Instruction Manual Version 2 01.14

Histone ChIP kit™

Histone Chromatin Immunoprecipitation kit

Cat. No. **C01010100** (kch-orgHIS-012)

diagenode

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Content

MANUAL

Introduction

Analysis of histone modifications in different cells within different selected regions of the genome is important to associate gene expression status with an exact distribution of epigenetic marks.

Chromatin Immunoprecipitation (ChIP) is a technique allowing the analysis of the histone modifications associated with specific genomic regions in the context of intact cells[1]. ChIP is then used to connect epigenetic marks to intergenic regions, active coding regions and/or silenced coding regions. The main steps of the ChIP technique are cell fixation, chromatin shearing, immunoselection, immunoprecipitation (IP), and analysis of the immunoprecipitated (IP'd) DNA.

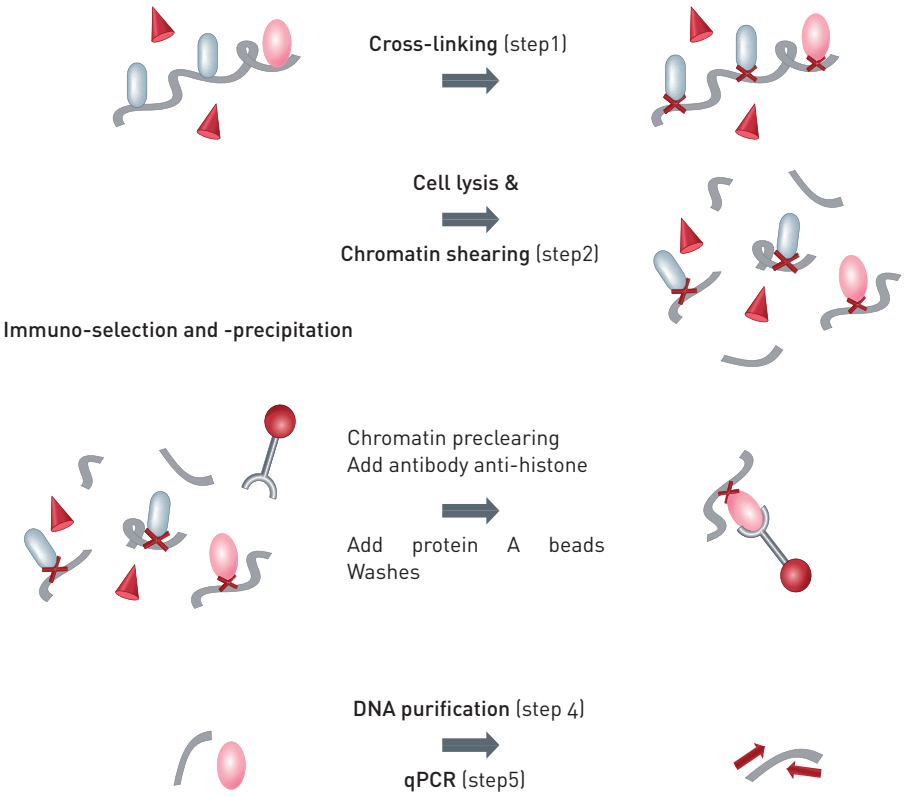
In short, cells are briefly fixed with a reversible cross-linking agent. Next, the cross-linked chromatin (DNA-Protein) is sheared and the DNA fragments associated with the protein of interest are immunoprecipitated using specific antibodies. Finally, the IP'd DNA is examined for the presence of particular sequences by quantitative polymerase chain reaction (qPCR). Enrichment of specific sequences in the precipitate indicates that the sequences are associated with the protein of interest in vivo.

The most widely used approach to fix DNA-Protein interactions in the living cell is by formaldehyde fixation (cross-linking) that generates covalent bonds between amino or imino groups of proteins and nucleic acids[2]. The formaldehyde cross-links protein-DNA as well as protein-protein complexes in situ. Following cross-linking, chromatin needs to be sheared very effectively into homogeneous small fragments that can subsequently be used in IP. The Bioruptor® from Diagenode provides you with high quality sheared chromatin ready for ChIP. Then, specific ChIP-grade antibodies and antibody binding beads are necessary to precipitate the proteins cross-linked to the genomic DNA fragments. Finally, the relative amount of a particular DNA fragment specifically IP'd is determined by qPCR as a measure of the occupancy of the protein at that particular position in the genome.

Although ChIP is a very versatile tool, the procedure requires tedious optimization of several reaction conditions. Diagenode provides kits with optimized reagents and simplified protocols for ChIP (www.diagenode.com). Each kit provides a different set of optimized tools allowing the researchers to approach ChIP assays based on their own needs and equipment.

In the Histone ChIP method the shearing protocol has been shortened and the ChIP method is improved to enhance the utility of the ChIP procedure for the specific study of histones and histone modifications. One major step has been greatly shortened: it is the preparation of the chromatin sample (see "Kit modules and protocol overview"). On the other hand, one chromatin preclearing step has been added -in comparison to the Transcription ChIP protocol- in order to add more specificity to the histone-ChIP assay (see "Time table" and "Kit assay protocol"). In addition, the Histone ChIP buffer, wash buffer stringencies and wash steps are suitable for the histone-ChIP. Finally, the controls provided such as control antibody and PCR primer pairs are relevant to histone-ChIPs (see "Results" section).

Kit Method Overview



The Histone ChIP kit from Diagenode includes four modules. The modules are for: 1/ Chromatin Shearing, 2/ Immuno-selection of histones, 3/ Chromatin IP and 4/ quantitative PCR. The content of the kit is more than sufficient to perform 18 ChIP assays: from cell collection to qPCR. Each module is provided with optimized buffers and protocol to perform ChIP assays on histone and histone modifications.

MANUAL

Kit materials

Kit Components

The content of the kit is sufficient to perform 18 histone-ChIP assays: from cell collection to qPCR.

Table 1: Kit components

Note: Upon receipt, store the components at the right temperature

SHEARING MODULE (steps 1 and 2)			
Component	Description	Quantity	Storage
1.25 M glycine	-	10 ml	4°C
Buffer A (Chromatin shearing)	Detergent and ion chelator mix included.	5 ml	4°C

ANTIBODY MODULE (step 3)			
Component	Description	Quantity	Storage
Antibody anti-histone modification: H3K4me3	0.5 µg/µl; 0.01% thimerosal included.	20 µl	-20°C

ChIP MODULE (step 3 & 4)			
Component	Description	Quantity	Storage
Buffer B (5x ChIP)	Detergent mix, salt and ion chelator mix included.	5 ml	4°C
Protease inhibitor mix (P.I.)	Dissolve the protease inhibitor mix tablet in 400 µl of water and store at -20°C. It is then a 25x stock solution.	1 tablet	-20°C
Pre-blocked protein A coated beads	1:2 suspension for 18 IPs and 18 preclearings; 0.02% azide included.	880 µl	4°C **Do not freeze**
Wash buffer-1	Detergent mix, salt and ion chelator mix included.	10 ml	4°C
Wash buffer-2	Detergent mix, salt and ion chelator mix included.	20 ml	4°C
Wash buffer-3	Ion chelator mix included.	20 ml	4°C
Buffer C (Elution)	Detergent included. Need to be placed at room temperature 1 hour before use.	10 ml	4°C
5 M NaCl	-	400 µl	4°C
DNA co-precipitant	-	100 µl	-20°C
DNA precipitant	-	1000 µl	4°C
H_20	-	10 ml	4°C

qPCR MODUE (step 5)			
Component	Description	Quantity	Storage
c-fos promoter primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
b-actin promoter primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
Myoglobin exon 2 primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
BMX primer pair	5 µM each (Rv & Fw).	50 µl	-20°C

Required Materials Not Provided

Reagents

- Gloves to wear at all steps
- Autoclaved tips
- RNAse/DNase-free 1.5 ml (and 2 ml) tubes
- Other tubes: PCR tubes, 15 ml and 50 ml conical tubes
- Cell scraper (**Step 1-B**: Scraping for chromatin shearing)
- Trypsin-EDTA (**Step 1-A**: Trypsinisation method for cell counting)
- Formaldehyde (37% stock, w/v)
- Ice-cold PBS buffer
- Water
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- Ethanol 100%
- Ethanol 70%
- RNAse (1 µg/µl)
- Agarose and TAE buffer
- DNA molecular weight marker

Equipment

- Centrifuges for 1.5 ml tubes (4°C), 15 ml and 50 ml tubes
- Shaking platform
- Cell counter
- Bioruptor Sonication apparatus from Diagenode (cat # UCD-200, website: http://www.diagenode.com)
- Rotating wheel
- Vortex
- Thermomixer (65°C)
- Cold room with microcentrifuge and rotating wheel
- Quantitative PCR facilities and reagents
- Agarose gel apparatus

Time table

Table 2 : Time table

		DAY	Time needed
STEP 1	Cell fixation and collection	1	30 minutes
STEP 2	Cell lysis and Chromatin shearing	1	20 minutes
STEP 3	Preclearing	1	75 minutes
	Immunoselection	1	15 minutes + O.N.
	Immunoprecipitation	2	1 hour
	Bead washes	2	1.5 hours
STEP 4	DNA purification	2	5 hours
STEP 5	qPCR	3	4 hours

Protocol

----------FIRST DAY----------

Starting material - Cell culture

Start the protocol below when cells in culture reach confluency.

- In the case of the human osteosarcoma U2OS cells grown to confluency, there are about 10 million cells per 140 mm culture dish. Other cell lines could give as little as 3x 10e6 cells per 15 cm dish. The protocol below is for a total of 1x 10e7 cells (step 1-A). The kit provides buffer for the preparation of chromatin from about 4x 10e7 cells.
- Cells can be submitted to inductions and/or treatments. Then treated as well as non treated cells can be prepared and studied in ChIP.
- 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: 1/ as input sample for the ChIP experiment (steps 4- and 5-) and 2/ for shearing efficiency (analysis step).

Each ChIP assay requires 100,000 cells; scale accordingly.

Table 3

Chromatin preparation	Cell number needed	Buffer A volume
For 1 histone-ChIP	1.25x 10e5	in 10 µl
For 6 histone-ChIPs	0.75 x 10e6	in 60 µl
For 12 histone-ChIPs	1.5 x 10e6	in 120 µl
For 18 histone-ChIPs	2.25 x 10e6	in 180 µl

- step 1-A point 7. (Cell suspension, as shown in Table 3 above).
- step 2- points 1. to 6. (Chromatin shearing)
- step 3- point 3. (ChIP)

STEP 1. Cell fixation and collection

The protocol below is for one 140 mm culture dish containing about 10 million cells (step 1-A). If working with more cells, scale accordingly. Keep one dish for counting the cells (step 1-B).

Step 1-A: The method described below is highly recommended for histone-ChIPs.

1. Add 37% formaldehyde (w/vol) directly to the cell culture medium in the flask containing the cells to reach a final concentration of formaldehyde of 1%. Mix immediately.
   - Add 0.65 ml of 37% formaldehyde to 24 ml of culture medium.
   - It is no necessary to fix with higher concentrations of formaldehyde when preparing chromatin for histone-ChIPs.

2. Incubate gently on a shaking platform for 10 minutes at room temperature.
   - It is no necessary to fix for longer when preparing chromatin for histone-ChIPs.

3. Add to the medium, 1/10 of volume of 1.25 M glycine giving a 125 mM final concentration of glycine. Mix. That is to quench the cross-linking reaction.
   - Add 2.5 ml of 1.25 M glycine to the cells (covered with about 25 ml of formaldehyde-culture medium mix.).

4. Remove the medium from the plate.

5. Wash the cells twice with 10 ml ice-cold 1x PBS. After the last wash, discard all the PBS.

6. Add then 500 µl of Buffer A to the plate and harvest the cells by scraping them from the plate and transfer to a 15 ml tube.
   - e.g.: a pellet of about 10 million cells is first resuspended in 500 µl. Scale according to the number of cells you have fixed.

7. The final concentration of the cell suspension should be 10x10e6 cells per 800 µl of Buffer A. Add more buffer if necessary.
   - The step 1-B is a protocol for cell counting (see below).
   - e.g.: when 10 million cells have been resuspended in the buffer at step 6., more buffer can be added in order to reach a total volume of [cells-buffer] mix of 800 µl.
   - Scale according to the number of cells you have fixed: eg. if using two plates of cultured cells: 25 million cells/ 2 ml buffer A.

8. This is the chromatin containing sample ready-to-shear.

Step 1-B: Trypsinisation method to count the cells.

1. Pre-warm PBS, culture media and trypsin-EDTA.

2. Remove old media and rinse cells with pre-warmed PBS (see Table 4 for volumes to use). Shake dishes for 2 minutes. Remove the PBS.

Table 4

Cell rinsing	3x 10e6 cells	10e7 cells
PBS	3.5 ml	10 ml

3. Add sterile trypsin-EDTA to tissue culture flask or dish still containing adherent cells (see Table 5 for volumes to use) Brief treatment with trypsin-EDTA removes adherent cells from tissue culture flask bottom.
   - Each cell line will differ in the degree of adherence it has, and this will be described in the literature that accompanies it. If necessary, place flask back into incubator for about 1-2 minutes.

Table 5

Cell detachment	3x 10e6 cells	10e7 cells
Trypsin-EDTA	1 ml	3 ml

4. Check after a minute to see if cells have come off the flask bottom.
   - Prolonged treatment with trypsin may damage cells. This can be observed macroscopically as sheets of floating cells will be visible.

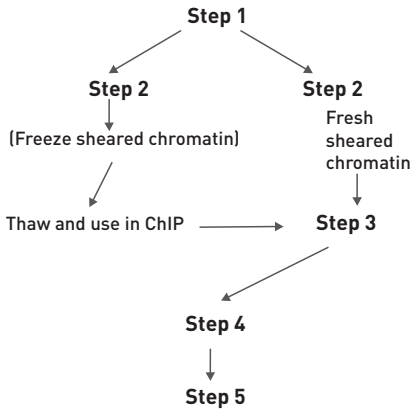
5. When cells are detached, add immediately culture medium to the cells (see Table 6 below). The addition of medium will inactivate the trypsin.

Table 6

Trypsin neutralisation	3x 10e6 cells	10e7 cells
Culture medium	2 ml	6 ml

6. Wash down sides of culture flask with a portion of the [cell/culture medium] mixture. Pipette the cells and transfer to a 15 ml or 50 ml centrifuge tube.

7. Count the cells.



It is best to freshly prepare samples of sheared chromatin and directly use them in ChIP (step3-). However, it is also possible to freeze the sheared chromatin (stop at step 2-pt. 6.) and perform the ChIP another day.

STEP 2. Cell lysis and chromatin shearing

Sonication is used to disrupt the cell and nuclear membranes and to fragment the cross-linked chromatin fibres. It is crucial to generate the appropriate length of chromatin fragments as it can greatly affect the ChIP results.

- This protocol is very successful for a large variety of mammalian cells. However you might want to optimize shearing conditions for your specific cell type and fixation protocol. Therefore, it is advised to start with a small sample (3x 10e6 cells) and to check the shearing efficiency.
- Once conditions have been optimized for your cells following this step 2- and the analysis step, prepare sheared chromatin for ChIP and proceed to steps 3- and 4-.

1. Transfer the chromatin containing sample in appropriate tubes:
- do not add more than 300 µl per 1.5 ml tubes.
- do not add more than 2 ml per 15 ml tubes.
- 10 µl of sheared chromatin is from 0.1 million cells (step 1-A).
- Keep some unsheared chromatin for future controls (analysis step-).

2. Shear the samples of chromatin using the Bioruptor® from Diagenode. Maintain temperature of the samples at 4°C. Samples are sonicated. 1 to 3 runs of 5 to 10 cycles [30 seconds "ON", 30 seconds "OFF"] at high power setting. Briefly vortex and spin between each run. These shearing conditions will work excellent for many cell types.
- Optimal shearing conditions are important for ChIP efficiency. The conditions of shearing are to be optimized for each cell type, fixation protocol and sonicator apparatus. A complete troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.

3. After shearing, if 15 ml tubes were used, transfer the content to new 2 ml tubes.

4. Centrifuge for 5 minutes at 14,000 g (13,000 rpm) at RT to remove debris. Keep the supernatant. That is the sheared chromatin sample.

5. Keep aliquots of sheared chromatin in 1.5 ml tubes (10 µl corresponds to about 0.1 x 10e6 cells) for future controls.
- e.g.: for the analysis of the quality of your sheared chromatin, follow the protocol described in this manual (analysis step).

6. Use the sheared chromatin directly in ChIP (step 3- point 3.).
- Some sheared chromatin will also be used as input sample (step 3- pt. 3.).
- Isolation of DNA from the ChIP samples and from the input sample will be performed in parallel (from step 4- pt. 3. onwards).

Alternatively, prepare aliquots of 75 or 150 µl of sheared chromatin into cryotubes, snap-freeze in liquid nitrogen and then store at -80°C.

- The chromatin can be stored in liquid nitrogen for several months or weeks depending on your ChIP target.
- You can use frozen sheared chromatin in ChIP but usually fresh chromatin gives the best results. If it gets older than 1 or 2 months (stored at -80°C) the quality goes down.
- To dialyse the chromatin sample O.N. against ChIP buffer 1x might be required for some histone modifications such as H3K27me3.
- Do not freeze/thaw.
- 10 µl of sheared chromatin is from 0.1 million cells (step 1-A).
- 10 µl of sheared chromatin is used per IP (step 3- point 3.).
- Samples of sheared chromatin of 75 µl will be for 6 IPs.
- Samples of sheared chromatin of 150 µl will be for 12 IPs.

STEP 3. Immuno-selection and -precipitation

First determine how many ChIPs will be performed, including positive and negative controls.

Preclearing and immunoselection
Preclear the sample of sheared chromatin and then, incubate the chromatin sample with the antibody anti-histone as described below.

1. Prepare IP-incubation mix. Mix the following components: P.I. mix, Buffer B, and water.

Table 7

IP-incubation mix included the volumes of:			
ChIP	P.I.	Buffer B (5x ChIP)	H_2O
1	4 µl	20 µl	76 µl

- P.I.: dissolve the tablet of protease inhibitor mix (P.I.) in 400 µl of water. It is then a 25x stock solution. After use, aliquot and store at -20°C (it is stable for 4 months at least).

2. Add 90 µl of the freshly prepared IP-incubation mix per IP tube.
   - Use one tube per ChIP assay. It is referred as IP tube in this protocol.

3. Then, per IP tube, add 10 µl of sheared chromatin (from step 2- point 6.). Also, keep 10 µl of diluted chromatin to use as input sample (step 4- point 3.).
   - Include control IP tubes.
   - 10 µl of sheared chromatin is from 0.1 million cells (step 1-A).
   - At this stage, the sheared chromatin is 10x diluted.
   - The input sample of sheared chromatin represents 10% of the material used per ChIP. Store at -20°C until being process together with the ChIP samples, from step 4- point 3. onwards.

4. Add 20 µl of the provided pre-blocked beads to the diluted chromatin and rotate for 60 minutes at 4°C. That is to preclear the sample of sheared chromatin.

5. Centrifuge for 2 minutes at 500 g (2,500-3,000 rpm). Keep the supernatant.

6. Transfer 100 µl of supernatant to new IP tubes.
   - 100 µl of precleared sheared chromatin is needed per IP.
   - Discard the beads.

7. Finally, per IP tube, add the antibody anti-histone of interest:
   - provided antibody anti-Histone H3 [K4me3]: use 0.5 µg (1 µl) per IP.
   - other antibody: use 0.5 to 2 µg of antibody (volume depending on the antibody used).

   - Include controls such as:
     "Negative Ctrl IgG", "no antibody added", "no chromatin added", "Positive Ctrl antibody", ...
   - Also, sheared chromatin samples from different cell types, or from cell types that were submitted to different inductions and/or treatments can be tested in parallel.

8. Mix by inverting several times and incubate overnight at 4°C on a rotating wheel.

----------SECOND DAY----------

Immuno-precipitation

This step consists in adding the pre-blocked antibody binding beads (1:2 suspension) to the [antibody-chromatin] mix as described below.

9. Resuspend the provided pre-blocked beads into a uniform suspension before use.

10. Add 20 µl of beads to each IP tube.

11. Incubate at 4°C on a rotating wheel for one hour.

Bead washes

Wash the [antibody-chromatin] complexes attached to the beads as described below.

12. After the incubation, pellet beads by centrifugation for 2 minutes at 500 g (2,500-3,000 rpm) at 4°C. Gently remove the supernatant.

- Pay attention not to remove beads.
- The beads are washed to isolate the [chromatin-antibody] complexes bound to the beads.
- Keep samples cold. It is highly recommended to perform the washing steps in a cold room.
- Also, place the Buffer C at room temperature (RT).

13. Proceed to the bead washes as follows. Add 400 µl of ice-cold wash buffer per IP tube. Incubate for 5 minutes at 4°C, with rotation. Pellet the beads by centrifugation for 2 minutes at 500 g (2,500-3,000 rpm) at 4°C. Gently remove supernatant. Repeat the washes as described above. Use the following buffers for the washes:

- with wash buffer-1, wash once
- with wash buffer-2, wash twice
- with wash buffer-3, wash twice

- Try to remove as much buffer as possible after each washing step without disturbing the pellet. After the last wash, remove carefully the last trace of buffer.

STEP 4. DNA purification

This step is to elute the DNA from the [antibody-chromatin] complexes bound to the beads (ChIP samples). After isolation of the DNA from the IP'd chromatin, the DNA is purified.

1. Elute the [DNA-histone-antibody] complex bound to the beads by adding 400 µl of Buffer C (elution buffer previously placed at RT) to the pelleted beads. Incubate for 20 minutes at room temperature with rotation.

2. Precipitate beads by centrifugation for 2 minutes at 500 g (2,500-3,000 rpm) at room temperature. Transfer supernatants to new clean 1.5 ml tubes. That corresponds to the DNA eluted from the beads and isolated by IP.
   - There are as many IP tubes as ChIP samples.

3. Add 390 µl of Buffer C (RT elution buffer) to 10 µl of diluted sheared chromatin sample (from step 3- point 3.) to bring the final volume to 400 µl. That corresponds to the input sample(s).
   - From this point, treat ChIP and input samples together.
   - Use either fresh or thawed up sheared chromatin from step 3- point 5..
   - The input sample of chromatin represents 10% of the material used per ChIP.

DNA recovery and purification from ChIP and input samples:

4. Add 16 µl of the provided 5 M NaCl, mix and incubate in a thermoshaker for 4 hours at 65°C to reverse cross-linking.
   - It is possible to reverse the cross-linking overnight.

5. Cool down samples to room temperature, add 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1), vigorously vortex for 5 seconds.
   - It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation.

6. Centrifuge for 2 minutes at 14,000 g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

7. Add 400 µl of chloroform/isoamyl alcohol (24:1), vigorously vortex for 5 seconds
   - It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation.

8. Centrifuge for 2 minutes at 14,000 g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

9. Per tube: add 5 µl of the provided DNA co-precipitant and 40 µl of the DNA precipitant. Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -20°C overnight.
   - It is possible to incubate the samples for only 30 minutes at -20°C. Then directly proceed to point 10. below.

----------THIRD DAY----------

10. Centrifuge for 25 minutes at 14,000 g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.

11. Centrifuge for 10 minutes at 14,000 g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 minutes at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that was purified from the sheared chromatin (Input sample(s)) and 2/ DNA that was isolated by ChIP (ChIP samples).
- Avoid leaving ethanol on tube walls.
- The sheared chromatin used as Input samples must correspond to the same preparation of sheared chromatin used in the ChIP assays.

12. Add 50 µl of water to the ChIP samples. To the input sample(s), add 50 µl of water as well.

13. Place the tubes in shaker for 30 minutes at 14,000 g (13,000 rpm) at room temperature to dissolve the pellets.
- Shake DNA to suspend evenly.
- During that incubation, prepare your PCR mix.

STEP 5. qPCR

- Chromatin immunoprecipitation yields very low amounts of DNA that can be amplified by qPCR using specific primers. The amounts of double stranded DNA generated during PCR cycling can be quantified by the fluorescence of an intercalating dye: such as SYBR Green.
- To obtain a reliable quantification several criteria should be carefully approached, see additional notes below.

1. Thaw the qPCR buffer mix on ice.

2. Add H_2O to the qPCR buffer mix. Per PCR sample: add 5.5 µl of H_2O to 12.5 µl of buffer mix. Vortex for 10 seconds.
   - The buffer mix could be IQ SYBR Green supermix or any other that is used in your lab. Follow the instructions of your supplier.

3. Per PCR sample: add 2 µl of primer pair set.
   - The primers in the qPCR module are provided as a mix containing both forward (FW) and reverse (RV) primers, each at 5 µM final concentration.

4. Prepare a dilution 1:10 of the input DNA sample(s) in water (prepared at step 4- point 13.). Mix by vortexing 5 seconds.
   - The aliquot of diluted input DNA sample corresponds to input DNA at a final concentration of 20 cells equivalent per microliter.

5. Per PCR sample: add 5 µl of DNA sample:
   - from ChIP(s) (prepared at step 4- pt. 13.)
   - from input(s) (dilution 1:10 prepared at step 5- pt. 4.)

Table 8

Per PCR Sample:			
qPCR buffer mix	water	Primer pairs	DNA
12.5 µl	5.5 µl	2 µl	5 µl

- The DNA input sample represents 1% of the material used per ChIP.

6. Proceed to the PCR using cycles and temperatures as described in the table below (see Figures 1 and 2 in the "Results" section).

Table 9

	Temperature	Time	Cycles
PCR Amplification	95°C	3 minutes	x1
	95°C	15 seconds	x40
	60°C	45 seconds	
	95°C	1 minute	x1
Melting curve	65°C and increment of 0,5°C per cycle	1 minute	x60

- We highly recommend these optimized qPCR parameters to be used with all the primer pairs supplied in this Histone ChIP kit.
- Primer pairs are targeting the c-fos and b-actin promoter regions; the BMX (bone marrow non-receptor tyrosine kinase, at 18 kb downstream of txn start) and the Myoglobin exon 2.
- Each double-stranded DNA has its own specific melting temperature (TBmB), which is defined as the temperature at which 50% of the DNA becomes single stranded. Even a single mismatch between the labeled probe and the amplicon will significantly reduce the melting temperature. By viewing a dissociation curve, you ensure that the desired amplicon was detected.

- **Your own primer design**
  - Self-complementarity and secondary structure of the primers can be tested using primer design programs. Annealing temperature of 60°C is recommended for qPCR primers.
  - Short length of amplified DNA fragment (50 - 100 bp) facilitates the PCR efficiency and reduces potential problems in amplification of G/C-rich regions.
  - Difference in melting temperature between forward and reverse primers should not exceed 2 to 3°C.
  - G/C stretches at the 3' end of the primers should be avoided.

- **Validation of your primers**
  - Test primer sets by in silico PCR: HThttp://genome.cse.ucsc.edu/cgi-bin/hgPcrTH. Primers should amplify unique DNA products from the genome.
  - Test every primer set in qPCR using 10 fold-serial dilutions of input DNA. Calculate amplification efficiency (AE) of primer set using the following by formula P(3)P: AE= 10^(-1 / slope).
  - The ideal amplification factor is 2. If it is not the case the qPCR reagents from different brand or new primers should be tested.

7. When the PCR is done, analyse the results. Some major advices are given below.

- **Background determination**
  The final goal of ChIP is to calculate the enrichment in the same ChIP sample of: 1/ the specific DNA fragments (corresponding to the binding sites of the protein of interest) in comparison with 2/ non-relevant genomic region (i.e. background).
  Background genomic region should be located far away (at least several thousands bp) from the binding sites of the protein of interest. Considering a complex structure of nuclear chromatin it is recommended to test several loci from different parts of the genome to ensure appropriate background levels. For example, in this kit: second exon of myoglobin is selected as a background region because this gene is tissue-specific and the locus is transcriptionally silent in most of the cell lines.

- **Data interpretation**
  To measure efficiency of chromatin immunoprecipitation of particular genomic locus can be calculated from qPCR data as a percentage of starting material (% of input, recovery):

% input= AE^(CtPinputP – CtPChIPP) B Bx Fd x 100%

Here AE is amplification efficiency as calculated above P(3)P; CtPChIPP and CtPinputP are threshold values obtained from exponential phase of qPCR; FBdB is a dilution compensatory factor to balance the difference in amounts of ChIP and input DNA taken for qPCR.

- Relative occupancy can be calculated as a ratio of specific signal over background:

Occupancy= % input (specific loci) / % input (background loci)

Relative occupancy is then used as a measure of the protein association with a specific locus; it provides clues about specificity of ChIP. Highly specific ChIP can result in about 10 fold enrichment over background and some antibodies can reach up to 1000 fold. This value not only depends on the antibody but also on the target. ChIP result can be considered as reliable in case of significant values for both efficiency and specificity.

- **Alternatively**
  Relative occupancy of the immunoprecipitated factor at a locus is calculated using the following equation:

2^(CtPNegCtlP – CtPTargetP)

where CtPNegCtlP and CtPTarget Pare mean threshold cycles of PCR done in triplicates on DNA samples from negative control ChIP (using non-immune IgG) and target ChIP (using specific antibody) P(4)P.

Analysis Step: Sheared chromatin analysis

If you wish to analyse the quality of your sheared chromatin, follow the protocol described below.

- Analysis step- pts 1. and 2. should be performed on Day 1 and Analysis step- pts 3. to 7. should be performed on Day 2.

1. In order to analyse the sheared chromatin on gel, take sheared chromatin (from step 2. point 5.).
   - One sample of 10 µl of sheared chromatin corresponds to the chromatin obtained from approximately 100,000 cells.
   - Some unsheared chromatin can be analysed on gel as well.

2. Add100 µl buffer C (pre-warmed elution buffer) and 4 µl of the provided 5 M NaCl. Incubate in a thermoshaker, for 4 hours at 65°C to reverse cross-linking (that can be incubated overnight).

3. Cool down to room temperature. Recover the DNA by phenol/ chloroform and chloroform extractions. Precipitate the DNA by adding cold 100% ethanol, co-precipitant and precipitant to the sample. Incubate at -20°C (step 4- points 5. to 9.).

4. Wash pellet with ethanol 70% and air dry (step 4- pts 10. and 11.).

5. Resuspend the pellet in water. That corresponds to the purified DNA from the sheared chromatin. Keep some in a new tube (step 5- points 12. and 13.).
   - If a ChIP is performed with the sheared chromatin, this sample of DNA corresponds to the input sample, as obtained at step 4- point 13..
   - The sample of DNA can be stored at 4°C (at -20°C for long period of time).

6. Treat 20 µl of the DNA sample with RNAse as follows. To the 20 µl of isolated DNA, add 2 µl of RNAse-DNAse free (1 µg/µl stock solution). Incubate for 30 minutes at 37°C.
   - If you use 20 µl of DNA that is not freshly prepared (point 5. above) but that have been kept at 4°C and/or -20°C, incubate the samples at 65°C in a thermomixer for 10 minutes before processing to the point 6..

7. Run samples on a 1% agarose gel along with DNA size marker to visualise shearing efficiency. Analyse on gel.

Results

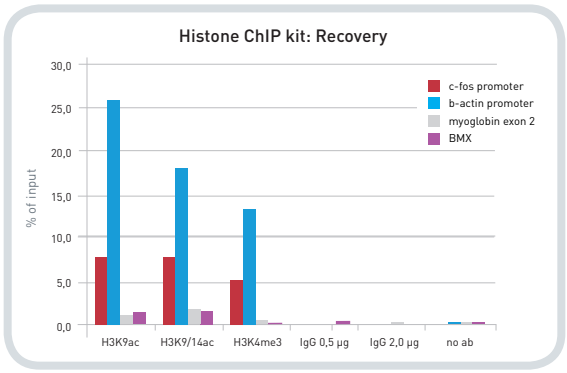


Figure 1: ChIP recovery

Analysis of the histone-ChIP

ChIP results obtained with the Diagenode Orange ChIP kit. ChIP assays were performed using the human osteosarcoma U2OS cells, Diagenode antibodies directed against histone modifications and four optimized PCR primer pairs for qPCR. The three histone-antibodies used were: antibody anti-histone H3 K9ac (2 µg/ChIP), antibody anti-H3K9/14ac (2 µg/ChIP) and antibody anti-histone H3 K4me3 (0.5 µg/ChIP). Three negative controls were included in the ChIP assay: negative control IgG (IgG 0.5 µg and IgG 2 µg) or no antibody added (no ab).

Recovery (above: Fig. 1) and Occupancy (below: Fig. 2) are shown.
Occupancy of the two promoters by the modified histones is evident based on fluorescent qPCR analysis of immunoprecipitated DNA. Controls for IP and PCR specificity include primers for the myoglobin exon 2 and BMX: giving very low background values. The histone-ChIP is specific and sensitive.

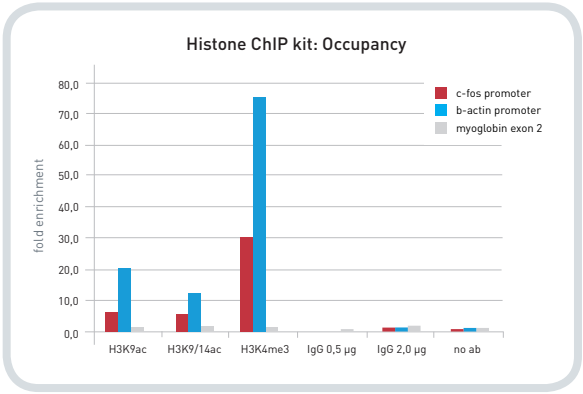


Figure 2: ChIP occurancy

Analysis of the histone-ChIP

ChIP results obtained with the Diagenode Orange ChIP kit. ChIP assays were performed as described in Fig.1.
Occupancy of the c-fos and b-actin promoters by modified histones is evident based on fluorescent qPCR analysis of immunoprecipitated DNA. Controls for IP and PCR specificity include primers for the myoglobin exon 2 and BMX (18 kb downstream of txn start).

Troubleshooting Guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the 10 minutes fixation step at the right temperature and with the correct formaldehyde concentration. Use high quality formaldehyde.
	Cross-linking is too strong.	
	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. Histones are tightly bound to DNA.	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. Histones do not need a long cross-linking step.
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol.
Cell number	The amount of cells required for one Histone- ChIP is important.	We recommend to use 0.1x 10e6 cells per histone-ChIP.
Chromatin shearing	Buffer composition.	Use the buffers provided in the kit. They are optimized containing essential key components. Keep samples cold.
High quality picture of the sheared DNA	For accurate size determination of the DNA fragments	Reverse the cross-linking and precipite the DNA after Phenol/ chloroform extraction.
	Gel electrophoresis of cross-linked samples could give smears.	Reversion of the cross-linking is advised.
	The migration of large quantities of DNA on agarose gel can lead to poor quality pictures which do not reflect the real DNA fragmentation.	Do not load too much on a gel. Do not load more than 5 µg/lane. Also treat the sample with RNAse.
	Agarose concentration.	Do not use more than 1% agarose gel and run slowly
	Running buffer concentration.	1X TAE or TBE is preferred to 0.5X TAE which can lead to smears on gel..
Beads in the IP	Beads centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500 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. 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.
	Bead binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human. (IgG1,2 and 4); and rat (IgG2c).

Antibody in IP	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
	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.
	Amount of antibody per ChIP to use?	To ensure efficient IP it is important to have an optimal ratio between amount of chromatin and amount of antibody. More antibody (or less chromatin) can be required in case of low affinity to antigen or high abundance of target protein (e.g. histones). The lack of antibody can result in low efficiency of ChIP whereas large excess of antibody might lead to lower specificity.
	Are my antibodies going to bind the protein A?	There is a significant difference in affinity of different types of immunoglobulins to protein A or G. For example, IgM can require a secondary antibody as a bridge to protein A or G.
PCR	primers	- length: 18 to 24 nucleotides - Tm: 60°C (+/- 3.0°C) - % GC: 50% (+/- 4%)
	Controls: –ve and +ve	-ve PCR control: PCR with primers specific for a DNA region at which your antigen of interest is not present using ChIP samples. +ve PCR control: PCR on input.
	Orange ChIP qPCR primer pairs	The provided qPCR primers are targeting human genomic loci
	Orange ChIP qPCR primers are provided for rapid checking of the ChIP efficiency.	Reverse cross-links and purify DNA from an aliquot of ChIP sample. Then, use the Histone ChIP qPCR specific primer pairs to amplify certain known promoter regions (c-fos, b-acttin) in order to check whether the ChIP is successful.
	Orange ChIP qPCR primers are provided for rapid checking of the ChIP specificity and efficiency.	Reverse cross-links and purify DNA from an aliquot of ChIP sample. Then, use the Histone ChIP qPCR specific primer pairs to amplify the provided promoter region as well as myoglobin exon2 and BMX. Analyse the data as described in the result section.
Freezing	Samples can be frozen at several steps of the protocol	- Step 2. points 5. and 6. (sheared chromatin) - Step 4. point 13. (ChIP and input: DNA) - Analysis Step. point 5. (Input: DNA)
	Avoid freeze/thawing	Snap freeze cells and thaw on ice.

Additional Protocols

PROTOCOL ADAPTED TO WORK WITH PARASITES

A. CROSSLINK PROTEIN-DNA complexes in vivo

1. Add to the cultures 37% formaldehyde to reach a final concentration of 1%. (e.g.: 270µl in 10 ml)
2. Incubate at 37°C for 5 minutes, gently shaking.
3. Add 1.25 M glycine to reach a final concentration of 0.125M (e.g. : 1ml in 10 ml).

B. LYSIS OF RED BLOOD CELLS (on ice)

4. Centrifuge the sample at 13,000 g for 10 minutes at 4°C.
5. Wash the pellet 1x with ice-cold PBS.
6. Resuspend erythrocytes in one culture volume of PBS.
7. Add and gently mix saponin to a final concentration of 0.05%.
8. Immediately centrifuge the tube at 6,000 g for 5 minutes after lysis is observed.
9. Remove the supernatant, and wash further with ice-cold PBS until clear.
10. Centrifuge at 1,000 g (4,000 rpm) for 10 minutes and wash in PBS.

C. LYSIS AND PREPARATION OF NUCLEI

11. Prepare nuclei by resuspending the cross-linked parasites in 3 ml of Buffer A and homogenizing gently with a douncer on ice (30 strokes).
12. Collect the nuclei by layering them on an equal volume of Buffer A containing 0.25 M sucrose. Spin in swing out rotor centrifuge at 500 g (2,000 rpm) for 10 minutes at 4°C.
13. Resuspend the nuclei in Buffer A and repeat step 12.

D. CHROMATIN SHEARING

14. Resuspend the pellet in a volume of Buffer A equivalent to have 3-5 x10e8 parasites in 150 µl of buffer.
15. Shear the samples, using the Bioruptor UCD-200 with cycles : 30 seconds « ON » and 30 seconds « OFF » for a total of 10 minutes.
16. Centrifuge at 13,000 g for 20 minutes to remove cell debris.
17. Collect the supernatants and freeze aliquots.

PROTOCOL ADAPTED TO re-ChIP

It is possible to ChIP with one antibody and re-ChIP the ChIP sample with a second antibody. That involves two ChIP rounds.

1. The first-round ChIP procedure is as described in this manual from step 3-pt. 1. to step 3- pt. 13..
2. After the washes of the beads (end of step 3-), elute the immune complexes in 50 µl of Buffer C for 10 minutes at room temperature (use 50 µl instead of 400 µl as at step 4- pt. 1.. Except for the volume used, perform as described at step 4- pt. 1. and step 4- pt. 2.)
3. Repeat the elution two more times to obtain 150 µl of eluate in total.
4. Prepare the DNA from 75 µl of the eluate as follows: reverse cross-link, purify and analyze by quantitative real-time PCR directly (to do for the first round ChIP only) (step 4- pt.4. to pt.13.)
5. Dilute 10x the remaining 75 µl of eluate (to a final volume of 750 µl) using ChIP buffer 1x buffer. That is the second-round ChIP.
6. Add antibody to the diluted ChIP samples (or eluates)
7. Incubate overnight at 4°C on a rotating wheel. Include controls (step 3- pts. 7. and 8.).
8. Proceed to immunoprecipitation and bead washes (as step 3- pts. 9. -13.).
9. Elution is performed as described for the first-round re-ChIP (using 3x 50 µl) (pts. 2. and 3. above). Proceed from step 4- pt.4. to step 5- as described in the manual (reverse cross-link, purify the DNA, and qPCR).

References

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3. HTPfaffl M.W.TH 2001 *Nucleic Acids Res.* 29(9):e45.
4. Pokholok D.K., Harbison C.T., Levine S., Cole M., Hannett N.M., Lee T.I., Walker K., Lewitter F., Rolfe P.A., Herbolsheimer E., Bell G.W., Zeitlinger J., Gifford D.K. and Young R.A. 2005. *Cell* 122(4):517-27.

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Here at Diagenode we are committed to remain the "best-in-class" epigenetic kits supplier by meeting the epigenetic research community needs.

Diagenode's kits are easy-to-use and will deliver rapid, sensitive and reproducible results. They are designed to support every step of your experiment, save you time and require minimal starting material. We have kits to perform individual steps of your experiment or cover the complete assay from start to finish. The two techniques that we are currently placing a large emphasis on are the ChIP and DNA methylation assays.

CHROMATIN FUNCTION

Chromatin Immunoprecipitation

Chromatin Immunoprecipitation (ChIP) is a method used to determine the location of DNA binding sites on the genome for a particular protein of interest. ChIP assay offers a huge potential to improve knowledge about the regulation of the genome expression. This technique is now used in a variety of life science disciplines and addresses several essential questions; for example cellular differentiation, tumor suppressor gene silencing as well as the effect of histone modifications on gene expression.

ChIP (crosslinked) procedure

Chromatin-bound proteins are formaldehyde fixed to the DNA. The chromatin is then sheared to small fragment sizes (200 bp – 1 kb) and immunoprecipitated using a specific ChIP-grade antibody. Following reverse crosslinking and proteinase K treatment, the purified DNA is analyzed to identify the genomic regions that the specific protein is bound to.

ChIP, ChIP-on-chip and **ChIP-Seq** are used to investigate interactions between proteins and DNA in vivo. It allows the identification of binding sites of DNA-binding proteins both efficiently and quantitatively. These protocols have been optimized to analyze proteins closely bound to the chromatin, including transcription factors, replication-related proteins, histones, histone variants and histone modifications.

ChIP combined with microarray hybridization (ChIP) or sequencing (Seq) can localize protein binding sites which may help in identifying functional elements in the genome (whole-genome or specific genomic regions).

All our products have been extensively validated in ChIP using various protein targets. The combination of our kits, reagents and equipment is the perfect starting point to your ChIP success.

Bringing it all together: Diagenode's ChIP-seq workflow

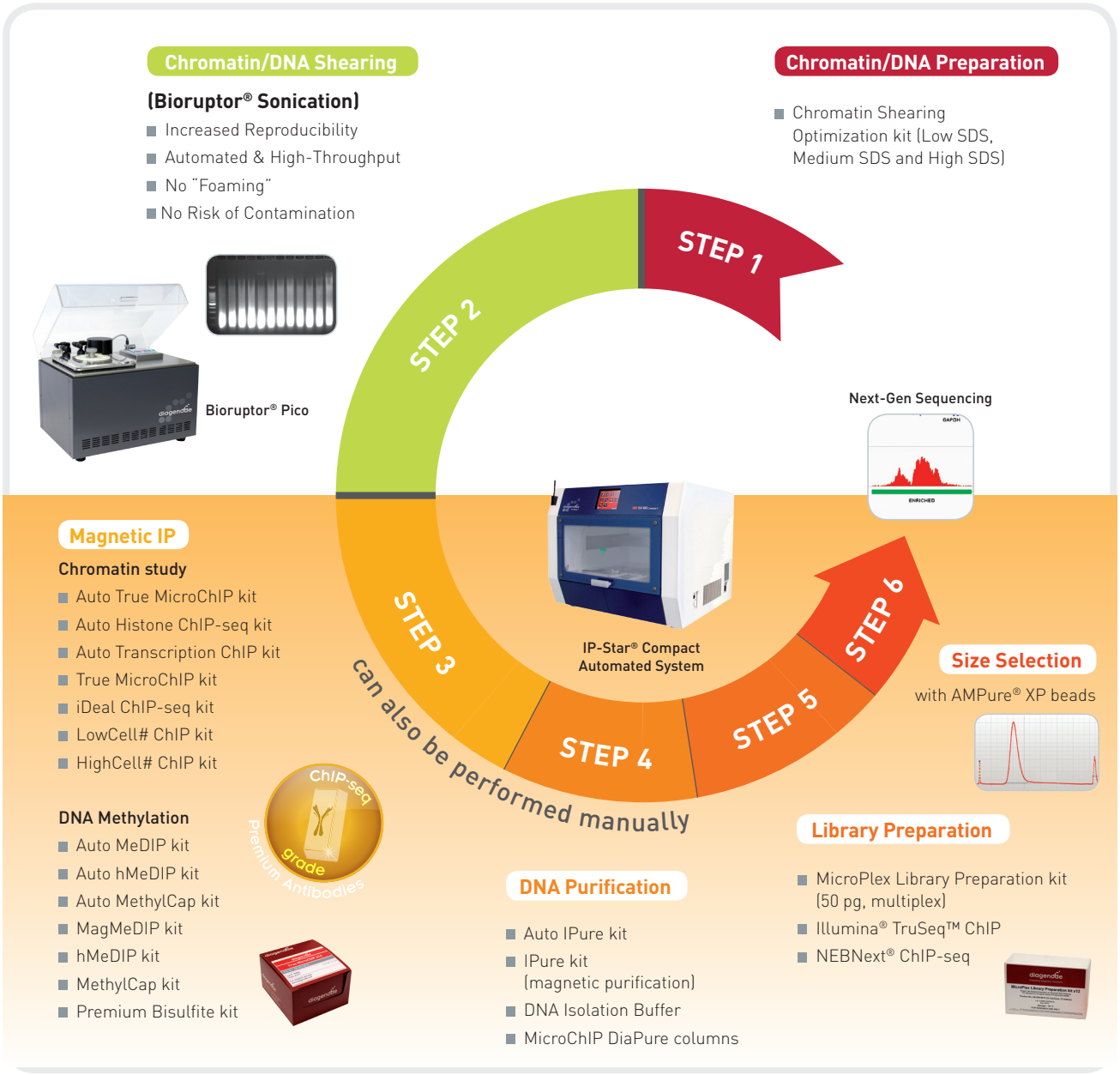

Figure 1. Diagenode provides a full suite of manual and automated solutions for ChIP experiments.

For Step 1, we offer products to isolate nuclei and chromatin. Step 2 describes reproducible sample shearing with the Bioruptor® product line. In Step 3 and Step 4, the Diagenode IP-Star® Compact provides error-free, walk-away automation for all your immunoprecipitation and antibody capture needs.

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