Instruction Manual Version 3 01.14

Transcription ChIP kit

Transcription Chromatin Immunoprecipitation kit

Cat. No. **C01010090** (kch-redTBP-012)

diagenode

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Contents

Introduction

Chromatin Immunoprecipitation (ChIP) is a technique allowing the analysis of the association of proteins with specific genomic regions in the context of intact cells. ChIP is used to determine changes in epigenetic signatures, chromatin remodeling and transcription regulator recruitments (1).

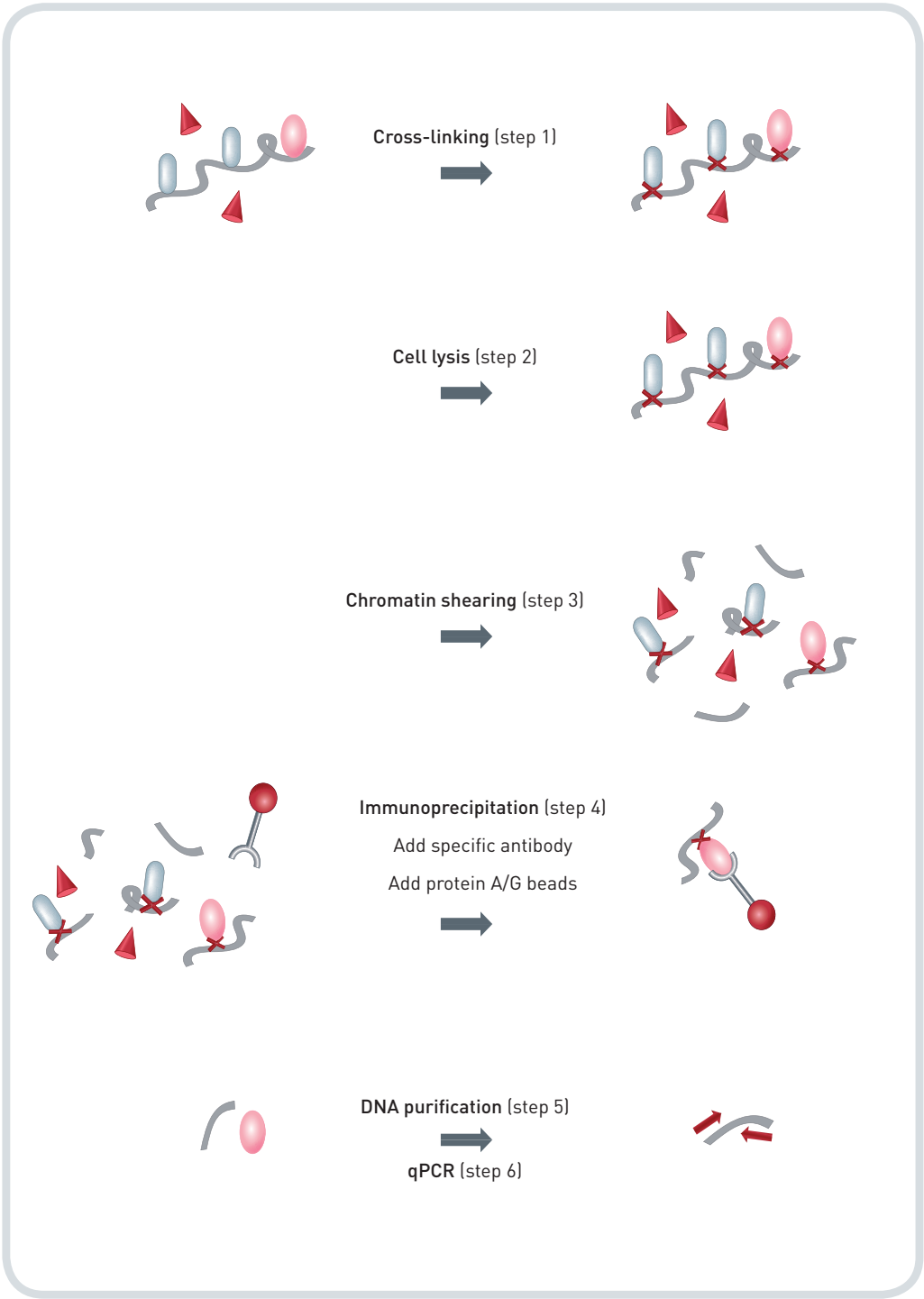
In brief, cells are fixed with a reversible cross-linking agent. Next, the cross-linked chromatin (DNA-Protein) is sheared and the DNA fragments associated with the protein of interest are immunoprecipitated (IP'd) using specific antibodies. Finally, DNA-Protein cross-links are reversed and the DNA is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

The most widely used approach to fix DNA-Protein interactions in the living cell is by formaldehyde fixation (cross-linking) that generates covalent bonds between amino or imino groups of proteins and nucleic acids (2). Following cross-linking, chromatin needs to be sheared very effectively into homogeneous small fragments that can subsequently be used in immunoprecipitation (IP). The BioruptorTM from Diagenode provides you with high quality sheared chromatin ready for ChIP. Moreover, a Shearing module is available from Diagenode to enable an easy and highly reproducible shearing method. Then, antibody binding beads and specific ChIP-grade antibodies are necessary to precipitate the proteins cross-linked to the genomic DNA fragments. Finally, the relative amount of a particular DNA fragment specifically IP'd is determined by quantitative PCR as a measure of the occupancy of the protein at that particular position in the genome. The occupancy factor is mostly determined by quantitative PCR using primers specific for different genomic loci. ChIP can also be used to simultaneously analyse a large subset of gene loci, chromosomes or entire genome (profiling) by hybridisation of immunoprecipitated, labeled DNA on genomic microarrays (ChIP-chip) (3, 4, 5) or sequencing (ChIP-Seq) (6).

ChIP grade beads, antibodies and qPCR primer pairs are available at Diagenode. In addition, we provide you with ChIP grade reagents now available separately, such as Protease Inhibitor Mix, DNA co-precipitant, RNAse, ... (see "Related Products" section). Note that some peptides can also be purchased for use in blocking experiments (negative ChIP controls).

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

Kit Method overview & Time Table



SHEARING MODULE
(step 1 to 3)

TBP ANTIBODY MODULE (step 4)

ChIP MODULE
(step 5)

qPCR MODULE
(step 6)

The Transcription ChIP kit from Diagenode includes four modules. The modules are for: 1/ Chromatin Shearing, 2/ Antibody detection, 3/ Chromatin IP and 4/ quantitative PCR. The content of the kit is sufficient to perform 18 ChIP assays: from cell collection to qPCR. Each module is provided with optimized buffers and protocol.

Table 1

		DAY	Time needed
STEP 1 (a. or b.)	Cell fixation and collection	1	1 hour
STEP 2	Cell lysis	1	30 minutes
STEP 3	Chromatin shearing	1	30 minutes
STEP 4 (pt. 1. to 4.)	IP	1	10 minutes once
STEP 4 (pt. 5. to 10.)		1	30 minutes + O.N.
STEP 5 (pt. 1. to 5.)	Bead washes	2	1.5 hours
STEP 5 (pt. 6. to 15.)	DNA purification	2	6.5 hours
STEP 6	qPCR	3	3 hours

SHEARED CHROMATIN ANALYSIS	DAY	Time needed
Analysis step- points 1. to 5.	DAY 2	Same as step 5- (pt 5. to 15.)
Analysis step- points 6. and 7.	DAY 3	2 hours
Alternatively, perform the: Analysis step- points 1. and 2. on DAY 1 (10 minutes+ O.N.) and Analysis step- points 3. to 7. on DAY 2 (3 hours)		

Kit Materials

Kit Components

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

Table 1
Note: Upon receipt, store the components at the right temperature

SHEARING MODULE (steps 1 to 3)			
Component	Description	Quantity	Storage
Buffer A (Cell collection)	Salt and ion chelator mix included. Add formaldehyde before use.	10 ml	4°C
1.25 M glycine	-	10 ml	4°C
Buffer B (Lysis 1)	Detergent and ion chelator mix included.	30 ml	4°C
Buffer C (Lysis 2)	Salt and ion chelator mix included.	30 ml	4°C
Buffer D (Chromatin shearing)	Detergent and ion chelator mix included.	3 ml	4°C

ANTIBODY MODULE (step 4)			
Component	Description	Quantity	Storage
Antibody anti-TBP	8 µg/µl; 0.02% thimerosal included.	10 µl	-20°C

Transcription ChIP MODULE (step 4 & 5)			
Component	Description	Quantity	Storage
Buffer E (5x ChIP)	Detergent mix, salt and ion chelator mix included.	5 ml	4°C
Protease inhibitor mix (P.I.)	Dissolve the protease inhibitor mix tablet in 400 µl of water and store at -20°C. It is then a 25x stock solution.	1 tablet	-20°C
Pre-blocked protein A/G coated beads	1:3 suspension for 18 IPs; 0.02% thimerosal included.	630 µl	4°C **Do not freeze**
Blocker for ChIP beads (BSA 5%)	50x stock solution.	200 µl	-20°C
Wash buffer-1	Detergent mix, salt and ion chelator mix included.	15 ml	4°C
Wash buffer-2	Detergent mix, salt and ion chelator mix included.	9 ml	4°C
Wash buffer-3	Detergent mix, salt and ion chelator mix included.	9 ml	4°C
Wash buffer-4	Ion chelator mix included.	15 ml	4°C
Buffer F (Elution)	Detergent included. Need to be placed at room temperature 1 hour before use.	10 ml	4°C
5 M NaCl	-	400 µl	4°C
DNA co-precipitant	-	100 µl	-20°C
DNA precipitant	-	1000 µl	4°C
H20	-	10 ml	4°C

qPCR MODULE (step 6)			
Component	Comments	Quantity	Storage
GAPDH TSS primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
c-fos promoter primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
b-actin promoter primer pair	5 µM each (Rv & Fw).	50 µl	-20°C
Myoglobin exon 2 primer pair	5 µM each (Rv & Fw).	50 µl	-20°C

Required Materials Not Provided

Reagents

- Gloves to wear at all steps
- Autoclaved tips
- RNAse/DNase-free 1.5 ml (and 2 ml) tubes
- Other tubes: PCR tubes, 15 ml and 50 ml conical
- Cell scraper (if following the Step1-a. Scraping method)
- Trypsin-EDTA (if following the Step1-b. Trypsinisation method)
- Formaldehyde (37% stock, w/v)
- Ice-cold PBS buffer
- Water
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- Ethanol 100%
- Ethanol 70%
- RNAse (0.5 µg/µl)
- Agarose and TAE buffer
- DNA molecular weight marker

Equipment

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

Protocol

Short protocol

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

Starting material - Cells culture

Each ChIP assay requires 1x 10e6 cells; scale accordingly (Table13).

Step 1- Cell fixation and collection

Cells can be fixed before scraping (step 1-a.) or cells can be fixed after collection (step 2-b.). Use the method that is the most suitable for your ChIP study. For more information refer to Step 1 in the detailed protocol.

Step 2- Cell lysis

Attention: Place Buffer D at room temperature before use.

1. After step 1, centrifuge for 5 minutes at 500 g (1,600 rpm) at 4°C. Discard the supernatant, keep the pellet.
2. Add 15 ml of ice-cold **Buffer C** per pellet (obtained from 5x 10e7 cells). Scale according to the amount of cells used. Resuspend the pellet by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
3. Pellet again by centrifugation for 5 minutes at 500 g (1,600 rpm) at 4°C, and discard supernatant.
4. Add **Protease Inhibitor mix** (P.I.) to **Buffer D**. The provided P.I. is 25x, add 4 µl of P.I. per 100 µl of Buffer D. This is the complete Buffer D. Keep the Buffer at room temperature until use.
5. Add 300 µl of freshly prepared [P.I.-buffer D] per ten million cells (Table 12). Resuspend the pellet in the [P.I.-buffer D]. This is the chromatin containing sample ready to shear (step 3). When using 1.5 ml microtubes for sonication we advice to shear in volumes from 100 to 300 µl. For bigger volumes (500 µl to 2 ml) use 15 ml tubes and Bioruptor® accessories.

Attention: Make sure when working with Buffer D, that there are no cristals left in solution. Otherwise heat up gently and mix until complete disappearance of such cristals.

Step 3- Chromatin Shearing

1. Transfer the chromatin containing sample in appropriate tubes.
2. Shear the samples of chromatin using the **Bioruptor®** from Diagenode. Maintain temperature of the samples at 4°C. Sonicate 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.
3. After shearing, transfer the content to new 2 ml tubes.

4. Centrifuge for 5 minutes at 14,000 g (13,000 rpm) and 4°C to remove debris. Keep the supernatant. That is the sheared chromatin sample.
5. Place an aliquot of 3 µl of sheared chromatin (corresponding to 10% Input) in 1.5 ml tube for preparation of **input** sample; it can stay at -20°C until being processed at step 5-5. together with the **ChIP** samples.
6. Prepare aliquots of 250 µl or 450 µl of sheared chromatin into cryotubes, snap-freeze in liquid nitrogen and then store at -80°C. Alternatively, the sheared chromatin can also be used directly in ChIP (step 4-).

Step 4- Immunoprecipitation

Bead conditioning: (Points 1. to 4. below need to be done only once.)

1. Prepare 2 ml of **P.I.-ChIP buffer 1x**.
2. Take the provided **pre-blocked protein A/G bead suspension**. Centrifuge for 2 minutes at 500 g (3,000 rpm) to pellet the beads.
3. Add 1 ml of freshly prepared [P.I.-ChIP buffer 1x] to the beads to wash the beads. Centrifuge for 2 minutes at 500 g (3,000 rpm) to pellet the beads again.
4. Add to the pelleted beads 415 µl of [P.I.-ChIP buffer 1x] to obtain a 1:3 bead suspension. The beads are ready for the chromatin immunoprecipitation experiments. Use directly in 12 to 18 IPs (next point, Table 14) or store at 4°C.

Immunoprecipitation:

5. Prepare **IP-incubation mix**. Mix the following components: BSA, P.I., Buffer E, beads and water. Two types of IP-incubation mix can be prepared (described here below and in Tables 14 and 15):

	IP-incubation mix includes volumes of:					TOTAL volume
	5% BSA	P.I.	Buffer E	Beads	H2O	
TBP ChIP	6 µl	10 µl	60 µl	30 µl	163.5 µl	269.5 µl
Other ChIP	6 µl	10 µl	60 µl	30 µl	(164–v.a.) µl	x

Note: "v.a." corresponds to the volume of antibody that will be added to the IP-incubation mix (at point 9. here after).

6. For each TBP-ChIP, add 269.5 µl of the freshly prepared IP incubation mix per IP tube.
7. For a ChIP using another antibody than the provided anti-TBP, add x µl of the freshly prepared IP incubation mix per IP tube (Table 15).
8. Per IP tube, add 30 µl of sheared chromatin (from step 3- point 6.).
9. Finally, per IP tube, add the antibody anti-TBP or your antibody of interest. The provided antibody anti-TBP: use 4 µg (0.5 µl) per IP. Other antibody: use 2 to 5 µg of antibody (volume depending on the antibody used.)
10. Mix by inverting several times and incubate overnight at 4°C on a rotating wheel.

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

Step 5- DNA purification

Bead washes and DNA elution from beads (ChIP samples):

1. After incubation, pellet beads by centrifugation for 2 minutes at 500 g (3,000 rpm) at 4°C. Gently remove the supernatant.
2. Proceed to the bead washes as follows. Add 350 µl of **ice-cold wash buffer** per IP tube. Incubate 5 minutes with rotation at 4°C. Pellet the beads by centrifugation for 2 minutes at 500 g (4,000 rpm) at 4°C. Gently remove supernatant. Repeat the washes as described above. Use the following buffers for the washes: 1/ with wash buffer-1, wash twice; 2/ with wash buffer-2, wash once; 3/ with wash buffer-3, wash once and 4/ with wash buffer-4, wash twice.
3. Elute the DNA-protein-antibody complex bound to the beads by adding 400 µl of **Buffer F** (elution buffer previously placed at RT) to the pelleted beads. Incubate for 20 minutes at room temperature with rotation.
4. Precipitate beads by centrifugation for 2 minutes at 500 g (3,000 rpm) at room temperature. Transfer supernatants to new clean 1.5 ml tubes. That corresponds to the eluted DNA, isolated by IP.
5. Add 390 µl of **Buffer F** (RT elution buffer) to 10 µl sheared chromatin sample (from step 3- point 5.) to bring the final volume to 400 µl. That corresponds to the input sample(s).

DNA recovery and purification from ChIP and input samples:

6. Add 16 µl of the provided **5 M NaCl**, mix and incubate in a thermoshaker for 4 hours at 65°C to reverse cross-linking.
7. Cool down samples to room temperature, add 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1), vigorously vortex for 5 seconds.
8. Centrifuge for 2 minutes at 14,000 g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
9. Add 400 µl of chloroform/isoamyl alcohol (24:1), vigorously vortex for 5 seconds
10. Centrifuge for 2 minutes at 14,000 g (13,000 rpm) at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
11. Per tube: add 5 µl of the provided DNA co-precipitant and 40 µl of the DNA precipitant. Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 minutes.
12. Centrifuge for 25 minutes at 14,000 g (13,000 rpm) at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.
13. Centrifuge for 10 minutes at 14,000 g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 minutes at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that was purified from the sheared chromatin (Input sample(s)) and 2/ DNA that was isolated by ChIP (ChIP samples).
14. Add 200 µl of water to the **ChIP samples** and to the **input sample(s)**, add 100 µl of water.
15. Place the tubes in shaker for 30 minutes at 12,000 rpm at room temperature to dissolve the pellets.

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

Step 6- qPCR

For more information refer to Step 6 in the detailed protocol.

MANUAL

Detailed protocol

Starting material- Cell culture

Start the protocol below when cells in culture reach confluency.

Notes:

- Use cell culture dishes of 140 mm diameter. In the case of U2OS cells grown to confluency, there are about 1x 10e7 cells per 140 mm culture dish. (Other cell types could give as little as 3x 10e6 cells per 15 cm dish.) The protocol below is for a total of 5x 10e7 cells.
- Cells can be submitted to inductions and/or treatments. Then treated as well as non treated cells can be prepared and studied in ChIP.

Each ChIP assay requires 1x 10e6 cells; scale accordingly (Table13).

STEP 1. Cell fixation and collection

Cells can be fixed before scraping (step 1-a.) or cells can be fixed after collection (step 1-b.). Both methods are described below: use the method that is the most suitable for your ChIP study.

Step 1-a. Scraping method:

1. Prepare cross-linking buffer by mixing Buffer A with 37% formaldehyde (w/v) and PBS as shown below (Table 3). The final concentration of formaldehyde is 1%.

   Note: Handle formaldehyde in a chemical fume hood.

Table 3

Cross-linking buffer	3x 10e6 cells	10e7 cells	5x 10e7 cells
37% formaldehyde (w:v)	90 µl	270 µl	1350 µl
Buffer A	210 µl	630 µl	3150 µl
PBS	3 ml	9 ml	45 ml
TOTAL VOLUME	3.3 ml	9.9 ml	49.5 ml

Note: Alternatively, the mixture of [formaldehyde 11% final in buffer A] can be prepared and added - at 1:10 (v/v)- directly to the cell culture medium in the cell containing flask. (If cells are grown in suspension, it is the method of choice). Mix immediately and incubate on a shaking platform for 10 minutes at room temperature. Then proceed to step1-a. point 4. Note.

2. Remove completely the culture media from the culture dishes or flasks.
3. Add the freshly prepared cross-linking buffer to the cells (Table 3). Add about 3 ml of buffer per 3x 10e6 cells. Mix immediately and incubate for 10 minutes at room temperature.

   Note: The duration of cross-linking varies between cell type and protein of interest. It is possible to optimize the fixation step by testing different incubation time, such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.

4. Add glycine to the cells to stop the cross-linking. Add about 300 µl of glycine per 3x 10e6 cells (Table 4). The provided stock solution of glycine is 1.25 M and is therefore diluted 10 times to reach a final concentration of 0.125 M. Mix and incubate for 5 minutes at room temperature (that is to quench the formaldehyde).

Table 4

To stop fixation	3x 10e6 cells	10e7 cells	5x 10e7 cells
1.25 M glycine	330 µl	990 µl	4950 µl

Note: Alternatively, if the mixture of [formaldehyde 11% final in buffer A] was added - at 1:10 - directly to the cell culture medium in the cell containing flask. (Method of choice for cells grown in suspension.). Then, add 1:10 (v/v) of 1.25 M glycine to the total volume of [(formaldehyde-buffer A-medium] mixture. Mix immediately and incubate for 5 minutes at room temperature. Then proceed to steps of centrifugation and washes.

5. Aspirate the media completely.

   Note: If cells are grown in suspension, collect the cells by centrifugation at this point.

6. Wash cross-linked cells twice with of ice-cold PBS. Use 5 ml of ice-cold PBS per wash per 3x 10e6 cells. For each wash, add the buffer to the cells and remove completely.

   Note: If cells are adherent, this washing step is done in the culture dish. If cells are grown in suspension, cells are collected by centrifugation after the washing step with PBS.

7. Add 500 µl of Buffer B per about 3x 10e6 cells (Table 5) and scrape cells from the culture dish or flask.

Table 5

Cell scraping	3x 10e6 cells	10e7 cells	5x 10e7 cells
Buffer B	500 µl	1.5 ml	7.5 ml

Note: If cells are grown in suspension, cells are collected by centrifugation at this point rather than by scraping.

8. Transfer to new conical tubes. Proceed to step 2-.

Step 1-b. Trypsinisation method

1. Pre-warm PBS, culture media and trypsin-EDTA.
2. Remove old media and rinse cells with pre-warmed PBS (Table 6). Shake dishes for 2 minutes. Remove the PBS.

Table 6

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

3. Add sterile trypsin-EDTA to tissue culture flask or dish still containing adherent cells (Table 7). Brief treatment with trypsin-EDTA removes adherent cells from tissue culture flask bottom.

   Note: Each cell line will differ in the degree of adherence it has, and this will be described in the literature that accompanies it. If necessary, place flask back into incubator for about 1-2 minutes.

Table 7

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

4. Check after a minute to see if cells have come off the flask bottom.

   Note: Prolonged treatment with trypsin may damage cells. This can be observed macroscopically as sheets of floating cells will be visible.

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

Table 8

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

6. Wash down sides of culture flask with a portion of the [cell/culture medium] mixture. Pipette the cells and transfer to a 15 ml or 50 ml centrifuge tube.
7. Count the cells.
8. Spin for 10 minutes at 500 g (1,600 rpm) at RT.
9. Resuspend the cells in culture medium. Use 1 ml of medium per 5 million cells (Table 9).

Table 9

Cell resuspension	3x 10e6 cells	10e7 cells	5x 10e7 cells
Culture medium	600 µl	2 ml	10 ml

10. Then, prepare a mixture of Buffer A plus formaldehyde as shown below (Table 10). Add the freshly prepared cross-linking buffer to the [cells and medium] for fixation. Incubate under gentle rotation for 10 minutes at room temperature. This is the cross-linking step.

    Notes: - Handle formaldehyde in a chemical fume hood.

    - After the cross-linking, keep the samples ice-cold at all times.

    - Prepare fresh buffers just before use.

Table 10

Cross-linking buffer	3x 10e6 cells	10e7 cells	5x 10e7 cells
Buffer A	45 ul	140 µl	700 µl
formaldehyde	20 µl	60 µl	300 µl

11. Add glycine to the cells (using the stock solution of 1.25 M) corresponding to about 1:10 (v/v) of the total volume of [cells and medium] solution (Table 11). Mix evenly. That is to quench the formaldehyde.

Table 11

To stop fixation	3x 10e6 cells	10e7 cells	5x 10e7 cells
1.25 M glycine	66 µl	220 µl	1100 µl

12. Centrifuge for 5 minutes at 500 g (1,600 rpm) at 4°C, to pellet cells. Discard the supernatant.
13. Wash the pelleted cells with ice-cold PBS. Add 15 ml of PBS per 5x 10e7 cells and resuspend the cells by pipetting up and down. Centrifuge immediately for 5 minutes at 500 g (1,600 rpm) at 4°C. Discard the supernatant.
14. Add 15 ml of ice-cold Buffer B per pellet of 5x 10e7 cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing. Proceed to step 2-.

STEP 2. Cell lysis

Notes: - Use a minimum of 3x 10e6 cells and a maximum of 5x 10e7 cells per sample.

- Keep the samples ice-cold at all times.

1. Centrifuge for 5 minutes at 500 g (1,600 rpm) at 4°C. Discard the supernatant, keep the pellet.
2. Add 15 ml of ice-cold Buffer C per pellet (obtained from 5x 10e7 cells). Scale according to the amount of cells used. Resuspend the pellet by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
3. Pellet again by centrifugation for 5 minutes at 500 g (1,600 rpm) at 4°C, and discard supernatant.

Note: During the centrifugation, dissolve the protease inhibitor mix tablet in 400 µl of water. It is then a 25x stock solution. After use, store at -20°C.

4. Add Protease Inhibitor mix (P.I.) to ice-cold Buffer D. The provided P.I. is 25x, add 4 µl of P.I. per 100 µl of Buffer D.

   Note: Prepare the volume of [P.I.-Buffer D] that is needed on the day (see Table 12)

5. Add 300 µl of freshly prepared [P.I.-buffer D] per ten million cells (Table 12). Resuspend the pellet in the [P.I.-buffer D]. This is the chromatin containing sample ready to shear (step 3-.). When using 1.5 ml microtubes for sonication we advice to shear in volumes from 100 to 300 µl. For bigger volumes (500 µl to 2 ml) use 15 ml tubes and Bioruptor® accessories.

Table 12

Cell resuspension	3x 10e6 cells	10e7 cells	5x 10e7 cells
P.I.- BufferD	90 µl	300 µl	1500 µl

Note: Use freshly prepared buffer and discard what is not used on the day

One ChIP experiment (step 4-) is done with chromatin sheared from one million cells. The Table 13 indicates the number of cells needed in order to prepare sheared chromatin ready for ChIP assay(s).

Table 13

IPs	Cell number/shearing	Volume of P.I.- Buffer D/shearing
1	1x 10e6	in 30 µl
6	6x 10e6	in 180 µl
12	1.2x 10e7	in 360 µl

Notes: - Each ChIP assay requires 1x 10e6 cells; scale accordingly.

- Determine how many ChIPs will be performed, including negative and positive controls (step 4- point 7.).
- Most of the sheared chromatin is to be used in the ChIP experiment, but remember that some of the sheared chromatin (10 µl) is needed as input sample for the ChIP experiment (steps 4- and 5-) and can also be checked on gel (analysis step).

STEP 3. Chromatin shearing

Note: This protocol is very successful for a large variety of mammalian cells. However you might want to optimize shearing conditions for your specific cell type and fixation protocol. Therefore, to start with a small sample (3x 10e6 cells) and check the shearing efficiency is advised. Once conditions have been optimized for your cells following step 3- and the analysis step, prepare sheared chromatin for ChIP and proceed to steps 4- and 5-.

1. Transfer the chromatin containing sample to appropriate tubes:

   - do not add more than 300 µl per 1.5 ml tubes

   - do not add more than 2 ml per 15 ml tubes.

   Note: Keep some unsheared chromatin for future controls (analysis step).

2. Shear the samples of chromatin using the Bioruptor®from Diagenode. Maintain temperature of the samples at 4°C. Sonicate 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.

3. After shearing, transfer the content to new 2 ml tubes.

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

   Note: At this point, the sheared chromatin can be stored for subsequent ChIP experiments (steps 4-. and 5-.) and for analysis of shearing efficiency (analysis step).

5. Place an aliquot of 10 µl of sheared chromatin (corresponding to 10 Input) in 1.5 ml tube for preparation of input sample; it can stay at -20°C until being processed at step 5-5. together with the ChIP samples.

6. Prepare aliquots of 250 µl or 450 µl of sheared chromatin into cryotubes, snap-freeze in liquid nitrogen and then store at -80°C. Alternatively, the sheared chromatin also can be used directly in ChIP (step 4-).

Notes: - The chromatin can be stored in liquid nitrogen for several months or weeks depending on your ChIP target. Do not freeze/thaw.

- Samples of sheared chromatin of 250 µl will be for 6 IPs

- Samples of sheared chromatin of 450 µl will be for12 IPs.

STEP 4. Immunoprecipitation

Bead conditioning: (Points 1. to 4. below need to be done only once.)

1. Prepare 2 ml of [P.I.-ChIP buffer 1x].

   Note: Add to 1520 µl of water, 400 µl of Buffer E (5x ChIP buffer) and 80 µl of P.I..

2. Take the provided pre-blocked protein A/G bead suspension. Centrifuge for 2 minutes at 500 g (3,000 rpm) to pellet the beads.

3. Add 1 ml of freshly prepared [P.I.-ChIP buffer 1x] to the beads to wash the beads. Centrifuge for 2 minutes at 500 g (3,000 rpm) to pellet the beads again.

4. Add to the pelleted beads 415 µl of [P.I.-ChIP buffer 1x] to obtain a 1:3 bead suspension. The beads are ready for the chromatin immunoprecipitation experiments. Use directly in 12 to 18 IPs (next point, Table 14) or store at 4°C.

   Note: Final volume of [beads plus buffer] is about 620 µl. Per IP: 30 µl is needed.

Immunoprecipitation:

5. Prepare IP-incubation mix. Mix the following components: BSA, P.I., Buffer E, beads and water. Two types of IP-incubation mix can be prepared as described here below:

   - Table 14 is for ChIP assay(s) performed with the TBP antibody provided with the Transcription ChIP Kit (TBP-ChIP).

   - Table 15 refers to ChIP experiments done with the antibodies of your choice. Adjust the volume of water to be added to prepare the IP-incubation mix, (see Table 15): it depends on the volume of antibody used per IP.

Table 14

Number of TBP-ChIPs	IP-incubation mix includes volumes of:					TOTAL volume
	5%BSA	P.I.	Buffer E (5x ChIP)	Beads for IP	H2O	
1	6 µl	10 µl	60 µl	30 µl	163.5 µl	269.5 µl
3	20 µl	33 µl	200 µl	100 µl	540 µl	893 µl
6	40 µl	66 µl	400 µl	200 µl	1080 µl	1786 µl
12	80 µl	132 µl	800 µl	400 µl	2160 µl	3572 µl

Note: Resuspend protein A/G beads into a uniform suspension before use.

Table 15

Number of ChIP	IP-incubation mix included the volumes of:					TOTAL volume
	5% BSA	P.I.	Buffer E (5x ChIP)	Beads for IP	H2O	
1	6 µl	10 µl	60 µl	30 µl	(164–v.a.) µl	x

Note: v.a." corresponds to the volume of antibody that will be added to the IP-incubation mix (at point 9. here after). Therefore, the "v.a." volume has to be substracted to the volume of water to add to the IP-incubation mix.

Per ChIP, the final volume of the mix including chromatin and antibody is 300 µl.

6. For each TBP-ChIP, add 269.5 µl of the freshly prepared IP incubation mix per IP tube.

Note: Use one tube per ChIP assay. It is referred as IP tube in this protocol.

7. For a ChIP using another antibody than the provided anti-TBP, add x µl of the freshly prepared IP incubation mix per IP tube (Table 15).

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

8. Then, per IP tube, add 30 µl of sheared chromatin (from step 3- point 6.).

9. Finally, per IP tube, add the antibody anti-TBP or your antibody of interest:

- provided antibody anti-TBP: use 4 µg (0.5 µl) per IP

- other antibody: use 2 to 5 µg of antibody (volume depending on the antibody used.)

Note: Include controls by omitting one component such as "no antibody added",...

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

Note: If you wish to analyse the quality of your sheared chromatin, follow the protocol described in this manual (analysis step-).

STEP 5. DNA purification

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

Bead washes and DNA elution from beads (ChIP samples):

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

   Notes: Pay attention not to remove beads: leave on top of the pellet of beads a small volume of buffer. The beads are washed to isolate the [chromatin-antibody-beads] complex. Keep samples cold. It is highly recommended to perform the washing steps in a cold room. Also, place the Buffer F at room temperature (RT).

2. Proceed to the bead washes as follows. Add 350 µl of ice-cold wash buffer per IP tube. Incubate 5 minutes with rotation at 4°C. Pellet the beads by centrifugation for 2 minutes at 500 g (4,000 rpm) at 4°C. Gently remove supernatant. Repeat the washes as described above. Use the following buffers for the washes:

   - with wash buffer-1, wash twice
   - with wash buffer-2, wash once
   - with wash buffer-3, wash once
   - with wash buffer-4, wash twice

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

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

4. Precipitate beads by centrifugation for 2 minutes at 500 g (3,000 rpm) at room temperature. Transfer supernatants to new clean 1.5 ml tubes. That corresponds to the eluted DNA, isolated by IP.

   Note: There are as many IP tubes as ChIP samples.

5. Add 390 µl of Buffer F (RT elution buffer) to 10 µl sheared chromatin sample (from step 3- point 5.) to bring the final volume to 400 µl. That corresponds to the input sample(s).

   Notes: - From this point onwards, treat ChIP and input samples together.

   - Use either fresh or thawed sheared chromatin from step 3- point 5..

DNA recovery and purification from ChIP and input samples:

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

   Note: It is possible to reverse the cross-linking overnight.

7. Cool down samples to room temperature, add 400 µl of phenol/chloroform/isoamyl alcohol (25:24:1), vigorously vortex for 5 seconds.

   Note: It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation.

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

9. Add 400 µl of chloroform/isoamyl alcohol (24:1), vigorously vortex for 5 seconds.

   Note: It is possible to incubate the samples at RT for 10 minutes on a rotating wheel before centrifugation.

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

11. Per tube: add 5 µl of the provided DNA co-precipitant and 40 µl of the DNA precipitant. Then, add 1 ml of ice-cold 100% ethanol. Mix well. Leave at -80°C for 30 minutes.

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

13. Centrifuge for 10 minutes at 14,000 g (13,000 rpm) at 4°C. Carefully remove the supernatant, leave tubes opened for 30 minutes at room temperature to evaporate the remaining ethanol. The pellets are: 1/ DNA that was purified from the sheared chromatin (Input sample(s)) and 2/ DNA that was isolated by ChIP (ChIP samples).

Notes: - Avoid leaving ethanol on tube walls.

- The sheared chromatin used as Input samples must correspond to the same preparation of sheared chromatin used in the ChIP assays.

14. Add 200 µl of water to the ChIP samples and to the input sample(s).

15. Place the tubes in shaker for 30 minutes at 600 rpm at room temperature to dissolve the pellets.

Note: Shake DNA to suspend evenly.

Note: If you wish to analyse the quality of your sheared chromatin, follow the protocol described in this manual (analysis step-).

STEP 6. qPCR

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

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

1. Thaw the qPCR buffer mix on ice.
2. Add H2O to the qPCR buffer mix. Per PCR sample: add 5.5 µl of H2O to 12.5 µl of buffer mix. Vortex for 10 seconds.

   Note: The buffer mix could be IQ SYBR Green supermix or any other that is used in your lab. Follow the instructions of your supplier.
3. Per PCR sample: add 2 µl of primer pair set

   Note: The primers in the qPCR module are provided as a mix containing both forward (FW) and reverse (RV) primers, each at 5 µM final concentration.
4. Prepare a 1:10 dilution of the input sample(s) with water (prepared at step 5- point 15.). Mix by vortexing 5 seconds.

   Note: The aliquot of diluted input DNA sample corresponds to 1% input DNA.
5. Per PCR sample: add 5 µl of DNA sample:
   - from ChIP(s) (prepared at step 5- pt. 15.)
   - from input(s) (1:10 dilution prepared at step 6- pt. 4.)

Table 16

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

6. Proceed to the PCR using cycles and temperatures as described in the table below (see Figures 2 and 3 in the Result section).

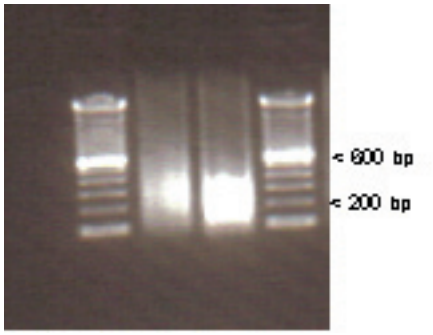
Table 17

	Temperature	Time	Cycles
PCR Amplification	95°C	3 minutes	x1
	95°C	15 seconds	x40
	60°C	45 seconds	
	95°C	1 minute	x1
Melting curve	65°C and increment of 0.5°C per cycle	1 minute	x60

Transcription ChIP Kit Results: Transcription Factor ChIP

Figure 1 (Sheared chromatin analysis):

Analysis on an agarose gel of the sheared chromatin obtained with the BioruptorTM and the shearing module of the Red ChIP kit from Diagenode.

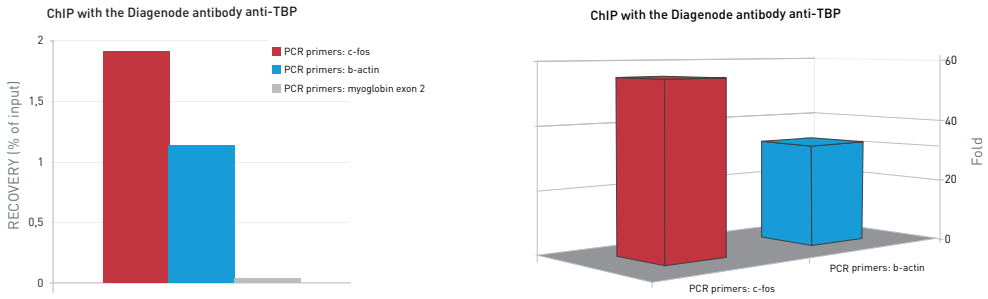


U2OS cells are fixed with 1% formaldehyde (10 minutes at RT). One million cells are resuspended per 30 µl of lysis buffer prior to chromatin shearing. Each sample consists on 5x 10e6 cells per 1.5 ml tube. Samples are sonicated for 15 cycles of: [30 seconds "ON" / 30 seconds "OFF"] with the BioruptorTM from Diagenode (cat # UCB-200). Samples 1 and 2 are duplicates. The sheared chromatin is submitted to cross-linking reversion, DNA purification and RNAse treatment. Then the samples are analysed on a 1% agarose gel. Two amounts of sheared chromatin are analysed as indicated in the legend (1.5 µl and 3 µl). The 100 bp DNA molecular weight marker is on lanes 1 and 4.

Note: Some unsheared chromatin can be analysed on gel as well (analysis step).

Figure 2 (ChIP results: qPCR):

Analysis of Transcription (TF) ChIP efficiency.

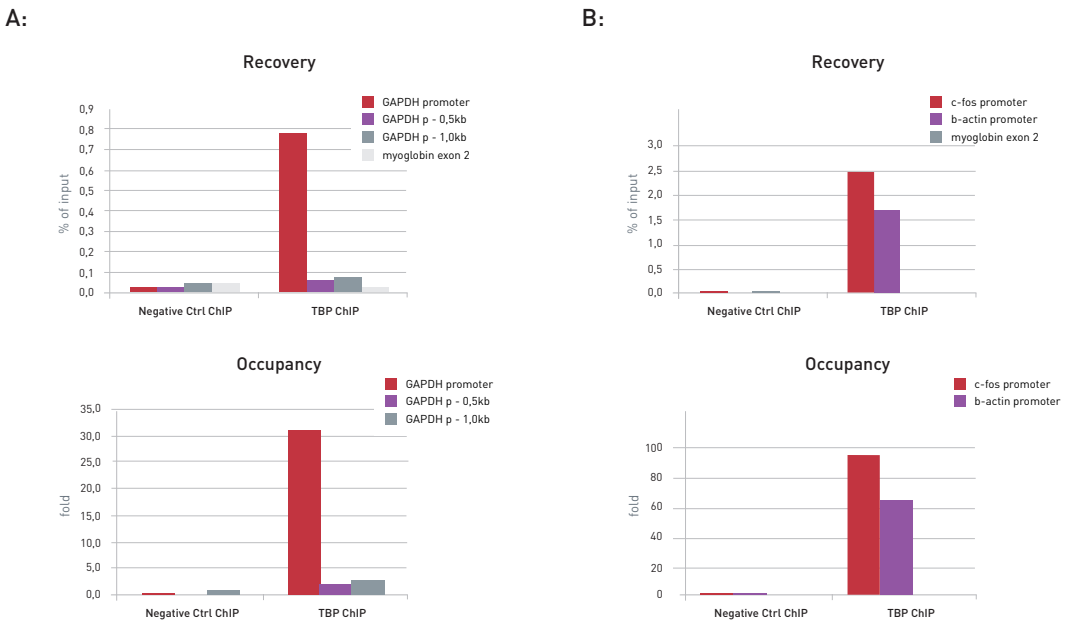


ChIP results obtained with the Diagenode Red ChIP kit for TF Chromatin IP including the TBP antibody. ChIP assays were performed using U2OS cells, the Diagenode antibody directed against TBP and optimized PCR primer pairs for qPCR.

Occupancy of the c-fos and b-actin promoters by TBP is evident based on fluorescent qPCR analysis of immunoprecipitated DNA. Controls for IP and PCR specificity include primers for the myoglobin exon 2. The Red ChIP controls reveal high ChIP efficiency.

Figure 3 (ChIP results: qPCR):

Analysis of TF ChIP resolution and TF ChIP efficiency.



ChIP results obtained with the Diagenode Red ChIP kit for TF ChIP including the TBP antibody. ChIP assays were performed using U2OS cells, the Diagenode antibody directed against TBP and optimized PCR primer pairs for qPCR. A negative control was included in the ChIP assay (no antibody added).

A: Occupancy of the GAPDH promoter by TBP. High ChIP resolution.

B: Occupancy of the c-fos and b-actin promoters by TBP.

Occupancy of the three promoters by TBP is evident based on fluorescent qPCR analysis of immunoprecipitated DNA. Controls for IP and PCR specificity include primers for the myoglobin exon 2 as well as GAPDH (promoter -0.5 kb) and GAPDH (promoter -1kb). The Red ChIP controls reveal high ChIP resolution as well as high ChIP efficiency.

Troubleshooting guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the 10 minutes fixation step at the right temperature and with the correct formaldehyde concentration. Use high quality formaldehyde.
	Cross-linking is too strong.	
	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 can lead to elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity accompanied with significant efficiency of ChIP.
	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.
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol.
Cell number	The amount of cells required for a ChIP experiment is determined by cell type, protein of interested and antibodies used.	We recommend to use 1x 10e6 cells per ChIP. For some cases, less than 10e6 cells could yielded successful results. .Also, up to 5 x106 cells could be used per ChIP.
Chromatin shearing	Buffer composition.	Use the buffers provided in the kit. They are optimized containing essential key components. Keep samples cold.
High quality picture of the sheared DNA	For accurate size determination of the DNA fragments	Reverse the cross-linking and precipite the DNA after Phenol/ chloroform extraction.
	Gel electrophoresis of cross-linked samples could give smears.	Reversion of the cross-linking is advised. 100 µl of the sheared chromatin is mixed with 300 µl of buffer F. Incubate at least 4 hours at 65°C. (minimum 4 hours and maximum O.N.).
	The migration of large quantities of DNA on agarose gel can lead to poor quality pictures which do not reflect the real DNA fragmentation.	Do not load too much on a gel. Do not load more than 5 µg/lane. Also treat the sample with RNAse.
	Agarose concentration.	Do not use more than 1% agarose gel and run slowly
	Running buffer concentration.	1X TAE or TBE is preferred to 0.5X TAE which can lead to smears on gel..
Beads in the IP	Beads centrifugation.	Don't spin the beads at high speed. Use gentle centrifugation (500 g for 2-3 minutes) as described in the manual protocol. g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm. It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Bead binding capacity	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).

	Bead binding capacity	rabbit and goat: polyclonal antibodies; human: IgG1, IgG2, IgG3 and IgG4; mouse: IgG1, IgG2a, IgG2b, IgG3 and IgA; rat: IgG1, IgG2a, IgG2b and IgG2c,
Antibody in IP	Why is my antibody not working in ChIP?	Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.
	Which antibody should I use in ChIP?	Use ChIP-grade antibodies. If not available, it is recommended to use several antibodies directed against different epitopes of the same protein
	How do I choose an antibody for ChIP?	Be aware of the possible cross-reactivity of antibodies. Verify by Western blot analysis the antibody specificity. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.
	Amount of antibody per ChIP to use?	To ensure efficient IP it is important to have an optimal ratio between amount of chromatin and amount of antibody. More antibody (or less chromatin) can be required in case of low affinity to antigen or high abundance of target protein (e.g. histones). The lack of antibody can result in low efficiency of ChIP whereas large excess of antibody might lead to lower specificity.
	Are my antibodies going to bind the protein A or the protein G?	There is a significant difference in affinity of different types of immunoglobulins to protein A or G. For example, IgM, IgY or IgG1 can require a secondary antibody as a bridge to protein A or G.
PCR	Primers	- length: 18 to 24 nucleotides - Tm: 60°C (+/- 3.0°C) - % GC: 50% (+/- 4%)
	Controls: –ve and +ve	-ve PCR control: PCR with primers specific for a DNA region at which your antigen of interest is not present using ChIP samples. +ve PCR control: PCR on input.
	Red ChIP qPCR primer pairs	The provided qPCR primers are targeting human genomic loci
	Red ChIP qPCR primers are provided for rapid checking of the ChIP efficiency.	Reverse cross-links and purify DNA from an aliquot of ChIP sample. Then, use the Red ChIP qPCR specific primer pairs to amplify certain known promoter regions (GAPDH, c-fos, b-acttin) in order to check whether the ChIP is successful.
	Red ChIP qPCR primers are provided for rapid checking of the ChIP resolution and chromatin shearing efficiency.	Reverse cross-links and purify DNA from an aliquot of ChIP sample. Then, use the Red ChIP qPCR specific primer pairs to amplify distant regions from the GAPDH promoter region (GAPDH -0.5 kb, GAPDH -1.0 kb) in order to estimate the resolution of the ChIP assay.
Freezing	Samples can be frozen at several steps of the protocol	- Step 3. points 5. and 6. (sheared chromatin) - Step 5. point 15. (ChIP and input: DNA) - Analysis Step. point 5. (Input: DNA)
	Avoid freeze/thawing	Snap freeze cells and thaw on ice.
ChIP resolution	How can I estimate the resolution of my ChIP assay?	Use the three GAPDH primer sets provided in the Red ChIP kit. The TATA-binding box in the promoter of GAPDH gene must reveal the highest enrichment in ChIP with the anti-TBP antibody because of the TBP-direct binding site included in the PCR amplified DNA region. DNA regions at 0.5 kb and 1 kb apart from the GAPDH promoter must reveal significant decrease of enrichment (→10 folds). Also indicating that chromatin shearing has been performed properly.

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