Transcription Factor Targeted ChIP-seq Kit – KOA0882 and KOA0883

Chromatin Immunoprecipitation (ChIP) coupled with high-throughput parallel sequencing as a detection method (ChIP-seq) is one of the primary methods for epigenomic research to investigate protein-DNA interaction on a genome-wide scale. This technique can be used in a variety of life science disciplines including cellular differentiation, tumor suppressor gene silencing, and the effect of histone modifications on gene expression. The transcription factor targeted ChIP-Seq Kit provides optimized reagents, simplified protocols and validation controls which facilitates successful ChIP-seq.

I. KIT COMPONENTS

ChIP-Seq Kit for Histones				
Component	**Catalog Number**	**Size**		**Storage**
		(X10) KOA0882	**(X24) KOA0883**	
Glycine Solution	KOA0880A	4.4mL	8.8mL	4°C
Lysis Buffer Two	KOA0880C	50mL	100mL	4°C
Protease Inhibitor Cocktail One	KOA0880E	38µl	80µl	-20°C
5% BSA (DNA free)	KOA0880G	380µl	800µl	-20°C
Protein-A Coated Magnetic Beads	KOA0880H	300µl	720µl	4°C Do NOT Freeze
Wash Buffer One	KOA0880I	3.5mL	8.4mL	4°C
Wash Buffer Two	KOA0880J	3.5mL	8.4mL	4°C
Wash Buffer Three	KOA0880K	3.5mL	8.4mL	4°C
Wash Buffer Four	KOA0880L	3.5mL	8.4mL	4°C
Elution Buffer One (Room temperature)	KOA0880M	1.5mL	3.4mL	4°C
Elution Buffer Two	KOA0880N	64µl	144µl	4°C
Rabbit IgG (control isotype antibody)	KOA0880O	4µl	8µl	4°C
Myoglobin Exon 2 Primer Pair (negative control)-Human-ChIP-Seq Grade	KOA0880R	42µl	96µl	-20°C
Water-ChIP-Seq Grade	KOA0880S	14mL	26.6mL	Ambient
CTCF Antibody ChIP-seq Grade	KOA0882B	4µg	8µg	-20°C
H19 imprinting control primer ChIP	KOA0882C	42µl	96µl	-20°C
Carrier Solution	KOA0882D	32µl	72µl	-20°C
Shearing Buffer Two	KOA0882E	3.4mL	6.7mL	4°C
Fixation Buffer One	KOA0882F	4mL	8mL	4°C
5X Chromatin IP Buffer Two	KOA0882K	3.4mL	6.9mL	4°C
Lysis Buffer Three	KOA0882L	30mL	60mL	4°C

DNA Purification Kit				
Component	**Catalog Number**	**Size**		**Storage**
		(X10) KOA0882	**(X24) KOA0883**	
Wash Buffer One w/o Isopropanol	KOA0882G	900µL	2mL	4°C
Wash Buffer Two w/o Isopropanol	KOA0882H	700µL	2mL	4°C
Buffer C Solution	KOA0882I	700µL	1.6mL	4°C
Magnetic Beads	KOA0882J	180µl	400µl	4°C

II. STORAGE AND STABILITY CONDITIONS

Store the components at the indicated temperature upon receipt. Store Protein-A Coated Magnetic Beads and Magnetic Beads at 4°C. DO NOT FREEZE magnetic beads because they may become damaged. Keep the beads in liquid suspension during storage as drying will result in reduced performance.

III. INTRODUCTION

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

In ChIP assay, living cells are first fixed with a reversible cross-linking 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 cross-linking, chromatin needs to be sheared very efficiently into homogeneous small fragments that can subsequently be used in immunoprecipitation (IP). The sonication device is most widely used for chromatin fragmentation and provides high quality sheared chromatin ready-to-ChIP. Shearing may also be accomplished with shearing kits which enable an easy 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 beads. Finally, the precipitated DNA fragments are released from the AB, and analyzed. 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.

IV. REQUIRED EQUIPMENT AND REAGENTS

Equipment:

- Magnetic rack (for 1.5mL microfuge tubes) (part number: TMS-06 or TMS-32)
- Sonication device

- Polymethylpentene microfuge tubes (1.5mL)
- Refrigerated centrifuge for 1.5mL, 15mL and 50mL tubes Cell counter
- Rotator (Rotating wheel)
- Cell counter
- Vortex
- Thermomixer
- Qubit system (Invitrogen)
- qPCR cycler

Reagents:

- Gloves to wear at all steps
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS) buffer
- 1 M Sodium butyrate (NaBu) (optional)
- RNase/DNase-free 1.5mL tubes
- 100% isopropanol
- Trypsin-EDTA
- qPCR SYBR® Green Mastermix
- Reagents for library preparation and sequencing
- Quant-IT dsDNA HS assay kit (Invitrogen)

V. KIT METHOD OVERVIEW AND TIME TABLE

ChIP Protocol Overview			
Step	**Description**	**Time needed**	**Day**
1	Cell collection and DNA-protein cross-linking (for cultured cells)	1 to 2 hours	1
2	Cell lysis and chromatin shearing (for cultured cells)	1 to 2 hours	1
3	Magnetic immunoprecipitation	Overnight	1-2
4	Elution, de-cross-linking 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

VI. GENERAL CONSIDERATIONS

1. Cell number

This protocol has been optimized for ChIP on 4,000,000 cells in 350µl ChIP reaction. For lower amounts of cells, simply dilute the chromatin in shearing buffer before adding it to the IP reaction. It is possible to use more cells, however, for optimal performance, we recommend performing separate ChIP assays and pooling the IP'd DNA before purification.

2. Shearing optimization and sheared chromatin analysis

Before starting the ChIP, the chromatin should be sheared into fragments of 100 to 600 bp. Our kits and protocols are optimized for chromatin shearing using the ChIP sonicator. The maximum volume for shearing is 300µl per 1.5mL Microtube (depending on the specific type). 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. It is important to perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation.

3. Magnetic beads

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

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

4. Negative and positive IP controls

The kit contains a negative (IgG) and a positive (CTCF) 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 CTCF antibody at least once. The kit also contains qPCR primer pairs for amplification of a positive and negative control target for CTCF (H19 imprinting control and hMyoglobin primer ChIP, 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 CTCF antibody on 4,000,000 HeLa cells is approximately 20 ng.

6. Quantitative PCR

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

In order to have sufficient DNA left for sequencing, we recommend using no 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. CTCF) the recovery of the positive control target (H19 imprinting control region) is expected to be approximately 5% although this will depend on the cell type used. The recovery of the negative control target (Myoglobin exon 2 locus) should be below 0.5%.

Criteria to decide whether the sample is good enough for sequencing will be largely target dependent.

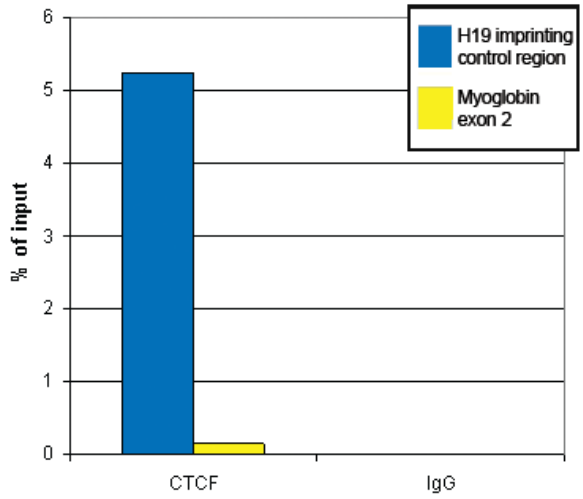

Figure 1. ChIP was performed on human HeLa cells using the control antibodies from the ChIP-seq kit. Sheared chromatin from 4 million cells, 0.5µl of the positive control antibody and 1µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control H19 imprinting control region 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).

VII. SHORT PROTOCOL FOR EXPERIENCED USERS

Step 1. Cell collection and DNA-protein cross-linking (for cultured cells)

1. Dilute formaldehyde in Fixation Buffer One to a final concentration of 11%.
2. Add 1/10 volume of diluted formaldehyde directly to the cell culture medium.
3. Incubate at room temperature for 10 to 20 minutes with gentle shaking.
4. Stop the fixation by adding 1/10 volume of Glycine Solution.

Step 2. Cell lysis and chromatin shearing (for cultured cells)

5a. For Adherent cells (~25 million cells)

- Remove the medium and wash the cells once with 20mL of PBS.
  - Keep everything at 4°C from this step on.

- Add 5mL of cold Lysis Buffer Two to the plate and collect the cells by scraping.
- Rinse the flask with an additional 20mL of Lysis Buffer Two.
- Incubate at 4°C for 20 minutes.

5b. For Suspension cells (~25 million cells)

- Collect the cells by centrifugation at 1,600 rpm and 4°C for 5 minutes.
- Wash the cells once with 20mL of PBS.
    - Keep everything at 4°C from this step on.
- Resuspend the cells in 25mL of cold Lysis Buffer Two.
- Incubate at 4°C for 20 minutes.

6. Discard the supernatant and resuspend the cells in 15mL Lysis Buffer Three. Incubate at 4°C for 10 minutes with gentle mixing.
7. Centrifuge at 1,600 rpm and 4°C for 5 minutes.
8. Add 1/200 volume protease inhibitor cocktail (PIC) to Shearing Buffer Two.
9. Discard the supernatant and resuspend the cells in Shearing Buffer Two + PIC to a final concentration of 15 million cells/mL. Resuspend by pipetting up and down.
10. Shear chromatin by sonication. An initial time course experiment is highly recommended.
    - Sonicate samples using either high power setting for 10-30 cycles (30 seconds ON, 30 seconds OFF; Stop the system after each run of 10 cycles, vortex and spin down sample) or sonicate for 5-15 cycles (30 seconds ON, 30 seconds OFF) depending on the sonicator. Vortexing is not required between runs.
11. Transfer samples to new 1.5mL tubes and centrifuge at 13,000 rpm for 10 min.
12. Collect the supernatant which contains the sheared chromatin.
13. Take an aliquot of 100µl for assessment of chromatin shearing. The remaining chromatin can be stored at -80°C for up to 2 months for further use in the immunoprecipitation.

Step 3. Magnetic Immunoprecipitation

14. Prepare 4mL of 1x ChIP Buffer Two. Add 80µl of 5% BSA DNA free.
15. Take the required amount of Protein A-coated magnetic beads (30µl/IP) and wash three times with 1mL of ice-cold 1x ChIP Buffer Two + BSA.
16. Resuspend the beads after the last wash in the original volume of 1x ChIP Buffer Two + BSA.
17. Set aside 1µl (1%) of the sheared chromatin to use as input sample and keep at 4°C.
18. Prepare the following ChIP reaction mix (per 1 IP):
    - 6µl of 5% BSA
    - 1.8µl of 200x protease inhibitor cocktail
    - 20µl of 5x ChIP Buffer Two
    - 20µl of Protein A-coated magnetic beads
    - Xµl ChIP-seq grade antibody
    - 42.2µl of ChIP-seq grade water
19. Incubate 2-4 hours (or overnight) at 4°C on a rotating wheel.

20. Add 250µl sheared chromatin. Keep 2.5µl chromatin aside to serve as input.
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 350µl ice-cold Wash Buffer One and incubate for 5 min at 4°C on a rotating wheel. Discard the wash buffer using the magnetic racks.
24. Repeat the wash as described above once with Wash Buffer Two, Wash Buffer Three, and Wash Buffer Four, respectively.

Step 4. Elution, de-cross-linking and DNA purification

25. After removing the last wash buffer, add 400µl of Elution Buffer One to the beads and incubate for 30 min on a rotating wheel at room temperature.
26. Briefly spin the tubes and place them in a magnetic rack. Transfer the supernatant to a new tube and add 16µl of Elution Buffer Two. Also add 397.5µl Elution Buffer One and 16µl of Elution Buffer Two to the 2.5µl input sample. Incubate for 4 hours or overnight in a thermomixer at 1300 rpm at 65°C.
27. Purify the DNA using the DNA purification kit.
28. Add 2µl of carrier solution to each IP and input sample. Vortex briefly and perform a brief spin.
29. Add 400µl of 100% isopropanol to each IP and input sample.
    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 15µl 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 with Isopropanol – One containing 50% isopropanol as follows:

Wash Buffer with Isopropanol – One		
Components	**24 reactions**	**100 reactions**
Wash Buffer One w/o Isopropanol	2mL	8mL
Isopropanol (100%)	2mL	8mL
Total Volume	4mL	16mL

33. Briefly spin the tubes, place the tubes in a magnetic rack, wait 1 min and discard the buffer. Add 100µl Wash Buffer with Isopropanol – One in each tube. Close the tubes, incubate for 5 minutes at room temperature on a rotating wheel (40 rpm).
34. Prepare the Wash Buffer Two containing 50% isopropanol as follows:

Wash Buffer with Isopropanol – Two		
Components	**24 reactions**	**100 reactions**
Wash Buffer Two w/o Isopropanol	2mL	8mL
Isopropanol (100%)	2mL	8mL
Total Volume	4mL	16mL

35. Briefly spin the tubes, place tubes in a magnetic rack, wait 1 min and discard the buffer. Keep the captured beads and add 100µl Wash Buffer with Isopropanol - Two per tube. Close the tubes, resuspend the beads and incubate for 5 min at room temperature on a rotating wheel (40 rpm).
36. Two elutions in 25µl (total volume 50µl)

    Briefly spin the tubes and place them in a magnetic rack, wait 1 minute and discard the buffer. Keep the captured beads and add 25µl Buffer C Solution per tube. 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 in a magnetic rack, wait 1 min and transfer the supernatants into a new labelled 1.5mL tube. Keep the bead pellets on ice.
38. Repeat the elution of the bead pellets for 15 minutes at room temperature on a rotating wheel (40 rpm) in 25µl Buffer C Solution.
39. Spin the tubes and place them in a magnetic rack, wait 1 min and pool the supernatant with the corresponding IP or input sample (1.5mL tube). Discard the beads.
40. Place the DNA on ice and proceed to any desired downstream application, or store the DNA at -20°C or -80°C until further use.

Step 5. Quantitative PCR analysis

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

Use the following PCR program:

- 3 to 10 min 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 optimization depending on the type of Master Mix or qPCR system used.

VIII. DETAILED PROTOCOL

Step 1. Cell Collection and DNA-Protein Cross-linking (for cultured cells)

(Day 1 ~30 min)

1. Dilute formaldehyde in Fixation Buffer One to a final concentration of 11%, e.g. add 5mL of a 37% formaldehyde solution to 11.8mL Fixation Buffer One. For a T175 culture flask you will need ~2mL of diluted formaldehyde.
2. Add 1/10 volume of the diluted formaldehyde directly to the cell culture medium.
3. Incubate the cells for 10 to 20 minutes at room temperature with gentle shaking. The fixation time can depend on your target of interest.
4. Add 1/10 volume of Glycine Solution to the cell culture medium to stop the fixation. Incubate for 5 minutes at room temperature with gentle shaking.

Note: The fixed cells can be stored at -80°C for up to 4 months. However, we strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP for ChIP-sequencing.

Step 2. Cell lysis and chromatin shearing (for cultured cells)

(Day 1 ~1-2hrs)

5a. For Adherent cells (~25 million cells)

- Remove the medium and wash the cells once with 20mL of PBS.
  - Keep everything at 4°C from this step on.
- Add 5mL of cold Lysis Buffer Two to the plate and collect the cells by scraping.
- Rinse the flask with an additional 20mL of Lysis Buffer Two and add this to the collected cells. The total volume of Lysis Buffer Two should be about 10mL per 10^7 cells (e.g. for a T175 culture flask (~25 million cells), rinse with an additional 20mL of Lysis Buffer Two.
- Incubate at 4°C for 20 minutes.

5b. For Suspension cells (~25 million cells)

- Collect the cells by centrifugation at 1600 rpm and 4°C for 5 minutes. Discard the cell culture medium.
- Wash the cells once with PBS. Resuspend the cells in 20mL of PBS, centrifuge at 1600 rpm and 4°C for 5 minutes and discard the supernatant.
  - Keep everything at 4°C from this step on.
- Resuspend the cells in 1 ml of ice-cold lysis buffer two by pipetting up and down several times. Add 24 ml of buffer two to obtain a total volume of 25 ml per 25 million cells (for up or down scaling use 1 ml of lysis buffer two per 1 million cells).
- Incubate at 4°C for 20 minutes with gentle mixing on a rotator. Pellet the cells by centrifugation at 1600rpm and 4°C for 5 minutes and discard the supernatant. Resuspend the cell pellet in 1 ml of ice-cold Lysis buffer three by pipetting up and down several times. Add 14 ml of Lysis buffer three and incubate for 10 minutes at 4°C with gentle mixing on a rotator (for up or down scaling, use 600 µl of Lysis buffer three per 1 million of cells).

6. Pellet the cells by centrifugation at 1,600 rpm for 5 minutes and 4°C and discard the supernatant.
7. Add 8.4µl of 200x protease inhibitor cocktail to 1.67ml of Shearing Buffer Two. Prepare 2mL of complete shearing buffer per tube of 25 million cells. Keep on ice.
8. Add 1mL of complete Shearing Buffer Two + PIC to 15 Million cells. Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1.5 Million cells per 100µl Shearing Buffer Two. Split into aliquots of 100-300µl and transfer the cell suspension to 1.5mL microtubes with caps optimized for chromatin shearing.
9. Shear chromatin by sonication. An initial time course experiment is highly recommended.
   - Sonicate samples using either high power setting for 10-30 cycles (30 seconds ON, 30 seconds OFF; Stop the system after each run of 10 cycles, vortex and spin down sample) or sonicate for 5-15 cycles (30 seconds ON, 30 seconds OFF) depending on the sonicator. Vortexing is not required between runs.
10. Transfer samples to new 1.5mL tubes and centrifuge at 13,000 rpm (16,000 x g) at 4°C for 10 minutes.
11. Pool the supernatant which contains the sheared chromatin.
12. Take an aliquot of 100µl for assessment of chromatin shearing. The remaining chromatin can be stored at -80°C for up to 2 months for further use in the immunoprecipitation.

Step 1. Tissue disaggregation and DNA-protein Cross-linking (For Tissues)

(Day 1 ~30min)

1. Equilibrate the Fixation Buffer One to room temperature before use.

2. Prepare the cross-linking solution in a fume hood by adding 54µl of 37% formaldehyde to 2mL of Fixation Buffer One to a final concentration of 1%. Use 2 mL of Fixation Buffer One for one chromatin preparation.
3. Put 200mg of fresh or frozen tissue in a petri dish on ice. Always keep samples on ice and minimize the time of manipulation to prevent sample degradation.
4. Chop the tissue into small pieces (btw 1-3mm^3) using a scalpel blade and disaggregate sample using a Dounce homogenizer or TissueLyzer.

   For Dounce homogenizer: Transfer tissue pieces into a Dounce homogenizer. Add 1 ml of formaldehyde diluted in Fixation buffer. Immediately disaggregate the tissue using a Dounce homogenizer (loose fitting pestle) to get a homogeneous suspension. Set the timer for a total fixation time of 15 minutes and start deducting the fixation time from this point.

   Transfer the tissue suspension into a 15 ml tube. Rinse the Dounce homogenizer (or 2 ml tubes for Tissue Lyser option) with an additional 1 ml of diluted formaldehyde and pool with the sample in the same 15 ml tube.

   For Tissue Lyser: Transfer tissue pieces to 2 ml tubes, supplemented with 2 stainless steel beads (5 mm) and immerse in liquid nitrogen for a few minutes. Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 8 hours at -80°c) and operate the TissueLyser for 2-4 minutes at 25-30 Hz.

   Add 2 ml of the cross-linking solution directly to the tissue lysate and start deducing the fixation time from this point, 15 minutes for the total fixation time.
5. Incubate for a total time of 15 minutes at room temperature with gentle rotation.

   NOTE: The fixation time might require an additional optimization.
6. Add 200 µl of Glycine to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing. Proceed to the next step immediately.

   NOTE: We strongly recommend using freshly fixed tissue for preparation of sheared chromatin prior to ChIP. If not possible, the fixed tissue can be stored at -80°C for up to 4 months. To freeze the tissue, remove the medium and wash the tissue once with 2 ml of PBS. Store the tissue pellet at -80°C.

Step 2. Cell Lysis and Chromatin shearing derived from tissue samples (For Tissues)

(Day 1 ~1-2hrs)

NOTE: An additional 100 µl of protease inhibitors cocktail is required per chromatin preparation

7. Centrifuge samples at 2100rpm for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet.
8. Wash the pellet with 10 ml of ice-cold PBS and centrifuge samples at 850 x g for 5 minutes at 4°C. Gently discard the supernatant. Keep everything at 4°C or on ice from now on.
9. Add 50 µl of 200x protease inhibitor cocktail to 10 ml of ice-cold Lysis buffer two. This is a complete lysis buffer two needed for 200 mg of tissue.
10. Add 1 ml of ice-cold complete Lysis buffer two to the pellet and resuspend by pipetting up and down several times. Add the remaining amount of complete buffer two.
11. Incubate at 4°C for 20 minutes with gentle mixing on a rotator.
12. Pellet the cells by centrifugation at 2100rpm for 5 minutes at 4°C and discard the supernatant.
13. Add 50 µl of 200x protease inhibitor cocktail to 10 ml of ice-cold Lysis buffer three. This is a complete Lysis buffer three needed for 200 mg of tissue. Add 1 ml of ice-cold complete Lysis buffer

three to the cell pellet and resuspend the cells by pipetting up and down several times. Add the remaining amount of complete buffer three.

14. Incubate at 4°C with gentle mixing on a Rotator for 10 minutes.
15. Pellet the cells again by centrifugation at 2100rpm for 5 minutes at 4°C and discard supernatant.
16. Add 8.4 µl of 200x protease inhibitor cocktail to 1.67 ml of Shearing buffer two. This is a complete Shearing buffer needed for 200 mg cells. Keep on ice.
17. Add the complete Shearing buffer two to the pellet. Resuspend the cells by pipetting up and down several times and incubate on ice 10 minutes.

Optional: if the suspension is not homogenous after pipetting, an additional homogenization using a Dounce homogenizer or Tissue Lyser could be performed. Homogenize using a dounce homogenizer (tight pestle) or TissueLyser.

For Dounce homogeniser, transfer the suspension to the homogeniser, perform several strokes to get a homogeneous suspension and proceed with the sonication as described below.

For TissueLyser, transfer the suspension to 2 ml tubes, supplemented with 2 stainless steel beads (5 mm) pre-cooled at 4°C. Insert 2 ml tubes into TissueLyser Adaptors and operate the TissueLyser for 2-4 minutes at 25 Hz. Proceed with the sonication as described below.

18. Split the cell suspension into aliquots of maximum 300 µl by transferring it to the appropriate 1.5 ml microtubes:

    a. When using the Bioruptor® Plus use 1.5 ml TPX Microtubes

    b. When using the Bioruptor® Pico use 1.5 ml Bioruptor® Microtubes with Caps

    NOTE: The maximum volume for shearing with the Bioruptor® is 300 µl per 1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.

19. Shear the chromatin by sonication using the Bioruptor®. Choose the protocol which is adapted to your device:
    19.1. When using the Bioruptor® Plus, shear for 10 to 30 cycles [30 seconds “ON”, 30 seconds “OFF”] each at High power setting.
    19.2. When using the Bioruptor® Pico shear for 5 to 12 cycles [30 seconds “ON”, 30 seconds “OFF”].
    19.3. NOTE: We recommend performing pilot experiments for each new sample type using the Chromatin EasyShear Kit - Low SDS
20. Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at x g at 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin.
21. Take an aliquot of 50 µl of sheared chromatin for the shearing assessment. The protocol for chromatin shearing analysis is described in a separate section.

    NOTE: We recommend analyzing the shearing for each batch of chromatin. This step can be omitted when optimal sonication settings for given experimental conditions (cell type, cell number and fixation) have been optimized previously. The analysis of chromatin shearing can be done in parallel with the following steps of the protocol or can be included in the main workflow starting from decross-linking. Store the chromatin aliquot at -20°C until analysis.

22. Use chromatin in immunoprecipitation or store it at -80°C for up to 2 months.

Step 1. Cell collection and DNA-protein Cross-linking (For Yeast)

1. Grow an overnight culture (50 ml) of yeast cells in appropriate liquid medium until the A600 reaches OD 0.6 – 1.0. 1.2.
2. Equilibrate the Fixation Buffer to room temperature before use.
3. Prepare the cross-linking solution in a fume hood by mixing 1.7 ml of 32% paraformaldehyde to 3.3 ml of Fixation Buffer and add these 5 ml of cross-linking reagent to the yeast culture. Incubate the cells for 15 minutes at room temperature with gentle shaking.

   NOTE: The fixation time might require an additional optimization.

4. Add 5.5 ml of Glycine to the yeast culture (proportion of 1:10 to stop the fixation). Incubate for 5 minutes at room temperature with gentle shaking. Proceed to the next step immediately.
5. Centrifuge in a 50 ml falcon tube for 5 minutes at 4500 rpm and remove supernatant.
6. Resuspend the cell pellet in 25 ml cold (4°C) TBS buffer (20 mM Tris-HCl pH 7.4, 150 mM NaCl).
7. Centrifuge in a 50 ml falcon tube for 5 minutes at 4500rpm and remove supernatant.

   NOTE: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the fixed cells can be stored at -80°C for up to 2 months. To freeze the cells, remove the medium, wash the cells once with 20 ml of PBS. Add another 5 ml of PBS, collect the cells by centrifugation at 500 x g for 5 minutes and 4°C and discard the supernatant. Store the pellet at -80°C.

Step 2. Cell Lysis and Chromatin shearing (For Yeast)

8. Resuspend the cells in 3 ml of ice-cold Lysis Buffer three by pipetting up and down several times.
9. Incubate at 4°C for 20 minutes with gentle mixing on a rotator. Pellet the cells by centrifugation at 4500rpm for 5 minutes at 4°C and discard the supernatant.
10. Resuspend the cell pellet in 2 ml of ice-cold Lysis Buffer three by pipetting up and down several times. and incubate for 10 minutes at 4°C with gentle mixing on a rotator.
11. Pellet the cells again by centrifugation for 5 minutes at 4500rpm and 4°C and discard supernatant.
12. Add 1.5 µl of 200x protease inhibitor cocktail to 298.5 µl of Shearing Buffer two. This is a complete Shearing Buffer needed for 1 sample. Keep on ice. 2.6. Transfer the liquid into a 2 ml screw cap tubes for Fastprep bead beater containing 300 µl of sterile glass beads (425-600 µm).
13. Break cells in a Bead beater Fastprep-24® device at 4°C (3 cycles of 30 sec at maximum speed. Put the tubes in ice for 2 minutes after each cycle).
14. Fix the Fastprep tubes at the top of 15 ml tubes. Punch a hole at the bottom of the Fastprep tubes using a needle. Centrifuge at 2150rpm for 1 minute at 4°C to collect the liquid in the 15 ml tubes.
15. Transfer into a 1.5 ml Bioruptor sonication tubes and shear by sonication. Choose the protocol which is adapted to your device and tubes:

    When using the Bioruptor Pico and 1.5 ml Bioruptor Microtubes, sonicate for 5-12 cycles 30 '' ON/30'' OFF.

    When using the Bioruptor Plus and 1.5 ml TPX Bioruptor Microtubes (cat # C30010010-300), sonicate for 10-30 cycles 30 '' ON/30'' OFF at High Power.

    NOTE: The maximum volume for shearing with the Bioruptor is 300 µl per 1.5 ml Microtube. The use of correct type of microtubes is essential for the efficiency of sonication.

16. Gently spin down sample at low speed and transfer the supernatant in a new 1.5 ml tube.

17. Centrifuge 10 minutes at 9000rpm and 4°C, then transfer the supernatant in a new 1.5 ml tube. The supernatant contains the sheared chromatin that can be used for immunoprecipitation and stored at -80°C for several weeks.

Step 3. Magnetic Immunoprecipitation (for Cells, Tissues, and Yeast)

(Day1-2 overnight)

18. Determine the total number of IP's in the experiment. Please note that we recommend to include one negative control in each experiment (IP with the IgG negative control).
19. Prepare 4mL of 1X Chromatin IP Buffer Two by adding 3.2mL Water ChIP-seg Grade to 0.8mL 5X Chromatin IP Buffer Two. Add 80µl of 5% BSA Solution DNA Free. Keep the diluted Chromatin IP Buffer Two on ice.
20. Take the required amount of Protein A-coated magnetic beads (30µl/IP) and wash three times with 1mL of ice-cold 1x Chromatin IP Buffer Two + BSA. To wash the beads, add 1x Chromatin IP Buffer Two + BSA, resuspend the beads by pipetting up and down several times and incubate at 4°C with gentle shaking for 5 min. Spin the tubes and place them in the magnetic rack for 1 minute and remove the supernatant. Repeat this two times. Alternatively, you can centrifuge the tubes for 5 minutes at 1,300 rpm, discard the supernatant and keep the bead pellet.
21. Resuspend the beads after the last wash in the original volume of 1x Chromatin IP Buffer Two + BSA.
22. Set aside 30µl of the sheared chromatin to use as input sample and keep at 4°C.
23. Prepare the ChIP reaction mix (per IP) according to the following table.

ChIP Reaction Mix					
Number of IPs	**5% BSA (µl)**	**200x PI Cocktail (µl)**	**5x Buffer Two (µl)**	**Water ChIP-seg Grade (µl)**	**ChIP-seq grade AB (x)**
1	6	1.8	20	42.2-x	X
2	12	3.3	40	84.4-x	X
4	24	7.2	80	168.8-x	X
6	36	10.8	120	253.2-x	X
8	48	14.4	160	337.6-x	X

24. Add the antibody to the reaction mix.

    Note: The total volume of the ChIP reaction mix per IP is 70 µl.
25. Use 1µl of the IgG negative control antibody for the negative control IP.

    If a positive control IP is included in the experiment, use 1µl of the CTCF positive control antibody.

If required, NaBu (20 mM final concentration) or other inhibitors can be added.

26. Add 70 µl of ChIP reaction mix to the individual tubes containing 30 µl of washed Magnetic Beads - Protein A Coated. Incubate the tubes for 2-4 hours at 4°C under constant rotation at 40 rpm on a rotating wheel.

27. Briefly spin tubes containing ChIP reaction then add 250µl of sheared chromatin. Keep 2.5µl chromatin aside to serve as an input the next day. Incubate the tubes overnight at 4°C under constant rotating at 40rpm on a rotation wheel.
28. The next day, briefly spin the tubes and perform washes:
    - 28.1. Place tubes in the magnetic rack for 1 minute and discard the supernatant.
    - 28.2. Add 350µl ice-cold Wash Buffer One to the tubes, resuspend beads by gently shaking.
    - 28.3. Incubate for 5 min at 4°C on a rotating wheel.
    - 28.4. Discard the wash buffer using the magnetic racks.
    - 28.5. Repeat the wash as described above once with Wash Buffer Two, Wash Buffer Three, and Wash Buffer Four, respectively.

Step 4. Elution, decross-linking and DNA purification (for Cells, Tissues, and Yeast)

(Day 2 ~5hrs)

NOTE: Before the first use of the kit, prepare Wash buffer isopropanol one and Wash buffer isopropanol two by adding an equal volume of isopropanol. Wash buffer isopropanol one and Wash buffer isopropanol two should be stored at 4°C. Never leave the bottle open during storage to avoid evaporation.

29. After removing the last wash buffer, add 100µl of Elution Buffer One to the beads and incubate for 30 min on a rotating wheel at room temperature.

NOTE: If a precipitation is observed in Elution buffer one, warm it at 37°C until it becomes clear. This will not impair the reaction.

30. Briefly spin the tubes and place them in a magnetic rack for one minute. Transfer the supernatant to a new tube and add 4µl of Elution Buffer Two. Also add 97.5µl Elution Buffer One and 4µl of Elution Buffer Two to the 2.5µl input sample. Incubate for 4 hours or overnight in a thermomixer at 1300 rpm at 65°C. If required, the incubation at 65°C can be performed overnight.
31. 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 purification and concentrate.

32. Add 2µl of carrier solution to each IP and input sample. Vortex briefly and perform a brief spin.
33. Add 100µl of 100% isopropanol to each IP and input sample. Vortex 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.
34. Resuspend the provided magnetic beads and transfer 10µl to each IP and input sample.
    - Keep magnetic beads in suspension during storage at 4°C and at all handling steps, as drying out will result in reduced performance.
    - The final volume is now 817µl per reaction.
35. Incubate IP and input samples for 10mins at room temperature on a rotating wheel (40 rpm).
36. Briefly spin the tubes, on the magnetic rack, wait 1 minute and discard the buffer.
37. Add 100 µl of Wash buffer isopropanol one (completed with isopropanol) per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room

temperature. Briefly spin the tubes and place in the magnetic rack, wait 1 minute and discard the buffers.

38. Add 100 µl of Wash buffer isopropanol two (completed with isopropanol) per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the magnetic rack, wait 1 minute and discard the buffer.
39. Do not disturb the captured beads attached to the tube wall.
40. Spin the tubes again and place them on the magnetic rack. Discard the remaining Wash buffer isopropanol two if necessary. Resuspend the beads pellet in 25 µl of Buffer C. Incubate at room temperature for 15 minutes on the rotator.

    NOTE: Buffer C is compatible with down-stream applications such as qPCR analysis and library preparation for Next-Generation sequencing.

41. Spin the tubes and place them into the rotator, wait 1 minute and transfer the supernatant containing the immunoprecipitated DNA into a new labelled 1.5 ml tubes. Discard the beads.
42. Place the DNA on ice and take 2 µl of IP'd DNA to determine the concentration with Qubit® dsDNA HS Assay Kit or a similar method.
43. Determine the total number of regions to be analyzed by qPCR for each sample. Take the required volume of INPUT and immunoprecipitated samples for qPCR analysis. Take into account that these samples will be diluted 1/10 and 5 µl will be used per PCR reaction.

    NOTE: The dilution of samples and the volume per PCR may vary depending on a sensitivity of a Master Mix and qPCR cycler used.

44. Store the remaining DNA at -20°C until further use.

Step 5. Quantitative PCR analysis (for Cells, Tissues, and Yeast)

(Day 2 ~2-3hrs)

Before sequencing the samples, we recommend analyzing the IP'd DNA by qPCR using at least 1 positive and 1 negative control target. The kit contains a positive (H19 imprinting control region) and negative (Myoglobin Exon 2) control primer pair which can be used for the positive control antibody provided in the kit (CTCF ChIP-seq grade antibody) in SYBR® Green qPCR assay using the protocol described below. Use your own method of choice for analyzing the appropriate control targets for your antibodies of interest.

45. Take an aliquot of immunoprecipitated DNA and a corresponding INPUT (step 4.13) and dilute them 1/10 using ChIP-seq grade water.
46. Prepare the qPCR mix as follows (20µl reaction volume using the provided control primer pairs):
    - 10µl of a 2x SYBR® Green qPCR master mix
    - 1µl of primer mix
    - 4µl of water
    - 5µl IP'd or input DNA
47. Use the following PCR program:

    NOTE: These conditions may require optimization depending on the type of Master Mix, qPCR system used and user provided primer pair.

Step	Time/Cycles		Temperature
1. Denaturation	3-10 min*		95°C
2. Amplification	30 sec	40 cycles	95°C

	30 sec		60°C
	30 sec		72°C (acquire fluorescence data)
3. Melting curve**	Follow qPCR instrument manufacturer recommendations		

* Please carefully check supplier's recommendations about Taq polymerase activation time.

** Include and inspect the melting curves based on the protocols recommended by the qPCR instrument manufacturer to ensure that primer pairs amplify only a single specific product.

48. Record the threshold cycles (Ct values) from the exponential phase of the qPCR for the IP'd DNA sample and input for each primer pair.
49. Calculate the relative amount of immunoprecipitated DNA compared to INPUT DNA for the control regions (% of recovery) using the following formula:

    % recovery = 2^((Ctinput – 6.64) - Ctsample) * 100%

    Ctsample and Ctinput are the threshold cycles from the exponential phase of the qPCR for the IP'd DNA sample and INPUT, respectively.

    2 is the amplification efficiency

    6.64 is a compensatory factor to correct the input dilution

    NOTE: This equation assumes that the PCR is 100% efficient (amplification efficiency = 2). For accurate results, the amplification efficiency with given primer pair has to be close to 100% meaning that for each cycle the amount of product is doubles (E=2). The real amplification efficiency, if known, should be used. The formula takes into account that 1% of INPUT was used as suggested in the protocol (2.5 µl INPUT vs 250 µl of chromatin per IP). If the amount of INPUT used is different from 1%, an introduction of a compensatory factor in the formula is required to correct the input dilution (x) as follows:

    %recovery = 2^[(Ct(input)-log2(X%) Ct(sample)]x100%

    Where: log2(x) accounts for the INPUT dilution

    Example: If you use an INPUT of 5 µl from 250 µl of chromatin used per IP, it corresponds to 50 X dilution. The compensatory factor is equal to log2(50)=5.64 and the formula to calculate the recovery will be as follows: %recovery = 2^[(Ct(input)-5.64 Ct(sample)]x100%.

IX. TROUBLESHOOTING

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/
	Cross-linking is too strong.	

		volume). Also, use high quality, fresh formaldehyde.
	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 cannot 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 57µl of 1.25M glycine per 513.5µl of sample. 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.
	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/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 10^6 cells or less is ok) 2) Keep samples cold (4°C) 3) High % SDS favors 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 sonicator.	Maintain temperature of the samples close to 0°C. The chromatin shearing needs to be optimized for each cell type.
Sheared chromatin analysis	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. (Add 870µl of complete Buffer A to the 130µl of sheared chromatin). Dilute the sheared chromatin at least 7 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	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. g = 11.18 x r x (rpm/1000) ^2; knowing that r is the radius of rotation in mm. It is possible to centrifuge the 1.5mL tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze

	Antibody binding capacity	Polyclonal antibody from rabbit, guinea pig, pig, human IgG. Monoclonal antibody from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage	Some inhibitors are unstable in solution. The provided protease inhibitor 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 could also be used as a negative ChIP control as well as non-immune IgG.
	Use antibody and specifically blocked antibody in parallel.	Use one antibody in ChIP 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 the antibody specificity by Western blot analysis. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.

	Are my antibodies going to bind the protein A or protein G?	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. **See table below:**
Immuno-selection incubation	What is the best incubation time for immuno-selection 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 immuno-selection work with using the ultrasonic water bath?	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?	Capacity: 5.5 liters. Size (L x W x H): 29 x 15 x 15 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 time of incubation ranges 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 (+/- 3.0ºC)/ % 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 properly perform 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 are critical.
	Using end-point PCR rather than quantitative PCR	If gel electrophoresis is used to estimate intensities of PCR products, then relative occupancy of a factor at a locus is the ratio of the intensity of the target IP band to the negative control IP band.
Freezing	Samples can be frozen at several steps of the protocol	Pellets of formaldehyde fixed cells can be stored at - 80°C for at least a year. Sheared chromatin can be stored at -80°C for months, depending on the protein of interest to be ChIP'd. Purified DNA from ChIP and input samples can be stored at -20°C for months.
	Avoid multiple freeze/thaw cycles	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin)

X. AFFINITY OF PROTEIN A and PROTEIN G TO DIFFERENT IgG SUBCLASSES

Species	Immunoglobulin isotype	Protein A	Protein G
Human	IgG1	++	++
	IgG2	++	++
	IgG3	-	++
	IgG4	++	++
	IgA	+	-
	IgD	+	-
	IgE	+	-
	IgM	+	-
Mouse	IgG1	+	++
	IgG2a	++	++
	IgG2b	++	++
	IgG3	+	++
	IgM	+/-	-
Rat	IgG	++	++
	IgG1	+/-	+
	IgG2a	+/-	++

	IgG2b	+/-	+
	IgG2c	+/-	+
	IgM	+/-	-
Rabbit	IgG	++	++
Hamster	IgG	+	++
Guinea pig	IgG	++	+
Bovine	IgG	+	++
Horse	IgG	+	++
Sheep	IgG	+/-	++
Goat	IgG	+/-	++
Pig	IgG	++	++
Chicken	IgG	-	+/-

XI. RELATED PRODUCTS

Catalog #	Product Name	Size
600-401-I57	Anti-Histone H3 [Monomethyl Lys4] (RABBIT) Antibody	50µg
MB-064-1000	ELISA Microwell Blocking Buffer with Stabilizer (Azide and Mercury Free)	1L
TMBE-100	TMB ELISA PEROXIDASE SUBSTRATE	100mL
TMS-06	6 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x6
TMS-15-50	15/50 Tube TrueBlot® Magnetic Separator	1x15, 1x50
TMS-32	32 position Microcentrifuge Tube TrueBlot® Magnetic Separator	1x32