Small Sample Targeted ChIP Kit – KOA0886

Chromatin Immunoprecipitation (ChIP) is one of the primary methods for epigenomic research to investigate protein-DNA interaction on a genome-wide scale. This technique can be used in a variety of life science disciplines including cellular differentiation, tumor suppressor gene silencing, and the effect of histone modifications on gene expression. The Small Sample Targeted ChIP Kit provides optimized reagents, simplified protocols and validation controls which facilitates successful ChIP.

I. KIT COMPONENTS

ChIP Kit			
Component	**Catalog Number**	**Size**	**Storage**
Lysis Buffer	KOA0884A	1mL	4°C
Protease Inhibitor Cocktail 200X	KOA0884B	225µL	-20°C
Chromatin Immunoprecipitation Buffer	KOA0884C	4.5mL	4°C
Bead Wash Buffer A	KOA0884D	13.6mL	4°C
Wash Buffer One	KOA0884E	4.5mL	4°C
Wash Buffer Two	KOA0884F	4.5mL	4°C
Wash Buffer Three	KOA0884G	4.5mL	4°C
Wash Buffer Four	KOA0884H	4.5mL	4°C
Elution Buffer One	KOA0884I	11mL	4°C
Elution Buffer Two	KOA0884J	410µL	4°C
Precipitant Solution	KOA0884K	1mL	-20°C
Co-Precipitant One	KOA0884L	50µL	-20°C
Co-Precipitant Two	KOA0884M	50µL	-20°C
Control IgG Antibody (1µg/mL)	KOA0884N	8µ8	-20°C
Glycine Solution	KOA0880A	4.5mL	4°C
Magnetic Beads - Protein A Coated	KOA0880H	180µL	4°C - Do NOT Freeze
H3K4Me3 ChIP-seq Grade (1µg/mL)	KOA0880P	8µg	-20°C
hGAPDH TSS primer ChIP positive control	KOA0880Q	40µL	-20°C
hMyoglobin primer ChIP negative control	KOA0880R	40µL	-20°C

II. STORAGE AND STABILITY CONDITIONS

Store the components at the indicated temperature upon receipt. Store Protein-A Coated Magnetic Beads and 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.

III. 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 remodeling and transcription regulator recruitment to specific genomic sites. 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 of 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, Small Sample Targeted ChIP Kit has optimized reagents and protocol to enable successful ChIP on as few as 10 000 cells. Moreover, the Small Sample Targeted ChIP Kit protocol has been thoroughly optimized for ChIP followed by high-throughput sequencing on Illumina® Next-Gen sequencers.

IV. REQUIRED EQUIPMENT AND REAGENTS

Equipment and reagents:

- Gloves to wear at all steps
- Phosphate Buffered Saline (PBS)
- Cell Culture Medium
- 1M Sodium butyrate (NaBu) (optional)
- Formaldehyde (fresh MolBiol Grade)
- Hank's balanced salt solution (HBSS)
- Trypsin-EDTA
- phenol/chloroform/isoamyl alcohol (25:24:1)
- 100% ethanol
- PCR tubes and reagents
- Magnetic rack (for 1.5 ml microfuge tubes) (part number: TMS-06 or TMS-32)
- Polymethylpentene microfuge tubes (1.5 ml)
- TE
- Qubit system
- Quant-IT dsDNA HS assay kit (Invitrogen)
- qPCR cycler
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- Rotator (Rotating wheel)
- Sonication device
- Vortex
- Thermomixer
- Cell counter
- Sonicator

V. KIT METHOD OVERVIEW AND TIMETABLE

IP Protocol Overview			
Step	**Description**	**Time needed**	**Day**
1	Cell collection and protein-DNA cross linking	1-2 hours	1
2	Cell lysis and chromatin shearing	1 hour	1
3	Magnetic immunoprecipitation and washes	Overnight + 3 hours	1-2
4	DNA de-crosslinking and purification	5 hours + overnight	2-3
5	qPCR and data analysis before amplification and sequencing	2-3 hours	3

VI. GENERAL CONSIDERATIONS

1. Cell Number and Sample Manipulation

This protocol has been optimized for shearing of 10,000 cells in 100µl and then subsequent immunoprecipitation on 10,000 cells in 200µl. Determine the number of IPs 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 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 Small Sample Targeted ChIP kit is also compatible with higher cell numbers. Efficient shearing has been validated with the Small Sample Targeted ChIP 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 - KOA0884A and HBSS to use) and cell lysate will then be split into 100µl 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 Chromatin Immunoprecipitation Buffer – KOA0884C 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. 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.

3. Antibodies

The optimal amount of antibody to use per ChIP experiment has to be optimized for each antibody. However, we recommend starting with 0.25µg of antibody per IP when performing ChIP on 10,000 cells. The kit contains a negative Control IgG Antibody – KOA0884N and a positive H3K4Me3 ChIP-seq Grade Antibody control antibody – KOA0880P. 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 (hGAPDH TSS primer ChIP positive control – KOA0884Q and hMyoglobin primer ChIP negative control – KOA0884R, respectively).

4. Magnetic beads

This kit includes Magnetic Beads - Protein A Coated – KOA0880H. Make sure the beads do not dry during the procedure as this will result in reduced performance. Always keep the beads homogenously in suspension 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 analyzing 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:

% 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 dependent. 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 5

VII. SHORT PROTOCOL

The protocol below is for use with 10,000 cells per ChIP. To perform ChIP with higher cell numbers, refer to General Considerations.

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 27µl of 36,5% formaldehyde per 1 ml sample. Invert tube and incubate 10 minutes at RT.
4. Add 115µl of **Glycine Solution - KOA0880A** to the sample. Invert the tube and incubate 5 minutes at RT.
5. Keep samples on ice from this point onwards.
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.

Cell Lysis and Chromatin Shearing

9. Add 25µl of complete Lysis Buffer (**Lysis Buffer - KOA0886A** + **Protease Inhibitor Cocktail - KOA0886B**) 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 75µl 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 sonicator. Optimization is needed depending on the cell type and the sonicator used.
12. Centrifuge at 14,000 x g for 10 minutes and collect the supernatant.

Magnetic Immunoprecipitation and Washes

13. Add 100µl of Complete ChIP Buffer (**Chromatin Immunoprecipitation Buffer - KOA0886C** + **Protease Inhibitor Cocktail 200X - KOA0886B**) to 100µl sheared chromatin.
14. Add the specific antibody/control antibodies to the 200µl of diluted chromatin.
15. Rotate at 40 rpm for 16 hours at 4°C.
16. Add 55µl of **Bead Wash Buffer A - KOA0886D** to 11µl 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 11µl **Bead Wash Buffer A - KOA0886D** per IP.
17. Add 10µl 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 100µl of **Wash Buffer One - KOA0886E** and rotate at 40 rpm for 4 minutes at 4°C.
19. Repeat Step 18 once with **Wash Buffer Two - KOA0886F**, **Wash Buffer Three - KOA0886G**, and **Wash Buffer Four - KOA0886H**, respectively.

DNA De-crosslinking and Purification

20. Place IP tubes in the magnetic rack. Keep the bead captured and remove the supernatant.
21. Add 200µl of **Elution Buffer One - KOA0886I** and rotate at 40 rpm for 30 minutes at room temperature. Also add 180µl of **Elution Buffer One - KOA0886I** to 20µl 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 8µl of **Elution Buffer Two - KOA0886J** and incubate at 65°C for 4 hours with shaking.

24. In a 1.5ml microcentrifuge tube, add 5 volumes of ChIP buffer to each volume of sample (1040µl: 208µl). 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 200µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.
28. Add 6-100µl DNA Elution buffer directly to the column matrix. Transfer the column to a new 1.5ml microcentrifuge tube and centrifuge at ≥ 10,000 x g for 30 seconds to elute the DNA.
29. Ultra-pure DNA is now ready for use for Library Preparation, PCR, arrays, DNA quantification, sequencing and other molecular applications.

Quantitative PCR Analysis

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

VIII. DETAILED PROTOCOL

The protocol below is for use with 10,000 cells per ChIP. To perform ChIP with higher cell numbers, refer to General Considerations.

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 10mls PBS. Detach cells by trypsinization. Collect cells by adding culture medium and transfer the medium with cells in a 15mls 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.
4. Add 27µl of 36.5% formaldehyde per 1ml 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 115µl of **Glycine Solution - KOA0880A** 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 30µl 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 PIC (**Protease Inhibitor Cocktail 200X - KOA0886B**; 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 stored at -80°C for up to 2 months.

Cell Lysis and Chromatin Shearing

1. Prepare Lysis Buffer. Add **Protease Inhibitor Cocktail 200X - KOA0886B** (1x final concentration) to **Lysis Buffer - KOA0886A** (RT). This is the Complete Lysis Buffer. 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 – KOA0886A before using. Gently heat and mix until crystals disappear.
2. Add Complete Lysis Buffer to the cells. Use 25µl of Complete Lysis Buffer 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.
3. Incubate on ice for 5 minutes to ensure complete cell lysis.
4. Add HBSS containing **Protease Inhibitor Cocktail 200X - KOA0886B** (1x final concentration) to the cell lysate. Use 75µl of complete HBSS per 10,000 cells. Scale accordingly when using higher numbers of cells.
5. Dispense 100µl 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 is no precipitate before splitting cell lysate into 100µl aliquots. Otherwise gently heat until crystals disappear.
6. Sonicate samples to shear the chromatin using the sonicator 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 model used.
    - We recommend using the temperature around 4°C during shearing. In brief: pre-cool the bath with ice, and then remove ice. Then add 4°C water and crushed ice to water level mark. 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.
7. 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 recommended before starting a ChIP, especially when a particular cell type is used for the first time.
8. Use the sheared chromatin directly in ChIP. However, if desired the chromatin can be store at -80°C for up to 2 months.

Magnetic Immunoprecipitation and Washes

1. Prepare Complete ChIP Buffer. Add **(Protease Inhibitor Cocktail 200X - KOA0886B** [final concentration 1x] t**o Chromatin Immunoprecipitation Buffer - KOA0886C**).
2. Add 100µl of Complete ChIP Buffer per 100µl of sheared chromatin.

3. Use 200µl of diluted sheared chromatin per tube for each IP. Use low retention 1.5 ml tube. Set aside 20µl as input sample and keep at 4°C.
4. 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.
5. Incubate the IP tubes at 40 rpm on a rotating wheel for 16 hours at 4°C.
6. Prepare magnetic beads. Each IP requires 11µl of beads. Add 55µl **Bead Wash Buffer A - KOA0886D** to 11µl 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 11µl of **Bead Wash Buffer A - KOA0886D** per IP reaction.
7. Add 10µl of pre-washed **Magnetic Beads - Protein A Coated – KOA0880H** to each IP tube.
8. Incubate the IP tubes at 40 rpm on a rotating wheel for 2 hours at 4°C.
9. 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 One - KOA0886E**. To wash the beads, add 100µl of **Wash Buffer One - KOA0886E**, gently shake the tubes to resuspend the beads and incubate for 4 minutes on a rotating wheel (40 rpm) at 4°C.
10. Repeat the wash as described above once with **Wash Buffer Two - KOA0886F**, **Wash Buffer Three - KOA0886G,** and **Wash Buffer Four - KOA0886H**, respectively.

DNA De-crosslinking and Purification

1. After removing the last Wash Buffer, add 200µl of **Elution Buffer One - KOA0886I** to the beads and incubate for 30 minutes on a rotating wheel at room temperature. Also add 180µl of **Elution Buffer One - KOA0886I** to 20µl of the input sample kept aside the day before.
2. Briefly spin the tubes and place them in the magnetic rack. Transfer the supernatant to a new tube and add 8µl of **Elution Buffer Two - KOA0886J**. Also add 8µl **Elution Buffer Two - KOA0886J** to the input sample. Incubate for 4 hours in a thermomixer at 1300 rpm and 65°C.
3. DNA purification using MicroChIP DiaPure columns. (Diagenode)
4. Alternatively, phenol chloroform extraction can be performed.
5. In a 1.5ml microcentrifuge tube, add 5 volumes of ChIP DNA Binding buffer to each volume of sample (1040µl: 208µl). Mix briefly.
6. Transfer mixture to a provided Spin column in a Collection tube.
7. Centrifuge at ≥ 10,000 x g for 30 seconds. Discard the flow-through.
8. Add 200µl DNA Wash buffer to the column. Centrifuge at ≥ 10,000 x g for 30 seconds. Repeat wash step.
9. Add 6-100µl 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.

Quantitative PCR Analysis

1. Before sequencing the samples, we recommend analyzing 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 recommend using one third of the total immunoprecipitated DNA for qPCR analysis.
2. Prepare the qPCR mix using SYBR Green master mix.
3. qPCR mix (total volume of 25µl/reaction):

4. 1µl of primer pair (stock: 5µM each: reverse and forward)
5. 12.5µl of master mix (e.g.: iQ SYBR Green supermix)
6. 5µl of purified diluted DNA sample and purified input(s)
7. 6.5µl of water
8. 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 optimization depending on the type of Master Mix or qPCR system used.

IX. CHIP SEQUENCING

The Small Sample Targeted ChIP kit protocol has been optimized for ChIP-seq on an Illumina® Next-Gen sequencer. The recommended amount of starting material for the Illumina® sample prep is 10-20ng of IP'd DNA. Depending on the cell type, the target protein abundance and the antibody used, you should recover between 500pg 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 Small Sample Targeted ChIP kit has been fully validated in ChIP-seq in association with the MicroPlex library preparation kit.

X. CHIP SEQUENCE 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 color 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 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 analyzing 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

XI. RELATED PRODUCTS

Catalog #	Product Name	Size
600-401-GV0	Anti-GAPDH (RABBIT) Antibody	100µg
MB-064-1000	ELISA Microwell Blocking Buffer with Stabilizer (Azide and Mercury Free)	1L
TMBE-100	TMB ELISA PEROXIDASE SUBSTRATE	100mL
TMS-06	6 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x6
TMS-15-50	15/50 Tube TrueBlot® Magnetic Separator	1x15, 1x50
TMS-32	32 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x32