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HighCell# ChIP kit

The High Cell Number Chromatin Immunoprecipitation kit

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USER GUIDE

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Please read this manual carefully before starting your experiment

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 to specific genomic sites. The main steps of the ChIP assay are cell fixation (cross-linlking), 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® 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: Transcription ChIP kit (Red) and Histone ChIP kit (Orange) but also for fast and magnetic bead-based (LowCell# ChIP kit and HighCell# ChIP kit) ChIP methods.

In the HighCell# ChIP kit, the protocol has been improved to allow researchers to work with high amount of cells.

Moreover, our Magnetic Rack together with our new HighCell# Magnetic ChIP protocol ensures the best IP conditions by working constantly at 4°C. The Diagenode Magnetic Rack has been designed to be used in IP experiments, keeping samples cool longer and allowing the use of standard 1.5 ml tubes.

The Diagenode HighCell# 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 mouse 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.

Kit method overview & time table

Table 1 : High Cell Magnetic ChIP protocol overview

		DAY	Time needed
STEP 1	Binding antibody to magnetic beads	1	2 hours hours (minimum) to 5 hrs
STEP 2	Cell collection and DNA-protein cross-linking	1	30 minutes
STEP 3	Cell lysis and chromatin shearing	1	2 hours
STEP 4	Magnetic Immunoprecipitation *	1	2 hours to O.N.
STEP 5	Washes of immune complexes	1 or 2	30 minutes
STEP 6	DNA purification	1 or 2	30 minutes (DIB)
STEP 7	Quantitative PCR and data analysis	1 or 2	2 hours

* The immunoselection at STEP 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 content of the kit is sufficient to perform 16 ChIP assays.

The DiaMag1.5 from Diagenode

The DiaMag1.5 magnetic rack from Diagenode has been specially designed for the HighCell# ChIP kit. It can hold 12 x 1.5ml tubes simultaneously and its efficiency is much better than classical magnetic supports. This rack may be used for other applications requiring a magnetic separation in 1.5 ml tube.

Kit Materials

Kit Content

The content of the kit is sufficient to perform 16 ChIP assays: from cell collection to immunoselected 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
- RNAse/DNase-free 1.5 ml tubes
- Agarose and TAE buffer
- DNA molecular weight marker, loading dye and DNA detection reagent
- Formaldehyde
- Glycine

Equipment

- DiaMag1.5 magnetic rack (Cat. No. kch-816-015)
- Centrifuge for 1.5 ml tubes (4°C)
- Cell counter
- Bioruptor®: Diagenode sonication apparatus (Cat. No. UCD-200)
- Rotating wheel (4°C)
- Vortex
- Floating rack for 1.5 ml tubes
- Boiling water
- Thermomixer (55°C or 65°C)
- Quantitative PCR facilities and reagents
- Agarose gel apparatus

Kit components

Table 2 : HighCell# ChIP kit content

High Cell Number ChIP kit				
Component	Description	Quantity (x16)	Quantity (x48)	Storage
ChIP buffer C1	Detergent mix, salt and ion chelator mix included.	90 ml	270 ml	4°C
Protein A-coated magnetic beads* or **Protein G-coated magnetic beads****	The beads are supplied for 16 IPs; detergent and 0.02% sodium azide included.	660 µl	2 ml	4°C **Do not freeze**
Negative control IgG (rabbit* or mouse)**	1 µg/µl	60 µl	180 µl	4°C
1.25 M Glycine	-	2 ml	6 ml	4°C
Lysis Buffer L1	Detergent and ion chelator mix included.	25 ml	75 ml	4°C
Lysis Buffer L2	Salt and ion chelator mix included.	25 ml	75 ml	4°C
Shearing Buffer S1	Detergent mix	5 ml	15 ml	4°C **Incubate at RT before use**
Protease Inhibitor	200x stock solution	100 µl	300 µl	-20°C
Buffer W1	Ion chelator mix included.	25ml	75 ml	4°C
DNA isolation Buffer (DIB)	-	4 ml	12 ml	4°C
Proteinase K	100 x stock solution	40 µl	120 µl	-20°C
GAPDH promoter primer pair	5 µM each (Rv & Fw)	50 µl	150 µl	-20°C
TSH2B primer pair	5 µM each (Rv & Fw)	50 µl	150 µl	-20°C

*: HighCell# ChIP kit protein A (Cat. No. kch-mahigh-A16) contains protein A-coated magnetic beads and rabbit IgG.
**: HighCell# ChIP kit protein G (Cat. No. kch-mahigh-G16) contains protein G-coated magnetic beads and mouse IgG.

Components available separatly				
Component	Cat. No.	Description	Quantity	Storage
DiaMag1.5 - magnetic rack & disc stand	kch-716-015	Holds 12x standard 1.5 ml tubes	1	RT
DiaMag1.5 - magnetic rack	kch-816-015	-		
Disc stand	kch-916-015	For bead resuspension		
1 M Sodium butyrate	kch-817-001	-	1 ml	-20°C
Protein A-coated magnetic beads	kch-802-220 kch-802-660 kch-802-150	-	200 µl 660 µl 1,500 µl	4°C **Do not freeze**
Protein G-coated magnetic beads	kch-818-220 kch-818-660 kch-818-150	-	200 µl 660 µl 1,500 µl	4°C **Do not freeze**
Negative Ctrl IgG from rabbit	kch-803-015	1 µg/µl	15 µl	4°C
Negative Ctrl IgG from mouse	kch-819-015	1 µg/µl	15 µl	4°C
Antibodies	www.diagenode.com	-		
Primer pairs	www.diagenode.com	5 µM each (Rv & Fw)		

Protocol

» Short The 6 steps in 3 pages p. 9
» Detailed The 6 steps in 9 pages p. 12

Short protocol

STEP 1. Binding antibodies to magnetic beads

1. Wash twice the Protein A-coated or protein G-coated magnetic beads with ice-cold ChIP buffer C1 as follows: To 28 µl of beads add 100 µl of ChIP Buffer C1 and resuspend the beads. Place the tube in the in the 1.5 ml magnetic rack (kch-816-015), wait 1 minute and discard the buffer. Keep the captured beads.
   • Alternatively, centrifuge for 5 minutes at 1,300 rpm, discard the supernatant and keep the bead pellet.
   • 25 µl of beads are needed per IP.

2. After washing, resuspend the beads in ChIP Buffer C1 (for 1 IP: 110 µl of ChIP Buffer C1).

3. Aliquot 100 µl of washed beads per 1.5 ml tube for each Magnetic ChIP reaction.

4. Add the specific antibody and control antibodies (positive and negative).
   • Use 6.3 µg of antibodies per 25 µl of beads in a volume of 100 µl.

5. 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

6. Use a trypsinisation method to collect the cells. Wash the cell suspension two times with PBS

7. Label new 1.5 ml tube(s), count the cells and put 1 million to 10 million cells in 500 µl of PBS.

8. Add 13.5 µl of 36.5% formaldehyde per 500 µl of sample (final concentration has to be approximatively 1%).

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

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

11. Mix by gentle vortexing. Incubate for 5 minutes at room temperature. This is to stop the fixation.
    • Work on ice from this point onwards

12. Centrifuge at 1,500 rpm (300 x g) for 5 minutes at 4°C.
    • We recommend the use of a swing-out rotor with soft settings for deceleration.

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

- These are the cross-linked cells ready for cell lysis and chromatin shearing.
- Do not disturb the pellet.

STEP 3. Cell lysis and Bioruptor chromatin shearing

14. Wash the cross-linked cells twice with 1 ml of ice-cold PBS.

15. After the last wash, discard the supernatant. Leave about 10 to 20 µl behind.

16. Add 1 ml of ice-cold Lysis Buffer L1 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.

17. Centrifuge for 5 minutes at 1,600 rpm (500 x g) at 4°C. Discard the supernatant, keep the pellet.

18. Add 1 ml of ice-cold Lysis Buffer L2 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.

19. Pellet again by centrifugation for 5 minutes at 1,600 rpm (500 x g) at 4°C, and discard the supernatant.

20. Add protease inhibitor to the Shearing Buffer S1 (RT). This is the complete shearing Buffer S1 for sonication. Keep the buffer at room temperature until use; discard what is not used during the day.

21. Add 200 µl of complete Shearing Buffer S1 (RT) to the cells. Vortex until resuspension. Incubate for 10 minutes on ice.

22. Shear the chromatin by sonication using the Bioruptor®. Shear for 1 to 3 runs of 5 to 10 cycles [30 seconds "ON", 30 seconds "OFF"] at high power setting. Briefly vortex and spin between each run. These shearing conditions will work excellent for many cell types. However, optimisation is required depending on the cell type and Bioruptor® system used.

23. Add 5 µl of Protease Inhibitor mix per ml of ChIP Buffer C1. If necessary, add NaBu (20 mM final) or any other inhibitor to ChIP Buffer C1.

24. Sheared chromatin analysis can be performed at this step. (See additional protocols p.20)

25. Add 800 µl of ChIP Buffer C1 to the 200 µl of sheared chromatin.

STEPS 4 and 5. Magnetic Immunoprecipitation and washes

26. Spin the diluted chromatin at 12,000 rpm (10,000 g) for 10 minutes. The supernatant is collected to perform the immunoprecipitation.

27. Briefly spin the tubes containing the antibody-coated beads to bring down liquid caught in the lid.

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

29. Discard the supernatant. Keep the pellet of antibody-coated beads.

30. Use 950 µl of diluted sheared chromatin per IP. Transfer 950 µl to each IP tube. Keep 9.5 µl as Input sample (1%), at 4°C.

31. Incubate under constant rotation on a rotator at 40 rpm for 2 hours up to overnight, at 4°C.

DAY 1 or 2

32. Spin and place the tubes in the Magnetic Rack, wait 1 minute and discard the supernatant.

33. Wash three times using 1 ml of ice-cold ChIP Buffer C1. Each wash is done as follows: add buffer, invert to mix, incubate for 5 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.

34. Wash one time with Buffer W1: add 1 ml Buffer W1 to the beads and invert to mix. Incubate on a rotating wheel for 5 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 W1.

STEP 6. DNA isolation

35. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.

36. Prepare **complete buffer DIB** as follows. Add 1 µl of **Proteinase K** per 100 µl of **DNA isolation buffer (DIB)**. Scale accordingly knowing that 100 µl are needed per IP'd DNA sample and 90.5 µl, per input DNA sample.

37. Remove the tubes from the **Magnetic Rack** and add 100 µl of **complete DIB** per IP'd DNA sample. Resuspend the beads and transfer the suspension into 1.5-ml tubes.

38. Add 90.5 µl of **complete DIB** to 9.5 µl of input DNA sample.

39. Incubate at 55°C for 15 minutes both IP'd DNA sample and input DNA sample.

40. Incubate at 100°C for 15 minutes all the samples.

41. Briefly spin the tubes to bring down the liquid caught in the lid.

42. Place the tubes in the ice-cold Magnetic Rack and wait for 1 minute.

43. Transfer the supernatants into new labeled tubes. That is the DNA ready for qPCR analysis. Store at -20°C. For alternative purification methods refer to page 20.

STEP 7. Quantitative PCR and Data analysis

44. Prepare the qPCR mix (total volume 25 µl / reaction).

45. When the PCR is done, analyse the results.

Detailed protocol

Starting material- Cells and sheared chromatin

1/ Cell number.
Cells and number of cells to use per experiment are shown in the protocol.
Each ChIP requires sheared chromatin from 1,000,000 or 10,000,000 cells; scale accordingly (see results section).

2/ Shearing method. Compatibility between protocols and kits.
Following cell harvesting, chromatin must be sheared to ~500 bp before it is used in ChIP. Note that our protocols are optimized for the use of the Bioruptor®.

The buffer for preparing sheared chromatin is included in the kit.
Depending on the target of interest, the cell number available and the number of PCRs: add 1 to 10 million cells in 200 µl Buffer S1. Scale volumes accordingly.

3/ Shearing optimization and Sheared Chromatin Analysis.
It is essential to optimize shearing conditions for your specific cell type and fixation protocol before starting a ChIP.

Therefore, 1) start with a small sample (eg. 1x 10e5 to 1x 10e6 cells) and 2) check the shearing efficiency. Protocol for analysis of chromatin shearing is given in the Chromatin Shearing Kit manual available on www.diagenode.com).

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 of 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 the Protein A-coated or protein G-coated magnetic beads twice with ice-cold ChIP buffer C1 as follows: To 28 µl of beads add 100 µl of ChIP Buffer C1 and resuspend the beads. Place the tube in the in the 1.5 ml magnetic rack (kch-816-015), wait 1 minute and discard the buffer.
   - Alternatively, centrifuge for 5 minutes at 1,300 rpm, discard the supernatant and keep the beads pellet.
   - 25 µl of beads are needed per IP.
   - Scale accordingly.

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

Note: If you see that the magnetic bead separation (at any step of this protocol) is not performing properly and some beads are remaining in the bottom of the tube, please proceed as follows:
- Cool down the sample (on ice) for 5 minutes.
- Centrifuge briefly (to spin down droplets that might be in the cap)
- Vortex gently
- Place the tube strip (tubes) again on the magnetic rack to perform the magnetic separation

2. After washing, resuspend the beads in ChIP Buffer C1 (for 1 IP: 110 µl of ChIP Buffer C1).
   - Do not freeze the beads.
   - Resuspend the beads before each use.

Note: This HighCell# ChIP kit has been optimized with Diagenode's high quality ChIP-grade antibodies and we use very low amounts of antibody per IP. The binding capacity of 25 µl of magnetic beads is 6.3 µg of antibody. If you plan to use more than 6.3 µg of antibody per IP we recommend that the quantity of beads is adjusted accordingly. Please contact us for advice if required.

3. Aliquot 100 µl of washed beads into labeled 1.5 ml tube for each Magnetic ChIP reaction.

4. Add the specific antibody and control antibodies (positive and negative).

   - Use 6.3 µg of antibodies per 25 µl of beads in a volume of 100 µl.

ATTENTION
If working with whole anti-serum or ascites fluid the amount of beads needs to be optimized.

   - See troubleshooting guide: for binding capacities of Protein A (and G).

5. 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

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

7. Remove old media and rinse cells with pre-warmed PBS (Table 6). Shake dishes for 2 minutes. Remove the PBS.

Table 6			
Cell rinsing	3x 10e6 cells	10e7 cells	5x 10e7 cells
PBS	3.5 ml	10 ml	50 ml

8. Add sterile trypsin-EDTA to tissue culture flask or dish still containing adherent cells (Table 7). Brief treatment with trypsin-EDTA removes adherent cells from tissue culture flask bottom.
   Note: Each cell line will differ in the degree of adherence it has, and this will be described in the literature that accompanies it. If necessary, place flask back into incubator for about 1-2 minutes.

Table 7			
Cell detachment	3x 10e6 cells	10e7 cells	5x 10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

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

10. When cells are detached, add immediately culture medium to the cells (Table 8). The addition of medium will inactivate the trypsin.

Table 8			
Trypsin neutralisation	3x 10e6 cells	10e7 cells	5x 10e7cells
Culture medium	2 ml	6 ml	30 ml

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

12. Wash two times the cell suspension with PBS.

13. Label new 1.5 ml tube(s), count the cells and put 1 million to 10 million cells in 500 µl of PBS.

    - 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.

14. Add 13.5 µl of 36.5% formaldehyde per 500 µl of sample (final concentration has to be approximatively 1%).

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

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

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

- Preferably work on a pre-chilled DiaMag1.5 magnetic rack or work on ice from this point onwards.

18. Centrifuge at 1,500 rpm (300 x g) for 5 minutes at 4°C.

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

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

- These are the cross-linked cells ready for chromatin shearing.
- Do not disturb the pellet.

STEP 3. Cell lysis, 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).

Work on ice unless otherwise stated.

20. Wash the cross-linked cells twice with 1 ml of ice-cold PBS (adding NaBu (20mM final concentration) 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.
- Resuspend with a pipette to ensure cells are thoroughly washed.
- In any case make sure that cells are in suspension before proceeding to the next point.

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

- These are the cross-linked cells ready for chromatin shearing.
- Avoid taking out too much as that could lead to material loss.

22. Add 1 ml of ice-cold Lysis Buffer L1 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.

23. Centrifuge for 5 minutes at 1,600 rpm (500 x g) at 4°C. Discard the supernatant, keep the pellet.

24. Add 1 ml of ice-cold Lysis Buffer L2 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.

25. Pellet again by centrifugation for 5 minutes at 1,600 rpm (500 x g) at 4°C, and discard supernatant.

26. Add protease inhibitor to the Shearing Buffer S1 (RT). This is the complete shearing Buffer S1 for sonication. Keep the buffer at room temperature until use; discard what is not used during the day.

27. Add 200 µl of complete Shearing Buffer S1 (RT) to the cells. Vortex until resuspention. Incubate for 10 minutes on ice.

28. Shear the chromatin by sonication using the Bioruptor®. Shear for 1 to 3 runs of 5 to 10 cycles (30 seconds "ON", 30 seconds "OFF") at high power setting. Briefly vortex and spin between each run. These shearing conditions will work excellent for many cell types. However, optimisation is required depending on the cell type and Bioruptor® system used.

29. Sheared chromatin analysis step. (see additional protocols p.20)

30. Add 5 µl of Protease Inhibitor mix per ml of ChIP Buffer C1. If necessary, add NaBu (20 mM final) or any other inhibitor to ChIP Buffer C1.

31. Add 800 µl of ChIP Buffer C1 to the 200 µl of sheared chromatin.

STEP 4 & 5. Magnetic Immunoprecipitation and washes

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

32. Spin the diluted chromatin at 12,000 rpm (10,000 g) for 10 minutes. The supernatant is collected to make the immunoprecipitation.

33. Briefly spin the tubes containing the antibody-coated beads to bring down liquid caught in the lid.

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

35. Discard the supernatant. Keep the pellet of antibody-coated beads.

36. Use 950 µl of diluted sheared chromatin per IP. Transfer 950 µl to each IP tube. Keep 9.5 µl as Input sample, at 4°C.

37. Incubate under constant rotation on a rotator at 40 rpm for 2 hours up to overnight, at 4°C.

DAY 1 or 2

38. Spin and place the tubes in the Magnetic Rack, wait 1 minute and discard the supernatant.

39. Wash three times using 1 ml of ice-cold ChIP Buffer C1. Each wash is done as follows: add buffer, invert to mix, incubate for 5 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.

40. Wash one time with Buffer W1: add 1 ml Buffer W1 to the beads and invert to mix. Incubate on a rotating wheel for 5 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 W1.

STEP 6. DNA isolation

Note: This kit includes a DNA isolation buffer for easy and very fast DNA isolation, which provides you with DNA suitable for qPCR analysis (see pt. 40-47).
If you need DNA of higher purity for next generation sequencing or other downstream application than PCR, we suggest to use the IPure kit (cat# AL-100-0100). Diagenode's IPure kit is the only DNA purification kit that is specifically optimized for extracting very low amounts of DNA after ChIP & MeDIP.

	DIB	IPure (Cat. No. AL-100-0100)
Time	1h15	5h40
DNA concentration	+	++ (possible to concentrate)
DNA purity	+	++
Subsequent analysis	qPCR	Next generation sequencing, microarray, qPCR amplification

41. Take the input samples, centrifuge briefly and from now onwards treat the input DNA samples and IP samples in parallel.

42. Prepare 100 µl complete buffer DIB per sample as follows. Add 1 µl of Proteinase K per 100 µl of Buffer DIB. Scale accordingly knowing that 100 µl are needed per IP'd DNA sample and 90.5 µl, per input DNA sample.

43. Remove the tubes from the Magnetic Rack and add 100 µl of complete DIB per IP'd DNA sample. Resuspend the beads.

44. Add 90.5 µl of complete DIB to 9.5 µl of input DNA sample.

45. Incubate at 55°C for 15 minutes both IP'd DNA sample and input DNA sample.

46. Next, incubate at 100°C for 15 minutes all the samples.

47. Briefly spin the tubes to bring down the liquid caught in the lid.

48. Place the tubes in the ice-cold DiaMag1.5 and wait for 1 minute.

49. Transfer the supernatants to new labeled tubes. That is the DNA ready for qPCR analysis. Store at -20°C.

STEP 7. Quantitative PCR & Data analysis

50. 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)
+ 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

51. 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 using primer design programs. 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 does not always pick up primer dimer 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).

  % (ChIP/ Total input)= 2^[(Ct(x%input) – log(x%)/log2) - Ct(ChIP)]x 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

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

Here AE is amplification efficiency as calculated above (5); CtChIP and Ctinput are threshold values obtained from exponential phase of qPCR; Fd 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.

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

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

- 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 protocols

Sheared chromatin analysis

This protocol refers to the Diagenode's Elution Module (Cat. No. mc-magme-002) that can be ordered separately.

Reagents not supplied:

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

1. Take an aliquot of 50µl of sheared chromatin and spin it at 12,000 rpm for 10 min at 4°C. Transfer the supernatant to a new tube for chromatin analysis.
2. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of water).
3. Add 2 µl of diluted RNase cocktail.
4. Incubate 1h at 37°C.
5. Prepare the Complete Elution Buffer by mixing thoroughly Buffers D, E and F as follows

Reagents	Volume
Buffer D	96 µl
Buffer E	10 µl
Buffer F	4 µl
Total volume	110 µl*

* enough volume for two chromatin samples

6. Add 54 µl of the Complete Elution Buffer to each chromatin sample.
7. Mix thoroughly before incubating sample at 65°C for 4h (or overnight).
8. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol. Incubate the sample at RT for 10 min on a rotating wheel.

9. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
10. Add 1 volume of chloroform/isoamyl alcohol {24:1}. Incubate the sample at RT for 10 min on a rotating wheel.
11. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
12. Precipitate the DNA by adding 10 µl of meDNA precipitant, 5 µl of meDNA coprecipitant, and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.
13. Centrifuge for 25 min at 13,000rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.
14. Centrifuge for 10 min at 13,000 rpm at 4°C. Carefully remove the supernatant, leave tubes opened for 30 min at RT to evaporate the remaining ethanol.
15. Re-suspend the pellet in 20 µl of TE buffer.
16. Run samples (20 µl of DNA + 4 µl of 6x loading dye) on a 1.5% agarose gel. As an alternative run 1 µl on a microfluidic chip (e.g. Bioanalyzer High Sensitivity DNA chip).

II. Use of a standard curve (see additional protocol)

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

Raw data (standard, Input and IP)			
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

Troubleshooting guide

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

Chromatin shearing	Optimal shearing conditions are important for ChIP efficiency.	Shearing conditions for each cell type must be optimized from cell collection, fixation to shearing method (settings of the sonicator apparatus).
	Critical points for shearing optimization.	1) Not to start with a too large amount of cells (1x 10e6 cells or less is ok) 2) Keep samples cold (4°C) 3) High % SDS favours better sonication but inhibits immunoselection (optimal range: 0,1 to 1%). Dilutions must be adapted accordingly prior to immunoselection; the final SDS concentration should not be higher than 0.15 to 0.20% (e.g. If the shearing buffer contains 0.75% SDS, the sheared chromatin is diluted 3.5 to 4.0 fold in the [P.I.-ChIP buffer 1x])
	Shear the samples of chromatin using the Bioruptor® from Diagenode (cat. No. UCD-200, UCD-300, UCD-400).	Maintain temperature of the samples close to 0°C. The chromatin shearing needs to be optimized for each cell type. A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.
Sheared chromatin analysis	Purify the DNA from the sheared chromatin as described in the kit protocol to analyse the shearing.	Extract total DNA from an aliquot of sheared chromatin and run on 1% agarose gel (stain with EtBr). In order to analyse the sheared chromatin on gel, take DNA purified from the sheared chromatin input -prepared at STEP 3 . Some unsheared chromatin can be analysed on gel as well (purify it as done with the input sample (see "6. Additional protocols" section). Chromatin eqvivalent to 100,000 cells, one million cells or more can for sure be visualized on a gel.
	Do not load too much DNA on a gel.	Loading of large quantities of DNA on agarose gel can lead to poor quality pictures, which do not reflect the real DNA fragmentation. The DNA amount to load depends on the size of the well and on the gel size.
	Agarose concentration.	Do not use more than 1-1.5% agarose gel and run slowly (Volt/cm and time depend on the gel size).
	Running buffer concentration.	1x TAE or TBE is preferred to 0.5x TAE, which can lead to smears on agarose gel.
Sheared chromatin amounts	How much sheared chromatin do I need to prepare?	Most of the sheared chromatin is to be used in the ChIP experiment, but remember that some of the sheared chromatin is needed as control as it corresponds to the input sample for the ChIP experiment and it can also be checked on agarose gel.
	Dilute the sheared chromatin in ChIP buffer for Immuno-selection incubation.	The sheared chromatin is diluted in complete Buffer A prior to the immunoselection incubation (see STEP 3: Add 870 µl of complete Buffer A to the 130 µl of sheared chromatin). Dilute the sheared chromatin at least 7 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	Beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm. (http://www.msu.edu/~venkata1/gforce.htm). It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage.	Store at 4°C. Do not freeze.
	Antibody binding capacity.	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage.	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C, and thawed before use.
Other enzyme inhibitors	Specific enzyme inhibitors are not included in the kit, such as phosphatase inhibitors.	Add phosphatase inhibitors or others to Buffers A and B, if necessary, depending on your research field and protein(s) of interest to be ChIP'd Add NaBu for histone ChIPs.

Negative ChIP control(s)	Use non-immune IgG in the IP incubation mix.	Use the non-immune IgG fraction from the same species the antibodies were produced in.
	Do not add antibody to the IP.	Incubation with beads, which were not coated with antibodies antibodies could also be used as a negative ChIP control 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.
Antibody in IP	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.
	Why is my antibody not working in ChIP?	Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.
	Which antibody should I use in ChIP?	Use ChIP-grade antibodies. If not available, it is recommended to use several antibodies directed against different epitopes of the same protein. Verify that the antibodies can work directly in IP on fresh cell extracts. Also, when testing new antibodies, include known ChIP-grade antibodies as positive control for your ChIP assay.
	How do I choose an antibody for ChIP?	Be aware of the possible cross-reactivity of antibodies. Verify by Western blot analysis the antibody specificity. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.
	Are my antibodies going to bind the protein A?	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.
	What are the water bath specifications?	Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W
	Can I use the kit w/o an ultrasonic water bath?	Yes, then a long incubation at 4ºC should be used. Depending on the antibody and target to be ChIP'd, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.

Polymerase Chain Reaction	Primer design.	Primer length: 18 to 24 nucleotides/ Primer Tm: 60°C (+/- 3.0°C)/ % GC: 50% (+/- 4%)
	Controls: negative and positive.	Negative PCR controls: PCR with DNA from samples IP'd with non-immune antibodies (negative IgG). Alternatively, PCR using DNA from ChIP samples and primers specific for a DNA region to which, your antigen of interest is not binding. Positive PCR control: PCR using input DNA.
	No PCR signal.	Include a positive PCR control as a control for your PCR mix (your primers, dNTP and Master Mix) using the Input DNA or a DNA sample of the same origin.
	High Ct values.	Use more input chromatin.
	CtNegCtl and CtTarget.	The ratio between target IP and negative control IP depends on the antibody used.
	Background is high.	Verify that you perform properly the following steps: Keep the antibody binding beads and DNA purifying slurry in suspension while adding to tubes. Check by eye that equal pellets of beads and slurry are present in each tube. Washes (step 5) are critical.
	Using end-point PCR rather than quantitative PCR.	If gel electrophoresis is used to estimate intensities of PCR products, then relative occupancy of a factor at a locus is the ratio of the intensity of the target IP band to the negative control IP band.
Freezing	Samples can be frozen at several steps of the protocol.	Pellets of formaldehyde fixed cells can be stored at - 80oC for at least a year. Sheared chromatin can be stored at - 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.
	Avo id multiple freeze/thawing.	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

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

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

CHROMATIN FUNCTION

Chromatin Immunoprecipitation

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

ChIP (crosslinked) procedure

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

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

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

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

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

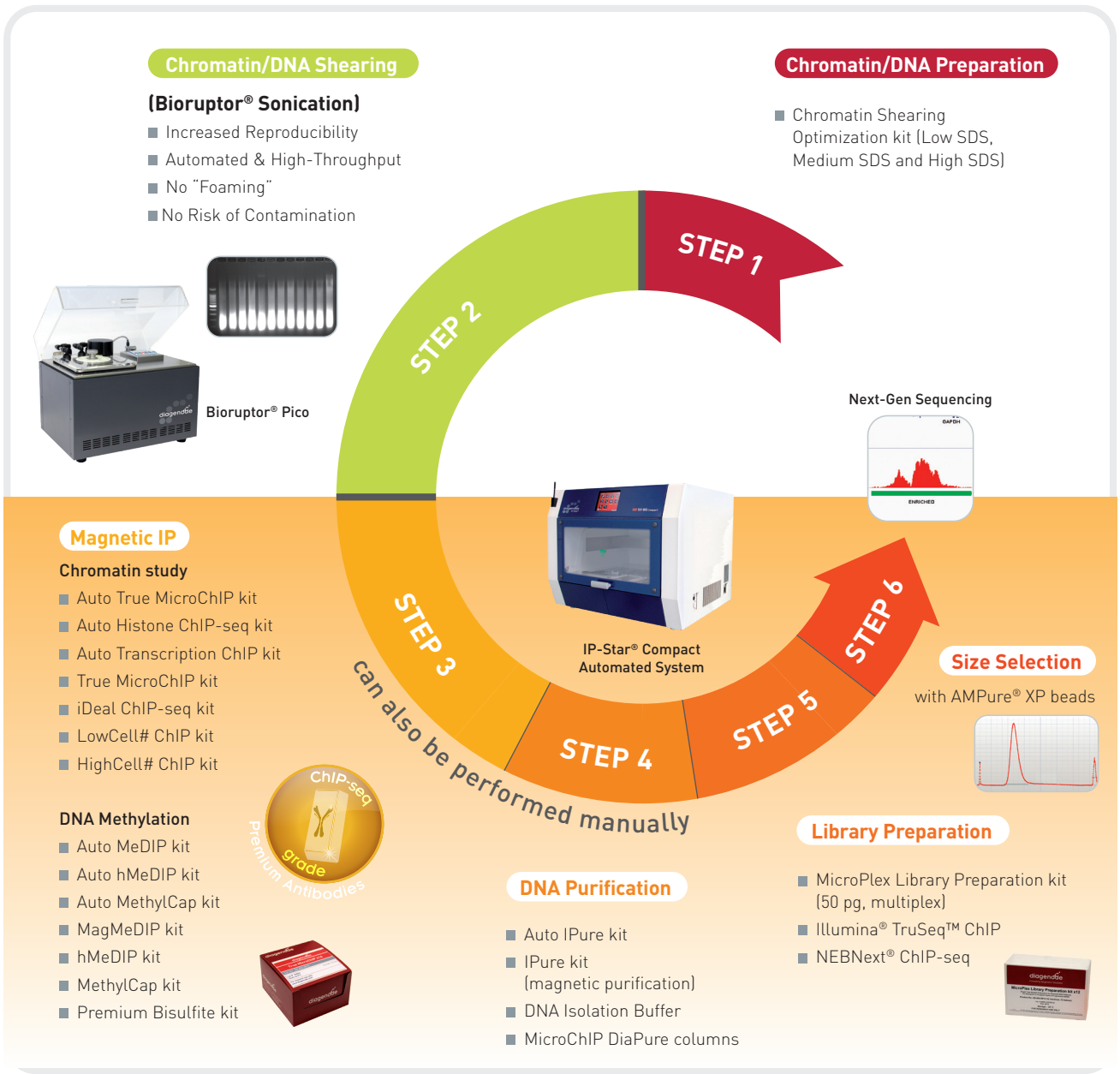

Figure 1. Diagenode provides a full suite of manual and automated solutions for ChIP experiments.

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