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KIT KEY WORDS:

ChIP chromatin from a low number (#) of cells.

Adapted Magnetic Rack available. Extensive validation and quality control.

**Once your chromatin is sheared, analyse your sample with a set of antibodies.
Use either 10,000 cells or 1,000 cells per IP. Test 6 (to 14) antibodies in 1 (or 2) rows.**

**Target either histones, histone modifications or others.
Low amount of all reagents per reaction, fewer buffers, fewer steps, easy to handle.
Can be performed in one day (or two). Controls included. qPCR validated primers.**

LowCell# ChIP Kit™

The Low Cell Number Magnetic Chromatin Immunoprecipitation Kit

Catalog #: kch-maglow-016

Instruction Manual (version 01)

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1. INTRODUCTION

Association between proteins and DNA is crucial for many vital cellular functions such as gene transcription and epigenetic silencing. It is important to know the genomic targets of DNA-binding proteins and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation.

Chromatin immunoprecipitation (ChIP) is a technique allowing analysis of the association of proteins with specific genomic regions in the context of intact cells. ChIP is used to determine changes in epigenetic signatures, chromatin remodeling and transcription regulator recruitment ⁽¹⁾ to specific genomic sites. The main steps of the ChIP assay are cell fixation (cross-linking), chromatin shearing, immunoselection, immunoprecipitation and analysis of the immunoprecipitated DNA.

In brief, cells are fixed with a reversible cross-linking agent. Next, the cross-linked chromatin (DNA-Protein) is sheared and DNA fragments associated with the protein of interest are immunoprecipitated (IP'd) using specific antibodies. Finally, the immunoprecipitated DNA is examined for the presence of particular sequences by quantitative polymerase chain reaction (qPCR), ChIP-chip or sequencing. 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 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 DNA-Protein 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 immunoprecipitation (IP). The **Bioruptor**TM from Diagenode provides you with high quality sheared chromatin ready-to-ChIP. Moreover, a **Shearing module** is available from Diagenode to enable an easy and highly reproducible shearing method. Then, antibody binding beads and specific ChIP-grade antibodies are necessary to precipitate the proteins cross-linked to genomic DNA fragments. Finally, the relative amount of a particular DNA fragment specifically IP'd is determined by quantitative PCR as a measure of the occupancy of the protein at that particular position in the genome. **ChIP grade beads, antibodies** and **qPCR primer pairs** are available from Diagenode.

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. Another major drawback of conventional ChIP assays is that the method is time consuming. It involves two overnight incubations (first for antibody binding to the target and then for the purification of the IP'd DNA). Therefore Diagenode offers kits not only for the traditional ChIP assay (such as the Red and Orange ChIP kits designed for transcription factors and histones, respectively) but also for the **rapid ChIP method (OneDay ChIP kit)**. The **LowCell# ChIP kit** utilizes some of the same advantages as the OneDay ChIP kit and can therefore also be carried out in one day.

In the **OneDay ChIP** method the protocol has been improved to enhance the utility of the ChIP procedure, allowing you to perform many more ChIPs per day and per week. The entire procedure can be performed in a day's work as the two overnight incubations have been eliminated. Two major steps have been greatly shortened: 1) antibody binding is

accelerated by incubating antibody with chromatin in an ultrasonic bath ⁽³⁾ and 2) DNA purification is rapid as it has been simplified by the use of a DNA purifying slurry and does not require multiple steps (see kit overview). Alternatively, the experiment can also be performed in two days including the traditional overnight antibody incubation with chromatin, if one does not want to purchase an ultrasonic bath. Two kit formats are available: one for 60 IPs, another for 180 IPs.

In the **LowCell# ChIP kit**, the protocol has been improved to allow researchers to work with a **much lower amount of cells** than traditionally used so far. Chromatin samples from as low as **8,000 cells** can be used for histone ChIPs for as many as 6 antibodies and two controls (one negative, another positive).

The LowCell# ChIP kit ensures the use of **low amount of reagents** per reaction (not only cells but also antibodies, inhibitors, buffers). The kit also includes **fewer** buffers in comparison with other kits. This makes this kit **cheaper to use** and **much simpler** to work with. The **number of steps is reduced** and **handling is easier** with our **LowCell# Magnetic procedure**.

Moreover, our **Magnetic Rack** together with our new **LowCell# Magnetic ChIP** protocol ensures the **best IP conditions** by working at a constantly cooler temperature. The Diagenode Magnetic Rack has been designed to be used in IP experiments, keeping samples cool longer and allowing the use of small tubes to reduce the reaction volumes and waste of reagents.

The kit protocol has been validated with our previous Shearing kits, and Bioruptor. Nevertheless, chromatin can be sheared with any in house protocol and sonication apparatus as long as efficiency is checked before use (see the « Important note » section: compatibility between protocols and kits).

The Diagenode LowCell# ChIP kit contains **protein A-coated paramagnetic beads** (and **negative IgG from rabbit**) to allow you to work with rabbit polyclonal antibodies. As positive control or target antibody, choose one of our **ChIP grade rabbit polyclonal antibodies** against main histone modifications or your protein of interest.

If performing ChIP with monoclonal antibodies, we also offer **the negative IgG from mouse** and **protein G-coated paramagnetic beads**. For positive ChIP controls, see our list of **ChIP grade monoclonal antibodies**.

In addition, Diagenode provides you with individual reagents of general use such as **inhibitors** (ChIP grade sodium butyrate), and **PBS**. We also offer several **primer pairs** validated in qPCR for analysis of the IP'd material. Peptides can also be purchased for use in blocking experiments (negative ChIP controls).

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

2. LowCell# ChIP KIT METHOD OVERVIEW & TIME TABLE.

Table 1: Low Cell Number Magnetic ChIP protocol overview.

STEP#	Step	DAY	Time needed
STEP 1	Binding antibody to magnetic beads	1	30 minutes + incubation
STEP 2	Cell collection and DNA-protein cross-linking	1	1 hour
STEP 3	Cell lysis and chromatin shearing one million cells in 130 µl of Buffer B or 100,000 cells in 130 µl Buffer B (or fewer cells, once shearing conditions are set up)	1	1 hour
STEP 4	Magnetic Immunoprecipitation Use 10,000 cells per IP or 1,000 cells per IP Add 870 µl Buffer A Add antibody-coated beads	1	30 minutes + 2 hours to O.N. incubation **
STEP 5	Washes of immune complexes	1 or 2	1 hour
STEP 6	DNA purification	1 or 2	2 hours
STEP 7	Quantitative PCR and data analysis	1 or 2	3 hours



The LowCell# ChIP kit and Magnetic Rack from Diagenode.

The LowCell# ChIP kit is provided with all necessary optimized reagents and buffers as well as a user-friendly protocol. One kit format can be purchased: the content of the kit is sufficient to perform 16 ChIP assays (2 rows of 8 IPs each).

Procedure. Bind antibodies to the magnetic beads at step 1. Using one row: perform 6 targeted ChIPs and include control antibodies (one positive and one negative). If using two rows: perform up to 14 ChIPs and two controls (STEP 1). Cross-link proteins to DNA (STEP 2). Shear to generate chromatin fragments (STEP 3). Incubate the sheared chromatin with antibody-coated beads and then the precipitation can take place (STEP 4). Wash the beads before IP'd DNA fragments are purified and amplified by qPCR (STEPS 5, 6 and 7).

****:** Alternatively: The immunoselection at SPEP 4 can be performed in 2 hours and can result in the same immuno-selection as with an overnight incubation, depending on the antibody used. This makes it possible to carry out the Magnetic ChIP in one day. Then, one can set up the PCR at the end of the day and view the results the following morning. The ultrasonic water bath would further shorten the time if applied. See the "Additional protocol" section.

3. KIT MATERIALS

Kit Content

The content of the kit is sufficient to perform 16 ChIP assays (2 rows of 8 IPs each): from cell collection to immuno-selected and precipitated DNA-ready to PCR. The kit content is described in Table 2. **Upon receipt, store the components at the temperatures indicated in Table 2.**

Required Materials Not Provided

Reagents

- Gloves to wear at all steps
- Phosphate buffered saline (PBS) buffer (available from Diagenode)
- 1 M Sodium butyrate (NaBu) (available from Diagenode)
- Trypsin-EDTA
- Formaldehyde
- RNase/DNase-free 1.5 ml tubes or 0.5 ml tubes
- RNase/DNase-free 0.2 ml tubes (optional, if not using 8 tube-strips)
- Ethanol 100%
- Ethanol 70%
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- Agarose and TAE buffer
- DNA molecular weight marker, loading dye and DNA detection reagent

Equipment

- Galaxy Mini with strip rotor (VWR International, cat. #: 521-2812) and inserts for 0.5 ml tubes (VWR International, cat #: 521-2817)
- Centrifuge for 1.5 ml tubes (4°C)
- Cell counter
- Bioruptor™: Diagenode sonication apparatus (cat. #: UCD-200) <http://www.diagenode.com/>
- Rotating wheel (4°C)
- Vortex
- Floating rack for 1.5 ml tubes
- Tube claps
- Boiling water
- Thermomixer (55°C)
- Quantitative PCR facilities and reagents
- Agarose gel apparatus

Table 2: LowCell# ChIP kit content.

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

Our LowCell# ChIP KIT: The Low Cell Number Magnetic ChIP			
Component	Comments	Quantity	Storage
Buffer A	Detergent mix, salt and ion chelator mix included.	25 ml	4°C
Protein A-coated paramagnetic beads	The beads are supplied for 16 IPs; detergent and 0.02% sodium azide included.	220 µl	4°C Do not freeze
Negative Ctrl IgG from rabbit	1 µg/µl.	15 µl	4°C
1.25 M Glycine	-	2 ml	4°C
Buffer B	Detergent and ion chelator mix included.	3 ml	4°C/RT Incubate at RT before use.
Protease Inhibitor mix (P.I. 200x)	200x stock solution	100 µl	-20°C
Buffer C	Ion chelator mix included.	4 ml	4°C
DNA purifying slurry	-	3 ml	4°C
Proteinase K	100 x stock solution.	30 µl	-20°C
PCR-grade H ₂ O	-	4 ml	4°C
PCR tube strips	For 1 row of 8 samples each.	4	RT
PCR strip caps	For 1 row of 8 samples each.	4	RT
Human SAT 2 primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
Human c-fos promoter primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
Human myoglobin exon 2 primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
Side products for Magnetic ChIP			
Component	Comments	Quantity	Storage
Magnetic rack	16 rxns.	1	RT
1 M Sodium butyrate	50 x stock solution.	1 ml	-20°C
Protein G-coated paramagnetic beads	-	220 µl	4°C
Negative Ctrl IgG from mouse	1 µg/µl.	15 µl	4°C
PBS	-	50 ml	-20°C
Antibodies	-	<i>Visit our website</i>	
Peptides	-	<i>Visit our website</i>	
Primer pairs	5 µM each (Rv & Fw).	<i>Visit our website</i>	

KIT ASSAY SHORT PROTOCOL (The 7 steps in 3 pages)

Our LowCell# Chromatin IP: *Magnetic ChIP*

STEP 1. Binding antibodies to magnetic beads

1. Wash twice the Protein A-coated paramagnetic beads with ice-cold **Buffer A** as follows: add Buffer A, suspend the beads in **Buffer A**, then centrifuge for 5 minutes at 1,300 rpm, discard the supernatant and keep the bead pellet. 10 μ l of beads are needed per IP. Scale accordingly.
2. After washing, resuspend in **Buffer A** to the same bead concentration as the stock.
3. Aliquot 90 μ l of **Buffer A** per 200 μ l PCR tube for each Magnetic ChIP reaction
4. Add 10 μ l of pre-washed Protein A-beads per IP tube.
5. Add the specific antibody and control antibodies (positive and negative).
6. Incubate the IP tubes at 40 rpm on a rotating wheel for at least 2 hours at 4°C.

STEP 2. Cell collection and DNA-protein cross-linking

7. Immediately before harvesting the cells, add inhibitors, if needed, to the culture medium and mix gently.
8. Prepare cells as described in section "4. Kit Assay Protocol"
9. Count the cells.
10. Label new 1.5 ml tube(s), add PBS (including inhibitors) to a final volume of 500 μ l after cells have been added. Transfer cells and wash the pipette tip thoroughly.
11. Add 13.5 μ l of 36.6% formaldehyde per 500 μ l sample.
12. Mix by gentle vortexing. Incubate for 8 minutes at room temperature to allow fixation to take place.
13. Add 57 μ l of 1.25 M Glycine to the sample.
14. Mix by gentle vortexing. Incubate for 5 minutes at room temperature. This is to stop the fixation
----- Work on ice from this point onwards -----
15. Centrifuge at 470 x g for 10 minutes at 4°C.
16. Aspirate the supernatant. Take care not to remove the cells. Aspirate slowly and leave approximately 30 μ l of the solution behind.

STEP 3. Cell lysis and Bioruptor chromatin shearing

17. Wash the cross-linked cells twice with 0.5 ml ice-cold PBS (adding NaBu and/or any other inhibitor of choice). Add the solution, gently vortex and centrifuge at 470 x g (in a swing-out rotor with soft settings for deceleration) for 10 minutes at 4 °C.
18. After the last wash, aspirate the supernatant. Leave about 10 to 20 μ l behind.
19. Add protease inhibitor and NaBu to **Buffer B (RT)**. This is the complete Buffer B. Keep the buffer at room temperature until use, discard what is not used during the day.
20. Add 130 μ l of complete **Buffer B (RT)** to the cells. Vortex until resuspension. Incubate for 5 minutes on ice.
21. Submit the samples to sonication to shear the chromatin using the Bioruptor™ for 12 cycles of: [30 seconds "ON", 30 seconds "OFF"] each.
22. Use the sheared chromatin directly in ChIP.

23. Add 5 μ l of **Protease Inhibitor mix** per ml of **Buffer A**. Add NaBu (20 mM final) or any other inhibitor to Buffer A.
24. Add 870 μ l complete **Buffer A** to the 130 μ l of sheared chromatin.
25. Analysis step. See "additional protocols" section. Once Shearing efficiency is assessed, proceed to the next Step.

STEPS 4 and 5. Magnetic Immunoprecipitation and washes

26. Briefly spin the 0.2 ml tubes containing the antibody-coated beads (STEP 1, Point 6.) to bring down liquid caught in the lid.
27. Place tubes in the ice-cold Magnetic Rack (cooled by placing on ice), wait for 1 minute.
28. Discard the supernatant. Keep the pellet of antibody coated beads.
29. Use 100 μ l of diluted sheared chromatin per IP (STEP 3, Point 24.). Transfer 100 μ l to each 0.2 ml IP tube. Keep 100 μ l as Input sample, keep at 4°C.
30. Close the tube caps and remove tubes from magnetic field.
31. Incubate under constant rotation on a rotator at 40 rpm for 2 hours up to overnight, at 4°C. See the "additional protocol" section, for the use of ultrasonic bath to further reduce the incubation time.

----- DAY 1 or 2 -----

32. Place tubes in the Magnetic Rack, wait 1 minute and discard the buffer. Wash three times using 100 μ l ice-cold **Buffer A** Each wash is done as follows: add buffer, invert to mix, incubate for 4 minutes at 4°C on a rotating wheel (40 rpm), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer. Keep the captured beads.
33. Wash one time with Buffer C: add 100 μ l **Buffer C** to the beads and invert to mix. Incubate on a rotating wheel for 4 minutes at 4°C (40 rpm). Spin and place the clean tubes now containing the beads in the Magnetic Rack after washing, capture the beads and remove **Buffer C**.

STEP 6. DNA purification

34. Put water to boil.
35. Label new 1.5 ml tubes. IP#1-8 (one row), IP# 1-8 and # 9-16 (2 rows).
36. Add 100 μ l of **DNA purifying slurry** directly to the washed beads and remove the 8 tube strips from the Diagenode Magnetic Rack. Mix by pipetting up and down and transfer the ChIP sample (beads and DNA purifying slurry) into the new labeled 1.5 ml tubes.
37. Add 100 μ l of **Input sample** (STEP 3, Point 24.) in a clean 1.5 ml tube and supplement with 100 μ l of **DNA purifying slurry**.
38. Invert the tubes and lock the tubes with tube claps.
39. Incubate the samples for 10 minutes in boiling water.

-----During boiling, do the following:-----

40. Turn on the thermomixer, set the temperature at 55°C.

41. Thaw the provided **proteinase K** on ice.
42. Label new 1.5 ml tubes. IP#1-8 (one row), IP# 1-8 and # 9-16 (2 rows).
-----When the 10 minute boiling is over, proceed to next point:-----
43. Take the tubes out of the boiling water (boiling water will be needed again) and spin briefly to bring down liquid caught in the lid.
44. Take off the tube claps. Wait for samples to cool down.
45. Add 1 μ l of **proteinase K** to each sample and 2 μ l for the **Input sample** (use barrier tips).
46. Vortex for 2 seconds at medium power.
47. Shake all the samples for 30 minutes at 1,000 rpm in the thermomixer at 55°C.
-----When the incubation of 30 minutes is over, proceed to next point:-----`
48. Spin briefly and lock the tubes with tube claps before boiling.
49. Incubate the samples for 10 minutes in boiling water.
50. Centrifuge for 1 minute at 14,000 x g (12,000 rpm) at 4°C.
51. Do not disturb the pellet. Transfer 50 μ l of the IP sample supernatant and 150 μ l of the input sample supernatant to the new labeled 1.5 ml tubes (Point 42.). The pellet of the input sample can be discarded.
52. Add 100 μ l of water to the pellet of the IP sample.
53. Vortex for 10 seconds at medium power.
54. Centrifuge for 1 minute at 14,000 x g (12,000 rpm) at 4°C.
55. Collect 100 μ l of supernatant and pool with the previous supernatant. Mix.

STEP 7. Quantitative PCR and Data analysis

- 1/ Dilute (1:100) the input sample(s).
- 2/ Prepare the **qPCR mix** (total volume of 25 μ l/reaction)
- 3/ When the PCR is done, **analyse** the results.

For more details: See section 4. Kit assay protocol.

4. KIT ASSAY Protocol

IMPORTANT NOTES

Starting material- Cells and sheared chromatin

1/ Cell number:

Cells and number of cells to use per experiment are shown in the protocol (Table 3).

Each ChIP requires sheared chromatin from 1,000 or 10,000 cells; scale accordingly (see results section and Table 3).

One can ChIP with as many as 6 antibodies in a row or 14 antibodies in 2 rows (including in each case: one negative and one positive ChIP control per assay).

e.g.: from as low cell # as 10,000 cells, once the sonication conditions for the cells are known, perform one row of 8 IPs.

!!: One advantage with the preparation of a common chromatin batch for all IPs is that it ensures that the same cells are analysed for the different markers/modifications (in several ChIPs).

2/ Shearing method. Compatibility between protocols and kits.

Following cell harvesting, chromatin must be sheared to ~500 bp before it is used in LowCell# Magnetic ChIP. Note that our protocols are optimized for the use of the Bioruptor™.

In the Magnetic ChIP kit, buffer is included to prepare sheared chromatin.

Depending on the target of interest, the cell number available and the number of

PCRs: add 10,000 to one million cells in 130 µl Buffer B. Scale volumes accordingly.

- it is also possible to use sheared chromatin obtained with our **Shearing module from the Red ChIP kit** (catalog # kch-redmod-100, kch-redmod-400) or to prepare your sheared chromatin following your protocol (see the “additional protocols” section).
- Be aware that using more chromatin per ChIP results in more template DNA for PCR analyses. Therefore it is recommended to take into consideration the number of loci one wants to assess by qPCR when determining the cell number to be used for each ChIP.

3/ Shearing optimization and Sheared Chromatin Analysis.

You might need to optimize shearing conditions for your specific cell type and fixation protocol before starting a ChIP. Therefore, 1) start with a small sample (1x 10⁵ to 1x 10⁶ cells) and 2) check the shearing efficiency. Protocol for quick analysis of shearing is given in the “additional protocol” section of this manual. (Alternatively, see in the Red ChIP kit manual).

4/ Shearing apparatus. Compatibility between protocols.

Our kits and protocols are adapted to Bioruptor™ chromatin shearing. It is possible to use another sonication apparatus as long as the buffer composition is adequate and efficient shearing is obtained (see the “additional protocols” section).

STEP 1. Binding antibodies to magnetic beads

This first step consists in binding the antibodies to the Protein A-coated paramagnetic beads. **!!**: Keep beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.

1. Wash twice the Protein A-coated paramagnetic beads with ice-cold **Buffer A** as follows: add Buffer A, suspend the beads in **Buffer A**, then centrifuge for 5 minutes at 1,300 rpm, discard the supernatant and keep the bead pellet.
 - ❖ Note that 10 µl of beads are needed per IP. Scale accordingly.
 - ❖ For 2 IPs: add a volume of 55 µl **Buffer A** to 22 µl stock solution of beads.
 - ❖ For 8 IPs: add a volume of 220 µl **Buffer A** to 88 µl stock solution of beads.
 - ❖ For 16 IPs: add a volume of 440 µl **Buffer A** to 176 µl stock solution of beads.

!!: Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility.

2. After washing, resuspend in **Buffer A** to the same bead concentration as the stock.
 - ❖ For 2 IPs: add 22 µl Buffer A; for 8 IPs: add 88 µl Buffer A; for 16 IPs: add 176 µl Buffer A.
 - ❖ Do not freeze the beads.

!!: Resuspend the beads before each use.

3. Aliquot 90 µl of **Buffer A** per 200 µl PCR tube for each Magnetic ChIP reaction
 - ❖ Use the 8-tube strips provided in the kit.
 - ❖ It is also possible to use individual tubes (0.2 ml)
 - ❖ Label your IP tubes (use one tube per IP: e.g.: IP#1 to IP#8 for one row).

4. Add 10 µl of pre-washed Protein A-beads per IP tube.

5. Add the specific antibody and control antibodies (positive and negative).
 - ❖ Add 1 to 3 µg of antibody or more (up to 10 µg per reaction), depending on the antibody used.
 - ❖ Antibody will bind to the beads.
 - ❖ See troubleshooting guide: for binding capacities of Protein A (and G).

6. Incubate the IP tubes at 40 rpm on a rotating wheel for at least 2 hours at 4°C.
 - ❖ If necessary, prolong incubation on the rotating wheel until chromatin samples are ready for immunoprecipitation (see next step).

STEP 2. Cell collection and DNA-protein cross-linking

Step 2 consists in harvesting and fixing the cells to prepare the sheared chromatin at the next step.

!!: Number of cells needed per IP is given Table 3, note that another one hundred thousand to one million cells are needed to double-check the shearing efficiency.

!!: Note that sodium butyrate (20 mM final concentration) is added to all solutions thereafter unless otherwise stated. Use other inhibitors if needed.

Table 3: number of cells needed per ChIP (not including cells needed for shearing efficiency test).

	# cells per ChIP	6 target ChIPs + negative IP Ctrl + positive IP Ctrl	14 target ChIPs + negative IP Ctrl + positive IP Ctrl
I.	100,000 cells	800,000 cells	1,600,000 cells
II.	10,000 cells	80,000 cells	160,000 cells
III.	1,000 cells	8,000 cells	16,000 cells

7. Immediately before cell harvesting, add sodium butyrate (NaBu) to a final concentration of 20 mM from a 1 M stock to the culture medium and mix gently. Add any other inhibitor.
8. Prepare cells as follows:
 - ❖ Place the PBS at room temperature (RT). The protocol below includes the use of NaBu-PBS for histone ChIPs. Use NaBu if needed and/or any other inhibitor that might be required based on your ChIP targets. The complete PBS mentioned below refers to PBS with inhibitor(s).
 - ❖ **If cells are growing adherent**, discard medium to remove dead cells. Wash cells by adding 10 ml complete PBS. Harvest cells by trypsinization using trypsin including inhibitor(s). Transfer cells* in a tube containing 10 ml complete PBS (RT), and centrifuge for 5 minutes at 1,300 rpm. Keep the cell pellet and discard the supernatant. (*As an option, before transferring, the trypsin can be inactivated by

addition of medium containing serum and inhibitors (20 mM NaBu and/or other). Then wash the cells in complete PBS to get rid of the medium again.).

- ❖ **If cells are growing in suspension**, centrifuge for 5 minutes at 1,300 rpm. Keep the cell pellet and discard the supernatant. Wash cells by adding 10 ml PBS (RT) containing 20 mM sodium butyrate (NaBu-PBS) or any other inhibitor.

9. Count the cells. e.g. determine the amount of cells in about 200 µl of your sample.

10. Label new 1.5 ml tube(s), add NaBu-PBS to a final volume of 500 µl after the cells have been added. Then, transfer cells and wash the pipette tip thoroughly in the sample.

- ❖ In order to preserve the cells, use either a 1000 µl pipette tip or a smaller tip that has been cut in order to increase the opening.

!!: At this stage, in 500 µl: you should end-up with 1 million cells, 100,000 cells, or 10,000 cells, depending on your experiment.

11. Add 13.5 µl of 36.6% formaldehyde per 500 µl of sample.

12. Mix by gentle vortexing. Incubate for 8 minutes at room temperature to allow fixation to take place.

13. Add 57 µl of 1.25 M Glycine to the sample.

14. Mix by gentle vortexing. Incubate for 5 minutes at room temperature. This is to stop the fixation.

----- Work on ice from this point onwards -----

15. Centrifuge at 470 x g for 10 minutes at 4°C.

- ❖ We recommend the use of a swing-out rotor with soft settings for deceleration.

16. Aspirate the supernatant. Take care not to remove the cells. Aspirate slowly and leave approximately 30 µl of the solution behind.

- ❖ These are the cross-linked cells ready for chromatin shearing. Proceed directly to STEP 3.

- ❖ Do not disturb the pellet.

STEP 3. Cell lysis and Bioruptor chromatin shearing

This section describes cell lysis and Bioruptor chromatin shearing. At this stage, it is essential to produce fragments of size suitable for ChIP and subsequent PCR analysis of the immunoprecipitated DNA. The average size is of 500 base pairs (bp) (range: 200 - 1,000 bp).

!!: Place the **Buffer B** at room temperature (RT) before use.

!!: Work on ice unless otherwise stated.

17. Wash the cross-linked cells twice with 0.5 ml ice-cold PBS (adding NaBu and/or any other inhibitor of choice).

- ❖ Add the NaBu-PBS solution, gently vortex and centrifuge at 470 x *g* (in a swing-out rotor with soft settings for deceleration) for 10 minutes at 4 °C.
- ❖ For 100,000 cells or more you might need to resuspend with a pipette to ensure cells are thoroughly washed.
- ❖ Smaller cell numbers are more easily washed and resuspended by vortexing.
- ❖ In any case make sure that cells are in suspension before proceeding to the next point.

18. After the last wash, aspirate the supernatant. Leave about 10 to 20 µl behind.

- ❖ Avoid taking out too much as that could lead to material loss.

19. Add protease inhibitor and NaBu to **Buffer B (RT)**. This is the complete Buffer B. Keep the buffer at room temperature until use, discard what is not used on the day.

20. Add 130 µl of complete **Buffer B (RT)** to the cells. Vortex until resuspension. Incubate for 5 minutes on ice.

- ❖ This is the cell lysis step prior to chromatin shearing.

21. Submit the samples to sonication to shear the chromatin using the Bioruptor™ for 12 cycles of: [30 seconds “ON”, 30 seconds “OFF”] each.

- ❖ Follow the Bioruptor instructions: In brief: pre-cool the bath with ice, remove ice and add water at 0 °C, and a little bit of ice crushed in small pieces, to water level mark. Set the Bioruptor to “High Power”. Replace the gradually heated water with new 0°C

water and ice every 4 cycles to maintain the temperature below 8°C. Place samples on ice while changing the water.

22. Use the sheared chromatin directly in ChIP.

- ❖ At this point, the sheared chromatin can also be stored for subsequent ChIP experiments and for analysis of shearing efficiency (analysis step).
- ❖ Alternatively, aliquots of 130 µl of sheared chromatin can be transferred into cryotubes, snap-frozen in liquid nitrogen and then stored at -80°C.
- ❖ The chromatin can be stored in liquid nitrogen for several weeks or months depending on your ChIP target. Do not freeze/thaw.

23. Add 5 µl of **Protease Inhibitor mix** per ml of **Buffer A**. Add NaBu (20 mM final) or any other inhibitor to Buffer A.

- ❖ This is the complete Buffer A to be used next.
- ❖ 870 µl of complete Buffer A is needed per sample, see next Point.

24. Add 870 µl complete **Buffer A** to the 130 µl of sheared chromatin.

- ❖ This dilution step is needed to reduce the SDS concentration (to ~0.1%) before the addition of antibodies.
- ❖ To scale down the number of cells per sample, samples can be diluted further.
- ❖ Chromatin from 1,000 cells is sufficient for one ChIP assay (depending on target and antibody used).

25. Analysis step. See “additional protocols” section. Once shearing efficiency is assessed, proceed to the next Step.

Each ml of chromatin is then used for 9 IPs + 1 Input sample (see next STEP).

- ❖ Sheared chromatin from one million cells /ml (to use 100,000 cell equivalent/IP)
- or Sheared chromatin from 100,000 cells /ml (to use 10,000 cell equivalent/IP)
- or Sheared chromatin from 10,000 cells /ml (to use 1,000 cell equivalent/IP)
- ❖ **Keep** on ice a chromatin sample equal to that used in the ChIP reactions to represent the **input** per reaction.
- ❖ Use chromatin from 100,000 to one million cells for **analysis of shearing efficiency**.

STEPS 4 and 5. Magnetic Immunoprecipitation and washes

STEP 4 consists in the immunoprecipitation of the protein-DNA complex of interest and washes of the IP'd material (STEP 5).

!!: Use in IP both the antibody coated beads (STEP 1, *Point 6.*) and the sheared chromatin (STEP 3, *Point 24.*)

26. Briefly spin the 0.2 ml tubes containing the antibody-coated beads (STEP 1, *Point 6.*) to bring down liquid caught in the lid.
27. Place tubes in the ice-cold Magnetic Rack (cooled by placing on ice), wait for 1 minute.
28. Discard the supernatant. Keep the pellet of antibody-coated beads.
29. Use 100 µl of diluted sheared chromatin per IP (STEP 3, *Point 24.*). Transfer 100 µl to each 0.2 ml IP tube. Keep 100 µl as Input sample at 4°C.
 - ❖ **I.** 100 µl of sheared chromatin were obtained from 100,000 cells
 - ❖ **or II.** 100 µl of sheared chromatin were obtained from 10,000 cells
 - ❖ **or III.** 100 µl of sheared chromatin were obtained from 1,000 cells
30. Close the tube caps and remove tubes from magnetic field.
31. Incubate under constant rotation on a rotator at 40 rpm for 2 hours up to overnight, at 4°C.
 - ❖ See the “additional protocol” section, for the use of ultrasonic bath to further reduce the incubation time.

Next:

----- DAY 1 or 2 -----

32. Place tubes in the Magnetic Rack, wait 1 minute and discard the buffer. Wash three times using 100 µl ice-cold **Buffer A**. Each wash is done as follows: add buffer, close the tube caps, invert the 8-tube strip to resuspend the beads, incubate for 4

minutes at 4°C on a rotating wheel (40 rpm), spin, place in the Magnetic Rack, wait 1 minute and discard the buffer. Keep the captured beads.

- ❖ Do not disturb the captured beads attached to the tube wall.
 - ❖ Always briefly spin the tubes to bring down liquid caught in the lid prior to positioning in the Diagenode Magnetic Rack.
 - ❖ Use 150 µl Buffer A for each wash if working in a 1.5 ml tube-Magnetic rack.
- !!! Washes of the [antibody-chromatin-beads] mix are performed to isolate the chromatin complexes that are specifically attached to the beads by antibody binding.

33. Wash one time with Buffer C: add 100 µl **Buffer C** to the beads. Close the tube caps, invert the 8-tube strip to resuspend the beads, incubate on a rotating wheel for 4 minutes at 4°C (40 rpm). Spin and place the clean tubes now containing the beads in the Magnetic Rack after washing, capture the beads and remove **Buffer C**.

- ❖ Use 150 µl Buffer C for each wash if working in a 1.5 ml tube-Magnetic rack.
 - ❖ Chromatin complexes specifically bound to the beads have been isolated.
 - ❖ Work at room temperature.
 - ❖ Use barrier filter tips, from this step onward.
 - ❖ Use the PCR-grade water provided in the kit from this step onwards.
- !!! From the washed beads, the bound DNA can now be purified (proceed to the next point).

STEP 6. DNA purification

The purpose of this step is to isolate the DNA from IP'd chromatin.

34. Put water to boil.

35. Label new 1.5 ml tubes. IP#1-8 (one row), IP# 1-8 and # 9-16 (2 rows).

36. Add 100 µl of **DNA purifying slurry** directly to the washed beads and remove the 8 tube strips from the Diagenode Magnetic Rack. Mix by pipetting up and down and transfer the CHIP sample (beads and DNA purifying slurry) into the new labeled 1.5 ml tubes.

- ❖ Keep the slurry in suspension while pipetting aliquots out.
- ❖ Use filtered tips. The tips could be cut.
- ❖ Pipet up-and-down before aliquoting into each tube and during the transfer of the CHIP material into the clean 1.5 ml tube.

37. Add 100 µl of **Input sample** (STEP 3, *Point 24.*) in a clean 1.5 ml tube and supplement with 100 µl of **DNA purifying slurry**.

38. Invert the tubes and lock the tubes with tube claps.

39. Incubate the samples for 10 minutes in boiling water.

-----During boiling, do the following:-----

40. Turn on the thermomixer, set the temperature at 55°C.

41. Thaw the provided **proteinase K** on ice.

42. Label new 1.5 ml tubes. IP# 1-8 (one row). IP# 1-8 and IP# 9-16 (2 rows).

-----When the 10 minute boiling is over, proceed to next point:-----

43. Remove the tubes from the boiling water (boiling water will be needed again) and briefly spin the tubes to bring down liquid caught in the lid.

44. Take off the tube claps. Wait for samples to cool down.
45. Add 1 μl of **proteinase K** to each sample and 2 μl for the **Input sample** (use barrier tips).
46. Vortex for 2 seconds at medium power.
47. Shake all the samples for 30 minutes at 1,000 rpm in the thermomixer at 55°C.

-----When the incubation of 30 minutes is over, proceed to next point:-----`
48. Briefly spin the tubes to bring down liquid caught in the lid and lock the tubes with tube claps.
49. Incubate the samples for 10 minutes in boiling water.
50. Centrifuge for 1 minute at 14,000 x g (12,000 rpm) at 4°C.
51. Do not disturb the pellet. Transfer 50 μl of the IP sample supernatant and 150 μl of the input sample supernatant to the new labeled 1.5 ml tubes (Point 42.). The pellet of the input sample can be discarded.
52. Add 100 μl of water to the pellet of the IP sample.
53. Vortex for 10 seconds at medium power.
54. Centrifuge for 1 minute at 14,000 x g (12,000 rpm) at 4°C.
55. Collect 100 μl of supernatant and pool with the previous supernatant. Mix.
The **total volume** for each sample (input and IP) is of **150 μl** .
 - ❖ The DNA from the ChIP and the input sample(s) is now purified and ready to be analysed by PCR.
 - ❖ Proceed to the PCR step immediately or freeze all the samples.
 - ❖ Store at -20°C.

STEP 7. Quantitative PCR and Data analysis

This last step consists in amplifying and analysing the IP'd DNA.

1/ Make the dilution (1:100) of the input(s) as follows:

to 1 µl of each purified DNA input, add 99 µl of water

2/ Prepare the **qPCR mix** using SYBR PCR Green master mix. **qPCR** cycles are given below.

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

- 1 µl of provided 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 DNA sample and diluted purified input(s) (see above for input dilution)
- + 6.5 µl of water

qPCR cycles:

	Temperature	Time	Cycles
PCR Amplification	95°C	3 minutes	x1
	95°C	30 seconds	x40
	60°C	30 seconds	
	72°C	30 seconds	
Melting curve	65°C and increment of 0.5°C per cycle	1 minute	x60

3/ When the PCR is done, **analyse** the results. Some major advices are given below.

❖ **Your own primer design**

- _ Self-complementarity and secondary structure of the primers can be tested for primer design (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi). Annealing temperature of 60°C is recommended for qPCR primers.
- _ Short length of amplified DNA fragment (50 - 150 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.

❖ **Advantages of the qPCR**

qPCR or Real time PCR enable fast, quantitative and reliable results. Visit: <http://www.gene-quantification.info/>. The Gene Quantification page describes and summarises all technical aspects involved in quantitative gene expression analysis using real-time qPCR & qRT-PCR. It presents a lot of applications, chemistries, methods, algorithms, cyclers, kits, dyes, analysis

methods, meetings, workshops, and services involved.

❖ **Validation of your primers**

_Test primer sets by in silico PCR: <http://genome.cse.ucsc.edu/cgi-bin/hgPcr>. 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⁽⁵⁾: **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.

_ qPCR products should also be run on a high resolution agarose gel since melting curve analysis in qPCR not always picks up primer dimmer or additional products.

❖ **Data interpretation**

The efficiency of chromatin immunoprecipitation of particular genomic locus can be calculated from qPCR data and reported as a percentage of starting material: % (ChIP/ Total input).

$$\% \text{ (ChIP/ Total input)} = 2^{[(Ct^{(x\%input)} - \log(x\%/log2) - Ct^{(ChIP)})]} \times 100\%$$

Here 2 is the amplification efficiency (AE) as calculated above⁽⁵⁾; Ct^(ChIP) and Ct^(x%input) are threshold values obtained from exponential phase of qPCR for the IP'd DNA sample and input sample respectively; the compensatory factor (logx%/log2) is used to take into account the dilution 1:x of the input. The **recovery** is the % (ChIP/ Total input).

Or

$$\% \text{ input} = AE^{(Ct^{input} - Ct^{ChIP})} \times F_d \times 100\%$$

Here AE is amplification efficiency as calculated above⁽⁵⁾; Ct^{ChIP} and Ct^{input} are threshold values obtained from exponential phase of qPCR; F_d is a dilution factor of the input DNA 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.

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

$$\text{Occupancy} = \% \text{ input (specific loci)} / \% \text{ input (background loci)}$$

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.

❖ Use of a **standard curve generated from fragmented genomic DNA**. A dilution series is made and qPCR is run on DNA with the primer one uses for ChIP. This will give the PCR efficiency. Most qPCR programs allow automatic calculation of the DNA quantity in the samples by comparing with the Ct and known quantities of DNA standards.

Additional notes: Data analysis

I. Use of a standard curve.

A standard curve is generated from fragmented genomic DNA. It is recommended to prepare DNA from the same species or the same cell type. Make serial dilutions of the DNA covering the area of concentration of the ChIP samples. Make 8 different concentrations with a broad range. qPCR program allows an automatic calculation of the quantity in the samples by comparing with the Ct and known quantities of the standards.

Raw data (standard, Input and IP))

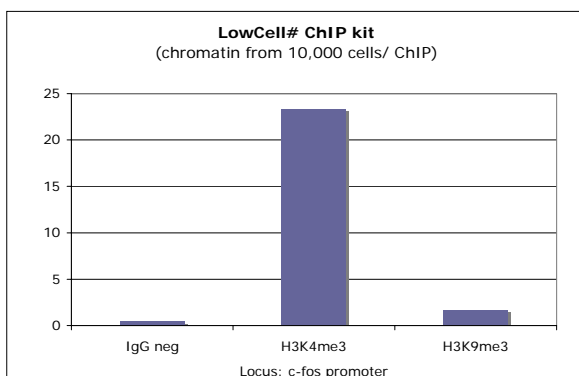
	Starting Quantity (SQ, ng)	Ct values
Standard 1	2.01 E+00	27.98
Standard 2	1.0 E+00	29.14
Standard 3	5.17 E-01	30.38
Standard 4	2.59 E-01	31.89
Standard 5	1.51 E-01	32.31
Standard 6	1.23 E-02	34.65
Standard 7	2.17E-02	34.75
Standard 8	9.58E-03	35.83

Identifier	Threshold Cycle (Ct)	Starting Quantity (SQ)	SQ Mean
INPUT 1/100	34.68	1.95E-02	1.25E-01
INPUT 1/100	31.36	2.30E-01	1.25E-01
IgG	33.36	5.20E-02	5.47E-02
IgG	33.23	5.74E-02	5.47E-02
H3K4me3	27.86	3.09E+00	2.92E+00
H3K4me3	28.02	2.75E+00	2.92E+00
H3K9me3	31.26	2.48E-01	2.09E-01
H3K9me3	31.76	1.71E-01	2.09E-01

Calculation: % of input= quantity of the IP/quantity of the INPUT (1%)

IgG	H3K4me3	H3K9me3
0.44	23.36	1.67

Graph (% of INPUT)



Use of standard curve to show ChIP results.

ChIP assays were performed using the Diagenode LowCell# ChIP kit (cat#: kch-maglow-016) and undifferentiated human teratocarcinoma (NCCIT) cells. Diagenode antibodies directed against H3K4me3 (cat# pAb-003-024) and against H3K9me3 (cat# pAb-056-050) as well as optimized qPCR primers to amplify a region of the c-fos promoter (cat#: pp-1004-050, -500) from the IP'd DNA were also used. Chromatin was sheared from 100,000 cells (STEP 3). Per ChIP experiment: chromatin from **10,000** cells and 1 µg of antibody

were used (STEP 4). A negative control antibody was included in the ChIP assay (negative IgG from rabbit: 1 µg/IP). A standard curve was used to present the data.

LowCell# ChIP kit: *The Low Cell Number Magnetic Chromatin IP*
Diagenode manual

II. Use directly the Ct values (without standard curve).

qPCR was made as indicated in STEP 7 (quantitative PCR and data analysis) without a standard curve and the Ct values were reported in the three tables (see raw data below). A compensatory factor ($\log(100)/\log 2$) was subtracted from the Ct values of the diluted input (1 %) in order to calculate the Ct values of the 100% input.

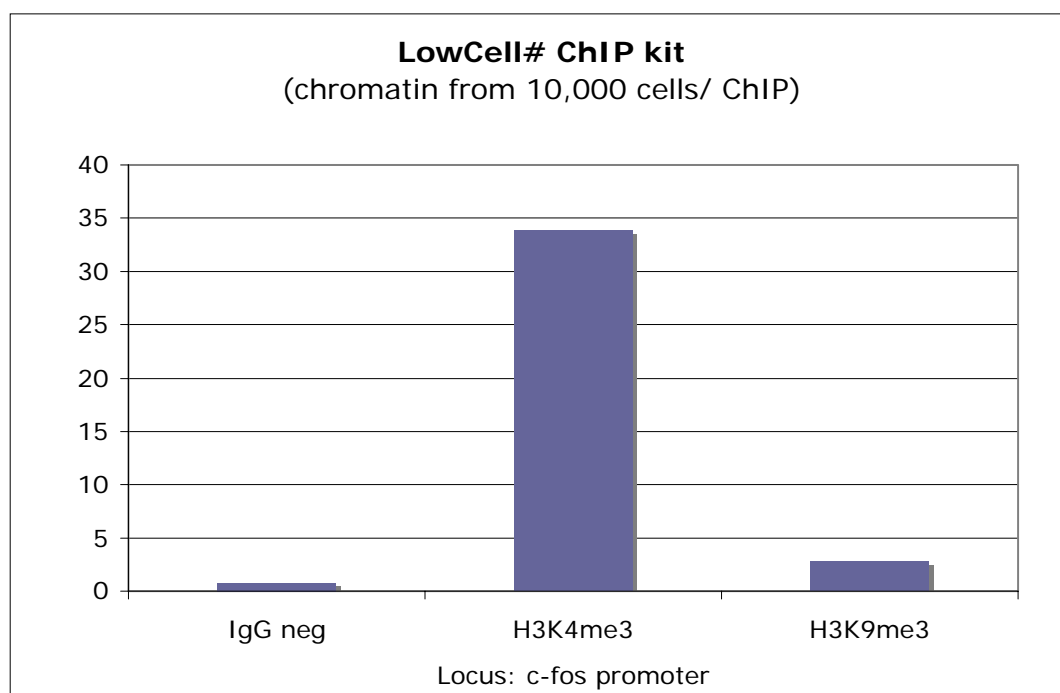
Raw data (Ct values)

Ct INPUT 1/100 Mean	Compensatory factor	Ct INPUT 100%	Ct IgG Mean
33.0	6.6	26.4	33.3
Ct INPUT 1/100 Mean	Compensatory factor	Ct INPUT 100%	Ct H3K4me3 Mean
33.0	6.6	26.4	27.9
Ct INPUT 1/100 Mean	Compensatory factor	Ct INPUT 100%	Ct H3K9me3 Mean
33.0	6.6	26.4	31.5

Calculation: $\% (\text{ChIP} / \text{Total input}) = 2^{[(\text{Ct}^{(x\% \text{input})} - \log(x\%)/\log 2) - \text{Ct}^{(\text{ChIP})}] \times 100\%$

IgG	H3K4me3	H3K9me3
0.8	33.8	2.8

Graph (% of INPUT)

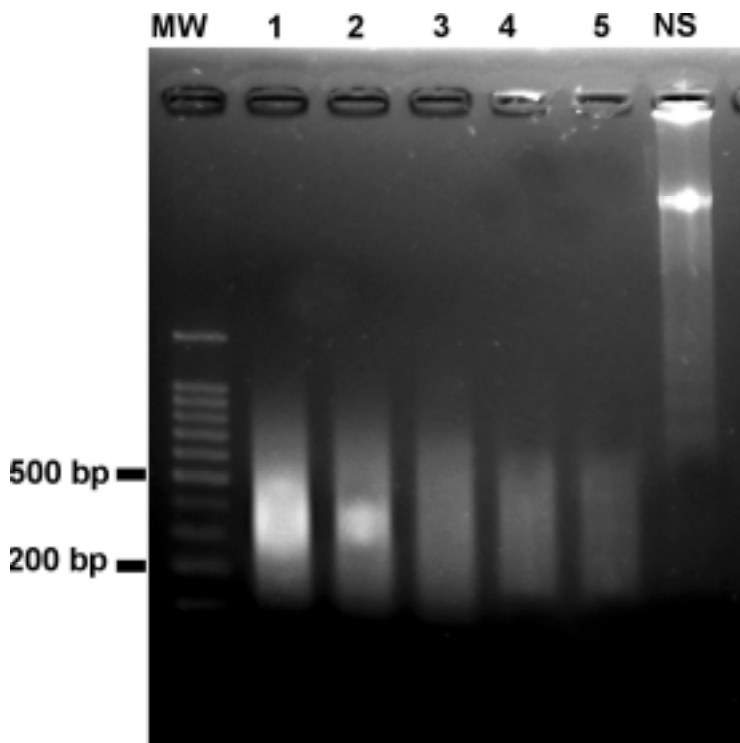


Analysis of ChIP results without standard curve

ChIP assays were performed using the Diagenode LowCell# ChIP kit (cat#: kch-maglow-016), undifferentiated human teratocarcinoma (NCCIT) cells. Diagenode antibodies directed against H3K4me3 (cat#: pAb-003-024) and against H3K9me3 (cat# pAb-056-050) as well as the optimized qPCR primer pair to amplify a region of the c-fos promoter (cat#: pp-1004-050, -500) from the IP'd DNA were also used. Chromatin was sheared from 100,000 cells (STEP 3). Per ChIP experiment: chromatin from **10,000** cells and 1 µg of antibody were used (STEP 4). A negative control antibody was included in the ChIP assay (negative IgG from rabbit: 1 µg/IP).

Diagenode LowCell# ChIP RESULTS

Figure 1: Sheared chromatin analysis prior to Magnetic ChIP:

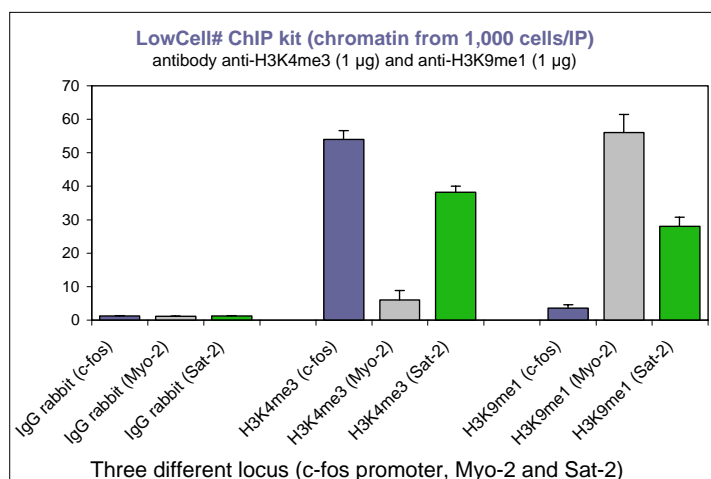
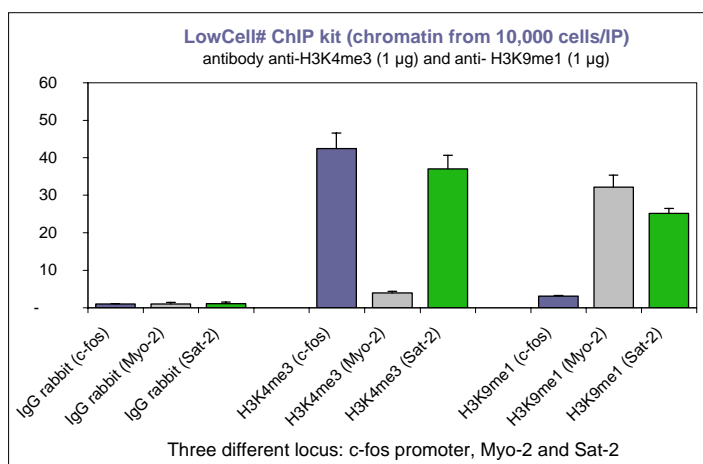
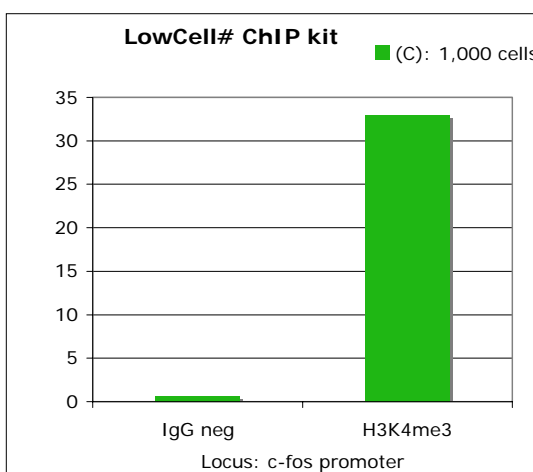
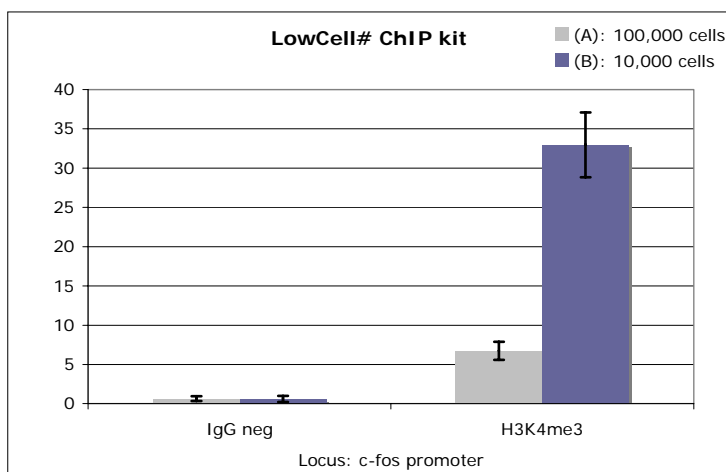


Analysis on an agarose gel of the DNA isolated from the sheared chromatin obtained with the shearing protocol of the LowCell# Magnetic ChIP kit from Diagenode.

Human breast adenomacarcinoma MCF7 cells were fixed with 1% formaldehyde (for 8 minutes at RT). Sheared chromatin from one million cells (lane 1), 750,000 cells (lane 2), 500,000 cells (lane 3) and 100,000 cells (lane 4 and 5) were analysed on gel.

One million cells (lanes 1, 2, 3 and 4) or 100,000 cells (lane 5) were resuspended in 130 μ l of **Buffer B** prior to chromatin shearing. Samples were sheared for 12 cycles of: [30 seconds "ON" / 30 seconds "OFF"] with the Bioruptor™ from Diagenode (cat.#: UCB-200). The shearing efficiency was assessed prior to Magnetic ChIP (see "shearing analysis step"). 10 μ l of DNA isolated from the sheared chromatin were analysed on a 1.5% agarose gel. The left lane shows the 100 bp DNA molecular weight marker (MW) and the right lane shows the DNA that was not sheared (NS).

Figure 2: LowCell# Magnetic ChIP performed with different amount of cells/IP (down-titration of cell#):



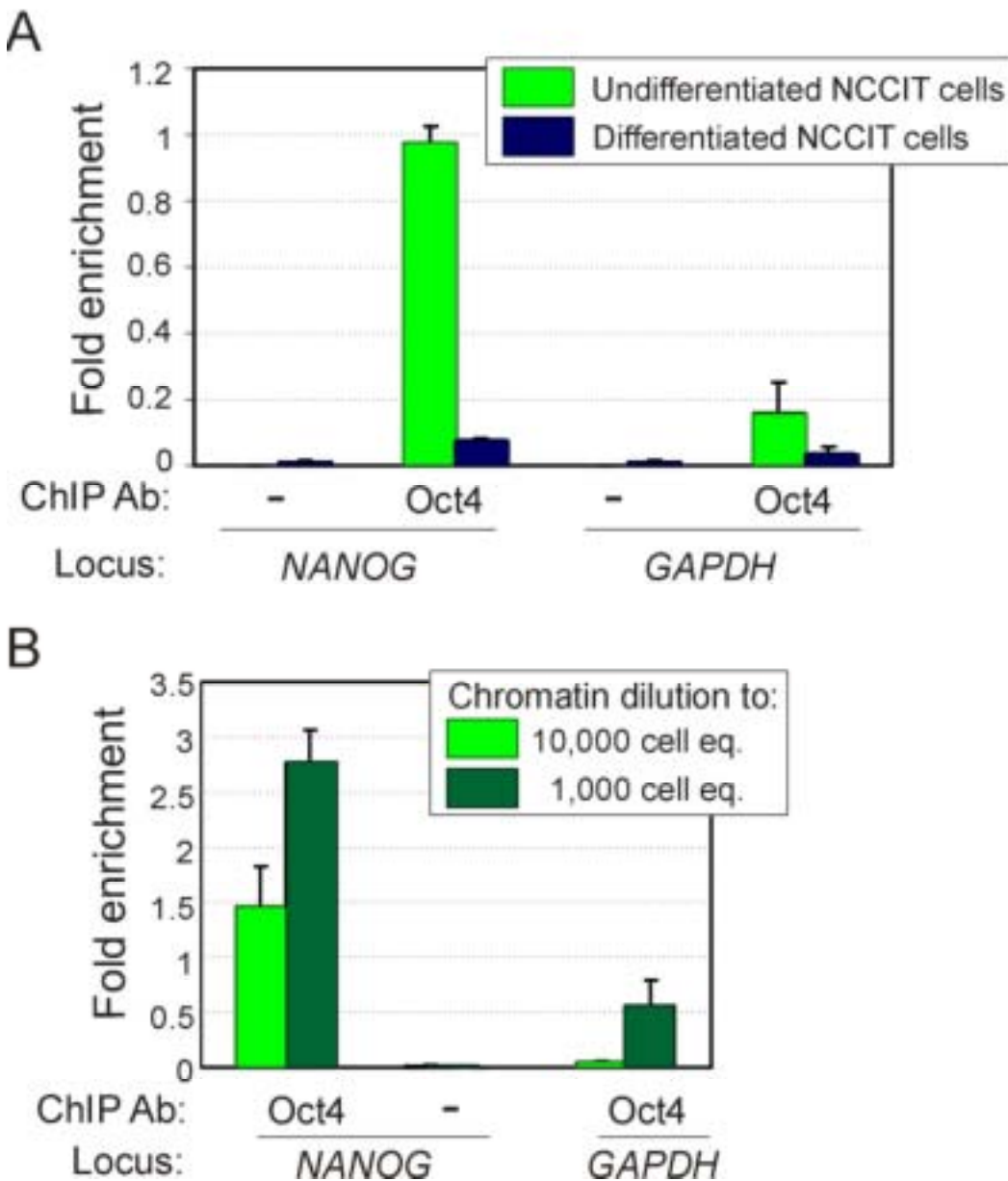
Down-titration of cells with Diagenode Magnetic ChIP protocol.

Top panels: ChIP assays were performed using the Diagenode LowCell# ChIP kit (cat#: kch-maglow-016) and NCCIT cells. Diagenode antibody directed against H3K4me3 (cat#: pAb-003-024) and the optimized qPCR primer pair to amplify a region of the c-fos promoter (cat#: pp-1004-050, -500) from the IP'd DNA were also used. Chromatin was sheared from 1 million cells (STEP 3) and **100,000** cell equivalent was used per ChIP (STEP 4)(see **A**). In two other experiments, chromatin was sheared from 100,000 cells (STEP 3) and either **10,000 (B)** or **1,000 (C)** cell equivalent was used per ChIP (STEP 4). One μg of antibody was used per ChIP experiment. A negative control antibody was included in the ChIP assay ($1 \mu\text{g}/\text{IP}$). Reproducibility is demonstrated and ChIP can be performed with as low cell number as **1,000/IP**. Results from four independent experiments are shown for A and B.

Middle and bottom panels: ChIP assays were also performed using the Diagenode antibody directed against the H3K9me1

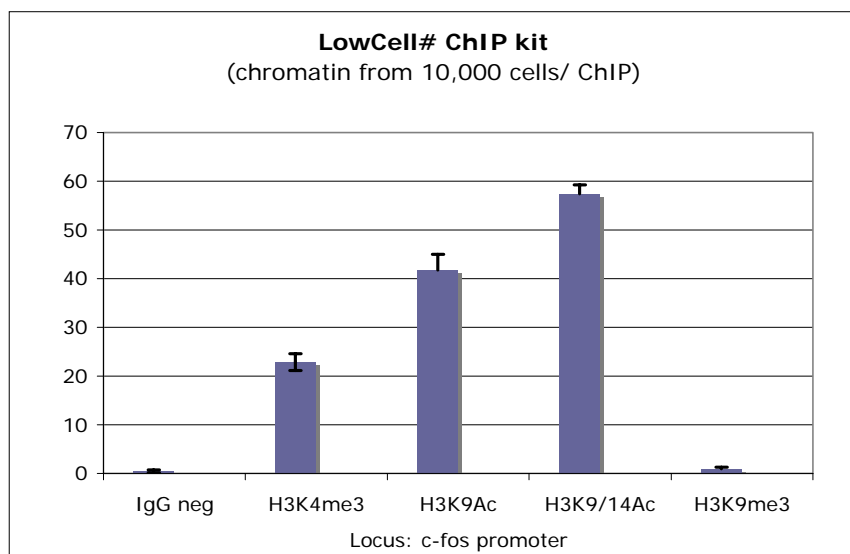
(cat#: pAb-065-100) and optimized qPCR primers to amplify three different loci from the IP'd DNA: a region of the c-fos promoter (cat#: pp-1004-050, -500), Myoglobin exon 2 (cat#: pp-1006-050, -500), and Sat-2 (cat#: pp-1040-050, -500). Per ChIP: chromatin from **10,000** cells (middle) or **1,000** cells (bottom panel) and $1 \mu\text{g}$ of antibody were used. A negative control antibody was included in the ChIP assay ($1 \mu\text{g}$ negative IgG from rabbit /IP). Each ChIP assay was performed in triplicate.

Figure 3: LowCell# Magnetic ChIP performed with different types and numbers of cells/IP (down-titration of cell#):



ChIP assays were performed using the Diagenode LowCell# ChIP kit (cat#: kch-maglow-016), NCCIT cells, antibody directed against Oct4 and the optimized qPCR primer pair to amplify specific loci from the IP'd DNA (NANOG and GAPDH). A. ChIP assays were performed using chromatin from 10,000 cells: either undifferentiated or differentiated NCCIT cells (light green and dark blue, respectively). B. Chromatin from either 10,000 or 1,000 undifferentiated cells (light green and dark green, respectively) was used per ChIP. A negative control antibody was included in the ChIP assay. Reproducibility is demonstrated and ChIP can be performed with as low cell number as 1,000/IP. Results from four independent experiments are shown. Results obtained in the laboratory of Philippe Collas (adapted from Freberg, Dahl, Timoskainen and Collas, 2007; Dahl and Collas, 2007).

Figure 4: LowCell# Magnetic ChIP analysis of a series of modified histones

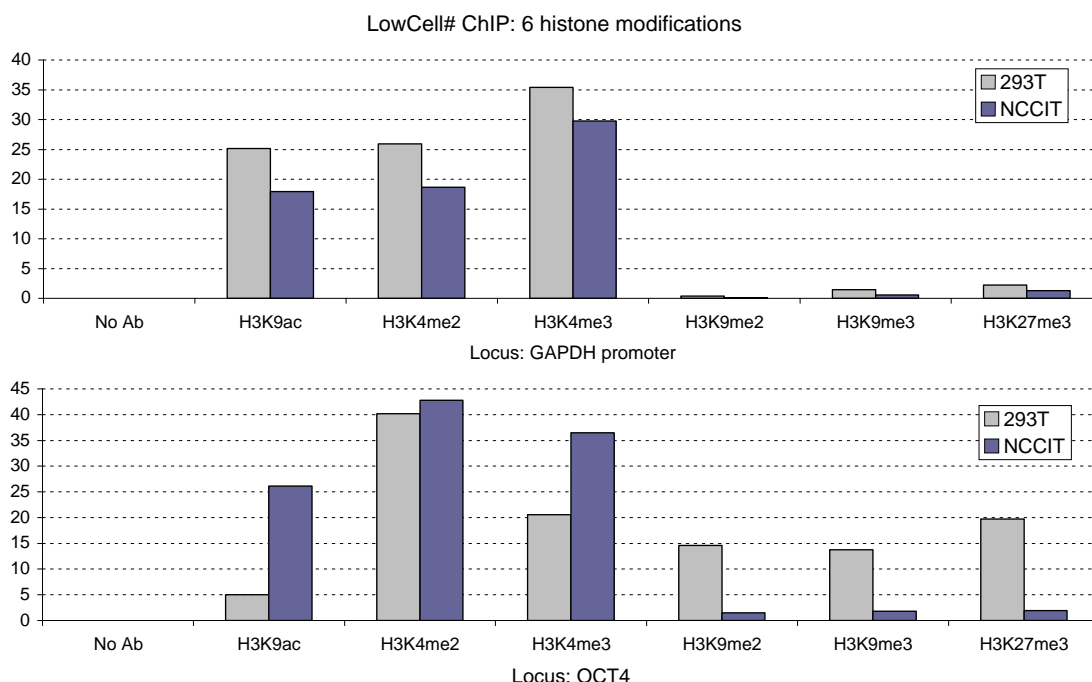


The Diagenode Magnetic ChIP kit is ideal to study a series of antibodies (8 or 16) on the same chromatin sample.

ChIP assays were performed using our LowCell# ChIP kit, undifferentiated human teratocarcinoma (NCCIT) cells, Diagenode antibodies directed against: H3K4me3 (cat.#: pAb-003-024), H3K9ac (cat.#: pAb-004-044), H3K9/14ac (cat.#: pAb-005-044) and H3K9me3 (cat.#: pAb-056-050). Optimized qPCR primers were used to amplify

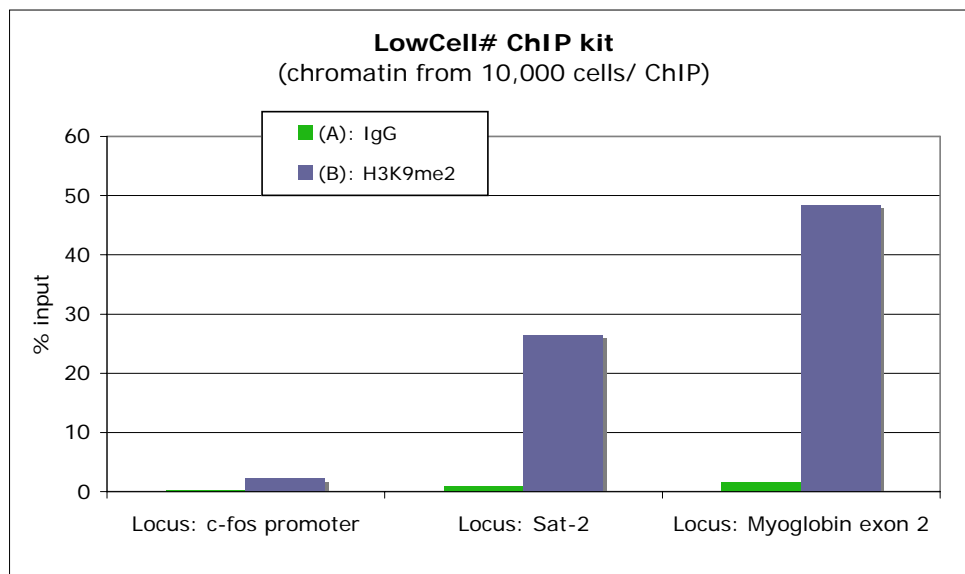
a region of the c-fos promoter (cat.#: pp-1004-050, -500) from the IP'd DNA. Chromatin was sheared from 100,000 cells. Per IP, chromatin from 10,000 cells and 1 µg of antibody were used. A negative control antibody was included in the ChIP assay (1 µg/IP). Results from 3 independent experiments are given.

Figure 5: LowCell# Magnetic ChIP analysis of a series of modified histones on different cell types



ChIP assays were performed using our LowCell# ChIP kit, NCCIT and 293T cells, and antibodies directed against modified histones. Optimized qPCR primers were used to amplify a region of the GAPDH promoter and OCT4 from the IP'd DNA. Chromatin was sheared from 100,000 cells. Per IP, 10,000 cell equivalent and 2.5 µg of antibody were used. A negative control antibody was included in the ChIP assay (2.5 µg/IP). Results were obtained in the laboratory of Philippe Collas (adapted from Freberg, Dahl, Timoskainen and Collas, 2007; Dahl and Collas, 2007).

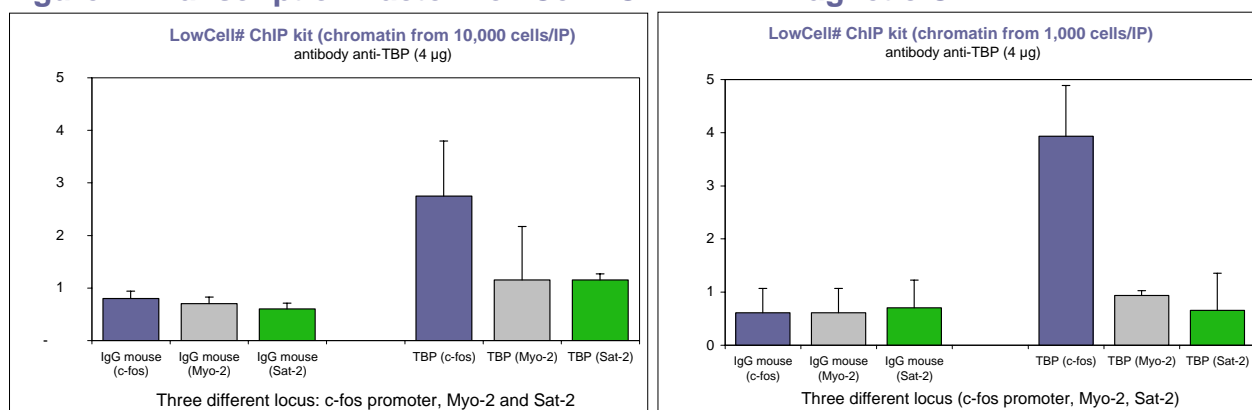
Figure 6: LowCell# Magnetic ChIP analysis of a series of different genomic regions



ChIP assays were performed using our LowCell# ChIP kit, NCCIT cells, the affinity purified antibody directed against H3K9me2 (cat.#: pAb-060-050) and optimized qPCR primer pairs. Sheared chromatin from 10,000 cells and 1 µg of antibody anti-H3K9me2 were used per ChIP experiment. IgG (1 µg/IP) is used as

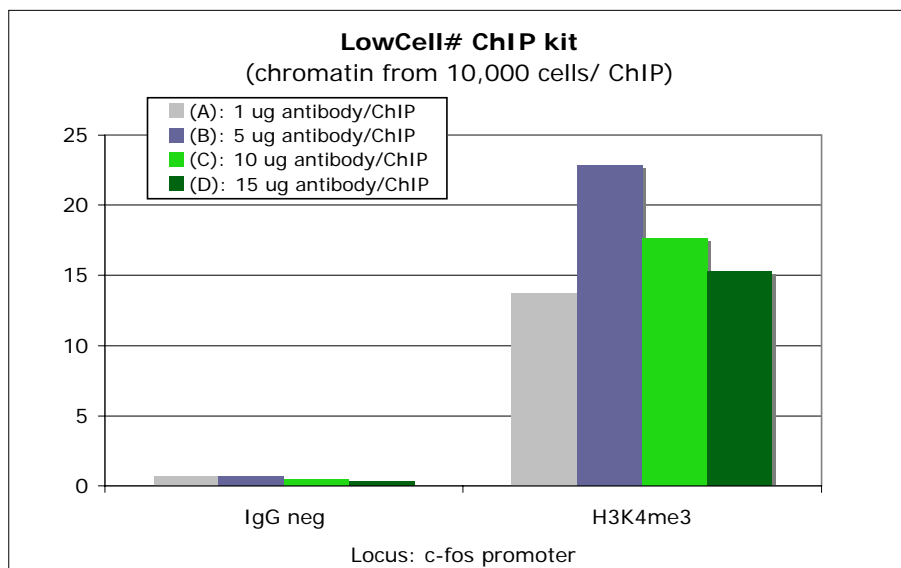
negative IP control. Methylation of H3K9 is related to silenced regions. Therefore, we used the promoter of a house-keeping gene: c-fos, which is under active transcription, as negative PCR control. Sat-2, present in heterochromatin and Myoglobin exon 2, which is inactive chromatin, are used as positive PCR controls. Recoveries (% of input) are shown here above. The % of recovery represents the relative amount of immunoprecipitated DNA compared to input DNA. The negative IP control gives low recoveries with all the primer pairs (bars 1, 3 and 5). Recoveries are high using the positive primer pairs: 'Sat-2' (bar 4) and 'Myoglobin exon 2' (bar 6), compared to the negative primer pair: 'c-fos promoter' (bar 2).

Figure 7: Transcription Factor LowCell# ChIP: TBP Magnetic ChIP



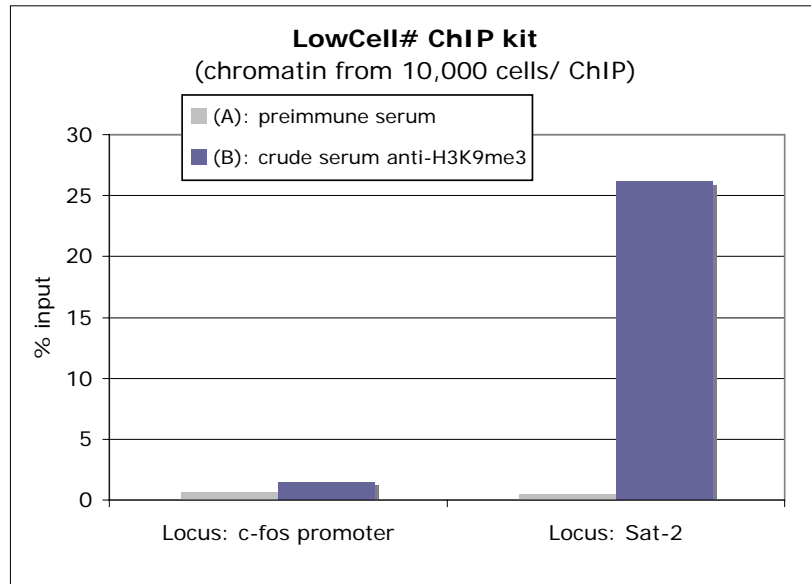
The ChIP assay were performed using our LowCell# ChIP kit, undifferentiated human teratocarcinoma (NCCIT) cells, the Diagenode antibody directed against the TATA box binding protein (TBP) (cat.#: MAb-002-100) and optimized qPCR primers to amplify from the IP'd DNA three different loci: a region of the c-fos promoter (cat.#: pp-1004-050, -500), Myoglobin exon 2 (cat.#: pp-1006-050, -500) and Sat-2 (cat.#: pp-1040-050, -500). Per ChIP: chromatin from 10,000 cells (left panel) or 1,000 cells (right panel) and 4 µg of antibody were used. A negative control antibody was included in the ChIP assay (4 µg negative IgG from mouse /IP). Each ChIP assay was performed in triplicate.

Figure 8: Example of LowCell# ChIP antibody titration



ChIP assays were performed using our LowCell# ChIP kit, undifferentiated teratocarcinoma (NCCIT) cells, the affinity purified antibody directed against H3K4me3 (cat.#: pAb-003-024) and the optimized PCR primer pair to amplify a region of the c-fos promoter (cat#: pp-1004-050, -500) by qPCR analysis from the IP'd DNA. Per ChIP, chromatin from 10,000 cells and different quantities of antibody anti-H3K4me3 were used (1 μ g, 5 μ g, 10 μ g and 15 μ g). Same quantity of negative control antibody (1 μ g, 5 μ g, 10 μ g and 15 μ g) was included in the ChIP assay (negative rabbit IgG).

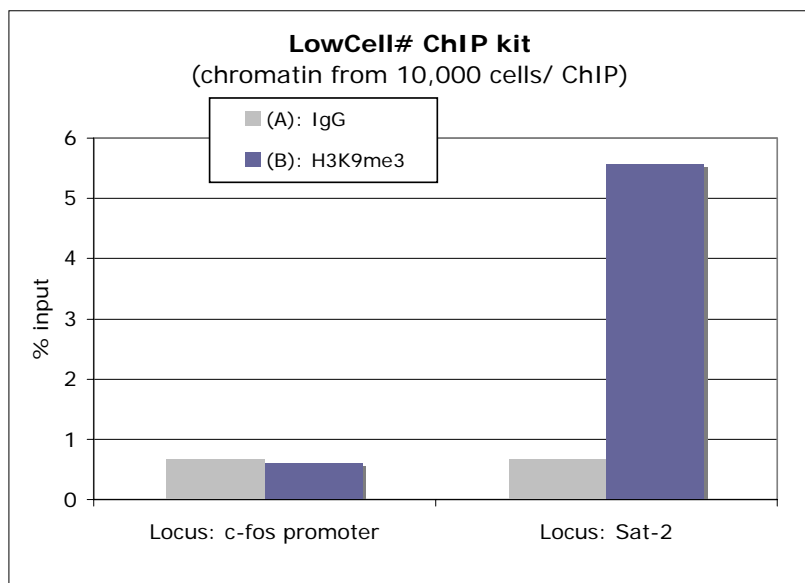
Figure 9: LowCell# ChIP kit using crude serum (and purified antibodies: next figure).



ChIP assays were performed using our LowCell# ChIP kit, undifferentiated human teratocarcinoma cells (NCCIT), the Diagenode crude serum directed against H3K9me3 (cat.#: CS-056-050) and optimized PCR primer pairs for qPCR. Sheared chromatin from 10,000 cells was used per ChIP experiment. Pre-immune serum and crude serum directed against H3K9me3 were used at dilution 1:5,000. The pre-immune serum is used as negative IP control. H3K9me3 is a marker for heterochromatin. Therefore, we used the promoter of a house keeping gene c-fos, which is under active

transcription, as negative PCR control. SAT 2, present in heterochromatin, is used as positive PCR control. The recovery (% of input) obtained with the antiserum anti-H3K9me3 is shown using primer pairs for the c-fos promoter as negative targeted PCR control and primer pairs for Sat-2 as positive PCR control (B). The recovery for the pre-immune serum is also shown using c-fos promoter and Sat-2 primer pairs, both being negative targets (A). The % of recovery represents the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analyses. Antibodies were affinity purified from the serum and tested in ChIP (see next figure).

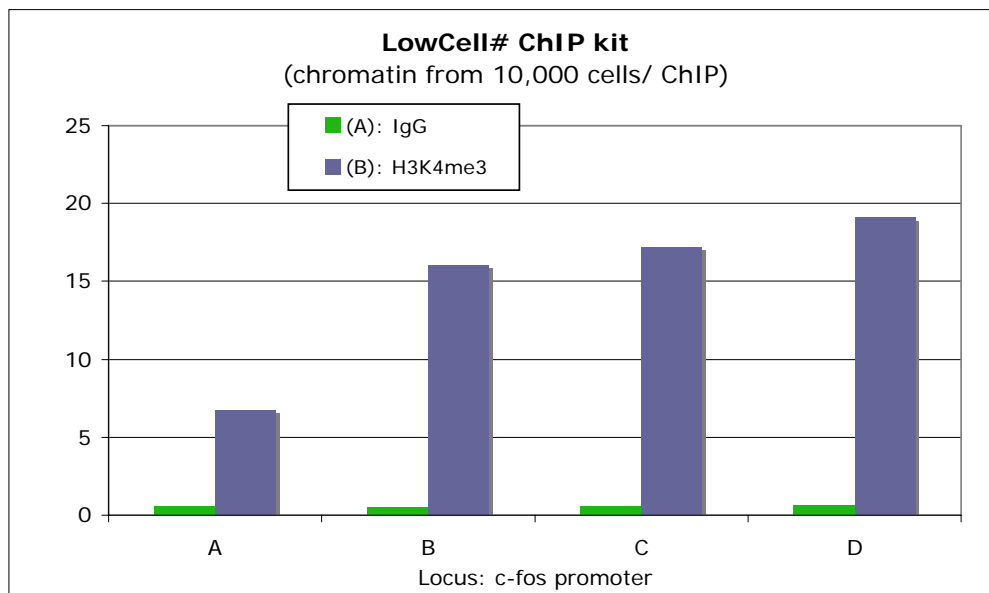
Figure 10: LowCell# ChIP kit results obtained with the Diagenode purified antibody directed against H3K9me3.



ChIP assays were performed using our LowCell# ChIP kit, NCCIT cells, the affinity purified antibody directed against H3K9me3 (cat# pAb-056-050) and same optimized qPCR primer sets as above. Sheared chromatin from 10,000 cells and 1 µg of antibody anti-H3K9me3 were used per ChIP experiment. IgG is used as negative IP control. Recoveries (% of input) obtained with the Diagenode antibody H3K9me3 are shown, analysing two loci: the c-fos promoter and Sat-2 (B). % of recovery of IgG are also shown (A). Similar results are obtained with both

crude and purified antibodies directed against H3K9me3 (compare figure 9 to 10).

Figure 11: LowCell# ChIP kit protocol performed in one day



ChIP assays were performed using our LowCell# ChIP kit, human cervical cancer cells (Hela cells), the affinity purified antibody directed against H3K4me3 (cat# pAb-MEHAHS-024) and the Diagenode primers to amplify a region of the c-fos promoter (cat#: pp-1004-050, -500). Sheared chromatin from 10,000 cells

and 1 µg of antibody anti-H3K4me3 were used per ChIP experiment. Negative IgG (1 µg/IP) is used as negative IP control. Chromatin and antibodies were incubated for 30 minutes in the ultrasonic water bath (as in OneDay ChIP kit) that was followed by another incubation with the paramagnetic beads: for 30 minutes (A), for 1 hour (B) and for 2 hours (C) (see “additional protocol” section of this manual). The LowCell# ChIP protocol as described in this manual was also used (D). In experiments B, C and D, results are comparable.

5. TROUBLESHOOTING GUIDE and NOTES

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the fixation step for the correct period of time, at the right temperature and with the correct formaldehyde concentration. e.g: incubate for 8 minutes at room temperature with 1 % formaldehyde final concentration (weight/ volume). Also, use high quality, fresh formaldehyde.
	Cross-linking is too strong.	
	Proteins have unique ways of interacting with the DNA. Some proteins are not directly bound to the DNA but interact with other DNA-associated proteins.	Very short or very long cross-linking time can lead to DNA loss and/or elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity and efficiency of ChIP.
	Both cross-linking time and formaldehyde concentration are critical.	Cross-linking can affect both efficiency of chromatin shearing and efficiency of specific antigen immunoprecipitation. Shorter cross-linking times (5 to 10 minutes) and/or lower formaldehyde concentrations (1%, weight/ volume) may improve shearing efficiency while, for some proteins especially those that do not directly bind DNA, this might reduce the efficiency of cross-linking and thus the yield of precipitated chromatin.
	The optimal duration of cross-linking varies between cell type and protein of interest.	It is possible to optimize the fixation step by testing different incubation times: such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.
	Efficient fixation of a protein to chromatin in vivo is a crucial step for ChIP. The extent of cross-linking is probably the most important parameter.	Two major problems concerning the subsequent immunoprecipitation step should be taken into account: 1/ an excess of cross-linking can result in the loss of material or reduced antigen availability in chromatin, or both. 2/ the relative sensitivity of the antigen epitopes to formaldehyde. It is essential to perform the cross-linking step with care.
	It is essential to quench the formadehyde.	Use glycine to stop the fixation: quench formaldehyde with 125 mM glycine for 5 minutes at room temperature (add 57 µl of 1.25M glycine per 513.5 µl of sample, see STEP 2). Alternatively, wash the fixed cells properly and make sure you get rid of ALL the formaldehyde.
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol (e.g.: 1 million cells or less/ 130 µl of complete Buffer B, see STEPS 2 and 3).
	Temperature is critical.	Perform cell lysis at 4°C (cold room) or on ice. Keep the samples ice-cold at all times during the cell lysis and use ice-cold buffers see STEP 3.
	Protein degradation during lysis can occur.	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Kit protocol validation	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells) and Kerationocytes have been used to validate the Magnetic ChIP protocol.
Cell number necessary per ChIP	The amount of cells required for a ChIP experiment is determined by cell type, protein of interest and antibodies used.	We recommend the use of chromatin from 1,000 to 10,000 cells per ChIP. For some cases, chromatin from up to 100,000 cells could be used per ChIP. Scale accordingly. One can also use other cell numbers than the ones exemplified in the protocol. (e.g. 50,000 cell equivalent of chromatin from a starting sample of 1 million cells).

Chromatin shearing	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.
	To get good shearing is critical.	You might want to optimize shearing conditions for your specific cell type and fixation protocol. Therefore, 1) to start with a small sample (1x 10e6 cells or less) and 2) check the shearing efficiency is advised. Also: 3) Keep samples cold. 4) Check the buffer composition, if using your own (e.g. If the shearing buffer contains 0.75% SDS, the sheared chromatin is diluted 3.5 to 4.0 fold in the complete BufferA prior to immunoselection. The final SDS concentration should not be higher than 0.15 to 0.20%).
	Shear the samples of chromatin using the Bioruptor™ from Diagenode (catalog # UCD-200).	Maintain temperature of the samples close to 0°C. Samples are sonicated for 10-12 cycles of: [30 seconds “ON” / 30 seconds “OFF”] each. Total time is of 10-12 minutes (these conditions were tested with many mammalian cell lines and were excellent for subsequent ChIP experiments). A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.
	Chromatin shearing with a probe sonicator – protocol.	Probe sonicator: Sonicate each sample for 3 x 30 seconds on ice. Allow a 30 seconds pause on ice between each pulsing session. Take care to avoid foaming.
Chromatin shearing buffers	Chromatin sheared with our modules	Note that it is also possible to use sheared chromatin obtained with our Shearing module from the Red ChIP kit (catalog # kch-redmod-100 or -400). When using sheared chromatin obtained from the shearing module (catalog #: kch-redmod-): 30 µl of shearing buffer is added per million cells. After shearing, transfer to a new tube the amount of chromatin needed (corresponding to a determined cell equivalent), and add some Buffer B from the LowCell# ChIP kit to reach a total volume of 130 µl as shown below: I. Add 30 µl of sheared chromatin to 100 µl of Buffer B (final : 1,000,000 cells / 130 µl). II. Add 3 µl of sheared chromatin to 127 µl of Buffer B (final : 100,000 cells / 130 µl)
	Shearing with “in house” protocols	Note that it is also possible to use sheared chromatin following your protocol (see “additional protocols” section). When using your “in house” protocol: check the composition of the shearing buffer that you use: as it should contain between 0.75% and 1% SDS, EDTA (1-10 mM) and/or EGTA (0-0.5 mM) and should be at pH 7.6 - 8.0. The sheared chromatin is to be diluted in the Buffer A and Buffer B prior to immunoselection: I. Add Buffer B to sheared chromatin (final: 1,000,000 cells / 130 µl). II. Add Buffer B to sheared chromatin (final: 100,000 cells / 130 µl).
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 equivalent to 100,000 cells, one million cells or more can for sure be visualized on a gel.
	Do not load too much DNA on a gel.	Loading of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation. The DNA amount to load depends on the size of the well and on the gel size.
	Agarose concentration.	Do not use more than 1-1.5% agarose gel and run slowly (Volt/cm and time depend on the gel size).

	Running buffer concentration.	1x TAE or TBE is preferred to 0.5x TAE, which can lead to smears on agarose gel.
Sheared chromatin amounts	How much sheared chromatin do I need to prepare?	Most of the sheared chromatin is to be used in the ChIP experiment, but remember that some of the sheared chromatin is needed as control as it corresponds to the input sample for the ChIP experiment and it can also be checked on agarose gel.
	Dilute the sheared chromatin in ChIP buffer for Immunoselection incubation.	The sheared chromatin is diluted in complete buffer A prior to the immunoselection incubation (see STEP 3: Add 870 µl complete Buffer A to the 130 µl of sheared chromatin). Dilute the sheared chromatin at least 7 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	Beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead centrifugation	Don't spin the beads at high speed. Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. $g = 11.18 \times r \times (\text{rpm}/1000)^2$; knowing that r is the radius of rotation in mm. (http://www.msu.edu/~venkata1/gforce.htm). It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Antibody binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAbs from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C, and thawed before use.
	Complete buffers	Add P.I. mix to buffers, just before use, in PBS (STEPS 2 and 3), Buffer B (STEP 3), in Buffer A (STEP 3). Prepare what is going to be used in the day's experiment.
Other enzyme inhibitors	Specific enzyme inhibitors are not included in the kit, such as phosphatase inhibitors.	Add phosphatase inhibitors or others to Buffers A and B, if necessary, depending on your research field and protein(s) of interest to be ChIP'd Add NaBu for histone ChIPs.
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 could also be used as a negative ChIP control as well as non-immune IgG. At STEP 4, the IP incubation mix includes sheared chromatin and beads but no antibody.
	Use antibody and specifically blocked antibody in parallel	Use one antibody in ChIP and, and the same antibody that is blocked with specific peptide. To specifically blocked one antibody: pre-incubate the antibody with saturating amounts of its epitope specific peptide for about 30 minutes at room temperature before use in the IP incubation mix. Use in ChIP, the blocked antibody as a negative control in parallel with the unblocked antibody.
	How many negative controls are necessary?	If multiple antibodies - of the same specie - are to be used with the same chromatin preparation then a single negative ChIP control is sufficient for all of the antibodies used.
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. 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.
	What is the amount of antibody per ChIP to use?	It should be determined empirically for each target and antibody. For abundant proteins, like histones, use 1 to 2 µg of affinity purified or monoclonal antibody per IP. For other targets, use up to 10 µg per ChIP. To ensure efficient IP it is important to have an optimal ratio between the amount of chromatin and the 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). Insufficient amount 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 immunoglobulin to protein A. For example, IgM or IgY can require a secondary antibody as a bridge to protein A.
Immuno-selection incubation	What is the best incubation time for immunoselection using the ultrasonic water bath?	To incubate the sheared chromatin with antibodies for 15 to 30 minutes works for many antibodies, however, the kinetics for reaching equilibrium of epitope-antibody binding may differ for each antibody and target. Optimization might improve the results (e.g. the incubation time may need to be increased for some antibodies).
	How does the immunoselection using the ultrasonic water bath work?	The rate-limiting step in many immunoassays is associated with the slow kinetics of binding of macro-molecular antigen to antibody. It was demonstrated that the use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to antibodies.
	Where can I buy the ultrasonic bath?	http://www.bransoninc.com/model_3510.asp : Branson cat# CPN-952-316. Fisher Scientific cat#15-337-22F.
	What are the water bath specifications?	Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W
	Can I use the kit w/o an ultrasonic water bath?	Yes, then a long incubation at 4°C should be used. Depending on the antibody and target to be ChIP'd, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.
	Polymerase Chain Reaction	Primer design
Controls: negative and positive		Negative PCR controls: PCR with DNA from samples IP'd with non-immune antibodies (negative IgG). Alternatively, PCR using DNA from ChIP samples and primers specific for a DNA region to which, your antigen of interest is not binding. Positive PCR control: PCR using input DNA.
No PCR signal		Include a positive PCR control as a control for your PCR mix (your primers, dNTP and Master Mix) using the Input DNA or a DNA sample of the same origin.
High Ct values		Use more input chromatin.
Ct^{NegCt} and Ct^{target}		The ratio between target IP and negative control IP depends on the antibody used.
Background is high		Verify that you perform properly the following steps: Keep the antibody binding beads and DNA purifying slurry in suspension while adding to tubes. Check by eye that equal pellets of beads and slurry are present in each tube. Washes (step 5) are critical.
Using end-point PCR rather than quantitative PCR		If gel electrophoresis is used to estimate intensities of PCR products, then relative occupancy of a factor at a locus is the ratio of the intensity of the target IP band to the negative control IP band.

Freezing	Samples can be frozen at several steps of the protocol.	- Pellets of formaldehyde fixed cells can be stored at - 80°C for at least a year. - Sheared chromatin can be stored at - 80°C for months, depending on the protein of interest to be CHIP'd. - Purified DNA from ChIP and input samples can be stored at - 20°C for months.
	Avoid multiple freeze/thawing	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

6. ADDITIONAL PROTOCOLS

Analysis Step: Sheared chromatin analysis

1. Add 370 μ l **Buffer A** to 130 μ l of sheared chromatin to have 1 million cells (I.) or 100,000 cells (II.) in 500 μ l.
2. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1).
It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation.
3. 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.
4. Add 1 volume of chloroform/isoamyl alcohol (24:1). It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation. Use gentle rotation.
5. 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.
6. Precipitate the DNA by adding co-precipitant (cat # kch-216-100), precipitant (cat # kch-217-001) and cold 100% ethanol to the sample. Incubate at -80°C for 30 minutes.
7. Centrifuge for 25 minutes at 14,000 x g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 500 μ l of ice-cold 70 % ethanol to the pellet.
8. 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.
9. Resuspend the pellet in 10 μ l or 20 μ l of TE. That corresponds to the purified DNA from the sheared chromatin. (Make sure that any DNA smear on the tube wall, this may sometimes occur, is also resolved).
10. Run samples (10 μ l of DNA + 2 μ l of 6x loading dye) in a 1.5% agarose gel along with DNA size marker to visualise shearing efficiency.

The use of ultrasonic bath to further reduce the incubation time:

1. Use 100 µl of diluted sheared chromatin per IP (STEP 3, *Point 24.*). Transfer 100 µl to each 0.2 ml IP tube. Keep 100 µl as Input sample, store at 4°C.
 - ❖ I. 100 µl of sheared chromatin were obtained from 100,000 cells
 - ❖ or II. 100 µl of sheared chromatin were obtained from 10,000 cells
 - ❖ or III. 100 µl of sheared chromatin were obtained from 1,000 cells

2. Add the specific antibody, control antibodies (positive and negative) and close the tube caps. Vortex, for 5 seconds, at medium power.
 - ❖ Add 1 to 3 µg of antibody or more (up to 10 µg per reaction), depending on the antibody used.
 - ❖ See troubleshooting guide: for binding capacities of Protein A (and G).

3. Briefly spin the 0.2 ml tubes containing the antibodies to bring down liquid caught in the lid.

4. Incubate for 30 minutes in the ultrasonic water bath. (4°C) (“TsG”)
 - ❖ Water bath description is in the “Kit Materials” section and troubleshooting guide.
 - ❖ You might want to optimize the incubation time (see troubleshooting guide).

5. Briefly spin the 0.2 ml tubes to bring down liquid caught in the lid and add 10 µl of pre-washed Protein A-bead per IP tube (STEP 1, *Point 2.*).

6. Incubate the IP tubes at 40 rpm on a rotating wheel for 1-2 hours at 4°C.

7. Briefly spin the 0.2 ml tubes containing the antibody coated beads to bring down liquid caught in the lid.

8. Place tubes in an ice-cold Magnetic Rack (cooled by placing it on ice), wait for 1 minute.

9. Discard the supernatant. Keep the pellet of antibody coated beads.

10. Proceed to the STEP 5, *Point 32. and 33.* (washes) before DNA purification (STEP 6).

DNA standard curve

Run samples of known DNA concentrations to get a standard curve. This must be done each time you run a qPCR plate. This is done by making up to eight serial dilutions of one unmanipulated cDNA sample (Make these fresh each time). This calibration is essential to allowing you to quantify the relative amount of DNA, this step instructs the machine as to what a dilution looks like and it will compare your experimental samples to these control samples.

- Prepare DNA from the same species or the same cell type, if possible, as the cell type used in ChIP.
- Prepare chromatin from the cells in the same manner as for ChIP.

In short:

- Cross-linking
- Lyse
- Sonicate to get fragments of 500 bp average length. Visualize by agarose gel analysis.
- Centrifuge at high speed to get rid of cellular debris.
- Take supernatant for cross-link reversal and Proteinase K digestion.
- Add elution buffer (20 mM Tris-HCl, pH 7.5, 5 mM EDTA, 20 mM sodium butyrate, 50 mM NaCl) containing 1% SDS, and 50 µg/ml proteinase K
- Incubate samples for 2 hours at 68°C, rotating at 1,300 rpm, in a thermomixer.
- Add elution buffer to make a total of 500 µl.

Purify DNA:

- Perform two Phenol/Chloroform/ isoamyl alcohol extractions followed by one Chloroform/ isoamyl alcohol extraction.
- Ethanol precipitate (add salt and ethanol 100%), incubate at -80 °C and wash.
- Resuspend the air-dried DNA pellet in TE pH 8.0 in e.g.: volume of 100 µl. (Volume is depending on the number of cells. If the solution gets "sticky", then increase the volume).
- To make sure all DNA is dissolved before concentration measurements, dissolve at room temperature for about 2 hours with repeated vortexing followed by centrifuging, and thereafter leave it at 4°C overnight.
- Measure DNA concentration by use of a spectrophotometer, A260, (and dilute to make sure the A260 is between 0.1 and 1 that is the linear range for a regular spectrophotometer).
- Calculate DNA concentration and make a dilution series to cover the area of concentration of your ChIP samples. Make 7 different concentrations with a broad range.

An example is given below. Using 100,000 NCCIT cells to start with, in 50 µl of TE, we got 3.02 ng of DNA /µl (or 15.1 ng/ 5 µl). The DNA sample was then diluted as shown below and 5 µl were used per qPCR well:

- Standard 1 : dilution 1/2 → 7.55 ng/ 5 µl
- Standard 2 : dilution 1/5 → 3.02 ng/ 5 µl
- Standard 3 : dilution 1/10 → 1.51 ng/ 5 µl
- Standard 4 : dilution 1/50 → 0.302 ng/ 5 µl
- Standard 5 : dilution 1/200 → 0.0755 ng/ 5 µl
- Standard 6 : dilution 1/500 → 0.0302 ng/ 5 µl
- Standard 7 : dilution 1/1000 → 0.0151 ng/ 5 µl
- Standard 8 : 0 ng

7. REFERENCES

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8. ORDERING INFORMATION

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Diagenode website: <http://www.diagenode.com/>

9. DIAGENODE RELATED PRODUCTS

Chromatin and DNA shearing

❖ Bioruptor™:

catalog #: UCD-200

Advantages of the Bioruptor - over the other sonicator apparatus -:

- With the Bioruptor the quality of the sheared chromatin (and DNA) is greater: a compact pack of chromatin (and DNA) fragments of the right size is obtained rather than a smear. In the chromatin (and DNA) sheared using the Bioruptor, the amount of different fragment sizes is reduced and the presence of too long fragments is also eliminated, these being a source of background in the subsequent ChIP (and Methyl DNA IP) experiments. Thus, the high quality of the sheared chromatin (and DNA) obtained with the Bioruptor is essential to get better results in ChIP (and Methyl DNA IP), by decreasing dramatically the background noise. The way to test the shearing efficiency is to look at the extent of DNA fragmentation using agarose gel electrophoresis.
- With chromatin (and DNA) sheared with the Bioruptor, the reproducibility is close to 100% (other sonicator apparatus gives good shearing in no more than 1 out of 5 trials)
- Note that the Bioruptor can also be used for many other applications.

❖ ChIP Sonication Ctl kit™:

catalog #: kch-sonctl-001

❖ Shearing-ChIP kit™:

catalog #: kch-shchro-040

❖ Shearing module:

catalog #: kch-redmod-100

❖ Large Shearing module:

catalog #: kch-redmod-400

LowCell# ChIP kit: *The Low Cell Number Magnetic Chromatin IP*
Diagenode manual

Chromatin IP kits

- ❖ **Red ChIP kit™ (TF-ChIP):** catalog #: kch-redTBP-012
- ❖ **Orange ChIP kit™ (Histone ChIP):** catalog #: kch-orgHIS-012
- ❖ **OneDay ChIP kit™ (Rapid ChIP):** cat #: kch-oneDIP-060,-180
- ❖ **LowCell# ChIP kit™ (Magnetic ChIP):** catalog #: kch-magfew-016

ChIP grade Reagents, separately available

- ❖ Protease Inhibitor Mix (20 µl): catalog #: kch-107-020
- ❖ Protease Inhibitor Mix (100 µl): catalog #: kch-502-100
- ❖ Protease Inhibitor Mix (300 µl): catalog #: kch-502-300
- ❖ Protease Inhibitor Mix (1 tablet): catalog #: kch-207-001
- ❖ Protease Inhibitor Mix (1 tablet): catalog #: kch-405-001
- ❖ Protease Inhibitor Mix (1 large tablet): catalog #: kch-306-001
- ❖ DNA co-precipitant (100 µl): catalog #: kch-216-100
- ❖ DNA precipitant (1000 µl): catalog #: kch-217-001
- ❖ Proteinase K (100 µl): catalog #: kch-507-100
- ❖ Proteinase K (250 µl): catalog #: kch-507-250
- ❖ Sterile Water (28 ml): catalog #: kch-508-028
- ❖ Sterile Water (85 ml): catalog #: kch-508-085
- ❖ GenDNA RNase (10 µl): catalog #: mcg-605-010
- ❖ 1 M Sodium butyrate (1 ml): catalog #: kch-817-001
- ❖ ProtG-coated paramagnetic beads: catalog #: kch-818-220
- ❖ Negative Ctrl IgG from mouse: catalog #: kch-819-015
- ❖ PBS (50 ml) : catalog #: kch-820-050

- ❖ **Blocked PA/G beads (630 µl) :** catalog #: kch-208-630
- ❖ **pre-Blocked PA beads (880 µl) :** catalog #: kch-406-880
- ❖ **unblocked Protein A beads (8 ml):** catalog #: kch-503-008
- ❖ **unblocked Protein A beads (2.8 ml):** catalog #: kch-503-028
- ❖ **unblocked Protein A beads (880 µl):** catalog #: kch-503-880

- ❖ Magnetic Rack (16 rxns): catalog #: kch-816-001

Some of our ChIP grade antibody directed against:

- TBP: catalog #: MAb-002-100
- Histone H3 [K4me3]: catalog #: pAb-003-024
- Histone H3 [K9ac]: catalog #: pAb-004-044
- Histone H3 [K9/14ac]: catalog #: pAb-005-044
- Histone H3 [K9me3]: catalog #: pAb-056-050
- Histone H3 [K9me3]: catalog #: CS-056-050
- Histone H4 [K20me3]: catalog #: pAb-057-050
- NF-YB: catalog #: pAb-007-100
- p63: catalog #: pAb-008-050
- ERalpha: catalog #: MAb-009-050
- GR : catalog #: MAb-010-050
- Ty1-tag : catalog #: AC-055-100
- HDAC1 : catalog #: pAb-053-050
- Pol II (RNA polymerase II): catalog #: MAb-054-050
- Plus many other antibodies: visit our website: <http://www.diagenode.com/>

Peptides

visit our website: <http://www.diagenode.com/>

Primer pairs

visit our website: <http://www.diagenode.com/>

Methylated DNA IP

- ❖ **Complete METHYL kit™:** catalog #: mc-green-003
- ❖ **Methyl DNA IP module:** catalog #: mc-green-001
- ❖ **GenDNA and qPCR modules:** catalog #: mc-green-002
- ❖ **meDNA IP blocked ProtA/G beads (300 µl):** catalog #: mcg-606-300

Methyl DNA IP grade antibody directed against:

- 5-methyl Cytidine: catalog #: MAb-5MECYT-100,-200,-500

DNA Methylation Detection kits™

- 25 rxns: catalog #: MEA-BISLPH-025
- 96 wells / vacuum: catalog #: MEA-BISWEV-096v
- 96 wells / centrifugation: catalog #: MEA-BISWEC-096c

Methyl Kit for methylated DNA immunoprecipitation (Methyl DNA IP)

Include in your IP the provided controls to directly estimate the methylation status of your DNA sample.

Kit designed to perform specific DNA methylation analysis and internal IP quality control.

- ❖ GenDNA module included for the preparation of DNA.
- ❖ Optimized protocol for DNA shearing.
- ❖ Brand new method: methylated DNA and unmethylated DNA internal IP controls are provided to be used together with your DNA sample - "All in one Tube" - allowing direct correlation between IP'd material and methylation status.
- ❖ One format: 10 IPs per kit.

RED ChIP Kit: The Transcription Factor-ChIP

Work with our straightforward and optimized ChIP method.

Kit designed to perform ChIP assays on transcription factors.

- ❖ Traditional method: 3 days from cell collection to PCR results (2 O.N. incubations).
- ❖ Includes a chromatin shearing module and a qPCR module.
- ❖ TBP: Control antibody.
- ❖ One Format: 18 IPs per kit.

ORANGE ChIP Kit: The Histone-ChIP

Work with our straightforward and optimized ChIP method.

Kit designed to perform ChIP assays on Histones and Histone Modifications.

- ❖ Simplified protocol.
- ❖ Traditional method: 3 days from cell collection to PCR results (2 O.N. incubations).
- ❖ Includes a chromatin shearing module and a qPCR module.
- ❖ Histone H3K4me3: Control antibody.
- ❖ One format: 18 IPs per kit.

OneDay ChIP Kit: The Rapid ChIP

Analyse many samples in a short period of time.

Kit designed to perform ChIP assays in abundance.

- ❖ ChIP in 2 hours.
- ❖ Recover the DNA ready-to-PCR in 2 hours.
- ❖ New method: one day from cell collection to PCR results.
- ❖ Two formats: 60 IPs or 180 IPs per kit.

LowCell# ChIP kit: The Magnetic ChIP

Work with a low number of cells per ChIP.

Kit designed to perform ChIP assays using magnetic IP and small volumes of reagents.

- ❖ Validated with low number of cells for histone- and TF-ChIP assays.
- ❖ Use chromatin from 10,000 cells or less to perform histone ChIPs.
- ❖ Ideal to test one sample with 6 to 14 antibody to modified histones or transcription factors.
- ❖ Magnetic rack available at Diagenode. Ensures the best ChIP conditions by providing cooling at all times to avoid degradation.
- ❖ Includes buffers for chromatin shearing and qPCR primer pairs.
- ❖ One Format: 16 IPs per kit.

Traditional ChIP versus LowCell# Magnetic ChIP

ChIP STEPS	THREE DAYS traditional ChIP	DIAGENODE Magnetic ChIP
Protocol steps	Numerous steps	Less steps and shorter steps
Reagents	Many buffers involved	Fewer buffers and less reagent is used per reaction
Antibody binding bead	Pre-block sepharose beads Pre-clear chromatin samples	Binding antibody to Magnetic Beads
Cell collection and DNA-protein cross-linking	Collect minimum 10 million cells Need: 1 to 2 million cells per IP	Collect as little as 1 million cells (or less) Work with 10,000 to 1,000 cells per IP
Cell lysis & chromatin shearing	Sonication apparatus other than Bioruptor, tedious shearing optimization with in house buffers and not standardized protocol.	Use the Bioruptor and QC'd reagents one million cells in 130 µl Buffer B or 100,000 cells in 130 µl Buffer B
Antibody incubation with chromatin	Incubate the chromatin and antibody first (4 hours to overnight) Add beads together with the chromatin and antibody or add beads subsequently and incubate again (for 1 to 3 hours more)	Magnetic Immunoprecipitation Add 870 µl Buffer A and antibody coated-beads to chromatin from 10,000 or 1,000 cells (per IP) Incubate (chromatin & antibodies coated-beads) for 2 hours to 16 hours or shorter (see Time table).
Washes of immune complexes	Wash by adding buffer, mixing, incubating and centrifuging using 3 to 4 different buffers performing 6 to 8 washes in total	Use one buffer, wash 3 times
DNA purification	Four long steps: 1/ elute chromatin from beads (30 minutes) 2/ cross-link reversal (4 hours, at 65°C) 3/ proteinase K digestion (1 to 2 hours, 37°C-42°C) 4/ phenol/chloroform extraction or column (1 hour to overnight)	One short step: DNA slurry (10 minutes)
PCR	End-point PCR	Quantitative PCR, ChIP-chip, ChIP-seq
Data analysis	No quantification	quantification (Raw data & formula in the manual)

10. LowCell# ChIP kit: catalog # of each component

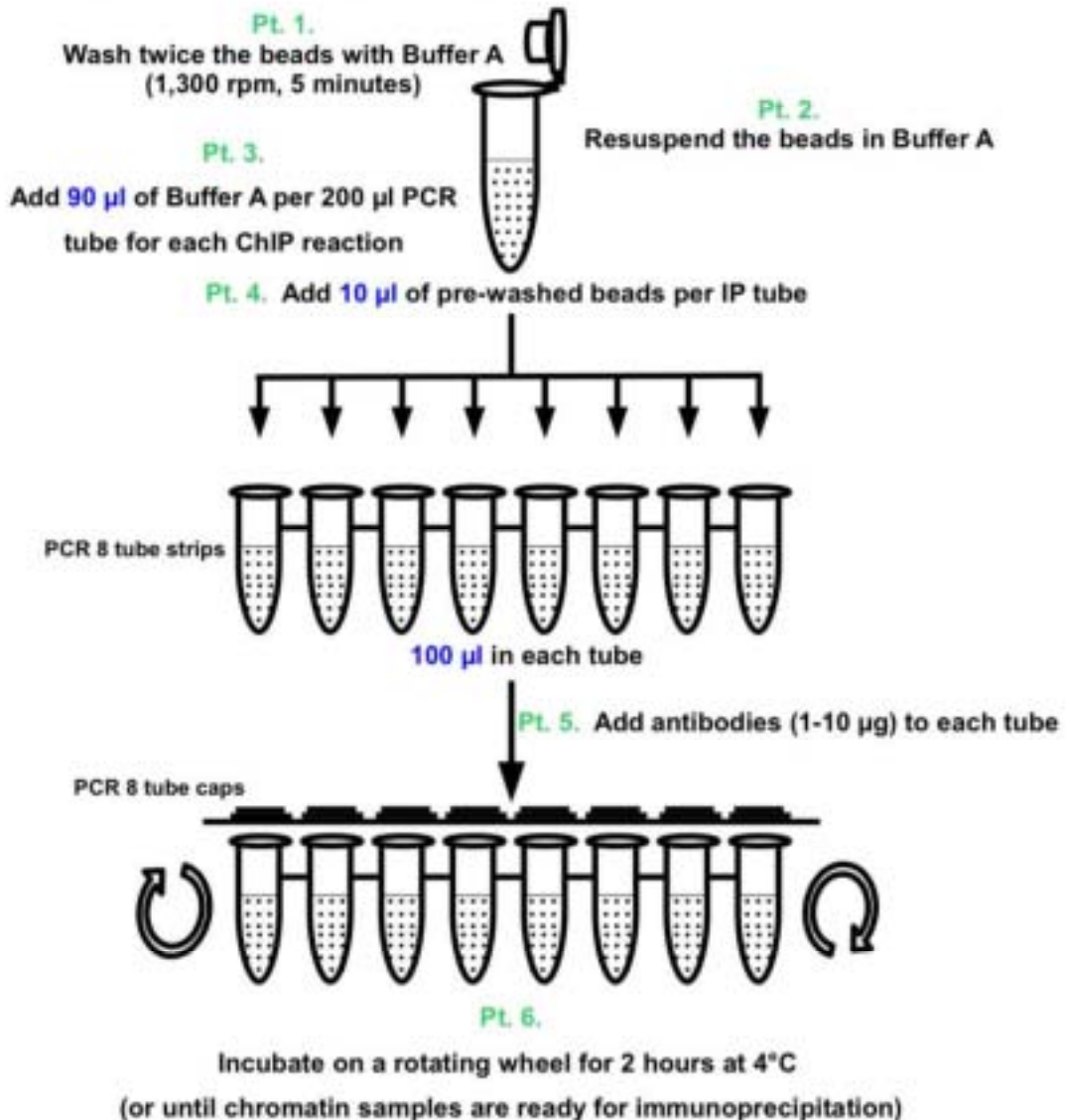
LowCell# ChIP KIT: Magnetic ChIP cat# kch-maglow-016			
kit component: name on the label	cat #	quantity	storage
Buffer A	kch-801-025	25 ml	4°C
Protein A-coated paramagnetic beads	kch-802-220	220 µl	4°C
Negative Ctrl IgG from rabbit	kch-803-015	15 µl	4°C
1.25 M Glycine	kch-804-002	2 ml	4°C
Buffer B	kch-805-003	3 ml	4°C/RT
Protease Inhibitor mix (P.I. 200x)	kch-806-100	100 µl	-20°C
Buffer C	kch-807-004	4 ml	4°C
DNA purifying slurry	kch-808-003	3 ml	4°C
Proteinase K	kch-809-030	30 µl	-20°C
PCR-grade H2O	kch-810-004	4 ml	4°C
SAT 2 primer pairs	kch-811-050	50 µl	-20°C
c-fos promoter primer pair	kch-812-050	50 µl	-20°C
Myoglobin exon 2 primer pair	kch-813-050	50 µl	-20°C
PCR tube strips	kch-814-004	4	RT
PCR strip caps	kch-815-004	4	RT

AVAILABLE SEPARATELY			
Magnetic rack (16 rxns)	kch-816-001	1	RT
1 M Sodium butyrate	kch-817-001	1 ml	-20°C
Protein G-coated paramagnetic beads	kch-818-220	220 µl	4°C
Negative Ctrl IgG from mouse	kch-819-015	15 µl	4°C
PBS	kch-820-050	50 ml	-20°C
Antibodies	www.diagenode.com		
Primer Pairs	www.diagenode.com		

11. LowCell# ChIP kit: QUICK CHARTS

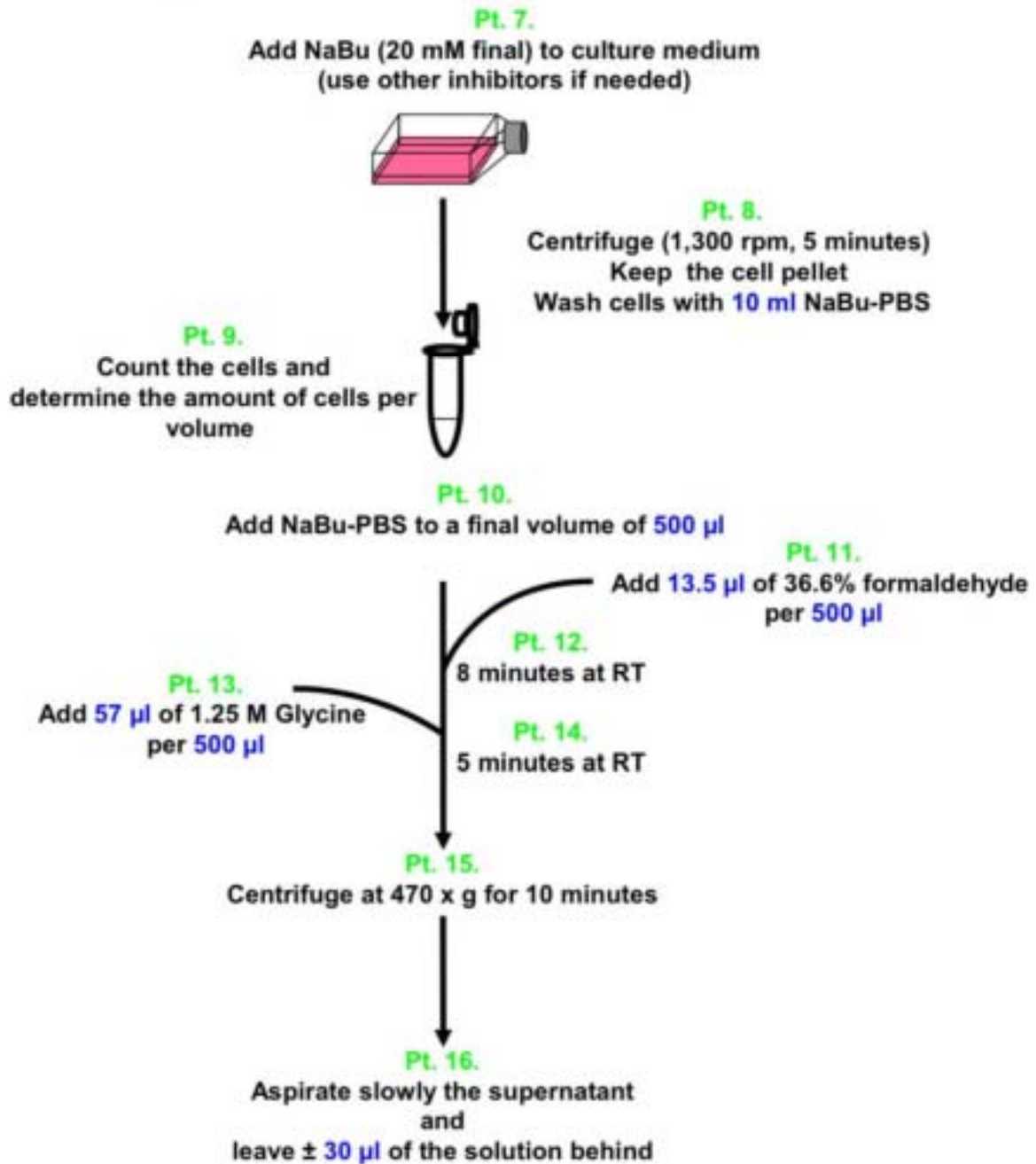
First the beads are incubated with the antibody of interest (STEP 1). Meanwhile, harvest the cells (STEP 2) and then shear the chromatin (STEP 3). The chromatin shearing efficiency can be double-checked (see analysis step). Next, the IP can take place: the sheared chromatin is incubated with the antibody-coated beads (STEP 4). Following IP, the beads are washed (STEP 5). The IP'd DNA is then purified (STEP 6) and analysed by qPCR (STEP 7).

STEP 1. Preparation of antibody-bead complexes



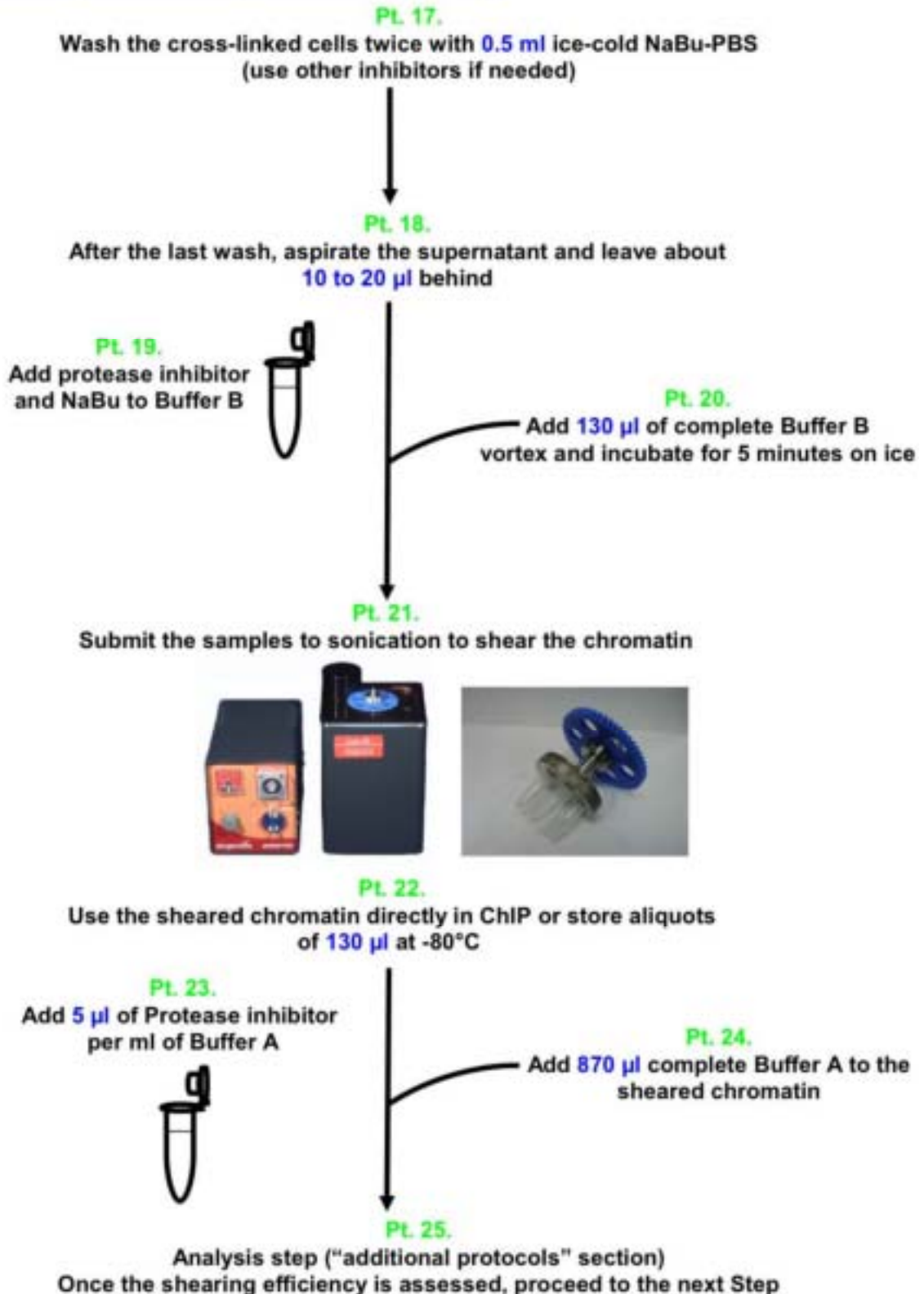
STEP 1 is shown above. Beads are first washed before they are added to individual IP tubes and incubated with the antibody of choice. Include the negative IP tube and a positive IP tube next to your IPs of interest. We highly recommend the use of antibodies that have been validated in ChIP. **!!:** When using a new antibody, it is worth titrating the antibody (e.g.: from 1 to 10 µg) to determine the best amount to use in ChIP (see trouble shooting guide and results section).

STEP 2. Cell collection and DNA-protein cross-linking



STEP 2 consists of harvesting and fixing the cells in order to prepare sheared chromatin (STEP 3) and use it subsequently in Chromatin IP experiments (STEP 4). **!!:** Note that sodium butyrate (20 mM final concentration) is added to all solutions thereafter unless otherwise stated. Use other inhibitors if needed.

STEP 3. Cell lysis and Bioruptor chromatin shearing

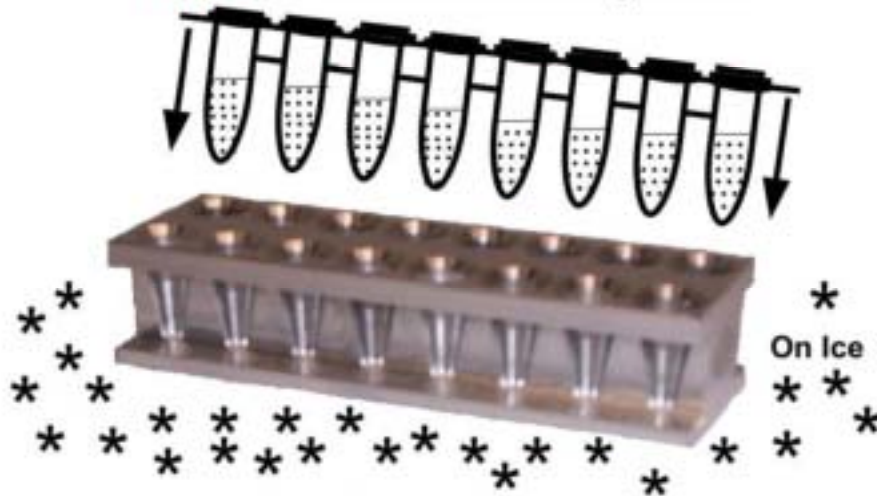


STEP 3 is shown above. This section describes cell lysis and Bioruptor chromatin shearing. At this stage, it is essential to produce fragments of size suitable for ChIP and subsequent PCR analysis of the immunoprecipitated DNA (200 - 1,000 base pairs). **!!**: Place the **Buffer B** at room temperature (RT) before use. **!!**: Work on ice at all following steps unless otherwise stated.

STEPS 4 and 5. Magnetic Immunoprecipitation and washes

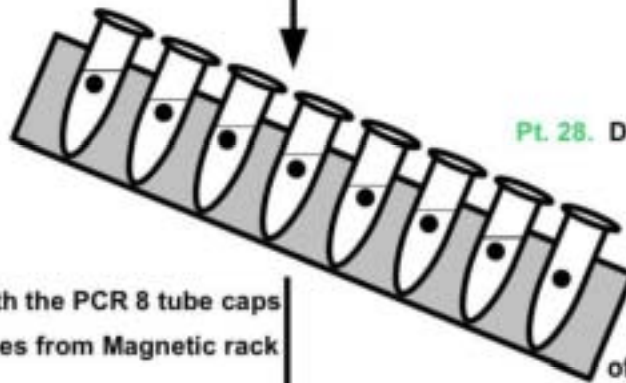
Pt. 26. Briefly spin PCR tubes containing the antibody-coated beads

Pt. 27. Place tubes in the ice-cold Magnetic rack



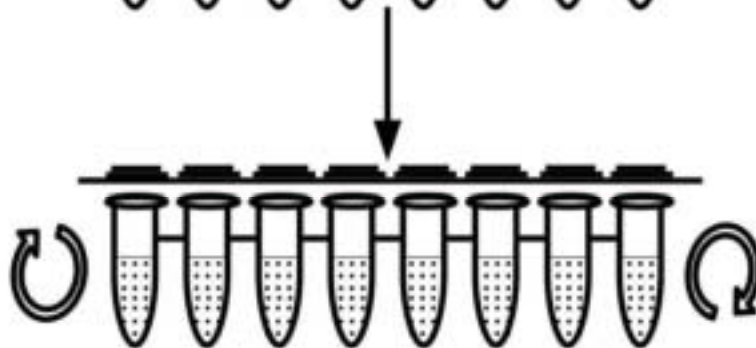
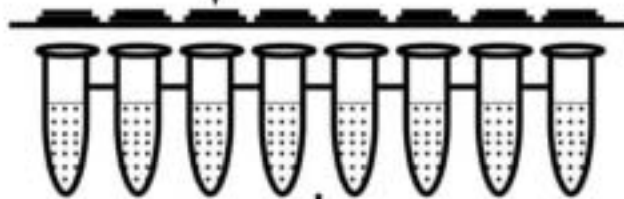
Wait for 1 minute

Pt. 28. Discard the supernatant



Pt. 30. Close with the PCR 8 tube caps and remove tubes from Magnetic rack

Pt. 29. Add 100 μ l of diluted sheared chromatin



Pt. 31. Incubate under constant rotation on a rotating wheel for 2 hours up to overnight at 4°C

STEPS 4 and 5: handling the beads in IP and at washing steps is simplified as shown above.