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Innovating Epigenetics Solutions

True MicroChIP kit

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USER GUIDE

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Content

Please read this manual carefully before starting your experiment

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 analyse 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 (crosslinking), chromatin shearing, immunoprecipitation, reverse crosslinking followed by DNA purification and analysis of the immunoprecipitated DNA. In ChIP, living cells are first fixed with a reversible crosslinking 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.

However conventional ChIP protocols require high numbers of cells (hundreds of thousands cells at least) limiting the application for ChIP technology to few cell samples. More recently, ChIP assays on smallest amount of cells have been reported. Nevertheless the procedure requires tedious optimization of several reaction conditions to face the increased background observed in ChIP performed with reduced amount of cells. That might consequently lead to considerable time and lab expenditures. To reduce these tedious steps, Diagenode provides the new True MicroChIP kit with optimized reagents and protocol to enable successful ChIP on as few as 10 000 cells. Moreover, the True MicroChIP kit protocol has been thoroughly optimised by Diagenode for ChIP followed by high-throughput sequencing on Illumina® Next-Gen sequencers. To allow the generation of consistant results in ChIP-seq on 10 000 cells, Diagenode has also optimised a library preparation protocol on limited amount of immunoprecipitated DNA. The new MicroPLEX library preparation kit allows the preparation of libraries for sequencing on picogram amount of immunoprecipitated DNA. Thus, association of the True MicroChIP kit with the MicroPLEX library preparation kit provides optimised solutions to perform ChIP-sequencing on limited amount of cells.

Kit Materials

Table 1: Components supplied with the True MicroChIP kit

Description	Quantity	Storage
Glycine	4.5 ml	4°C
Lysis Buffer tL1	1 ml	4°C
Protease inhibitor cocktail 200x (PIC)	225 µl	**-20°C**
ChIP Buffer tC1	4,5 ml	4°C
Beads Wash Buffer tBW1	13.6 ml	4°C
Protein-A coated magnetic beads	180 µl	4°C **Do NOT freeze !**
Wash Buffer tW1	4.5 ml	4°C
Wash Buffer tW2	4.5 ml	**4°C**
Wash Buffer tW3	4.5 ml	4°C
Wash Buffer tW4	4.5 ml	4°C
Elution Buffer tE1	11 ml	4°C
Elution Buffer tE2	410 µl	4°C
Precipitant tP1	1 ml	4°C
Co-precipitant tCP1	50 µl	**-20°C**
Co-precipitant tCP2	50 µl	**-20°C**
Control 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	40 µl	**-20°C**
ChIP-seq grade Myoglobin exon 2 primer pair	40 µl	**-20°C**

Store DiaMag Protein A-coated 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

- MicroChIP DiaPure columns (Cat. No. C03040001)
- Gloves to wear at all steps
- Phosphate buffered saline (PBS)
- Cell culture medium
- 1 M Sodium butyrate (NaBu) (Cat. No. kch-817-001) (optional)
- Trypsin-EDTA
- Formaldehyde (fresh MolBiol Grade)
- Hank's balanced salt solution (HBSS)
- Ethanol
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- qPCR reagents
- Quant-IT dsDNA HS assay kit (Invitrogen)
- TE

Equipment and accessories

- DiaMag 1.5 magnetic rack (Cat. No. kch-816-015)
- Refrigerated centrifuge for 1.5 ml tubes
- Cell counter
- Eppendorf Low retention 1.5 ml Tubes (VWR 525-0130)
- Bioruptor® sonication apparatus
- Diagenode 1.5 ml TPX microtubes (optimized for chromatin shearing with Bioruptor) (Cat. No. M-50050/M-50001)
- DiaMage Rotator (rotating wheel) (Cat.No. VL-100-0001)
- Thermomixer (65°C)
- Vortex
- Qubit system
- qPCR cycler

Kit method overview & time table

Table 2: True MicroChIP kit protocol overview

Step		Time needed	Day
1	Cell collection and protein-DNA crosslinking	1 to 2 hours	1
2	Cell lysis and chromatin shearing	1 hour	1
3	Magnetic immunoprecipitation and washes	Overnight + 3 hours	1-2
4	DNA decrosslinking and purification	5 hours + overnight	2-3
5	qPCR and data analysis before amplification and sequencing	2 to 3 hours	3

Remarks before starting

1. Cell number and sample manipulation

This protocol has been optimized for shearing of 10 000 cells in 100 µl using the Diagenode's Bioruptor® and then subsequent immunoprecipitation on 10 000 cells in 200 µl. Determine the number of IP you will perform and start with fixation of a unique batch of chromatin. For example, if you would like to perform 4 ChIP on the same chromatin, start with fixation of 40 000 cells. Add also an extra chromatin preparation to use for the input.

Due to the low amount of starting material it is critical to avoid sample loss throughout the experiment to ensure reproducible and consistent results. Avoid pipetting up and down when adding buffers to samples. It is also recommended to use low retention Eppendorf tubes at each step of the protocol to minimize sample lost.

The use of an automated cell counter is also recommended to reduce variations in the amount of the starting cell number.

The True MicroChIP kit is also compatible with higher cell numbers. Efficient shearing has been validated with the True MicroChIP kit in a cell range from 10 000 cells to 100 000 cells to allow performing ChIP assay on 10 000 to 100 000 cells. Determine the number of cells you would like to use per ChIP reaction (between 10 000 and 100 000 cells) and perform shearing on that cell number. Fixation can be done on larger cell numbers (scale accordingly volumes of Lysis Buffer tL1 and HBSS to use) and cell lysate will then be split into 100 µl aliquots (corresponding to the number of cells that will be use per IP reaction) before shearing. Then sheared chromatin will be diluted two times with ChIP Buffer tC1 before performing the immunoprecipitation.

2. Shearing optimization and sheared chromatin analysis.

Before starting the ChIP, the chromatin should be sheared to fragments in the 100 to 600 bp range. Our kit is optimized for chromatin shearing using the Bioruptor®. We recommend using Diagenode's micro tubes as shearing has been shown to be more efficient and reproducible using these tubes. The shearing conditions mentioned in the protocol are adequate for a variety of cell types. However, you should optimize shearing conditions for your specific cell type and fixation protocol before starting a ChIP. Nevertheless analysis of shearing efficiency is not obvious when working with 10 000 cells due to the low amount of DNA recovered after sonication and crosslinking reversion for subsequent analysis on agarose gels. Therefore at least 6 replicates should be performed to check the shearing efficiency and pooled before loading onto agarose gel. The protocol for shearing analysis is described in "Additional Protocols".

3. Antibodies

The optimal amount of antibody to use per ChIP experiment has to be optimized for each antibody. However, we recommend to start with 0.25 µg of antiboby per IP when performing ChIP on 10 000 cells. 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 human qPCR primer pairs for amplification of a positive and negative control target for H3K4me3 (GAPDH-TSS and Myoglobin exon 2, respectively).

4. 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.

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 one quarter of the IP'd material for quantification (when working with 10 000 cells). 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.

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. In order to have sufficient DNA left for sequencing, we recommend not using more than one third of the total IP'd DNA for qPCR. You can dilute the DNA (1/4 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).

% recovery =100* 2^[(Ct(input) – log(X%)/log2) – Ct(sample)]

- Ct (sample) and Ct (input) are threshold values obtained from exponential phase of qPCR for the IP'd DNA sample and input sample respectively
- (logx%/log2) accounts for the dilution 1/x of the input.

If the amount used for the input was 10% of the amount used for ChIP, the recovery can be calculated as follows:

% recovery =100* 2^[(Ct(input) – 3,32) – Ct(sample)]

This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results the real amplification efficiency, if known, should be used.

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 ratio of the positive versus the negative control target should be at least 5Short protocol for experienced users

The protocol below is for use with 10 000 cells per ChIP. To perform ChIP with higher cell numbers refer to « Notes Before Starting ».

Short protocol for experienced users

STEP 1. Cell collection and DNA-protein crosslinking

1. Harvest and count the cells.
2. Add medium to cells to a final volume of 1 ml.
3. Add 27 µl of 36,5% formaldehyde per 1 ml sample. Invert tube and incubate 10 minutes at RT.
4. Add 115 µl of Glycine to the sample. Invert the tube and incubate 5 minutes at RT.
5. Work on ice from this point anwards.
6. Centrifuge at 300 x g for 10 minutes at 4°C. Aspirate the supernatant slowly.
7. Wash cells with 1 ml ice-cold HBSS with inhibitors. Invert the tube to resuspend the cells and centrifuge at 300

x g for 10 minutes at 4°C.

8. Aspirate the supernatant and keep the cell pellet on ice.

STEP 2. Cell lysis and chromatin shearing

9. Add 25 µl of complete Lysis Buffer tL1 (Lysis Buffer tL1 + Protease Inhibitor Cocktail - PIC) per 10 000 cells and agitate manually the bottom of the tube to resuspend the cells.
10. Incubate on ice for 5 minutes.
11. Add 75 µl of complete HBSS (HBSS + PIC) per 10 000 cells and sonicate aliquots of 10 000 cells (in 100 µl) for 1 to 5 runs of 5 cycles of: [30 seconds "ON", 30 seconds "OFF"] using the Bioruptor®. Optimization is needed depending on the cell type and the Bioruptor® model used.
12. Centrifuge at 14,000 x g for 10 minutes and collect the supernatant.

STEP 3. Magnetic immunoprecipitation and washes

13. Add 100 µl of complete ChIP Buffer tC1 (ChIP Buffer tC1 + PIC) to 100 µl sheared chromatin.
14. Add the specific antibody/control antibodies to the 200 µl of diluted chromatin.
15. Rotate at 40 rpm for 16 hours at 4°C.
16. Add 55 µl of Beads Wash Buffer tBW1 to 11 µl of beads and resuspend. Place the beads in the magnetic rack. Discard the supernatant and keep bead pellet. Repeat this wash once. Then resuspend bead pellet in 11 µl Beads Wash Buffer tBW1 per IP.
17. Add 10 µl of washed beads to each IP. Rotate at 40 rpm for 2 hours at 4°C.
18. Place IP tubes in the magnetic rack. Keep the bead captured and remove the supernatant. Add 100 µl of Wash Buffer tW1 and rotate at 40 rpm for 4 minutes at 4°C.
19. Repeat Step 18 once with Wash Buffer tW2, tW3 and tW4, respectively.

STEP 4. DNA decrosslinking and purification

20. Place IP tubes in the magnetic rack. Keep the bead captured and remove the supernatant.
21. Add 200 µl of Elution Buffer tE1 and rotate at 40 rpm for 30 minutes at room temperature. Also add 180 µl of Elution Buffer tE1 to 20 µl of input. Work with both input and IP sample in parallel for remaining steps.
22. Place tubes in the magnetic rack. Transfer the supernatant into new tubes.
23. Add 8 µl of Elution Buffer tE2 and incubate at 65°C for 4 hours with shaking.
24. In a 1.5 ml microcentrifuge tube, add 5 volumes of ChIP DNA Binding buffer to each volume of sample (1040 µl: 208 µl). Mix briefly.
25. Transfer mixture to a provided Spin column in a Collection tube.
26. Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.
27. Add 200 µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.
28. Add 6-100 µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.

Ultra-pure DNA is now ready for use for Library Preparation, PCR, arrays, DNA quantification, sequencing and other molecular applications.

STEP 5. Quantitative PCR analysis

29. Prepare the qPCR mix (total volume of 25 µl/reaction), perform PCR and analyze.

Detailed protocol

The protocol below is for use with 10 000 cells per ChIP. To perform ChIP with higher cell numbers refer to « Notes Before Starting ».

STEP 1. Cell collection and DNA-protein crosslinking

1. Prepare and harvest cells as follows:
   - Place PBS, cell culture medium and trypsin-EDTA at room temperature (RT).
   - If using adherent cells, discard medium to remove dead cells. Wash cells by adding 10 ml PBS. Detach cells by trypsinization. Collect cells by adding culture medium and transfer the medium with cells in a 15 ml centrifugation tube. Use culture medium containing serum (you can the same medium as the one used for culturing the cells). Centrifuge 5 minutes at 1,300 rpm. Keep the cell pellet and discard the supernatant.
   - If using suspension cells, centrifuge for 5 minutes at 1,300 rpm. Keep the cell pellet and discard the supernatant.
2. Resuspend the cells in cell culture medium. You should have at least 10 000 cells per ml of cell culture medium. Count the cells.
3. Label new 1.5 ml tube(s). Add medium to a final volume of 1 ml after the cells have been added. To determine the amount of cells to use for fixation, determine the number of immunoprecipitation you will perform and start fixation of a unique batch of chromatin (see also notes before starting).
4. Add 27 µl of 36.5% formaldehyde per 1 ml of sample (final concentration should be ~1%) and invert tubes immediately two to three times to ensure complete mixing.
5. Incubate for 10 minutes at room temperature to enable fixation with occasional manual agitation. Optimization of fixation time may be required depending cell type, it could be 8-10 minutes.
6. Add 115 µl of Glycine to the sample.
7. Mix by inversion of the tube four to five times. Incubate for 5 minutes at room temperature to stop the fixation. Work on ice from this point onwards.
8. Centrifuge at 300 x g for 10 minutes at 4°C.
   - We recommend the use of a swing-out rotor with soft settings for deceleration.
9. Aspirate the supernatant slowly and leave approximately 30 µl of the solution. Do not disturb the pellet. Take care to not remove these cross-linked cells.
10. Wash the cross-linked cells with 1 ml of ice cold HBSS containing protease inhibitor cocktail (PIC, 200x; final concentration 1x).
    - Add 1 ml of HBSS and invert the tube four to five times to resuspend the cells.
    - When working with higher cell numbers (100 000 cells and more) you should gently vortex to completely resuspend the cells.
    - Centrifuge at 300 x g for 10 minutes at 4°C (in a swing-out rotor with soft settings for deceleration).
11. Discard the supernatant and keep the cell pellet on ice. Proceed directly to cell lysis or if desired the cell pellets can be store at -80°C for up to 2 months.

STEP 2. Cell lysis and chromatin shearing

12. Prepare Lysis Buffer. Add protease inhibitor cocktail (1x final concentration) to Lysis Buffer tL1 (RT). This is the complete Lysis Buffer tL1. Keep the buffer at room temperature until use. Discard what is not used within a day.
    - **Attention**: Make sure that there are no crystals in the Lysis Buffer tL1 before using. Gently heat and mix until

crystals disappear.

13. Add complete Lysis Buffer tL1 to the cells. Use 25 µl of complete Lysis Buffer tL1 per 10 000 cells. Agitate manually the bottom of the tube to resuspend the cells and allow bubbles to form. Scale accordingly when using higher numbers of cells.
14. Incubate on ice for 5 minutes to ensure complete cell lysis.
15. Add HBSS containing protease inhibitor cocktail (PIC, 200x; final concentration 1x) to the cell lysate. Use 75 µl of complete HBSS per 10 000 cells. Scale accordingly when using higher numbers of cells.
16. Dispense 100 µl of cell lysate (equivalent to 10 000 cells) into 1,5 ml microtubes. If cell lysis was performed on more than 10 000 cells, make sure that there are no precipitate before spliting cell lysate into 100 µl aliquots. Otherwise gently heat until crystals disappear.
17. Sonicate samples to shear the chromatin using the Bioruptor® for 1-5 runs of 5 cycles of: [30 seconds "ON", 30 seconds "OFF"] each. Optimization may be required depending on cell type and density, and depending on the Bioruptor® model used. – refer to Bioruptor® manual.
    - We recommend using the Bioruptor® Water Cooler to maintain the temperature around 4°C during shearing. Otherwise, follow the Bioruptor® instructions: In brief: pre-cool the bath with ice, and then remove ice. Then add 4°C water and crushed ice to water level mark. Set the Bioruptor® to "High Power". Replace water with 4°C water and ice every 5 cycles to maintain the temperature below 8°C. Place samples on ice while changing the water.
18. Centrifuge at 14,000 x g (13,000 rpm) for 10 minutes and collect the supernatant which contains the sheared chromatin. Analysis of shearing efficiency is recommanded before starting a ChIP, especially when a particular cell type is used for the first time. See aditional protocols and see also notes before starting.
19. Use the sheared chromatin directly in ChIP. However, if desired the chromatin can be store at -80°C for up to 2 months.

STEP 3. Magnetic immunoprecipitation and washes

20. Prepare complete ChIP Buffer tC1. Add protease inhibitor cocktail (PIC, 200x; final concentration 1x) to ChIP Buffer tC1. This is complete ChIP Buffer tC1.
21. Add 100 µl of complete ChIP Buffer tC1 per 100 µl of sheared chromatin.
22. Use 200 µl of diluted sheared chromatin per tube for each IP. Use low retention 1.5 ml tube. Set aside 20 µl as input sample and keep at 4°C.
23. Add the specific antibody or control antibodies (positive and negative) to each tube. We recommend including one IgG negative control in each series of ChIP reaction.
24. Incubate the IP tubes at 40 rpm on a rotating wheel for 16 hours at 4°C.
25. Prepare magnetic beads. Each IP requires 11 µl of beads. Add 55 µl Beads Wash Buffer tBW1 to 11 µl stock solution of beads for each IP and scale accordingly. Resuspend the beads and place them in the magnetic rack. Discard the supernatant and keep the beads captured. Repeat this wash once. Then resuspend the bead pellet in 11 µl of Beads Wash Buffer tBW1 per IP reaction.
26. Add 10 µl of pre-washed Protein A-coated beads to each IP tube.
27. Incubate the IP tubes at 40 rpm on a rotating wheel for 2 hours at 4°C.
28. Briefly spin the tubes and place them in the magnetic rack. Wait for one minute and remove the supernatant. Wash the beads with Wash Buffer tW1. To wash the beads, add 100 µl of Wash Buffer tW1, gently shake the tubes to resuspend the beads and incubate for 4 minutes on a rotating wheel (40 rpm) at 4°C.
29. Repeat the wash as described above once with Wash Buffer tW2, tW3 and tW4, respectively.

STEP 4. DNA decrosslinking and purification

30. After removing the last Wash Buffer, add 200 µl of Elution Buffer tE1 to the beads and incubate for 30 minutes on a rotating wheel at room temperature. Also add 180 µl of Elution Buffert tE1 to 20 µl of the input sample kept aside the day before.

31. Briefly spin the tubes and place them in the magnetic rack. Transfer the supernatant to a new tube and add 8 µl of Elution Buffer tE2. Also add 8 µl Elution Buffer tE2 to the input sample. Incubate for 4 hours in a thermomixer at 1300 rpm and 65°C.

32. DNA purification using MicroChIP DiaPure columns (Cat. No. C03040001)

(Alternatively phenol chloform extraction can be performed. See additiona protocol (start at point 8))

1. *In a 1.5 ml microcentrifuge tube, add 5 volumes of ChIP DNA Binding buffer to each volume of sample (1040 µl: 208 µl). Mix briefly.*
2. *Transfer mixture to a provided Spin column in a Collection tube.*
3. *Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.*
4. *Add 200 µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.*
5. *Add 6-100 µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.*

STEP 5. Quantitative PCR analysis and data analysis

Before sequencing the samples, we recommend analysing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. In order to have sufficient DNA left for quantification and sequencing, we recommand to use one third of the total immunoprecipitated DNA for qPCR analysis.

33. Prepare the qPCR mix using SYBR Green master mix.

    qPCR mix (total volume of 25 µl/reaction):

    - 1 µl of primer pair (stock: 5 µM each: reverse and forward)
    - 12.5 µl of master mix (e.g.: iQ SYBR Green supermix)
    - 5.0 µl of purified diluted DNA sample and purified input(s)
    - 6.5 µl of water

34. Use the following PCR program: 3 to 10 minutes denaturation step at 95°C (please check carefully supplier's recommendations about Taq polymerase activation time), followed by 45 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72 °C. These conditions may require optimisation depending on the type of Master Mix or qPCR system used.

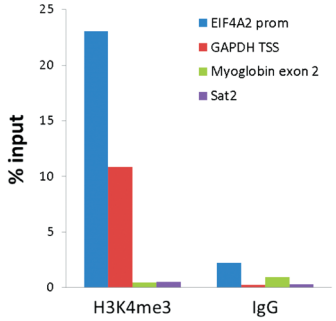

Figure 1: ChIP was performed on human HeLa cells using the Diagenode antibody H3K4me3 (Cat No. pAb-003-050). Sheared chromatin from 10 000 cells, 0.25 µg of the H3K4me3 antibody and 0.25 µg of the negative IgG control were used per IP. Quantitative PCR was performed with the positive controls GAPDH-TSS and EIF4A2 promoter and the negative controls Myoglobin exon 2 and Sat 2 primer sets. The recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis) is shown in figure 1.

ChIP-sequencing

The True MicroChIP kit protocol has been optimised for ChIP-seq on an Illumina® Next-Gen sequencer. The recommended amount of starting material for the Illumina® sample prep is 10-20 ng of IP'd DNA. Depending on the cell type, the target protein abundance and the antibody used, you should recover between 500 pg to a few nanograms of IP'DNA when starting with 10 000 cells. Moreover, after quantification and qPCR analysis, you could have only picogram amounts of immunoprecipitated DNA left for sequencing. Therefore an amplification step is necessary before sequencing the sample using a classical library preparation protocol. Thus, to provide a complete solution for ChIP-sequencing on 10 000 cells, Diagenode has developed a library preparation protocol for use with limited quantity of DNA. The MicroPlex library preparation kit requires only picogram amounts of ChIP'd DNA to start library preparation. This kit allows for rapid amplification of few DNA picograms combined with the conversion of DNA into a sequencing-ready preparation for the Illumina® platform. The True MicroChIP kit has been fully validated in ChIP-seq in association with the MicroPlex library preparation kit.

Figure 2

A: ChIP has been peformed with H3K4me3 antibody, amplification of 17 pg of DNA ChIP'd from 10.000 cells and amplification of 35 pg of DNA ChIP'd from 100.000 cells (control experiment). The IP'd DNA was amplified and transformed into a sequencing-ready preparation for the Illumina platform with the True MicroPlex library preparation kit. The library was then analysed on an Illumina® Genome Analyzer. Cluster generation and sequencing were performed according to the manufacturer's instructions.

B: We observed a perfect match between the top 40% of True MicroChIP peaks and the reference dataset. Based on the NIH Encode project criterion, ChIP-seq results are considered reproducible between an original and reproduced dataset if the top 40% of peaks have at least an 80% overlap ratio with the compared data set.

ChIP-seq data analysis recommendations

To find the captured regions of the genome after the 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
- **SI CER**: 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

Aditional Protocols

Sheared chromatin analysis

Reagents not supplied

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24 : 1)
- 100% Ethanol
- 70% Ethanol
- Agarose and TAE buffer
- TE

1. Take an aliquot of 100 µl of sheared chromatin and spin the chromatin at 14,000 x g (13,000 rpm) for 10 min at 4°C. Transfer the supernatant to a new tube for chromatin analysis.

   A minimum of 60 000 cells is needed to be vizualized onto agarose gel. If each 100 µl of sheared chromatin correspond to 10 000 cells, then perform 6 reactions in parallel and pool the DNA pellets obtained at Step 14 during respuspension in TE.
2. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1 µl of cocktail in 150 µl of water).
3. Add 2 µl of diluted RNase cocktail to the chromatin.
4. Incubate 1h at 37°C.
5. Add 100 µl of the Elution Buffer tE1 and 8 µl of Elution Buffer tE2 to each chromatin sample.
6. Mix thoroughly and incubate samples at 65°C for 4 hours (or overnight).
7. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Incubate the samples at RT for 10 minutes on a rotating wheel.
8. Centrifuge for 2 minutes at 14,000 xg (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
9. Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the samples at RT for 10 minutes on a rotating wheel.
10. Centrifuge for 2 minutes at 14,000 x g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
11. Precipitate the DNA by adding precipitant tP1 (1/10 of the volume) and 2 µl of co-precipitant tCP1. (OPTIONAL: when DNA is isolated from low cell amounts, add also 2 µl of co-precipitant tCP2). Add 1 ml of cold 100% ethanol. Vortex and incubate at -80°C for 30 minutes.

12. Centrifuge for 25 minutes at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70 % ethanol to the pellet.
13. Centrifuge for 10 minutes at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 minutes at RT to evaporate the remaining ethanol.
14. Resuspend the pellet in 10 µl of TE. That corresponds to the purified DNA from the sheared chromatin. Several DNA pellets can be pooled at this step to have DNA corresponding to a minimum of 60 000 cells in 10 µl of TE.
15. Run samples (10 µl of DNA + 2 µl of 6x loading dye) in a 1.5% agarose gel along with DNA size marker to visualise shearing efficiency.

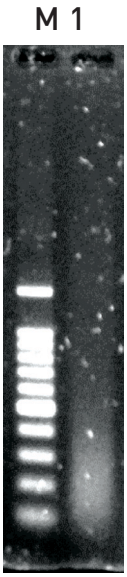

Figure 3: Hela cells were fixed with 1% formaldehyde (for 10 minutes at RT). Cell lysis are performed using the Lysis Buffer tL1 of the Diagenode True MicroChIP kit. Samples corresponding to 10 000 cells are sheared during 5 rounds of 5 cycles of 30 seconds "ON" / 30 seconds "OFF" with the Bioruptor® Plus combined with the Bioruptor® Water cooler (Cat No. BioAcc-cool) at HIGH power setting (position H). For optimal results, samples are vortexed before and after performing 5 sonication cycles, followed by a short centrifugation at 4°C. 10 µl of DNA (equivalent to 60 000 cells) are analysed on a 1.5% agarose gel. (lane 1; lane M: 100 bp DNA Molecular Weight Marker)

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 10-20 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 1/10 volume of 1.25M glycine). 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.
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 high concentration of cells (15 x 10e6 cells/ml or less is ok) 2) Keep samples cold (4°C)
	Shear the samples of chromatin using the Bioruptor® Pico from Diagenode.	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.
Antibody binding beads	Beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead storage.	Store at 4°C. Do not freeze.
	Antibody binding capacity.	~ 10 µg / 30 µl
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.
Negative ChIP control(s)	Use non-immune IgG in the IP incubation mix.	Use the non-immune IgG fraction from the same species the antibodies were produced in.
	Do not add antibody to the IP.	Incubation with beads, which were not coated with antibodies antibodies could also be used as a negative ChIP control.
	Use a specifically blocked antibody in parallel.	Use one antibody in ChIP and, the same antibody that is blocked with specific peptide. To specifically block an 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 the blocked antibody as a negative control in parallel with the unblocked antibody.
Antibody in IP	How many negative controls are necessary?	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.
	Why is my antibody not working in ChIP?	Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.
	Which antibody should I use in ChIP?	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.
	How do I choose an antibody for ChIP?	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.
	Are my antibodies going to bind the protein A or protein G?	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.
Freezing	Avoid multiple freeze/thawing.	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

Species	Immunglobulli Isotype	Protein A	Protein G
Human	IgG1	+++	+++
	IgG2	+++	+++
	IgG3	-	+++
	IgG4	+++	+++
	IgGM	Use anti Human IgM	
	IgGF	-	+
	IgGA	-	+
Mouse	IgG1	+	+++
	IgG2a	+++	+++
	IgG2b	++	++
	IgG3	+	+
	IgGM	Use anti Mouse IgM	
Rat	IgG1	-	+
	IgG2a	-	+++
	IgG2b	-	++
	IgG2c	+	++
Chicken	All Isotypes	-	++
Cow	All Isotypes	++	+++
Goat	All Isotypes	-	++
Guinea Pig	All Isotypes	+++	++
Hamster	All Isotypes	+	++
Horse	All Isotypes	++	+++
Pig	All Isotypes	+	++
Rabbit	All Isotypes	+++	++
Sheep	All Isotypes	-	++