Instruction Manual

iDeal ChIP-seq kit for Histones & Library Preparation kit x24 (incl. Index Primer Set 1)

Chromatin Immunoprecipitation and Library Preparation kit for ChIP-seq analysis

Cat. No. C01010053

- **Proven:** We consistently deliver expertise in our ChIP-seq tools
- **Robust:** Delivers efficient and reproducible results for ChIP-seq

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Content

Introduction

Association between proteins and DNA is a major mechanism in many vital cellular functions such as gene transcription and epigenetic silencing. It is crucial to understand these interactions and the mechanisms by which they control and guide gene regulation pathways and cellular proliferation. Chromatin immunoprecipitation (ChIP) is a technique to analyse the association of proteins with specific genomic regions in intact cells. ChIP can be used to study changes in epigenetic signatures, chromatin remodelling and transcription regulator recruitment to specific genomic sites.

The different steps of the ChIP assay are cell fixation (crosslinking), chromatin shearing, immunoprecipitation, reverse crosslinking followed by DNA purification and analysis of the immunoprecipitated DNA.

In ChIP, living cells are first fixed with a reversible crosslinking agent to stabilize protein-DNA interactions. The most widely used reagent to fix cells is formaldehyde which generates covalent bonds between amino or imino groups of proteins and nucleic acids. Formaldehyde treatment crosslinks both DNA-protein as well as protein-protein complexes.

Following crosslinking, chromatin needs to be sheared very efficiently into homogeneous small fragments that can subsequently be used in immunoprecipitation (IP). The Bioruptor® series (UCD-Standard, Plus, Twin and XL) from Diagenode provides you with high quality sheared chromatin ready-to-ChIP. Currently, the Bioruptor® is the most widely used and the most cited chromatin fragmentation system as evidenced by more than 250 publications. Shearing may also be accomplished with shearing kits from Diagenode which enable an easy and highly reproducible shearing process for any cell type. After fragmentation, the sheared chromatin is precipitated with a specific antibody (Ab) directed against the protein of interest. The chromatin-Ab complex is isolated using magnetic or sepharose beads. Finally, the precipitated DNA fragments are released from the Ab, and analysed. Enrichment of specific sequences in the precipitated (IP'd) DNA indicates that these sequences were associated with the protein of interest in vivo. Analysis of specific regions can be performed by quantitative polymerase chain reaction (qPCR). In recent years, ChIP combined with high-throughput Next-Generation Sequencing (ChIP-seq) has become the gold standard for whole-genome mapping of protein-DNA interactions.

Although ChIP-seq is a powerful tool, the procedure requires tedious optimization of several reaction conditions that might lead to considerable time and lab expenditures. To reduce these tedious steps, Diagenode provides kits with optimized reagents and protocols for ChIP which enable successful ChIP-seq. The new iDeal ChIP-seq kit protocol has been thoroughly optimised by Diagenode for ChIP followed by high-throughput sequencing on Illumina® GAIIx and Ion Torrent™ PGM™ systems.

Diagenode also offers a wide range of additional tools and products for ChIP analysis including the Bioruptor® for chromatin shearing and nucleic acid fragmentation, the SX-8G IP-Star® for automated ChIP and DNA methylation analysis, magnetic racks for 0.2 or 1.5 ml tubes which enable you to work with magnetic beads under optimal conditions (4°C), and ChIP and ChIP-seq grade antibodies against many epigenetic targets. In addition, Diagenode provides individual reagents, such as magnetic beads, negative IP controls (mouse and rabbit IgG), protease inhibitors and deacetylase inhibitors (sodium butyrate). Diagenode also offers several qPCR primer pairs for the analysis of IP'd DNA. Peptides can additionally 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 a perfect starting point that will lead to your success.

Kit method overview & time table

Table 1 : iDeal ChIP-seq protocol overview

Step		Time needed	Day
1	Cell collection and DNA-protein crosslinking	1 to 2 hours	1
2	Cell lysis and chromatin shearing	1 to 2 hours	1
3	Magnetic immunoprecipitation	overnight	1-2
4	Elution, decrosslinking and DNA purification	6 hours	2
5	Quantitative PCR and data analysis prior to Library preparation and Next-Generation Sequencing	2 to 3 hours	3
6	Library preparation	2 to 3 hours	3

Kit Materials

The content of the kit is sufficient to perform 24 ChIP-seq assays from cell collection to library preparation. Store the components at the indicated temperature upon receipt.

Table 2: Components supplied with the iDeal ChIP-seq kit

Description	Quantity (x10)	Quantity (x24)	Storage
Glycine	175 µl	420 µl	4°C
Lysis buffer iL1	15 ml	36 ml	4°C
Lysis buffer iL2	15 ml	36 ml	4°C
Shearing buffer iS1	2 ml	4 ml	4°C
Protease inhibitor cocktail	30 µl	65 µl	-20°C
5x ChIP buffer iC1	1.7 ml	4 ml	4°C
5% BSA (DNA free)	70 µl	175 µl	-20°C
Protein A-coated magnetic beads	240 µl	580 µl	4°C **Do NOT freeze !**
Wash buffer iW1	4 ml	10 ml	4°C
Wash buffer iW2	4 ml	10 ml	4°C
Wash buffer iW3	4 ml	10 ml	4°C
Wash buffer iW4	4 ml	10 ml	4°C
Elution buffer iE1 (warm to room temp. before use)	6 ml	15 ml	4°C
Elution buffer iE2	240 µl	580 µl	4°C
Rabbit IgG (control isotype antibody)	10 µl	15 µl	-20°C
ChIP-seq grade antibody H3K4me3	10 µg	10 µg	-20°C
ChIP-seq grade GAPDH TSS primer pair (positive control)	25 µl	50 µl	-20°C
ChIP-seq grade Myoglobin exon 2 primer pair (negative control)	25 µl	50 µl	-20°C
ChIP-seq grade water	4 ml	10 ml	Room temperature

Table 3: IPure kit content

Description	Quantity	Storage
Wash buffer 1 w/o iso-propanol (IPure)	2 ml	4°C
Wash buffer 2 w/o iso-propanol (IPure)	2 ml	4°C
Buffer C (IPure)	4 ml	4°C
Magnetic beads (IPure)	400 µl	4°C
Carrier (IPure)	55 µl	-20°C

Store DiaMag Protein A-coated magnetic beads and IPure Magnetic beads at 4°C. Do NOT freeze magnetic beads because they may become damaged. Keep the beads in liquid suspension during storage as drying will result in reduced performance.

Table 4: iDeal Library Preparation kit

Description	Storage
iDeal Library End Repair/dA-Tailing Enzyme Mix (green)	-20°C
iDeal Library End Repair/dA-Tailing Buffer (green)	-20°C
iDeal Library Ligation Master Mix (red)	-20°C
iDeal Library Ligation Enhancer (red)	-20°C
iDeal Library PCR Master Mix (blue)	-20°C
iDeal Library Adaptor for Illumina (red)	-20°C
iDeal Library Uracil Excision Reagent (red)	-20°C
iDeal Library Universal PCR Primer for Illumina (blue)	-20°C

Table 5: iDeal Library Index Primers for Illumina (Primer Set 1)

Product	Index Primer Sequence	Expected Index Primer Sequence Read	Quantity	Storage
iDeal Library Index 1 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**CGTGAT**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ATCACG	10 µl	-20°C
iDeal Library Index 2 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**ACATCG**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CGATGT	10 µl	-20°C
iDeal Library Index 3 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGA-T**GCCTAA**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TTAGGC	10 µl	-20°C
iDeal Library Index 4 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**TGGTCA**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TGACCA	10 µl	-20°C
iDeal Library Index 5 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**CACTGT**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACAGTG	10 µl	-20°C
iDeal Library Index 6 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**ATTGGC**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GCCAAT	10 µl	-20°C
iDeal Library Index 7 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGA-T**GATCTG**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CAGATC	10 µl	-20°C
iDeal Library Index 8 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGA-T**TCAAGT**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	ACTTGA	10 µl	-20°C
iDeal Library Index 9 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGA-T**CTGATC**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GATCAG	10 µl	-20°C
iDeal Library Index 10 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**AAGCTA**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	TAGCTT	10 µl	-20°C

iDeal Library Index 11 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT**GTAGCC**GTGAC TGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	GGCTAC	10 µl	-20°C
iDeal Library Index 12 Primer for Illumina	5´-CAAGCAGAAGACGGCATACGAGAT-**TACAAG**GTGA CTGGAGTTCAGACGTGTGCTCTTCCGATC-s-T-3´	CTTGTA	10 µl	-20°C

Where -s- indicates phosphorothioate bond.

Note: If fewer than 12 indexes are used in a lane for sequencing, it is recommended to use the following indexes:

- Pool of 2 samples: Index #6 and 12
- Pool of 3 samples: Index #4, 6 and 12
- Pool of 6 samples: Index #2, 4, 5, 6, 7 and 12

Required Materials Not Provided

Reagents

- Gloves to wear at all steps
- Formaldehyde, 37%, Molecular Biology Grade
- Phosphate buffered saline (PBS) buffer
- 1 M Sodium butyrate (NaBu) (Diagenode Cat. No. kch-817-001) **(optional)**
- 100% isopropanol
- Trypsin-EDTA
- RNase/DNase-free 1.5 ml tubes
- qPCR SYBR® Green Mastermix
- Reagents for library preparation, cluster generation (Illumina®) or ePCR (Ion Torrent™ PGM™) and sequencing
- Quant-IT dsDNA HS assay kit (Invitrogen)
- 100% Ethanol
- Nuclease-free Water
- 10 mM Tris-HCl, pH 8.0 or 0.1X TE
- AMPure® XP Beads (Beckman Coulter, Inc. #A63881)
- **Optional: iDeal Library Index Primer Set 2 (Diagenode, Cat. No. C05010021)**

Equipment

- Optional: ChIPettor(tm) System for Histones (Cat.. No. C01010162)
- Diagenode DiaMag1.5 magnetic rack (Cat. No. kch-816-015)
- Diagenode Bioruptor®: sonication device (Cat. No. UCD-200, UCD-300, UCD-400, UCD-500)
- Diagenode 1.5 ml TPX microtubes (optimized for chromatin shearing with Bioruptor®) (Cat No. M-50001)
- Refrigerated centrifuge for 1.5 ml, 15 ml and 50 ml tubes
- Cell counter
- DiaMag Rotator (Rotating wheel) (Cat. No. VL-100-0001)
- Vortex
- Thermomixer
- Qubit system (Invitrogen)
- qPCR cycler
- DNA LoBind Tubes (Eppendorf #022431021)
- Magnetic rack (Diagenode, Cat. No. B04000001)

Remarks before starting

1. Cell number

This protocol has been optimised for ChIP on 1,000,000 cells in 300 µl ChIP reaction. It is possible to use more cells. However, for optimal performance, we recommend performing separate ChIPs and pool the IP'd DNA before purification.

2. Shearing optimization and sheared chromatin analysis.

Before starting the ChIP, the chromatin should be sheared to fragments in the 100 to 600 bp range. Our kits and protocols are optimized for chromatin shearing using the Bioruptor® (UCD-Standard, Plus and Pico). The maximum volume for shearing with the Bioruptor® is 300 µl per 1.5 ml microtube (depending on the specific type). We recommend using TPX tubes (C30010010) for Bioruptor® UCD-Standard and Plus as shearing has been shown to be more efficient and reproducible using these tubes. For Bioruptor® Pico we recommend using 1.5 ml Microtubes with caps (C30010016). The shearing conditions mentioned in the protocol are adequate for a variety of cell types. However, given that cell types are different, we recommend optimizing sonication conditions for each cell type before processing large quantities of cells or samples. Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation. A protocol to assess the shearing efficiency can be found in the "additional protocols" section.

3. Magnetic beads

This kit includes DiaMag Protein A-coated magnetic beads. Make sure the beads do not dry during the procedure as this will result in reduced performance. Keep the beads homogenously in suspension at all times when pipetting. Variation in the amount of beads will lead to lower reproducibility. Do not freeze the beads.

The amount of beads needed per IP depends on the amount of antibody used for the IP. The protocol below uses 20 µl of beads. The binding capacity of this amount is approximately 5 µg of antibody. With most of Diagenode's high quality ChIP-seq grade antibodies the recommended amount to use is 1 to 2 µg per IP reaction. However, if you plan to use more than 5 µg of antibody per IP we recommend increasing the amount of beads accordingly.

4. Negative and positive IP controls (IgG and control Ab)

The kit contains a negative (IgG) and a positive (H3K4me3) control antibody. We recommend including one IgG negative IP control in each series of ChIP reactions. We also recommend using the positive control ChIP-seq grade H3K4me3 antibody at least once. The kit also contains qPCR primer pairs for amplification of a positive and negative control target for H3K4me3 (GAPDH-TSS and Myoglobin exon 2, respectively).

5. Quantification

Determine the concentration of the IP'd DNA after the ChIP with a highly sensitive method such as the 'Quant-IT dsDNA HS assay kit' on the Qubit system from Invitrogen. PicoGreen is also suitable but UV spectrophotometric methods such as the NanoDrop are usually not sufficiently sensitive. In most cases it is sufficient to use approximately 10% of the IP'd material for quantification. The expected DNA yield will be dependent on different factors such as the cell type, the quality of the antibody used and the antibody target. The expected DNA yield obtained with the positive control H3K4me3 antibody on 1,000,000 HeLa cells is approximately 10 ng.

6. Quantitative PCR

Before sequencing the samples, we recommend analysing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. The kit contains a positive and negative control primer pair which can be used for the H3K4me3 positive control antibody in SYBR® Green qPCR assay using the protocol described in the manual. Use your own method of choice for analysing the appropriate control targets for your antibodies of interest.

In order to have sufficient DNA left for sequencing, we recommend not using more than 10% of the total IP'd DNA for qPCR. You can dilute the DNA (1/10 or more) to perform sufficient PCR reactions. PCR reactions should be performed at least in duplicate although performing them in triplicate is recommended to be able to identify potential outliers.

7. Quantitative PCR data interpretation

The efficiency of chromatin immunoprecipitation of particular genomic loci can be expressed as the recovery of that locus calculated as the percentage of the input (the relative amount of immunoprecipitated DNA compared to input DNA). If the amount used for the input was 1% of the amount used for ChIP, the recovery can be calculated as follows:

% recovery = $2^{(Ct_{input} - Ct_{sample})}$

Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and input, respectively. This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results the real amplification efficiency, if known, should be used.

For the positive control antibody (e.g. H3K4me3) the recovery of the positive control target (GAPDH TSS locus) is expected to be between 10 and 20% although this will depend on the cell type used. The recovery of the negative control target (Myoglobin exon 2 locus) should be below 1%.

Criteria to decide whether the sample is good enough for sequencing will be largely target dependant. Therefore, the following are only general guidelines:

- the recovery of the positive control target should be at least 5%
- the recovery of the negative control target should be below 1%
- the ratio of the positive versus the negative control target should be at least 5

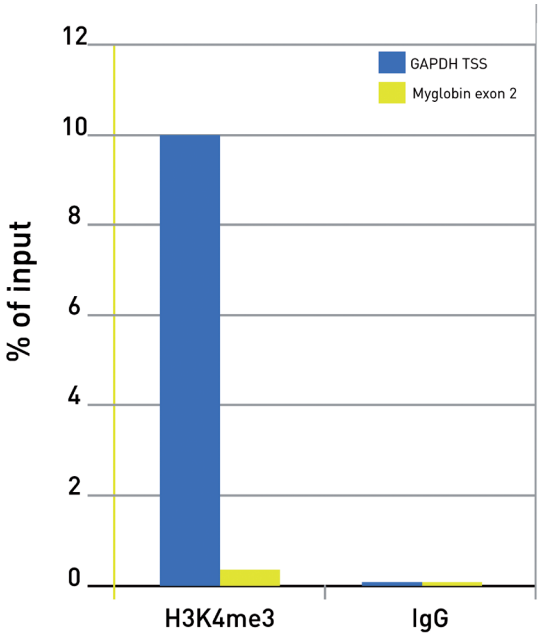


Figure 1: ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit. Sheared chromatin from 1 million cells, 1 µl of the positive control antibody and 2 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control GAPDH-TSS and the negative control Myoglobin exon 2 primer sets from the kit. The recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

Short protocol for experienced users

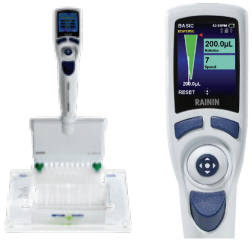
STEP 1. Cell collection and DNA-protein crosslinking

1. Collect the cells by trypsinisation and wash two times with PBS.
2. Count the cells and resuspend them in PBS to obtain up to 10 million cells in 500 µl of PBS. Aliquot 500 µl of cell suspension in 1.5 ml tubes.
3. Add 13.5 µl of formaldehyde 37%. Mix by gentle vortexing and incubate for 8 minutes at room temperature to allow fixation to take place.
4. Stop the fixation by adding 57 µl of **Glycine** solution. Mix by gentle vortexing and incubate for 5 minutes at room temperature. Work on ice from this point onwards.
5. Centrifuge at 1,600 rpm (500 x g) for 5 minutes at 4°C and gently aspirate the supernatant without disturbing the cell pellet.
6. Wash the cells twice with 1 ml PBS.

STEP 2. Cell lysis and chromatin shearing

7. Add 10 ml of ice-cold **Lysis buffer iL1** to the cell pellet corresponding to 10 million cells. Resuspend the cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing. Scale down accordingly when using fewer cells.
8. Centrifuge for 5 minutes at 1,600 rpm (500 x g) and 4°C and discard the supernatant.
9. Add 10 ml of ice-cold **Lysis buffer iL2** to the cell pellet. Resuspend the cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
10. Centrifuge for 5 minutes at 1,600 rpm (500 x g) and 4°C and discard the supernatant.
11. Add 200x **protease inhibitor cocktail** to the **Shearing buffer iS1**. Keep on ice.
12. Add 1 ml of **Shearing buffer iS1** containing protease inhibitor to 10 million cells. Resuspend by pipetting up and down and incubate on ice for 10 minutes.
13. Shear the chromatin by sonication using the **Bioruptor®** for 2 to 3 runs of 10 cycles [30 seconds "ON", 30 seconds "OFF"] at High Power setting. Briefly vortex and spin the tubes between the runs.
14. Centrifuge at 13,000 rpm (16,000 x g) for 10 minutes and collect the supernatant which contains the sheared chromatin.

STEP 3. Magnetic immunoprecipitation



The immunoprecipitation step can also be performed using the semi-automated **ChIPettor™ System.** If doing so, please refer to the corresponding protocol delivered with this product. Alternatively, the protocol can also be downloaded from the website www.diagenode.com

15.

16. Dilute the **5x ChIP buffer iC1** and with ChIP-seq grade water to obtain **1x ChIP buffer iC1**. Place on ice.
17. Take the required amount of **DiaMag Protein A-coated magnetic beads** (20 µl/IP) and wash four times with twice the volume of ice-cold **1x ChIP buffer iC1**.
18. Resuspend the beads after the last wash in the original volume **1x ChIP buffer iC1**.
19. Set aside 1 µl (1%) of the sheared chromatin to use as input sample and keep at 4°C.
20. Prepare the following ChIP reaction mix (1 IP) :
    6 µl of **5% BSA**
    1.5 µl of 200x **protease inhibitor cocktail**
    56 µl of **5x ChIP buffer iC1**
    100 µl of sheared chromatin
    20 µl of **DiaMag Protein A-coated magnetic beads**
    x µl **ChIP-seq grade antibody**
    add **ChIP-seq grade water** to a total volume of 300 µl
    If required, NaBu (HDAC inhibitior, 20mM final concentration) or other inhibitors can also be added.
21. Incubate overnight at 4°C on a rotating wheel.
22. The next day, briefly spin the tubes, place them in the ice-cold magnetic rack and discard the supernatant.
23. Add 350 µl ice-cold **Wash buffer iW1** and incubate for 5 minutes at 4°C on a rotating wheel. Discard the wash buffer using the **Diagenode magnetic rack**.
24. Repeat step 21 and 22 once with **Wash buffer iW2, iW3 and iW4**, respectively.

STEP 4. Elution, decrosslinking and DNA purification

25. After removing the last wash buffer, add 400 µl of **Elution buffer** iE1 to the beads and incubate for 30 minutes on a rotating wheel at room temperature.
26. Briefly spin the tubes and place them into the Diagenode magnetic rack. Transfer the supernatant to a new tube and add 16 µl of **Elution buffer iE2**. Also add 399 µl **buffer iE1** and 16 µl of **buffer iE2** to the 1 µl input sample. Incubate for 4 hours or overnight in a thermomixer at 1300 rpm and 65°C.
27. Purify the DNA using the IPure kit.
28. Add 2 µl of **carrier** to each IP and input sample. Vortex briefly and perform a short spin.
29. Add 400 µl of 100% isopropanol to each IP and input sample. Vortex briefly and perform a short spin.
    ATTENTION: Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.
30. Resuspend the provided **Magnetic beads** and transfer 15 µl to each IP and input sample.
31. Incubate IP and input samples for 1 hour at room temperature on a rotating wheel (40 rpm).
32. Prepare the **Wash buffer 1** containing 50% isopropanol:

Wash buffer 1	
Wash buffer 1 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	4 ml

33. Briefly spin the tubes, place into the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 1**. Close the tubes, the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).
34. Prepare the **Wash buffer 2** containing 50% isopropanol as follows:

Wash buffer 2	

Wash buffer 2 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	4 ml

35. Briefly spin the tubes, place into the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add 100 µl **Wash buffer 2** per tube. Close the tubes, resuspend the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).

36. **Two elutions in 25 µl (total volume 50 µl)**

    Briefly spin the tubes and place them into the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 25 µl buffer C. Close the tubes, resuspend the beads and incubate for 15 minutes at room temperature on a rotating wheel (40 rpm). Resuspend the pelleted beads using the pipet and make sure that you drop them on the bottom of the tube.

37. Spin the tubes and place them into the **DiaMag1.5**, wait 1 minute and transfer the supernatants into a new labelled 1.5 ml tube. Keep the bead pellets on ice.

38. Repeat the elution of the bead pellets for 15 min at room temperature on a rotating wheel (40 rpm) in 25 µl **buffer C**.

39. Spin tubes and place them in the DiaMag1.5, wait 1 minute and pool the supernatant with the corresponding IP or input sample (1.5 ml tube). Discard the beads.

40. Place the DNA on ice and proceed to any desired downstream applications, or store it at -20°C or -80°C until further use.

STEP 5. Quantitative PCR analysis

41. Prepare the qPCR mix as follows (20 µl reaction volume using the provided **control primer pairs**):
    10 µl of a 2x SYBR® Green qPCR master mix
    1 µl of primer mix
    4 µl of water
    5 µl IP'd or input DNA

42. Use the following PCR program: 3 to 10 minutes denaturation step at 95°C (please check carefully supplier's recommendations about Taq polymerase activation time), followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72 °C. These conditions may require optimisation depending on the type of Master Mix or qPCR system used.

STEP 6. Library Preparation

Symbols

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

Starting Material

5 ng–1 µg fragmented DNA.

iDeal Library Preparation End Prep

1. Mix the following components in a sterile nuclease-free tube:

*If DNA Input is less than 55.5 µl, complete with water up to 55.5 µl.

iDeal LibraryEnd Repair/dA-Tailing Enzyme Mix (green)	3.0 µl

iDeal Library End Repair/dA-Tailing Buffer (green)	6.5 µl
Fragmented DNA	55.5 µl*
Total volume	**65 µl**

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.
3. Place in a thermocycler, with the heated lid on, and run the follwing program:
   - **30 minutes at 20°C**
   - **30 minutes at 65°C**
   - Hold at 4°C

Adaptor Ligation

If DNA input is < 100 ng, dilute the **iDeal Library Preparation Adaptor for Illumina*** 1:10 in sterile water use immediately to a final concentration of 1.5 µM.

1. Add the following components directly to the iDeal Library End Prep reaction mixture and mix well:

iDeal Library Ligation Master Mix (red)	15 µl
iDeal Library Adaptor for Illumina* (red)	2.5 µl
iDeal Library Ligation Enhancer (red)	1 µl
Total volume	**83.5 µl**

2. Incubate at **20°C for 15 minutes** in a thermal cycler.
3. Add **3 µl of iDeal Library Uracil Excision Reagent** (red) to the ligation mixture from step 3.
4. Mix well and incubate at **37°C for 15 minutes**.

Size Selection of Adaptor-ligated DNA

Size selection is optional. If the starting material is less than 50 ng, size selection is not recommended. If you are not performing size selection, proceed to the next page and perform clean up step prior to PCR amplification. The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to Table 1 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µl.

1. Add **13.5 µl dH_2O** to the ligation reaction for a 100 µl total volume.
2. Add **55 µl of resuspended AMPure XP beads** to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).
5. Add **25 µl resuspended AMPure XP beads** to the supernatant, mix well and incubate for 5 minutes at room temperature.
6. Wash twice with **200 µl of 80% freshly prepared ethanol** to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
7. Air the dry beads for 10 minutes while the tube is on the **magnetic rack** with the lid open.
8. Elute the DNA target from the beads into **28 µl of 10 mM Tris-HCl or 0.1 X TE, pH 8.0**. Transfer 23 µl to a new PCR tube for amplification.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the **iDeal Library PCR Master Mix** in the subsequent PCR step.

Table 1: Recommended conditions for bead based size selection.

LIBRARY PARAMETERS	Approximate insert size	150 bp	200 bp	250 bp	300 - 400 bp	400 - 500 bp	500 - 700 bp
	Total Library size (insert + adaptor)	270 bp	320 bp	400 bp	400 - 500 bp	500 - 600 bp	600-800 bp
VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

Alternatively, Cleanup of Adaptor-ligated DNA without Size Selection

1. Add **86.5 µl resuspended AMPure XP beads** to the ligation reaction. Mix well by pipetting up and down at least 10 times. Vortex AMPure XP beads to resuspend.
2. Incubate for 5 minutes at room temperature.
3. Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).
4. Wash twice with **200 µl of 80% freshly prepared ethanol** to the tube while in the **magnetic rack**. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
5. Air the dry beads for 10 minutes while the tube is on the magnetic stand with the lid open.
6. Elute the DNA target from the beads by adding **28 µl of 10 mM Tris-HCl, pH 8.0 or 0.1X TE**.
7. After the solution is clear (about 5 minutes), transfer **23 µl to a new PCR** tube for amplification.

PCR Amplification

8. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	23 µl
iDeal Library PCR Master Mix (blue)	25 µl
iDeal Library Index Primer* (blue)	1 µl
iDeal Universal PCR Primer* (blue)	1 µl
Total volume	**50 µl**

* These primers (Index Primer Set 1) are included. Index Primer Set 2 may be purchased separately (Cat. No. C05010021).

PCR cycling conditions

9. Transfer tubes to a pre-programmed thermal cycler and incubate as follows.

Cycle Step	Temp	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	6 - 15*
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

* Suggestion: 6 PCR cycles for 1 µg DNA input 10 cycles for 50 ng, and 13-15 for 5 ng DNA input. Further optimization of PCR cycle number may be required

Cleanup of PCR Amplification

10. Add **50 µl of resuspended AMPure XP beads** to the PCR reactions (~ 50 µl). Mix well by pipetting up and down at least 10 times.
11. Incubate for 5 minutes at room temperature.
12. Carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads)
13. Wash twice with **200 µl of 80% freshly prepared ethanol** to the PCR plate while in the DiaMag02 - magnetic rack (Cat. No. B04000001). Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
14. Air dry the beads for 10 minutes while the PCR plate is on the magnetic stand with the lid open.
15. Elute DNA target from beads into **33 µl 10 mM Tris-HCl, pH 8.0 or 0.1X TE**. Mix well by pipetting up and down at least 10 times.
16. Dilute the library 5 fold with **water**, and check the size distribution on an Agilent high sensitivity chip.

Detailed protocol

STEP 1. Cell collection and DNA-protein crosslinking

The protocol below is intended for adherent cells. Collect suspension cells by centrifugation and continue with the protocol starting from step 6.

1. Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.
2. Remove the medium and rinse the cells with pre-warmed PBS (10 ml for a 75 cm2 culture flask). Gently shake the flask for 2 minutes.
3. Remove the PBS and add sterile trypsin-EDTA to the culture flask to detach adherent cells from the bottom. Table 4 shows the required amount of trypsin for different numbers of cells. Gently shake the culture flask for 1-2 minutes or until the cells start to detach. The time needed may depend on the cell type but do not continue trypsin treatment longer then necessary as prolonged treatment with trypsin may damage the cells. Regularly check if the cells start to detach.

Table 4			
# of cells	3x 10e6 cells	10e7 cells	5x 10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

4. Immediately add fresh culture medium to the cells when they are detached (Table 5). This will inactivate trypsin. Tansfer cell suspension to a 50 ml tube.

Table 5			
# of cells	3x 10e6 cells	10e7 cells	5x 10e7cells
Culture medium	2 ml	6 ml	30 ml

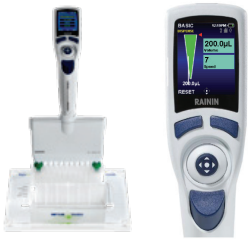
5. Rinse the flask by adding 10 ml of PBS. Add this volume to your 50 ml tubes containing cells from point 4.
6. Centrifuge for 5 minutes at 1600 rpm and 4°C and remove the supernatant.
7. Resuspend the cells in 20 ml of PBS and count them. Collect the cells by centrifugation for 5 minutes at 1600 rpm and 4°C.
8. Resuspend the cells in PBS to obtain a concentration of up to 10 million cells per 500 µl of PBS. If desired, the cell concentration can be decreased down to 1 million per 500 µl. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.
9. Add 13.5 µl of formaldehyde 37% to each tube containing 500 µl of cell suspension. Mix by gentle vortexing and incubate for 8 minutes at room temperature to allow fixation to take place.
10. Add 57 µl of **Glycine** to the cells to stop the fixation. Mix by gentle vortexing and incubate for 5 minutes at room temperature. Keep the cells on ice from this point onwards.
11. Collect the cells by centrifugation at 1600 rpm for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.
12. Wash the cells twice with 1 ml of cold PBS.

STEP 2. Cell lysis and chromatin shearing

13. Add 1 ml of ice-cold **Lysis buffer iL1** to the 1.5 ml tube containing 10 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 9 ml of **buffer iL1** and incubate for 10 minutes at 4°C with gentle mixing. If the starting amount of cells was less than 10 million, scale down accordingly (e.g. use a total of 5 ml **buffer iL1** for 5 million cells).
14. Pellet the cells by centrifugation at 1,600 rpm for 5 minutes and 4°C and discard the supernatant.

15. Add 1 ml of ice-cold **Lysis buffer iL2** and resuspend the cells by pipetting up and down several times. Add another 9 ml of **buffer iL2** and incubate for 10 minutes at 4°C with gentle mixing. Scale down accordingly when using less then 10 million cells.
16. Pellet the cells again by centrifugation for 5 minutes at 1,600 rpm (500 x g) and 4°C and discard supernatant.
17. Add 200x **protease inhibitor cocktail** to **Shearing buffer iS1**. Prepare 1 ml of complete shearing buffer per tube of 10 million cells. Keep on ice.
18. Add 1 ml of complete **Shearing buffer iS1** to 10 million cells. Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1 million cells per 100 µl **buffer iS1**. Split into aliquots of 100 to 300 µl and transfer the cell suspension to **1.5 ml TPX microtubes** (Diagenode Cat. No. M-50001). Incubate on ice for 10 minutes. Vortex and spin down the samples.
19. 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.Optimization is needed depending on the cell type and Bioruptor® system used.
20. Centrifuge at 13,000 rpm (16,000 x g) for 10 minutes and collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. If desired, the chromatin shearing effciciency can be analysed at this step (see the protocol in Additional protocols).

STEP 3. Magnetic immunoprecipitation



The immunoprecipitation step can also be performed using the semi-automated **ChIPettor™ System.** If doing so, please refer to the corresponding protocol delivered with this product. Alternatively, the protocol can also be downloaded from the website www.diagenode.com

This protocol has been optimised for 1 million cells per ChIP. Although it is possible to use more cells, we recommend performing separate ChIP reactions and pool the samples before purification of the DNA.

21. Determine the total number of IP's in the experiment. Please note that we recommend including one negative control in each experiment (IP with the IgG negative control). Take the required amount of **DiaMag Protein A-coated magnetic beads** (20 µl/IP). Dilute the **5x ChIP buffer iC1** with ChIP-seq grade water to obtain **1x ChIP buffer iC1**. The total amount of **1x ChIP buffer iC1** needed is 9 times the volume of beads required for the experiment. Place the diluted **ChIP buffer iC1** on ice.
22. Wash the beads 4 times with twice the volume of ice-cold **1x ChIP buffer iC1**. To wash the beads add **1x ChIP buffer iC1**, resuspend the beads by pipetting up and down several times and place the tubes in the **1.5 ml Diagenode magnetic rack** (Cat. No. kch-816-015). Wait for one minute to allow the beads to be captured by the magnet and remove the supernatant. Repeat this 3 times. Alternatively, centrifuge the tubes for 5 minutes at 1,300 rpm, discard the supernatant and keep the bead pellet.
23. After the last wash, resuspend the beads in the original volume **1x ChIP buffer iC1**.
24. Prepare the ChIP reaction mix according to Table 5. If required, NaBu (20 mM final concentration) or other inhibitors can also be added. Use 2 µl of the **rabbit IgG control antibody** for the negative control IP. If a positive control IP is included in the experiment, use 1 µl of the **H3K4me3 ChIP-seq grade control antibody**. When preparing the reaction mix, place 1 µl of the sheared chromatin aside to be used as an input the next day.

Table 6. ChIP reaction mix							
Number. of IP's	5% BSA (µl)	200x Protease inhibitor cocktail (µl)	5x buffer iC1 (µl)	Sheared chromatin (1e10 cells) (µl)	Magnetic beads (µl)	ChIP-seq grade water (µl)	Antibody (µl)
1	6	1.5	56	100	20	116.5-x	x
2	12	3	112	200	40	233-x	x
4	24	6	224	400	80	466-x	x
6	36	9	336	600	120	699-x	x
8	48	12	448	800	160	932-x	x

25. Incubate the tubes overnight at 4°C under constant rotation at 40 rpm on a rotating wheel.

26. The next morning, after the overnight incubation, briefly spin the tubes and place them in the **magnetic rack**. Wait for one minute and remove the supernatant. Wash the beads with **Wash buffer iW1**. To wash the beads, add 350 µl of iW1, gently shake the tubes to resuspend the beads and incubate for 5 minutes on a rotating wheel at 4°C.

27. Repeat the wash as described above once with **Wash buffer iW2, iW3 and iW4** using the same buffer volume, respectively.

STEP 4. Elution, decrosslinking and DNA isolation

28. After removing the last wash buffer, add 400 µl of **Elution buffer iE1** to the beads and incubate for 30 minutes on a rotating wheel at room temperature.

29. Briefly spin the tubes and place them in the **magnetic rack**. Transfer the supernatant to a new tube and add 16 µl of **iE2 buffer**. Also add 399 µl **buffer iE1** and 16 µl **buffer iE2** to 1 µl of the input sample kept aside the day before. Incubate for 4 hours in a thermomixer at 1300 rpm and 65°C. If required, the incubation at 65°C can be performed overnight.

30. Pool samples if necessary.

    Note: Up to 2 samples can be easily pooled. If more than 2 samples need to be pooled, process each sample purification individually, pool final eluates at the end of the IPure purification and concentrate (e.g. using Microcon®)

31. Add 2 µl of **carrier** to each IP and input sample. Vortex briefly and perform a short spin.

32. Add 400 µl of 100% isopropanol to each IP and input sample. Vortex briefly and perform a short spin.

    Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.

33. Resuspend the provided **Magnetic beads** and transfer 15 µl to each IP and input sample.

    - Keep **Magnetic beads** in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
    - The final volume is now 817 µl per IPure reaction.

34. Incubate IP and input samples for 1 hour at room temperature on a rotating wheel (40 rpm).

35. Prepare the **Wash buffer 1** containing 50% isopropanol:

Wash buffer 1	
Wash buffer 1 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	4 ml

    - Never leave the bottle open to avoid evaporation.

36. Briefly spin the tubes, place in the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 1**. Close the tubes, invert the 8-tube strip to resuspend the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).
    - 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 into the **Diagenode Magnetic Rack**.
37. Prepare the **Wash buffer 2** containing 50% isopropanol as follows:

Wash buffer 2	
Wash buffer 2 w/o isopropanol	2 ml
Isopropanol (100%)	2 ml
Total volume	4 ml

    - Never leave the bottle open to avoid evaporation.
38. Wash the IP and input samples with the Wash buffer 2 as follows. Briefly spin the tubes, place into the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 100 µl **Wash buffer 2**. Close the tubes, invert the 8-tube strip to resuspend the beads and incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).
    - 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 DiaMag rack.

Note: This **Elution buffer (buffer C)** is suitable for direct qPCR analysis, whole genome amplification, chip hybridization and next generation sequencing.

39. Two elutions in 25 µl each (standard procedure.
    a. Briefly spin the tubes and place them into the **DiaMag1.5**, wait 1 minute and discard the buffer. Keep the captured beads and add per tube, 25 µl **buffer C**. Close the tubes, invert the 8-tube strip to resuspend the beads and incubate for 15 minutes at room temperature on a rotating wheel (40 rpm). Resuspend the pelleted beads using the pipet and make sure that you drop them on the bottom of the tube.
    b. Spin the tubes and place them into the **DiaMag1.5**, wait 1 minute and transfer the supernatants into a new labelled 1.5 ml tube. Keep the bead pellets on ice.
    c. Repeat the elution of the bead pellets for 15 min at room temperature on a rotating wheel (40 rpm) in 25 µl buffer C.
    d. Spin the tubes and place them into the **DiaMag1.5**, wait 1 minute and pool the supernatant with the corresponding IP or input sample (1.5 ml tube). Discard the beads.
        - Total elution volume for both IP and input samples is 50 µl (1.5 ml tube).
    e. Place the DNA on ice and proceed to any desired downstream applications, or store it at -20°C or -80°C until further use.
40. Take 5 µl (10%) of IP'd DNA and determine the concentration with 'Quant-IT dsDNA HS assay kit' using the Qubit system or a similar method.
41. Store the DNA at -20°C until you are ready to analyse it with qPCR or by high throughput sequencing.

STEP 5. Quantitative PCR analysis

Before sequencing the samples, we recommend analysing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. The kit contains a positive (GAPDH TSS) and negative (Myoglobin Exon 2) control primer pair which can be used for the positive control antibody provided in the kit (H3K4me3 ChIP-seq grade antibody) in SYBR®

Green qPCR assay using the protocol described below. Use your own method of choice for analysing the appropriate control targets for your antibodies of interest.

42. Prepare the qPCR mix as follows (20 µl reaction volume using the provided **control primer pairs**):

- 10 µl of a 2x SYBR® Green qPCR master mix
- 1 µl of primer mix
- 4 µl of water
- 5 µl IP'd or input DNA

Use the following PCR program: 3 to 10 minutes denaturation step at 95°C (please check carefully supplier's recommendations about Taq polymerase activation time), followed by 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72 °C. These conditions may require optimisation depending on the type of Master Mix or qPCR system used.

STEP 6. Library Preparation

Symbols

This caution sign signifies a step in the protocol that has multiple paths leading to the same end point but is dependent on a user variable, like the amount of input DNA.

Starting Material

5 ng–1 µg fragmented DNA.

iDeal Library Preparation End Prep

1. Mix the following components in a sterile nuclease-free tube:

*If DNA Input is less than 55.5 µl, complete with water up to 55.5 µl.

iDeal LibraryEnd Repair/dA-Tailing Enzyme Mix (green)	3.0 µl
iDeal Library End Repair/dA-Tailing Buffer (green)	6.5 µl
Fragmented DNA	55.5 µl*
Total volume	**65 µl**

2. Mix by pipetting followed by a quick spin to collect all liquid from the sides of the tube.

3. Place in a thermocycler, with the heated lid on, and run the follwing program:

- **30 minutes at 20°C**
- **30 minutes at 65°C**
- Hold at 4°C

Adaptor Ligation

If DNA input is < 100 ng, dilute the **iDeal Library Preparation Adaptor for Illumina*** 1:10 in sterile water use immediately to a final concentration of 1.5 µM.

1. Add the following components directly to the iDeal Library End Prep reaction mixture and mix well:

iDeal Library Ligation Master Mix (red)	15 µl
iDeal Library Adaptor for Illumina* (red)	2.5 µl
iDeal Library Ligation Enhancer (red)	1 µl
Total volume	**83.5 µl**

2. Incubate at **20°C for 15 minutes** in a thermal cycler.
3. Add **3 µl of iDeal Library Uracil Excision Reagent** (red) to the ligation mixture from step 3.
4. Mix well and incubate at **37°C for 15 minutes**.

Size Selection of Adaptor-ligated DNA

Size selection is optional. If the starting material is less than 50 ng, size selection is not recommended. If you are not performing size selection, proceed to the next page and perform clean up step prior to PCR amplification. The following size selection protocol is for libraries with 200 bp inserts only. For libraries with different size fragment inserts, refer to Table 1 for the appropriate volume of beads to be added. The size selection protocol is based on a starting volume of 100 µl.

1. Vortex **AMPure XP beads** to resuspend.
2. Add **13.5 µl dH_2O** to the ligation reaction for a 100 µl total volume.
3. Add **55 µl of resuspended AMPure XP beads** to the 100 µl ligation reaction. Mix well by pipetting up and down at least 10 times.
4. Incubate for 5 minutes at room temperature.
5. Quickly spin the tube and place the tube on the **DiaMag02 - magnetic rack** (Cat. No. B04000001) to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully transfer the supernatant containing your DNA to a new tube ***(Caution: do not discard the supernatant)***. Discard the beads that contain the unwanted large fragments.
6. Add **25 µl resuspended AMPure XP beads** to the supernatant, mix well and incubate for 5 minutes at room temperature.
7. Quickly spin the tube and place it on the **magnetic rack** to separate the beads from the supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant that contains unwanted DNA. Be careful not to disturb the beads that contain the desired DNA targets ***(Caution: do not discard beads)***.
8. Add **200 µl of 80% freshly prepared ethanol** to the tube while in the magnetic rack. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
9. Repeat Step 8 twice for a total of three washes.
10. Air the dry beads for 10 minutes while the tube is on the **magnetic rack** with the lid open.
11. Elute the DNA target from the beads into **28 µl of 10 mM Tris-HCl or 0.1 X TE, pH 8.0**. Mix well on a vortex mixer or by pipetting up and down. Quickly spin the tube and place it on a magnetic stand. After the solution is clear (about 5 minutes), transfer 23 µl to a new PCR tube for amplification.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the **iDeal Library PCR Master Mix** in the subsequent PCR step.

Table 1: Recommended conditions for bead based size selection.

LIBRARY PARAMETERS	Approximate insert size	150 bp	200 bp	250 bp	300 - 400 bp	400 - 500 bp	500 - 700 bp
	Total Library size (insert + adaptor)	270 bp	320 bp	400 bp	400 - 500 bp	500 - 600 bp	600-800 bp
VOLUME TO BE ADDED (µl)	1st Bead Selection	65	55	45	40	35	30
	2nd Bead Selection	25	25	25	20	15	15

Alternatively, Cleanup of Adaptor-ligated DNA without Size Selection

1. Vortex **AMPure XP** beads to resuspend
2. Add **86.5 µl resuspended AMPure XP beads** to the ligation reaction. Mix well by pipetting up and down at least 10 times. Vortex AMPure XP beads to resuspend.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it on the **DiaMag02 - magnetic rack** (Cat. No. B04000001) to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads).
5. Add **200 µl of 80% freshly prepared ethanol** to the tube while in the **magnetic rack**. Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 twice, for a total of three washes.
7. Air the dry beads for 10 minutes while the tube is on the magnetic stand with the lid open.
8. Elute the DNA target from the beads by adding **28 µl of 10 mM Tris-HCl, pH 8.0 or 0.1X TE**.

Note: Be sure not to transfer any beads. Trace amounts of bead carry over may affect the optimal performance of the polymerase used in the **iDeal Library PCR Master Mix** in the subsequent PCR step.

9. Mix well by pipetting up and down, or on a vortex mixer.
10. Quickly spin the tube and place it on the magnetic stand.
11. After the solution is clear (about 5 minutes), transfer **23 µl to a new PCR** tube for amplification.

PCR Amplification

1. Mix the following components in sterile strip tubes:

Adaptor Ligated DNA Fragments	23 µl
iDeal Library PCR Master Mix (blue)	25 µl
iDeal Library Index Primer* (blue)	1 µl
iDeal Universal PCR Primer* (blue)	1 µl
Total volume	**50 µl**

* These primers (Index Primer Set 1) are included. Index Primer Set 2 may be purchased separately (Cat. No. C05010021).

PCR cycling conditions

1. Mix the following components in sterile strip tubes:

Cycle Step	Temp	Time	Cycles
Initial Denaturation	98°C	30 seconds	1
Denaturation	98°C	10 seconds	6 - 15*
Annealing	65°C	30 seconds	
Extension	72°C	30 seconds	
Final Extension	72°C	5 minutes	1
Hold	4°C	∞	

* Suggestion: 6 PCR cycles for 1 µg DNA input 10 cycles for 50 ng, and 13-15 for 5 ng DNA input. Further optimization of PCR cycle number may be required

Cleanup of PCR Amplification

1. Vortex **AMPure XP beads** to resuspend.
2. Add **50 µl of resuspended AMPure XP beads** to the PCR reactions (~ 50 µl). Mix well by pipetting up and down at least 10 times.
3. Incubate for 5 minutes at room temperature.
4. Quickly spin the tube and place it on DiaMag02 - magnetic rack (Cat. No. B04000001) to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully remove and discard the supernatant. Be careful not to disturb the beads that contain DNA targets (Caution: do not discard beads)
5. Add 200 µl of 80% ethanol to the PCR plate while in the DiaMag02 - magnetic rack (Cat. No. B04000001). Incubate at room temperature for 30 seconds, and then carefully remove and discard the supernatant.
6. Repeat Step 5 once.
7. Air dry the beads for 10 minutes while the PCR plate is on the magnetic stand with the lid open.
8. Elute DNA target from beads into **33 µl 10 mM Tris-HCl, pH 8.0 or 0.1X TE**. Mix well by pipetting up and down at least 10 times. Quickly spin the tube and place it on an appropriate magnetic stand to separate beads from supernatant. After the solution is clear (about 5 minutes), carefully transfer 28 µl supernatant to a new PCR tube. Store libraries at –20°C.
9. Dilute the library 5 fold with **water**, and check the size distribution on an Agilent high sensitivity chip.

Quantitative PCR data interpretation

The efficiency of chromatin immunoprecipitation of particular genomic loci can be expressed as the recovery of that locus calculated as the percentage of the input (the relative amount of immunoprecipitated DNA compared to input DNA). If the amount used for the input was 1% of the amount used for ChIP, the recovery can be calculated as follows:

% recovery = $2^{(Ct_{input} - Ct_{sample})}$

Ct_{sample} and Ct_{input} are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and input, respectively. This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results the real amplification efficiency, if known, should be used.

For the positive control antibody (e.g. H3K4me3) the recovery of the positive control target (GAPDH TSS locus) is expected to be between 10 and 20% although this will depend on the cell type used. The recovery of the negative control target (Myoglobin exon 2 locus) should be below 1%.

Criteria to decide whether the sample is good enough for sequencing will be largely target dependant. Therefore, the following are only general guidelines:

- the recovery of the positive control target should be at least 5%
- the recovery of the negative control target should be below 1%
- the ratio of the positive versus the negative control target should be at least 5

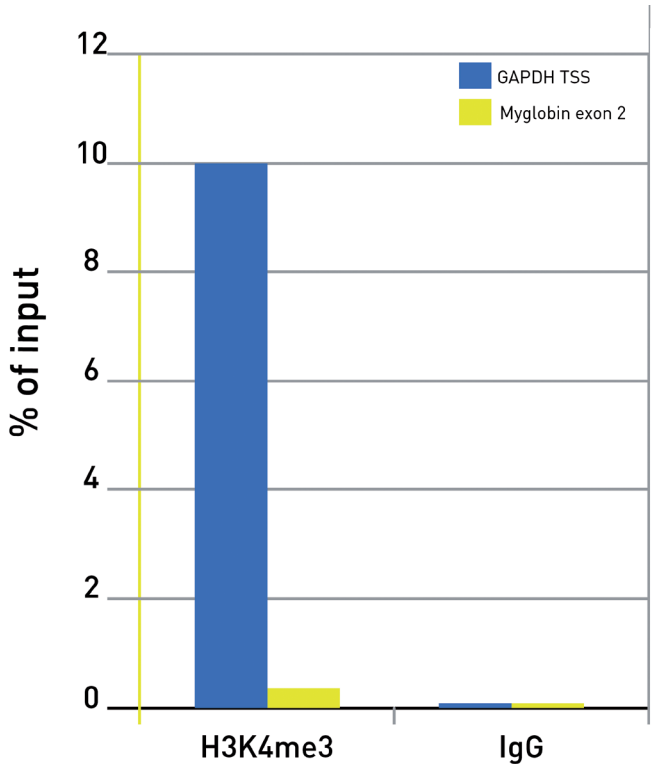


Figure 2: ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit. Sheared chromatin from 1 million cells, 1 µl of the positive control antibody and 2 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control GAPDH-TSS and the negative control Myoglobin exon 2 primer sets from the kit. The recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

ChIP-sequencing

The iDeal protocol has been optimised for ChIP-seq on Illumina® platforms.

Please, do not hesitate to contact our customer support team if you have any questions about the design of your ChIP-seq experiment or the bioinformatics data analysis.

Contact for Europe, Asia, Oceania and Africa:
custsupport@diagenode.com

Contact for North and South America:
custsupport.na@diagenode.com

ChIP-seq data analysis recommendations

To find the captured regions of the genome after the sequencing you must perform a) a reference alignment followed by b) a peak calling, then c) further data analysis (annotation, visualization etc.) to help you find what you are looking for. There are abundant software tools for each task that use different approaches to the same problem; choose your preferred one considering your dataset and scientific goals. The workflows for different sequencers basically differ only in the alignment step, since every sequencer has its own characteristic read set (short or long, fixed or variable length, nucleotide or colour code etc.).

a) The built-in aligners with default settings worked very well for our ChIP-seq experiments (e.g. **ELAND** for Illumina®®, **TMAP** for PGM). If you cannot access them, open source tools are also available; we have positive experience with **BWA**: http://bio-bwa.sourceforge.net. If you use a multipurpose aligner, do not forget to use settings appropriate to your dataset; please consult with the manual of your software.

b) The purpose of the peak calling is to find the enriched regions in the alignment. Take extreme care when you choose and set up your peak caller, since the outcome can vary widely depending on the used software and its settings. We advise you to read the comparative literature and the software manuals to fully understand how a certain program works. One of the key features of your data is the expected length of the enrichment regions. Transcription factors tend to produce short and sharp peaks, while histone marks create broad islands of enrichment. A remarkable tool for sharp peak detection is **MACS**, while **SICER** is dedicated to histone marks, and tools like **ZINBA** can be used for both with decent outcomes. **MACS 2** is reported to be better suited for histone marks than previous versions.

The availability of the mentioned softwares:

- **MACS**: http://liulab.dfci.harvard.edu/MACS
- **MACS 2**: https://github.com/taoliu/MACS/tree/master/MACS2
- **SICER**: http://home.gwu.edu/~wpeng/Software.htm
- **ZINBA**: http://code.google.com/p/zinba

We are extensively using **MACS 1.4.1** for our experiments. While it is a prominent tool for shorter peaks, sometimes it has difficulties with broader regions, therefore we recommend you to set a wider local peak background and lower the pvalue cutoff if necessary for histone marks. In some cases turning off the local lambda calculation provides a better coverage of broad enrichment islands, though this can result in more false positive peaks detected. Please refer to the MACS manual (http://liulab.dfci.harvard.edu/MACS/README.html) if you are not sure how to tweak the parameters.

c) Having your peaks you can start decrypting the epigenetic code.

The visual inspection is a common first step, especially if the aim of your experiment was to see if certain genes have certain histone modifications/transcription factors attached, or you want to check some positive/negative control sites for enrichment. Choose the appropriate viewer software according to the output format of your peak caller and your preferences.

Annotation is always very useful, since you can identify biological features that are relevant to your peaks, or check if you have the peaks at the expected loci, like H3K4me3 enrichments in the promoter regions of active genes. You can expand the annotation with a gene ontology/pathway analysis of the peak associated genes, thus discovering how your transcription factor/histone modification is involved in the cell's or the whole organism's life.

Motif search is almost an obligatory analysis for the sequence specific transcription factors, but you may find common motifs among histone modification sites as well, so you can check for example if you indeed have promoter specific motifs in your theoretically promoter specific enrichments.

A lot of programs, including peak callers themselves output descriptive statistics of the peaks, measuring for example their enrichment ratios, significances, width, heights, reads in peaks. This characterization helps you better understand your data, which is essential for most applications; a typical example is the comparison of performance of different sample preparation protocols or different sequencer setups.

The final recommended analysis type is the comparative analysis. We encourage scientists to use replicates in their experiments; removing peaks that are not common could effectively reduce false positives. You can also use a validated reference set of peaks for comparisons, but that is rarely available. Additionally, if you have other biologically relevant data from your samples, it is wise to compare and integrate them. For example, an RNA-seq dataset is a great source of validation for histone marks that are supposed to regulate gene expression.

Recommended free tools for the peak analysis:

- **IGV** (visualization): http://www.broadinstitute.org/igv
- **UCSC Genome Browser** (visualization): http://genome.ucsc.edu
- **HOMER** (motif search, annotation, gene ontology, comparison, statistics): http://biowhat.ucsd.edu/homer
- **PinkThing** (annotation, conservation, comparison, gene ontology, statistics): http://pinkthing.cmbi.ru.nl
- **GREAT** (annotation, statistics): http://great.stanford.edu

When analysing ChIP-seq, please always keep an eye on sequencing quality and the performance of the software tools used for analysis. For example with a low quality sequencing with a lot of read errors you will have a hard time finding the peaks you are looking for, despite your excellent IP'd DNA. To control the quality use the **vendor supplied software** and metrics, like the ones available in the Illumina®® pipeline for GA II. Open source tools can also be used, e.g. the **FastQC** by Babraham Institute: http://www.bioinformatics.bbsrc.ac.uk/projects/fastqc.

Throughout this chapter we recommended some free tools, because they are accessible for everyone and we have tested most of them. Please note that there are commercial softwares for the same purposes as well, most of them capable of performing several tasks, or even a complete ChIP-seq workflow. Here are a few examples that we know of (but we have not tested them):

- **CLC Genomics Workbench**: http://clcbio.com
- **Partek Genomics Suite**: http://www.partek.com/partekgs
- **NextGENe**: http://www.softgenetics.com/NextGENe.html
- **Avadis NGS**: http://www.avadis-ngs.com
- **Geneious**: http://www.geneious.com/web/geneious/geneious-pro
- **GenoMiner**: http://www.astridbio.com/genominer.html
- **GenoMatix**: http://www.genomatix.de

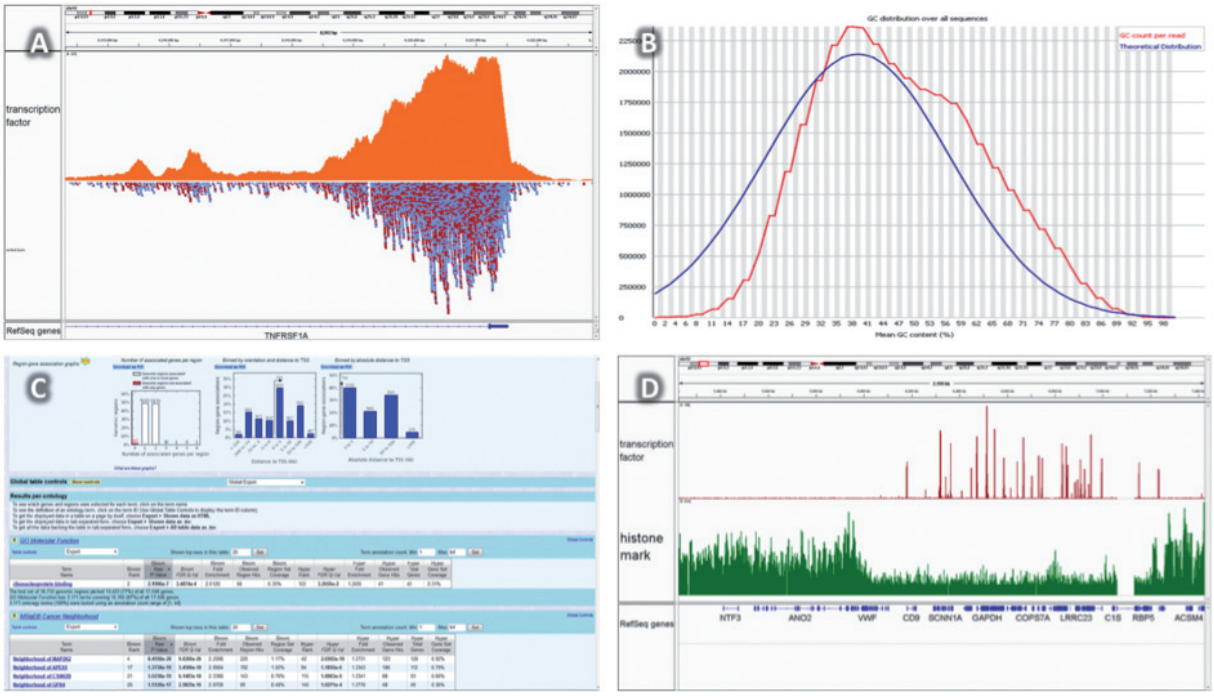


Figure 5

Various stages of bioinformatics data analysis

Representative images made during bioinformatics analysis of ChIP-seq data.

A: The reads are accumulating around the binding site to form a peak like structure in the coverage graph. Peak callers are used to detect these peaks.

B: A quality control software (like FastQC) anlyses numerous parameters that can help us assess the goodness of sequencing. Here we can monitor the GC content distribution.

C: Descriptive statistics and annotation output by GREAT.

D: Transcription factors tend to produce sharp peaks (upper red band), while broad enrichments are characteristic of many histone modifications (lower green band).

Aditional Protocols

Sheared chromatin analysis

Reagents not supplied with the iDeal ChIP-seq kit

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24 : 1)
- 100% Ethanol
- 70% Ethanol
- DNA precipitant (Cat. No. C03030002)
- DNA co-precipitant (Cat. No. C03030001)

1. Take an aliquot of 50µl of sheared chromatin and spin the chromatin 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 ChIP-seq grade water)
3. Add 2 µl of diluted RNase cocktail
4. Incubate 1h at 37°C.
5. Add 50µl of elution buffer iE1.
6. Add 4µl of elution buffer iE2, mix thoroughly.
7. Incubate samples at 65°C for 4h (or overnight)
8. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25 : 24 : 1). 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 DNA precipitant, 5 µl of co-precipitant, 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-suspended the pellet in 20 µl of TE buffer.
16. Run samples (20 µl of DNA + 4 µl of 6x loading dye) in a 1.5% agarose gel.

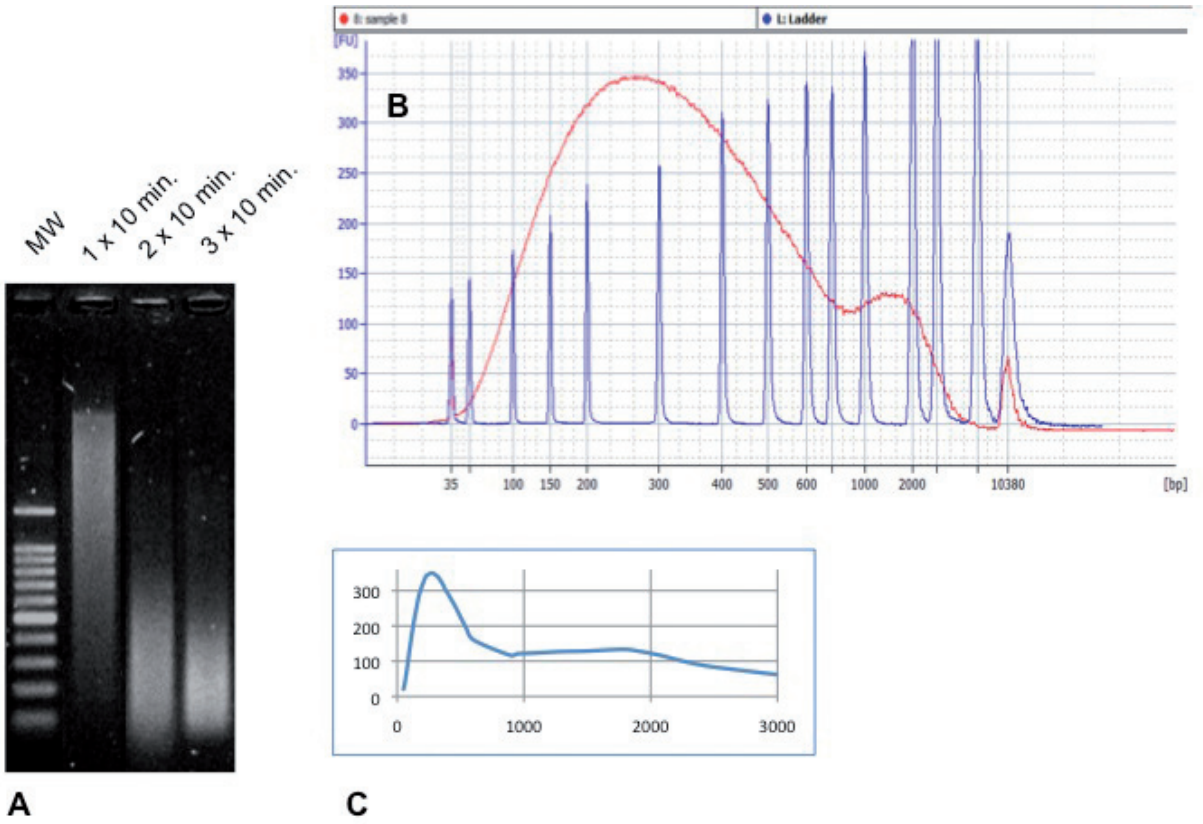


Figure 6

Superior chromatin shearing results with the Bioruptor® Plus using buffers and protocol of the Diagenode iDeal ChIP-seq kit

Hela cells were fixed with 1% formaldehyde (for 8 minutes at RT). Nucleus isolation of five million fresh or frozen (stored at -80°C) cells are performed using Buffers of the Diagenode iDeal ChIP-seq kit (Cat. No. AB-001-0001) and are then resuspended in 200µl of Shearing Buffer iS1 prior to chromatin shearing.

Samples are sheared during 1, 2 or 3 rounds of 10 cycles of 30 seconds "ON" / 30 seconds "OFF" with the Bioruptor® Plus combined with the Bioruptor® Water cooler (Cat No. BioAcc-cool) at HIGH power setting (position H). For optimal results, samples are vortexed before and after performing 10 sonication cycles, followed by a short centrifugation at 4°C. All samples were treated with RNase (see Additional Protocols).

Panel A: 10 µl of DNA (equivalent to 300 ng) are analysed on a 1.5% agarose gel.

Panel B and C: Sample 3 (3x 10 min.) was analysed on Bioanalyzer 2100 using DNA High Sensitivity chip. The default log scaled Bioanalyzer output can be seen in Panel A, while Panel C represents their linear transformation for better vizualisation. Out of range fragments were not shown on this graph.

In this example, the optimal shearing condition corresponds to 3 rounds of 10 cycles (30''ON/30'' OFF) corresponding to the optimal DNA size range for ChIP-seq analysis (100 to 600 bp).

Troubleshooting guide

Process	Protocol step	Issues and resolutions
Crosslinking and fixation	Optimize crosslinking time	Poor crosslinking causes DNA loss, elevated background, and/or reduced antigen availability in chromatin. Emperically determine optimal crosslinking time for maximal specificity and efficiency of ChIP. The optimal duration of cross-linking varies between cell type and protein of interest. Short crosslinking time (5-10 minutes) may improve shearing efficiency. Crosslinking duration should not exceed 30 minutes or shearing will be inefficient.
	Assure proper fixation time with formaldehyde	Crosslinking may be too weak or too strong without proper fixation time. Optimize fixation step e.g: incubate for 8 minutes at room temperature with high-quality, fresh 1% formaldehyde final concentration (weight/volume).
	Optimize formaldehyde concentration	Lower formaldehyde concentrations (1%weight/volume) may improve shearing efficiency. For some proteins, however, especially those that do not directly bind DNA, this might reduce crosslinking efficiency and thus the yield of precipitated chromatin. Empirically determine the formaldehyde concentration as some antigen epitopes may be more sensitive to formaldehyde.
Cell lysis	Make sure cells disrupt completely	Do not use too many cells per amount of lysis buffer (w/v) so that cells can be completely disrupted. Follow the instructions in the protocol (e.g.: 1 million cells or less/130 µl of complete Buffer B). See steps 2 and 3.
	Maintain cold temperature during lysis	Perform cell lysis at 4°C (cold room) or on ice. Always keep the samples ice cold during cell lysis and use cold buffers as in Step 3.
	Prevent protein degradation	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Determine which cell types have previously been validated with the kit	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells), U2OS and kerationocytes have been used to validate this magnetic ChIP protocol.
Number of cells required	Determine number of cells for ChIP	The number of cells for ChIP is determined by cell type, protein of interest, and antibodies used. Use chromatin from 1,000 to 10,000 cells per ChIP. (In some cases, chromatin from up to 100,000 cells may be needed). You may need to empirically determine the optimal number.
Chromatin shearing	Maintain 4°C temperature during shearing	Keep samples cold at 4°C before sonication to maintain sample integrity.
	Maintain 0ÐC temperature during sonication	Maintain temperature of the samples at 4°C to maintain sample integrity.
	Optimize SDS concentration	High % SDS favours better sonication but inhibits immunoselection (optimal range: 0.1% to 1%). 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 ChIP buffer.
	Determine amount of sheared chromatin needed for ChIP	Most of the sheared chromatin will be used for ChIP and the input control. A small amount will 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). Dilute the sheared chromatin at least 7-fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Sonication tips	Determine cell number	Start with 1x10e6 cells or less.
	Sonication conditions with the Bioruptor tips	Shear the samples of chromatin using the Bioruptor® for 1 to 3 runs of 5 to 10 cycles: [30 seconds "ON" / 30 seconds "OFF"]. 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.
	Chromatin shearing with a probe sonicator tips	Probe sonicator: Sonicate each sample for 3 x 30 seconds on ice. Allow 30 seconds pause on ice between each pulsing session. Avoid foaming.
	Chromatin shearing with Diagenode modules tips	You can also use the Chromatin shearing optimization kit - Low SDS (C01020010) for shearing.
	Shearing with other protocols tips	When using your own protocol,make sure the shearing buffer contains between 0.75% and 1% SDS, EDTA (1-10 mM) and/or EGTA (0-0.5 mM) with pH 7.6-8.0. The sheared chromatin is to be diluted in the Buffer A and Buffer B prior to immunoselection.

Gel analysis of sheared chromatin	Load enough DNA on gel	Chromatin equivalent to at least 100,000 cells can be be visualized on a gel. Do not use an excessive amount or it will obscure the visualization. The DNA amount to load depends on well size and on the gel size.
	Use correct agarose concentration	Use a 1-1.5% agarose gel.
	Use correct running buffer concentration and run time	1x TAE or TBE is preferred to 0.5x TAE (which can lead to smears). Run slowly.
Antibody-bead binding	Make sure beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Use proper bead centrifugation methods	Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. g= (1.118 x 10-5) x r x rpm2 where r is the radius (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.
	Store beads at 4°C	Store at 4°C. Do not freeze.
	Determine 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 and other inhibitors	Store protease inhibitors properly	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C and thawed before use.
	Always use fresh, complete buffers	Add protease inhibitor mix to buffers, just before use, in PBS (Steps 2 and 3), Buffer B (Step 3), in Buffer A (Step 3). Discard within 24 hours.
	Use other inhibitors as needed.	Add phosphatase inhibitors or others to Buffers A and B, if necessary, depending on your research field and protein(s) of interest. Add NaBu for histone ChIPs.
Negative ChIP controls	Use non-immune IgG in the IP incubation mix.	Use the non-immune IgG fraction from the same species the antibodies were produced in as a negative control.
	Do not add antibody to the IP to serve as a negative control	Incubation with uncoated beads could also be used as a negative ChIP control (see Step 4).
	Use an unblocked antibody and specifically blocked antibody in parallel	Use one antibody in ChIP and the same antibody that is blocked with specific peptide. To specifically block 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.
	Determine number of negative controls needed	If multiple antibodies of the same species are to be used with the same chromatin preparation then a single negative ChIP control is sufficient for all of the antibodies used.
	Antibody-antigen recognition may affect ChIP	Antibody-antigen recognition can be significantly affected by crosslinking resulting in loss of epitope accessibility and/or recognition.
Antibody in IP	Use ChIP-grade antibodies, include controls, and test antibodies before ChIP	Use ChIP-grade antibodies or several antibodies directed against different epitopes of the same protein. Verify that the antibodies work directly in IP on fresh cell extracts. When testing new antibodies, include known ChIP-grade antibodies as a positive control. 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.
	Determine amount of antibody per ChIP	Empirically determine amount of target and antibody. For abundant proteins, like histones, use 1-2 µg of affinity purified or monoclonal antibody per IP. For other targets, use up to 10 µg per ChIP. Efficient IPs result from optimal ratios between the amount of chromatin and the amount of antibody. More antibody (or less chromatin) can be required with 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.

<table>
<tr><td></td><td>Concerns about antibodies binding to protein A or protein G.</td><td>There is a significant difference in affinity of different types of immunoglobulins to protein A or G. Therefore, in function of the antibody used for your ChIP, it is recommended to choose either protein A or protein G coated beads.

<table>
<tr><th>Species</th><th>Immunglobulli Isotype</th><th>Protein A</th><th>Protein G</th></tr>
<tr><td>Human</td><td>IgG1</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG2</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG3</td><td>-</td><td>+++</td></tr>
<tr><td></td><td>IgG4</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgGM</td><td colspan="2">Use anti Human IgM</td></tr>
<tr><td></td><td>IgGF</td><td>-</td><td>+</td></tr>
<tr><td></td><td>IgGA</td><td>-</td><td>+</td></tr>
<tr><td>Mouse</td><td>IgG1</td><td>+</td><td>+++</td></tr>
<tr><td></td><td>IgG2a</td><td>+++</td><td>+++</td></tr>
<tr><td></td><td>IgG2b</td><td>++</td><td>++</td></tr>
<tr><td></td><td>IgG3</td><td>+</td><td>+</td></tr>
<tr><td></td><td>IgGM</td><td colspan="2">Use anti Mouse IgM</td></tr>
<tr><td>Rat</td><td>IgG1</td><td>-</td><td>+</td></tr>
<tr><td></td><td>IgG2a</td><td>-</td><td>+++</td></tr>
<tr><td></td><td>IgG2b</td><td>-</td><td>++</td></tr>
<tr><td></td><td>IgG2c</td><td>+</td><td>++</td></tr>
<tr><td>Chicken</td><td>All Isotypes</td><td>-</td><td>++</td></tr>
<tr><td>Cow</td><td>All Isotypes</td><td>++</td><td>+++</td></tr>
<tr><td>Goat</td><td>All Isotypes</td><td>-</td><td>++</td></tr>
<tr><td>Guinea Pig</td><td>All Isotypes</td><td>+++</td><td>++</td></tr>
<tr><td>Hamster</td><td>All Isotypes</td><td>+</td><td>++</td></tr>
<tr><td>Horse</td><td>All Isotypes</td><td>++</td><td>+++</td></tr>
<tr><td>Pig</td><td>All Isotypes</td><td>+</td><td>++</td></tr>
<tr><td>Rabbit</td><td>All Isotypes</td><td>+++</td><td>++</td></tr>
<tr><td>Sheep</td><td>All Isotypes</td><td>-</td><td>++</td></tr>
</table>
</td></tr>
<tr><td rowspan="5">Immunoselection incubation</td><td>Understanding the benefits of using an ultrasonic water bath</td><td>The use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to antibodies, the typical rate-limiting step in ChIP.</td></tr>
<tr><td>Buying an ultrasonic bath</td><td>See http://www.bransonic.com/model_3510.asp: Branson Cat. No. CPN-952316 or Fisher Scientific Cat. No.15-337-22F.</td></tr>
<tr><td>Determine water bath specifications</td><td>Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W</td></tr>
<tr><td>Optimize the incubation time with an ultrasonic bath</td><td>Incubation of 15-30 minutes is usually sufficient but may differ depending on antibody-target kinetics. A longer incubation may be required in some cases.</td></tr>
<tr><td>Using the kit without an ultrasonic water bath</td><td>Without the bath, a long incubation at 4°C should be used. Depending on the antibody and target, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.</td></tr>
<tr><td rowspan="5">PCR tips</td><td>Optimize primer design</td><td>Primer length: 18 to 24 nucleotides and primer Tm: 60°C (+/-3.0°C)/ % GC: 50% (+/-4%)</td></tr>
<tr><td>Include negative and positive controls</td><td>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.</td></tr>
<tr><td>Troubleshoot high Ct values</td><td>Use more input chromatin in the case of high Ct values.</td></tr>
<tr><td>Determine the ratio between Ct(NegCtl) and Ct(Target)</td><td>The ratio between target IP and negative control IP depends on the antibody used.</td></tr>
<tr><td>Minimize high background</td><td>Keep the antibody binding beads in suspension during the experiment. Check by eye that equal pellets of beads are present in each tube. Washes (step 4) are critical.</td></tr>
<tr><td>Sample storage and freezing</td><td>Using end-point PCR analysis rather than quantitative PCR</td><td>If gel electrophoresis is used to estimate intensities of PCR products, the 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.</td></tr>
<tr><td></td><td>Samples can be frozen at several steps of the protocol</td><td>Snap freeze and thaw on ice (e.g. fixed cell pellets and sheared chromatin). 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. Purified DNA from ChIP and input samples can be stored at -20°C for months. Avoid multiple freeze/thawing.</td></tr>
</table>

Ordering information

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