	224	400	80	466-x	x
6	36	9	336	600	120	699-x	x
8	48	12	448	800	160	932-x	x

47. Incubate the tubes overnight at 4°C under constant rotation at 40 rpm on a rotating wheel.

48. The next morning, after the overnight incubation, briefly spin the tubes and place them in the **magnetic rack**. Wait for one min and remove the supernatant. To wash the beads, add 350 µl of **wash buffer iW1**, gently shake the tubes to resuspend the beads and incubate for 5 min on a rotating wheel at 4°C.

49. Repeat the wash as described above once with **Wash buffer iW2, iW3 and iW4** using the same buffer volume, respectively.

STEP 4. Elution, decross-linking and DNA isolation

50. After removing the last wash buffer, add 100 µl of **Elution buffer iE1** to the beads and incubate for 30 min on a rotating wheel at room temperature.

51. Briefly spin the tubes and place them into the Diagenode **magnetic rack**. Transfer the supernatant to a new tube and add 4 µl of **Elution bufferiE2**. Also add 99 µl **buffer iE1** and 4 µl of **buffer iE2** to the 1 µl input sample. Incubate for 4 hours or overnight at 65°C.

52. Pool samples if necessary.

    NOTE: Up to 2 samples can be easily pooled. If more than 2 samples need to be pooled, process each sample purification individually, pool final eluates at the end of the IPure purification and concentrate (e.g. using Microcon®)

53. Add 2 µl of **carrier** to each IP and input sample.

54. Add 100 µl of 100% isopropanol to each IP and input sample.

    Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

55. Resuspend the provided **IPure beads v2** and transfer 10 µl to each IP and input sample.

- Keep **IPure beads v2** in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
- The final volume is now 216 µl per IPure reaction.

56. Incubate IP and input samples for 10 minutes at room temperature on a rotating wheel (40 rpm).Prepare the **Wash buffer 1** containing 50% isopropanol:

Wash buffer 1	
Wash buffer 1 w/o isopropanol	700 µl
Isopropanol (100%)	700 µl
Total volume	1.4 ml

- Never leave the bottle open to avoid evaporation.

57. Briefly spin the tubes, place in the **DiaMag1.5**, wait 1 min and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 1**. Close the tubes, invert the 8-tube strip to resuspend the beads and incubate for 5 min at room temperature on a rotating wheel (40 rpm).

- Do not disturb the captured beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning into the **Diagenode Magnetic Rack**. Prepare the **Wash buffer 2** containing 50% isopropanol as follows:

Wash buffer 2	
Wash buffer 2 w/o isopropanol	700 µl
Isopropanol (100%)	700 µl
Total volume	1.4 ml

- Never leave the bottle open to avoid evaporation.

58. Wash the IP and input samples with the Wash buffer 2 as follows. Briefly spin the tubes, place into the **DiaMag1.5**, wait 1 min and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 2**. Close the tubes, invert the 8-tube strip to resuspend the beads and incubate for 5 min at room temperature on a rotating wheel (40 rpm).

- Do not disturb the captured beads attached to the tube wall.
- Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the DiaMag rack.

NOTE: This **Elution buffer (buffer C)** is suitable for direct qPCR analysis, whole genome amplification, chip hybridization and Next-Generation sequencing.

59.

a. Briefly spin the tubes and place them into the DiaMag1.5, wait 1 min and discard the buffer. Spin the tubes again and place them on the DiaMag1.5 magnetic rack. Discard the remaing Wash buffer 2 if necessary. Resuspend the beads pellet with 25 µl of buffer C. Incubate at room temperature for 15 minutes on a rotating wheel (40 rpm).
b. Spin the tubes and place them into the DiaMag1.5, wait 1 min and transfer the supernatants into a new labelled 1.5 ml tube and discard the beads.
c. Place the DNA on ice and proceed to any desired downstream applications, or store it at -20°C or -80°C until further use.

40. Take 5 µl (10%) of IP'd DNA and determine the concentration with 'Quant-IT dsDNA HS assay kit' using the Qubit system or a similar method.

41. Store the DNA at -20°C until you are ready to analyze it with qPCR or by high throughput sequencing.

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

STEP 5. Quantitative PCR analysis

- 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.

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

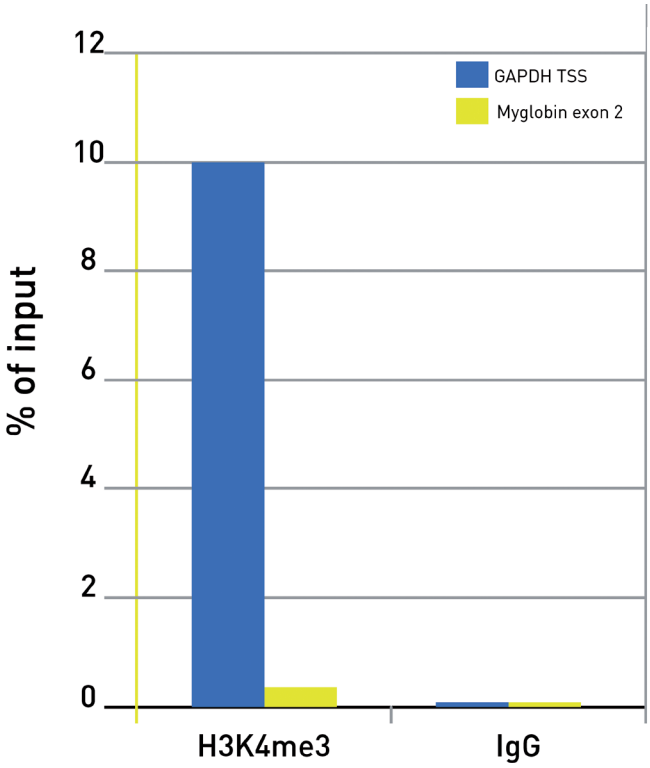


Figure 2: ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit. Sheared chromatin from 1 million cells, 1 µl of the positive control antibody and 2 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control GAPDH-TSS 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).

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ChIP-sequencing

The iDeal protocol has been optimized for ChIP-seq on an Illumina® GAIIx and Ion Torrent™ PGM™ Next-Gen sequencers. However, other sequencing systems such as the Illumina® HiSeq 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.

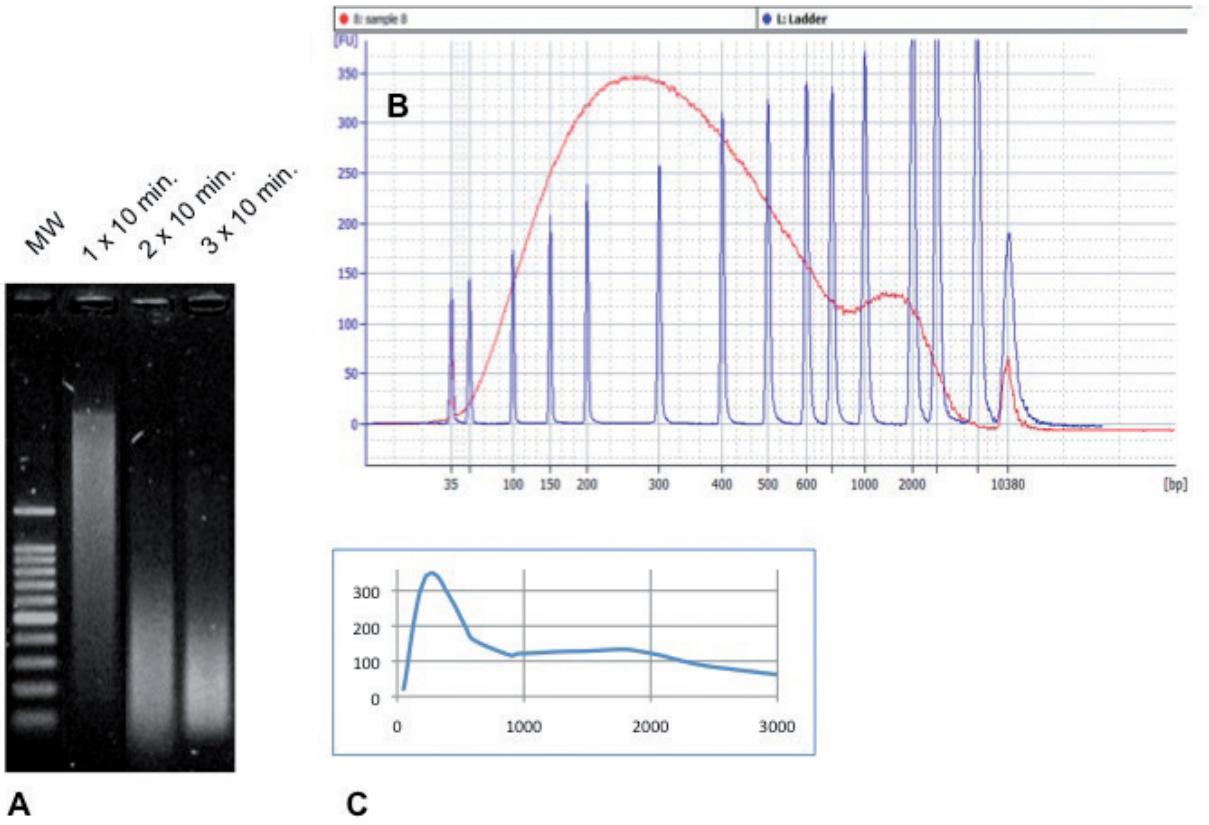

Figure 6

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).

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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).

Sheared chromatin analysis	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.
	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="3">Negative ChIP control(s)</td><td>Use non-immune IgG in the IP incubation mix.</td><td>Use the non-immune IgG fraction from the same species the antibodies were produced in.</td></tr>
<tr><td>Do not add antibody to the IP.</td><td>Incubation with beads, which were not coated with antibodies antibodies could also be used as a negative ChIP control as well as non-immune IgG. At STEP 4, the IP incubation mix includes sheared chromatin and beads but no antibody.</td></tr>
<tr><td>Use antibody and specifically blocked antibody in parallel.</td><td>Use one antibody in ChIP and, and the same antibody that is blocked with specific peptide. To specifically blocked one antibody: pre-incubate the antibody with saturating amounts of its epitope specific peptide for about 30 minutes at room temperature before use in the IP incubation mix. Use in ChIP, the blocked antibody as a negative control in parallel with the unblocked antibody.</td></tr>
<tr><td rowspan="5">Antibody in IP</td><td>How many negative controls are necessary?</td><td>If multiple antibodies - of the same specie - 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>Species</th><th>Immunglobulli Isotype</th><th>Protein A</th><th>Protein G</th></tr>
<tr><td>Human</td><td>IgG1</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG2</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG3</td><td>-</td><td>+++</td></tr>
<tr><td></td><td>IgG4</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgGM</td><td colspan="2">Use anti Human IgM</td></tr>
<tr><td></td><td>IgGF</td><td>-</td><td>+</td></tr>
<tr><td></td><td>IgGA</td><td>-</td><td>+</td></tr>
<tr><td>Mouse</td><td>IgG1</td><td>+</td><td>+++</td></tr>
<tr><td></td><td>IgG2a</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG2b</td><td>++</td><td>++</td></tr>
<tr><td></td><td>IgG3</td><td>+</td><td>+</td></tr>
<tr><td></td><td>IgGM</td><td colspan="2">Use anti Mouse IgM</td></tr>
<tr><td>Rat</td><td>IgG1</td><td>-</td><td>+</td></tr>
<tr><td></td><td>IgG2a</td><td>-</td><td>+++</td></tr>
<tr><td></td><td>IgG2b</td><td>-</td><td>++</td></tr>
<tr><td></td><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>
</table>

Immuno-selection incubation	What is the best incubation time for immunoselection using the ultrasonic water bath?	To incubate the sheared chromatin with antibodies for 15 to 30 minutes works for many antibodies, however, the kinetics for reaching equilibrium of epitope-antibody binding may differ for each antibody and target. Optimization might improve the results (e.g. the incubation time may need to be increased for some antibodies).
	How does the immunoselection using the ultrasonic water bath work?	The rate-limiting step in many immunoassays is associated with the slow kinetics of binding of macro-molecular antigen to antibody. It was demonstrated that the use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to antibodies.
	What are the water bath specifications?	Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W
	Can I use the kit w/o an ultrasonic water bath?	Yes, then a long incubation at 4ºC should be used. Depending on the antibody and target to be ChIP'd, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.
Polymerase Chain Reaction	Primer design.	Primer length: 18 to 24 nucleotides/ Primer Tm: 60°C (+/- 3.0ºC)/ % GC: 50% (+/- 4%)
	Controls: negative and positive.	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.
	No PCR signal.	Include a positive PCR control as a control for your PCR mix (your primers, dNTP and Master Mix) using the Input DNA or a DNA sample of the same origin.
	High Ct values.	Use more input chromatin.
	CtNegCtl and CtTarget.	The ratio between target IP and negative control IP depends on the antibody used.
	Background is high.	Verify that you perform properly the following steps: Keep the antibody binding beads and DNA purifying slurry in suspension while adding to tubes. Check by eye that equal pellets of beads and slurry are present in each tube. Washes (step 5) are critical.
	Using end-point PCR rather than quantitative PCR.	If gel electrophoresis is used to estimate intensities of PCR products, then relative occupancy of a factor at a locus is the ratio of the intensity of the target IP band to the negative control IP band.
Freezing	Samples can be frozen at several steps of the protocol.	Pellets of formaldehyde fixed cells can be stored at - 80oC for at least a year. Sheared chromatin can be stored at - 80ºC for months, depending on the protein of interest to be ChIP'd. Purified DNA from ChIP and input samples can be stored at -20ºC for months.
	Avo id multiple freeze/thawing.	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

MANUAL

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