

Instruction Manual Version 2 02.14

OneDay ChIP kit

The rapid Chromatin Immunoprecipitation kit

Cat. No. C01010080 (kch-oneDIP-060)

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Introduction

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

Chromatin immunoprecipitation (ChIP) is a technique allowing 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 recruitment (1). The steps of this technique are cell fixation, chromatin shearing, immunoselection, immunoprecipitation and analysis of the immunoprecipitated DNA.

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

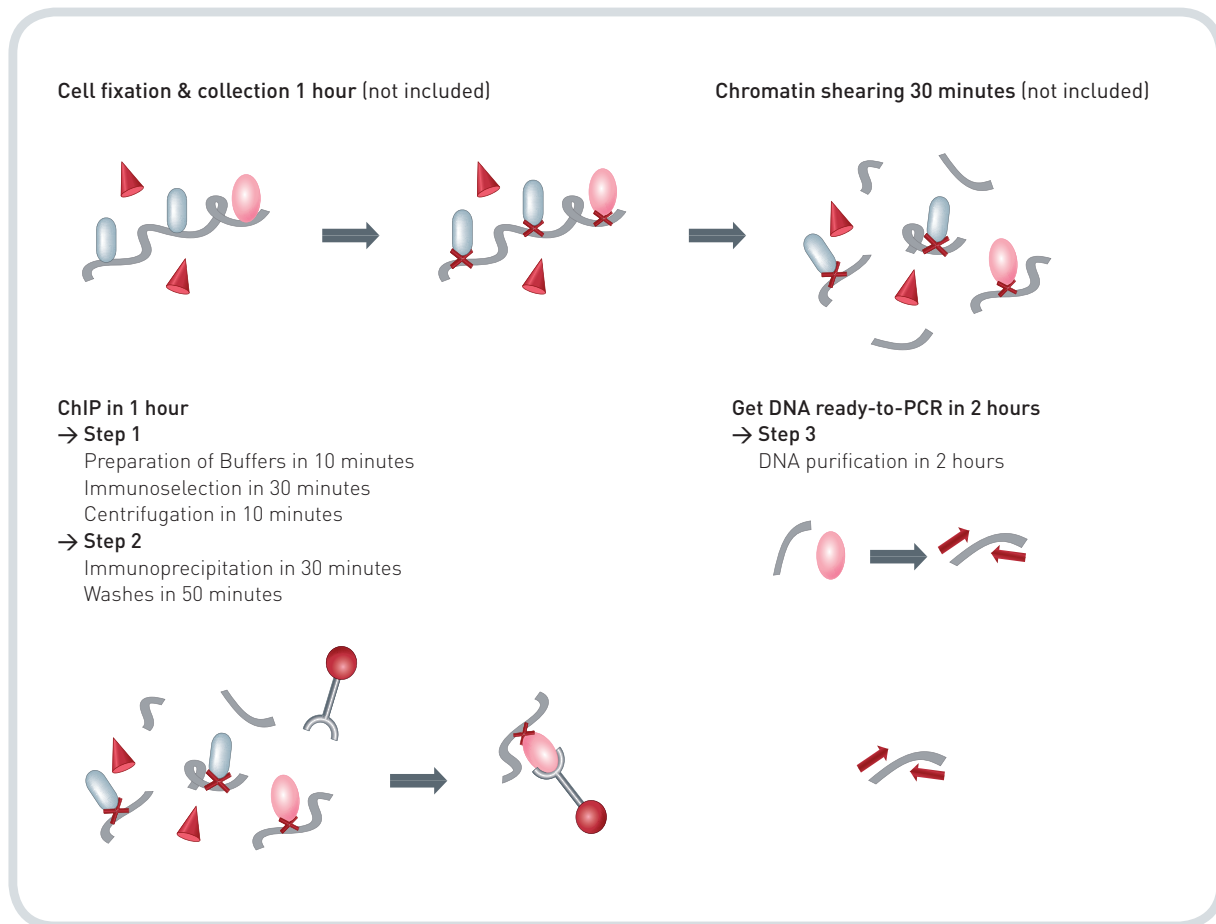
The most widely used approach to fix DNA-Protein interactions in the living cell is by formaldehyde fixation (cross-linking) that generates covalent bonds between amino or imino groups of proteins and nucleic acids (2). The formaldehyde cross-links DNA-Protein as well as protein-protein complexes in situ. Following cross-linking, chromatin needs to be sheared very effectively into homogeneous small fragments that can subsequently be used in immunoprecipitation (IP). The **Bioruptor™** from Diagenode provides you with high quality sheared chromatin ready-to-ChIP. Moreover, at Diagenode one **Shearing module** is available and brings an easy and highly reproducible shearing method. Then, antibody binding beads and specific ChIP-grade antibodies are necessary to precipitate the proteins cross-linked to genomic DNA fragments. Finally, the relative amount of a particular DNA fragment specifically IP'd is determined by quantitative PCR as a measure of the occupancy of the protein at that particular position in the genome.

Although ChIP is a very versatile tool, the procedure requires tedious optimization of several reaction conditions. Diagenode provides kits with optimized reagents and simplified protocols for ChIP. Another major drawback to ChIP assays is that the traditional ChIP method is time consuming. It involves two overnight incubations (first for antibody binding to the target and then for the purification of the IP'd DNA). Therefore Diagenode offers kits not only for the traditional ChIP method (such as the Transcription and Histone ChIP kits) but also for the **rapid ChIP method (OneDay ChIP kit)**.

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

1. The antibody binding is accelerated by incubating antibody with chromatin in an ultrasonic bath (3) and
2. The DNA purification is rapid as it has been simplified by the use of a DNA purifying slurry and does not require multiple steps (see kit overview).

Kit method overview



The OneDay ChIP kit from Diagenode allows you to perform the immuno-selection and -precipitation in 2 hours and to get the DNA ready-to-PCR in 2 hours.

The OneDay ChIP kit is provided with all necessary optimized reagents and buffers as well as user-friendly protocol.

- Prepare sheared chromatin ready-to-ChIP using the Bioruptor™ and our Shearing module. Alternatively use your own method.
- Once you have your sheared chromatin and ChIP grade antibodies, use our novel OneDay ChIP kit, it is simple, fast and optimized. Take one day instead of three to perform your ChIP with the Diagenode OneDay ChIP kit (see pages 5 and 6).

Representation above: Proteins are represented as rectangles, triangles and oval. The protein of interest is in blue. Proteins are attached or not to the DNA (black line). Cross-linked proteins to DNA are indicated with a bold cross. Shearing generates chromatin fragments. The sheared chromatin is then incubated with antibody (step 1) and precipitated with antibody binding beads (step 2). The IP'd DNA fragments are purified (step 3) and amplified by PCR (step 4).

Kit Materials

Kit Content

The content of the kit is sufficient to perform 60 ChIP assays: from immuno-selection and precipitation to DNA-ready to PCR *. The kit content is described in Table 1. **Upon receipt, store the components at the temperatures indicated in Table 1.**

Table 1.

One Day ChIP cat# kch-onedIP-060			
Component	Comments	Quantity	Storage
ChIP buffer 5x	Salt, detergent mix and ion chelator included	300 ml	RT
Protease Inhibitor mix (P.I. 200x)	200x stock solution	100 µl	-20°C
Unblocked Protein A beads	Suspension 1:4	2800 µl	4°C
Negative Ctrl IgG from rabbit (1 µg/µl)	0.02% azide included.	100 µl	4°C
BSA 5%	100x stock solution	200 µl	-20°C
DNA purifying slurry	-	10 ml	4°C
Proteinase K	100 x stock solution	100 µl	-20°C
Sterile Water	-	28 ml	4°C

Required Materials Not Provided

Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml tubes
- Other tubes: PCR tubes or plates, 15 ml tubes
- Autoclaved tips and Barrier tips
- Tube claps
- Deionized water
- Ethanol 100% and ethanol 75% (vol/vol)

Equipment

- Pipette
- Refrigerated centrifuges for 1.5 ml tubes (4°C), 15 ml tubes (4°C)
- Ultrasonic water bath ** (B3510-MT http://www.bransonics.com/model_3510.asp CPN-952-316)
- Rotating wheel (4°C)
- Thermomixer or shaking heat-block (55°C)
- Boiling water (98°C to 100°C)
- Quantitative PCR (or end-point PCR) facilities and reagents
- Agarose gel apparatus

Important notes:

- * : the kit does not include the reagents necessary for chromatin shearing and for PCR, but protocols from cell harvesting to PCR data analysis are included in this manual. Use our Shearing module (catalog # kch-redmod-100) and Bioruptor™ to prepare sheared chromatin-ready-to-ChIP. Alternatively, use your “in house” protocol (see “additionnal protocols” section). A complete troubleshooting guide is also provided. (“TsG”)
- ** : (4°C), sold as ultrasonic cleaner (115/120V, 50/60Hz), built-in sweep frequency. If not available, step 1 will take longer. See troubleshooting guide. (“TsG”)

ABBREVIATION LIST

(“TsG”): refer to the troubleshooting guide.

(“STOP”): it is possible to stop at that stage, refer to the troubleshooting guide for more details.

(4°C): it is essential to work on ice or in cold rooms or with refrigerated centrifuges.

(RT): work at room temperature. Do not need to work on ice anymore.

(“Input”): input from sheared chromatin used in ChIP.

(“!”): important step, make sure you do it as described in the protocol.

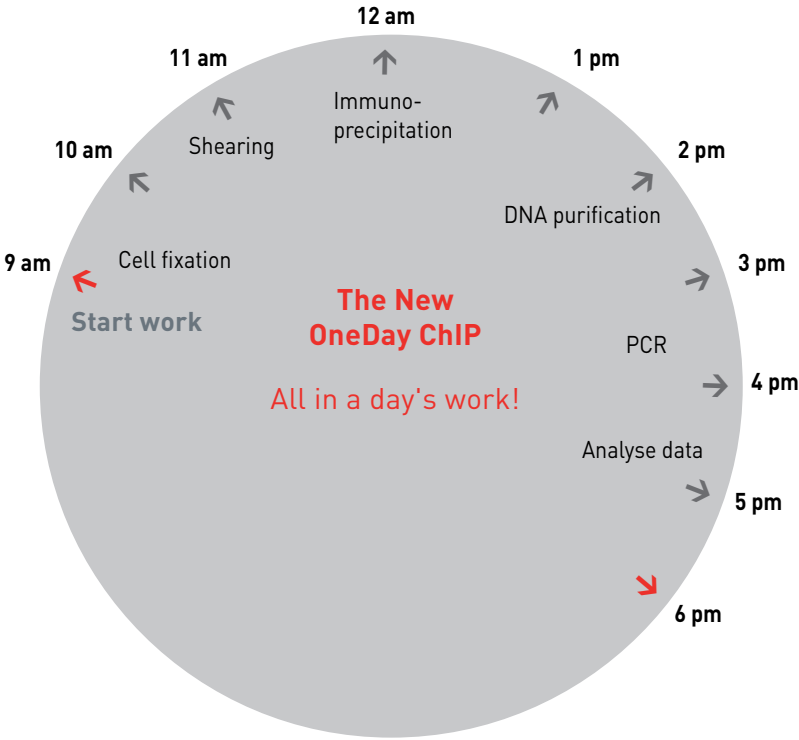
Kit Protocol Overview

Table 2.

Chromatin shearing STEPS (using our Shearing module: catalog # kch-redmod-100)		Time needed
Cell fixation and collection		1 hour
Cell lysis		30 minutes
Chromatin shearing		30 minutes

Table 3.

OneDay ChIP kit STEPS		DAY	Time needed
Step 1	Preparation of Buffers, ...	DAY 1	10 minutes
	Immunoselection	DAY 1	30 minutes
	Centrifugation	DAY 1	10 minutes
Step 2	Immunoprecipitation	DAY 1	30 minutes
	Washes	DAY 1	50 minutes
Step 3	DNA purification	DAY 1	2 hours
Step 4	qPCR and data analysis	DAY 1	2 hours



Protocol

OneDay ChIP: Chromatin Immunoprecipitation Procedure

Starting material- Sheared chromatin

Sheared chromatin must be prepared to be used in the OneDay ChIP kit protocol (starting on the page after next).

We highly recommend the use of our Shearing module from the Transcription ChIP kit (Cat. No. kch-redmod-100): below find a quick overview and the main steps for chromatin shearing with our Shearing module. Alternatively, prepare your sheared chromatin following your protocol (see “additional protocols” section). Note that our Shearing-ChIP kit works as well and can be used prior to OneDay ChIP (see “additional protocols” section). Also, refer to the troubleshooting guide. (“TsG”)

Important notes:

Each ChIP requires sheared chromatin from 1.5 to 2x 10⁶ cells; scale accordingly.

With our Shearing module of the Transcription ChIP kit™: prepare sheared chromatin ready-to-ChIP, then ChIP with either the Transcription ChIP traditional protocol or the OneDay rapid method.

From Diagenode, the **Shearing module** is now available to prepare sheared chromatin ready-to-use in OneDay ChIP. The Shearing module provides you with an easy and highly reproducible chromatin shearing method. Then, ChIP with our rapid ChIP method using our **OneDay ChIP kit™**. Using the two **standard protocols and kits** lead to a significant decrease in potential variables that can occur from shearing to ChIP, which makes interpretation and analysis from experiment to experiment much more accessible.

To obtain sheared chromatin of high quality is a prerequisite for subsequent successful results in Chromatin Immunoprecipitation (ChIP) assays. Establish optimal conditions required for shearing cross-linked chromatin (protein-DNA) is usually laborious; the protocol of the Shearing module is fast, easy-to-use and optimized to get the best results possible.

When cells in culture reach confluency, start the protocol of the Shearing module at step 1 (protocol and reagents as in the Transcription ChIP kit manual). Note that cells can be submitted to inductions and/or treatments. Then treated as well as non treated cells can be prepared and studied in ChIP. Cells can be fixed before scraping (step 1-a.) or cells can be fixed after collection (step 1-b.). Both methods are described in the Transcription ChIP kit manual (Cat. N. kch-redTBP-012). Use the method that is the most suitable for your ChIP target.

To prepare your sheared chromatin, use the optimized buffers provided in the Shearing module of the Transcription ChIP kit™. The buffers are provided for chromatin shearing from up to 100 million cells and ready-to-ChIP chromatin to be used in 50 to 60 IPs. It is possible to shear chromatin from as many as 50 million cells in one go. If working with more cells, scale accordingly. The sheared chromatin is prepared knowing that 45 to 60 µl of sheared chromatin will be used per immunoprecipitation (IP) following our OneDay ChIP protocol (Table 4).

Additional notes:

- Each ChIP assay requires the shearing of 1.5 to 2 million cells; scale accordingly.
- In the case of HeLa cells grown to confluency, there are about 30 million cells per 175 cm² culture flask and that is sufficient for 20 IPs.
- Number of samples will depend on the number of different type of cells and/or conditions to be tested. Cells can be submitted to inductions and/or treatments. Then, sheared chromatin from treated as well as non treated cells can be prepared and studied in ChIP.
- Size of the sample will depend on the number of ChIPs to be tested per sample. One sample is about 3 million cells at a minimum and 50 million cells at a maximum (see manual from the Red ChIP kit). If samples are bigger, scale accordingly.
- Number of IPs: 2 million cells/ IP are needed, e.g.: with a sample of about 50 million cells, 25 IPs can be performed at a maximum (Table 4).

Table 4.

Red ChIP kit : Shearing Module (steps 1 to 3)		
Steps and provided buffers	Quantity provided	Comments
Starting material: 100 million cells	-	Work with 2 samples of 50 million cells each
Cell collection: Buffer A	10 ml	Vol used depends on step followed: Step1-a. or -b.
1.25 M glycine	10 ml	
Lysis 1: Buffer B	30 ml	
Lysis 2: Buffer C	30 ml	50 million cells/ 15 ml
Chromatin shearing: Buffer D	3 ml	50 million cells/ 1.5 ml
Number of OneDay ChIPs that can be performed:	50 IPs (max.)	Use 60 µl of sheared chromatin per OneDay ChIP (corresponding to 2 million cells)
	60 IPs (max.)	Use 45 µl of sheared chromatin per OneDay ChIP (corresponding to 1.5 million cells)

The use of the Diagenode Shearing module and Bioruptor is the key for a successful ChIP. It is fast, optimized and user-friendly. After shearing, the chromatin is ready-to-use in OneDay ChIP.

The Diagenode OneDay Package for Standardized Chromatin IP includes the Bioruptor, the Shearing module and the OneDay ChIP kit.

- **Ensure reproducibility of your Chromatin IP results:** use our OneDay Package!
First, prepare high quality Sheared Chromatin ready-to-ChIP, then ChIP with our Rapid ChIP method:
1/ use standardized protocols,
2/ work with optimized reagents. Therefore, decrease the number of potential variables that can occur from shearing to ChIP, which is essential to greatly facilitate data analysis from experiment to experiment.
- **Save time:** All done in one day's work from cell harvesting to ChIP data analysis!
- Our products are **validated** with positive and negative controls (antibodies and PCR primer pairs, also available at Diagenode).

Protocol

STEP 1. Immunoselection



This first step consists in adding the antibody to the sheared chromatin. During the immunoselection incubation, antibody binding beads are washed to be used in IP at the following step.

Before starting the ChIP assay, determine which samples of sheared chromatin, and which antibodies and controls are going to be used.

(e.g. perform 20 ChIPs in parallel, using 1.5 ml tubes labeled from IP: #1 to #20).

The protocol below is given for 20 IPs to do in parallel on the same day. To do more, scale accordingly.

For each chromatin sample to test in ChIP, test the following:

- antibody directed against the target(s) of interest: one or more.
- negative control: one or more (1. non-immune IgG or 2. no antibody added or 3. antibody blocked with specific peptide).
- positive antibody: one or more (used in previous experiments).

1. Prepare the ChIP buffer 1x from the provided 5x stock solution, see table below.

- To prepare the 1x ChIP buffer use deionized water.
- Store the ChIP buffer 1x at 4°C.

Table 5.

IP #	ChIP buffer 5x	Deionized water	Total volume of ChIP 1x
60	300 ml	1.2 liter	1.5 liter

2. Thaw on ice the protease inhibitor mix (P.I. 200x). Thaw on ice the antibodies that are going to be used in ChIP including negative and positive antibody controls. (4°C)

- One sheared chromatin preparation can be analysed by ChIP with several antibodies and/or amounts of antibodies in parallel. Determine how many ChIPs will be performed with the same sheared chromatin.
- As a negative control, use the non-immune IgG fraction from the same species the antibodies were produced in.

3. Thaw the sheared chromatin on ice or use freshly sheared chromatin from the day.

- 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.
- Per IP: sheared chromatin from 2 million cells is needed (e.g. 60 µl). (60 µl are used at point 7.) It is also possible to use sheared chromatin from 1.5 million cells (e.g. 45 µl). Adjust volumes if necessary. ("TsG")
- Per Input: keep sheared chromatin from 0.2 million cells (point 28.). ("Input") (6 µl are used at point 28.. Adjust volumes if necessary).

During thawing, do the following:

4. For each ChIP, you will need one 1.5 ml tube. Take the necessary new tubes and label them (e.g. 20 tubes for IP: #1 to #20). Place the labeled tubes on ice on your bench. They will be used at point 8. below. (4°C)

5. Label new tubes of 15 ml (one tube per ChIP; use the same numbers as described above at point 4.). Add 12 ml ChIP buffer 1x to each tube. Place the tubes in a cold room or fridge. They will be used at point 39. below. (4°C)
6. Add the protease inhibitor mix (P.I. 200x) to the ChIP buffer 1x. (4°C) That is [P.I.-ChIP buffer 1x] to be used freshly prepared.
 - Add 25 µl of P.I. to 5 ml of ChIP buffer 1x (e.g. volume needed for 20 IPs).
 - [P.I.-ChIP buffer 1x] is used only for the immunoselection incubation (points 7. to 11. below).
7. Add [P.I.-ChIP buffer 1x] to the sheared chromatin (Table 6). Vortex, for 5 seconds, at medium power. (4°C)
 - Knowing how many ChIPs will be performed with the same sheared chromatin (see points 2. and 3. above), prepare a total volume of diluted chromatin including an excess of 10% (as described in Table 6).
 - Using sheared chromatin prepared with our Shearing module: use 60 µl of sheared chromatin per IP, corresponding to 2 million cells.
 - It is important to dilute the sheared chromatin in [P.I.-ChIP buffer 1x] (use a maximum of 28% (vol/vol) of sheared chromatin sample in the mix). ("TsG")
8. Dispense 280 µl per IP tube in the appropriate labeled 1.5 ml tubes (labeled at point 4.). (4°C)
 - Volume of the diluted chromatin per single IP is 280 µl (including 60 µl of sheared chromatin and 220 µl of buffer).

Table 6.

IP #	Sheared Chromatin	P.I.-ChIP buffer 1x	Diluted chromatin
1	66 µl	242 µl	308 µl
2	132 µl	484 µl	616 µl
4	264 µl	968 µl	1232 µl
5	330 µl	1210 µl	1540 µl
10	660 µl	2420 µl	3080 µl
20	1320 µl	4840 µl	6160 µl

9. Freeze the excess. That corresponds to the input sample (28 µl).
10. Add antibody to the samples. Vortex, for 5 seconds, at medium power. (4°C) ("TsG")
 - The amount of antibody to add depends on the antibody and on the target (see troubleshooting guide).
11. Incubate for 30 minutes in the ultrasonic water bath. (4°C) ("TsG")
 - Water bath description is in the "Kit Materials" section and troubleshooting guide.
 - You might want to optimize the incubation time (see troubleshooting guide).
 - If the water bath is not available, step 1 will take longer. The incubation is then depending on your antibody and target (see troubleshooting guide).

During the immunoselection incubation, wash the beads:

12. Take the provided antibody binding beads. Resuspend the beads into a uniform suspension before each use.
 - The beads need a quick wash (points 12. to 21.) and then will be used on the day or can be stored for up to one week at 4°C.
 - Use 840 µl for 20 IPs (e.g. IP: #1 to #20).
 - Cut off the tip of a 1000 µl pipette-tip to pipet the bead suspension.
 - Keep the beads in suspension when pipetting aliquots out. ("!")

13. Transfer 840 µl of bead suspension to a 15 ml tube. Add 10.5 ml of ChIP buffer 1x. Invert the tube twice to mix. That is to wash the beads.
14. Centrifuge for 3 minutes at 500 x g (1,500 - 2,000 rpm) to pellet the beads. (4°C) ("TsG")
15. During the centrifugation, label new tubes of 1.5 ml. For each ChIP, you will need one 1.5 ml tube. Take the necessary new tubes and label them (e.g. IP tubes: #1 to #20). Place the labeled tubes on ice. They will be used at point 18. (4°C)
16. When the centrifugation is finished, do not disturb the bead pellet. Discard the supernatant. ("!")
17. Add to the pelleted beads, 10.5 ml** of ChIP buffer 1x again. That is the second wash.
 - **: If you work with yeast rather than with cultured cell lines, you might need to block the beads with BSA. To block the beads, do as follows: add 5 ml** ChIP buffer 1x to the pellet of beads rather than 10.5 ml. Then add 50 µl of blocker to the beads (to reach a final concentration of 5 mg/ml). Rotate at 4°C for 15 minutes. Centrifuge the beads at low speed (500 x g) for 2 to 3 minutes. Discard the supernatant. Add 10.5 ml ChIP buffer 1x to the pellet of blocked beads.
18. Dispense 0.5 ml of washed antibody binding beads per new labeled IP tube.
 - Keep the antibody binding beads in suspension while aliquoting into the tubes.
19. Centrifuge for 2 minutes at 500 x g (2,500 - 3,000 rpm) to pellet the beads. (4°C) ("TsG")
 - Check by eye that equal pellets of beads are present in each tube. ("!")
20. Do not disturb the bead pellet. Discard the supernatant. (4°C)
 - Aspirate the buffer that could be in the cap. ("!")
21. Keep on ice the pellets of washed antibody binding beads - until point 25. below -.
 - The beads are ready for the chromatin immunoprecipitation incubation. (4°C)

When the immunoselection incubation of 30 minutes is over, do the following:

22. Take out of the ultrasonic bath the tubes that contain the [antibody-chromatin] mix.
23. Centrifuge for 10 minutes at 14,000 x g (12,000 rpm). (4°C)
At this stage it is very important to get rid of any unspecific aggregates. ("!")
24. Do not disturb the pellet. ("!"). The supernatant contains the formed [antibody-chromatin] complexes.

STEP 2. Immunoprecipitation



This second step consists in adding the formed [antibody-chromatin] complexes to antibody binding beads. Then, the IP incubation is followed by washes.

25. Transfer 250 µl of the supernatant (from point 24.) to the pellets of washed antibody binding beads (prepared at point 21.). (4°C)
 - Do not add all the [antibody-chromatin] mix to the pellet of beads.
 - Do not take more than 250 µl out of the tubes and discard the rest.
 - Check the # on the tubes. Make sure you transfer to the correct labeled tube.
26. Incubate the [antibody-chromatin-beads] mix for 30 minutes on a rotating wheel. (4°C)
 - Incubations of up to one hour can be performed.

During the immunoprecipitation incubation, do the following:

27. Label new tubes of 1.5 ml. For each ChIP, you will need one 1.5 ml tube. Take the necessary new tubes and number them (e.g. IP tubes: #1 to #20). Place the labeled tubes on ice. They will be used at point 45. below. (4°C)
28. Take 6 µl of input (sheared chromatin aliquot as described at point 3.). Add 30 µl of 100% ethanol. That is to precipitate the total DNA from the aliquoted input. ("Input chromatin")
 - 6 µl of sheared chromatin corresponds to 10% of the amount used per IP (see point 7.).
 - Alternatively use the 28 µl excess prepared at point 9.
29. Incubate for 10 minutes on ice. (4°C)
30. Centrifuge for 10 minutes at 10,000 x g. (4°C)
31. Do not disturb the pellet. Aspirate the supernatant. Keep the pellet on ice. (4°C)
 - It is possible to wash the pellet with ethanol 75% as follows: add 50 µl of 75% ethanol (vol/vol) to the pellet. Centrifuge for 10 minutes at 10,000 x g again. (4°C) Do not disturb the pellet. Discard the supernatant. Keep the pellet.
32. Leave the cap open to air-dry the pellet - until point 51. -. ("Input DNA")

When the IP incubation of 30 minutes is over, proceed to the next point:

33. Take the tubes that contain the [antibody-chromatin-beads] mix out of the rotating wheel.
34. Open all the tubes, add 1 ml ice-cold ChIP-buffer 1x to each tube. Close them all.
 - The [antibody-chromatin-beads] mix will be submitted to washes.
 - Wash to isolate the [chromatin-antibody] complexes that are attached to the beads.
 - After each centrifugation remove the supernatant without disturbing the pellet.
35. Invert tubes twice.
36. Centrifuge for 2 minutes at 500 x g (2,500 - 3,000 rpm) to pellet the beads. (4°C)

37. Do not disturb the bead pellet. Aspirate the supernatant. (4°C)
- Aspirate the buffer that could be in the cap. (!)
38. Keep the bead pellets on ice. Add 1 ml of ice-cold ChIP buffer 1x to each bead pellet.
39. Take the labeled 15 ml tubes containing 12 ml of cold ChIP 1x buffer (prepared at point 5.). Open all the caps.
40. One IP tube at a time: resuspend the beads in the 1 ml of cold buffer that was just added to the beads - point 38.
- by pipetting twice up-and-down and transfer to the corresponding labeled 15 ml tube.
 - Check the # on the tubes. Make sure you transfer to the correct labeled tube.
 - Add the 1 ml of [buffer-bead] suspension at the very top, on the wall of the 15 ml tube.
 - Beads will go down and will be washed while going through the 12 ml of ice-cold ChIP buffer 1x.
41. Incubate the 15 ml tubes in a refrigerated centrifuge (or cold room) for 5 minutes. (4°C)
42. Centrifuge for 3 minutes at 500 x g (1,500 - 2,000 rpm) to pellet the beads. (4°C)
43. Check by eye, that you can see a pellet of beads at the bottom of each tube.
44. Take all the caps off, throw them away. Aspirate 12 ml of buffer. About 1 ml of buffer is left in each tube.
45. Take new labeled 1.5 ml tubes (e.g. IP: #1 to #20) - prepared at point 27. -. Open the caps.
46. One IP tube at a time: resuspend the bead pellet in the buffer left (pipette twice up-and-down) and transfer the 1ml [buffer-bead] suspension to the new 1.5 ml tube.
- Check the # on the tubes.
47. Centrifuge for 2 minutes at 500 x g (2,500 - 3,000 rpm) to pellet the beads. (4°C)
- Check by eye, that you can see a pellet of beads in each tube.
48. Do not disturb the bead pellet. Aspirate the supernatant. (4°C)
- Aspirate the buffer that could be in the cap. (!)
 - After this last wash, remove carefully the last trace of buffer.
49. Keep the pellets of beads. Leave the tubes at room temperature with the cap open. (RT)
- [chromatin-antibody] complexes attached to the beads have been isolated.
 - Do not work on ice anymore.
 - Use barrier tips, from this step onwards.
 - Use the PCR-grade water provided in the kit, from this step onwards.
 - From the washed beads, the DNA can now be purified (proceed to the next point).

STEP 3. DNA purification



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

50. Put water to boil.
51. Add 100 µl of water to the dried pellet of "Input DNA" (point 32.). Suspend the dried pellet. Incubate at RT - until point 54. -.
52. Take the provided DNA purifying slurry. Resuspend into a uniform suspension before each use.
 - Keep the slurry in suspension while pipetting aliquots out. ("!")
 - Use filtered tips. The tips do not need cutting.
 - Pipet up-and-down before aliquoting into each tube.
53. Add 100 µl of DNA purifying slurry directly to the washed beads - from point 49. -.
 - These are the IP samples.
 - The positive IP samples will include [chromatin-antibody-bead] complexes.
 - Keep the slurry in suspension while adding to the tubes. ("!")
 - Wait for one minute. Meanwhile, proceed to point 54..
 - Afterwards, check by eye that aliquots are equal in each tube.
54. Add 100 µl of DNA purifying slurry directly to the input sample - from point 51. -.
 - This is the "Input" sample.
 - Keep the slurry in suspension while adding to the tubes. ("!")
 - Wait for one minute. Meanwhile, go back to check the pellets at point 53..
 - Afterwards, check by eye that aliquots are equal in each tube.
55. Invert the tubes from points 53. and 54..
 - From this step onwards, work with both IP and Input samples in parallel.
56. Lock the tubes with the tube claps. ("!")
57. Incubate the samples for 10 minutes in boiling water.

During boiling, do the following:

58. Turn on the thermomixer, set the temperature at 55°C.
59. Thaw on ice the provided proteinase K.
60. Label new tubes of 1.5 ml. Take the necessary new tubes and label them (e.g. IP tubes: #1 to #20 and input sample(s)). Tubes will be used at point 71. (RT)

When the 10 minutes boiling are over, proceed to next point:

61. Take the tubes out of the boiling water.
 - Boiling water will be needed at point 69. again.
62. Take off the tube claps. Wait for samples to cool down.

63. Add 1 µl of proteinase K to each sample.
 - Use barrier tips.
64. Vortex, for 2 seconds, at medium power.
65. Shake samples for 30 minutes at 1,000 rpm in the thermomixer at 55°C.

During the 30 minutes incubation, do the following:

66. If you plan to do the PCR on the same day: determine which PCR primer pairs will be used, including controls:
 - target of interest to be tested,
 - Negative control: one or more,
 - Positive control: one or more.
67. Thaw on ice the primer pairs. Prepare the tubes and/or plates.

When the incubation of 30 minutes is over, proceed to the next point:

68. Lock the tubes with the tube claps. ("!")
69. Incubate the samples for 10 minutes in boiling water.
70. Centrifuge for 1 minute at 14,000 x g (12,000 rpm). (4°C)
71. Do not disturb the pellet. Transfer 70 µl of the supernatant to new labeled 1.5 ml tubes - prepared at point 60.
 - Do not transfer the pellet. Any trace of the pellet in the collected sample will interfere with the subsequent PCR. ("!")
72. Add 130 µl of water to the pellet.
73. Vortex for 10 seconds at medium power.
74. Centrifuge for 1 minute at 14,000 x g (12,000 rpm). (4°C)
75. Collect 130 µl of supernatant and pool with the previous supernatant (point 71.). Mix. The total volume for each sample is 200 µl.
 - The DNA from the ChIP and the input sample(s) is now purified and ready to be analysed by PCR.
 - Proceed to the PCR step immediately or freeze all the samples. ("STOP")
 - Store at -20°C.

STEP 4. Quantitative PCR and data analysis



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

Quantitative PCR

Chromatin immunoprecipitation yields very low amounts of DNA but that can be amplified by real-time polymerase chain reactions (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 the "qPCR additional notes" below.

Each ChIP yields sufficient amounts of DNA for 80 qPCR.

- Use 2.5 µl of purified DNA from ChIP in a 10 µl final PCR-volume per well, in a 384-well plate.

Input DNA can be analysed by PCR as well, but needs to be diluted before hand (e.g. use a dilution 1:10 corresponding to input DNA at a final concentration of 100 cells equivalent per microliter).

- Use 2.5 µl of diluted purified DNA from input in a 10 µl final PCR-volume per well, in a 384-well plate.

One PCR mix contains: 2.5 µl DNA template, 5 µl qPCR buffer mix, 2.2 µl H₂O and 0.3 µl primer pair (10 µM each). Perform each PCR in triplicate.

76. Thaw the qPCR buffer mix on ice. (4°C)

- The buffer mix could be IQ SYBR Green supermix or any other that is used in your lab. Follow the instructions of your supplier.

77. Add H₂O to the qPCR buffer mix as follows: (4°C)

Mix reagents for n+1 reactions, where n is the number of PCR reactions to be performed. For example, per three PCR reactions: add 8.8 µl of H₂O to 20.0 µl of buffer mix.
Vortex for 4 seconds, at medium power.

78. Per three PCRs: add 1.2 µl of primer set (10 µM). (4°C)

79. Check that the volume of [buffer-H₂O-primers] mix is prepared with an excess and that the final volume is correct (e.g. 30 µl is prepared, 22.5 µl will be used). (4°C)

80. Add 2.5 µl of DNA template per well in the PCR plate, in triplicate. (4°C)

81. Dispense 7.5 µl of [buffer-H₂O-primers] mix per well, in the PCR plate. (4°C)

82. Cover the plate with an optical adhesive.

83. Spin for 2 minutes at 500 x g (2,500 – 3,000 rpm). (4°C)

84. Proceed to the PCR using cycles and temperatures according to your primer pairs.

Data analysis

85. Data are acquired using the PCR apparatus program (e.g. ABI). For each primer pair the threshold and baseline are set automatically.

86. The data is exported to Excel spreadsheets.

87. Relative occupancy of the immunoprecipitated factor at a locus is calculated using the following equation:

$$2^{-(CtNegCtl - CtTarget)}$$

Where CtNegCtl and CtTarget are mean threshold cycles of PCR done in triplicates on DNA samples from negative control ChIP (using non-immune IgG) and targeted ChIP (using specific antibody) [4] (Fig. 1 and 2). ("TsG")

qPCR additional notes:

Your own primer design

- Self-complementarity and secondary structure of the primers can be tested using primer design programs. Annealing temperature of 60°C is recommended for qPCR primers.
- Short length of amplified DNA fragment (50 - 100 bp) facilitates the PCR efficiency and reduces potential problems in amplification of G/C-rich regions.
- Difference in melting temperature between forward and reverse primers should not exceed 2 to 3°C.
- G/C stretches at the 3' end of the primers should be avoided.

Validation of your primers

- Test primer sets by in silico PCR: <http://genome.cse.ucsc.edu/cgi-bin/hgPcr>. Primers should amplify unique DNA products from the genome.
- Test every primer set in qPCR using 10 fold-serial dilutions of input DNA. With the slope of the obtained curve, calculate amplification efficiency (AE) of primer set by formula (5): $AE = 10^{-1 / \text{slope}}$

An amplification factor of 2 is highly reliable. If it is not the case the qPCR reagents from different brand or new primers should be tested.

Data interpretation

- Positive IP/ Negative IP can be used to determine the ChIP efficiency as well as ChIP specificity and to present ChIP data as described above (see point 87. above and Fig. 1).
- Alternatively, efficiency of chromatin immunoprecipitation of particular genomic locus can be calculated from qPCR data as a percentage of starting material [% of input as recovery]:

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

Here AE is amplification efficiency as calculated above (5); CtChIP and Ctinput are threshold values obtained from exponential phase of qPCR; Fd is a dilution factor of the input DNA to balance the difference in amounts of ChIP and input DNA taken for qPCR.

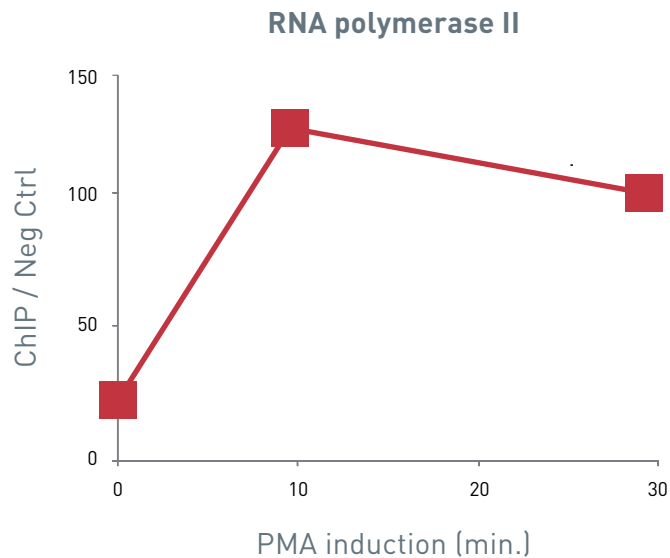
Relative occupancy can be calculated as a ratio of specific signal over background:

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

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

OneDay ChIP Kit Results:

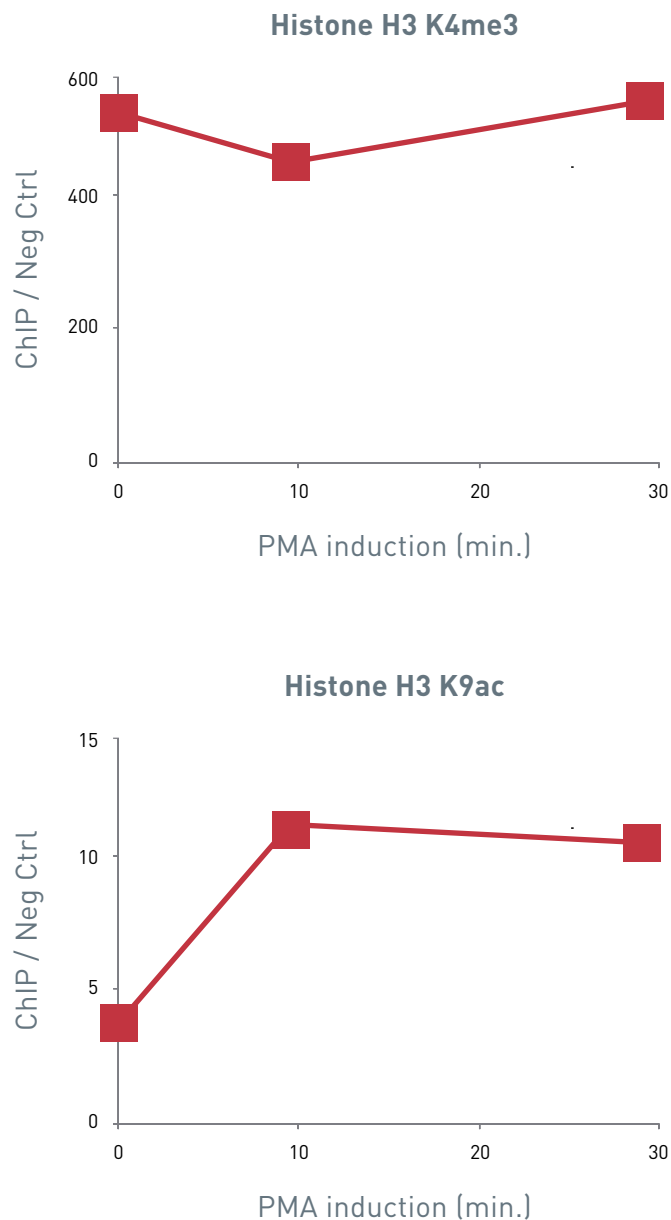
Figure 1 (OneDay ChIP results: qPCR): OneDay ChIP kit



ChIP results obtained with the Diagenode OneDay ChIP kit.

ChIP assays were performed using hepatoma HTC-IR cells and antibodies directed against RNA polymerase II. Cells were non-induced and induced with 0.1 μ M PMA (phorbol 12-myristate 13-acetate) for 10 and 30 minutes as indicated in the graph. Sheared chromatin was obtained with an "in house" protocol (see the "starting material" and "additional protocols" sections). Optimized qPCR primer pairs to amplify the promoter of the PMA-inducible *egr* gene were used. A negative control was included in the ChIP assay (Negative Ctrl IgG from rabbit). Data analysis was performed as described in the manual ("qPCR" section: step 4- point 87.).

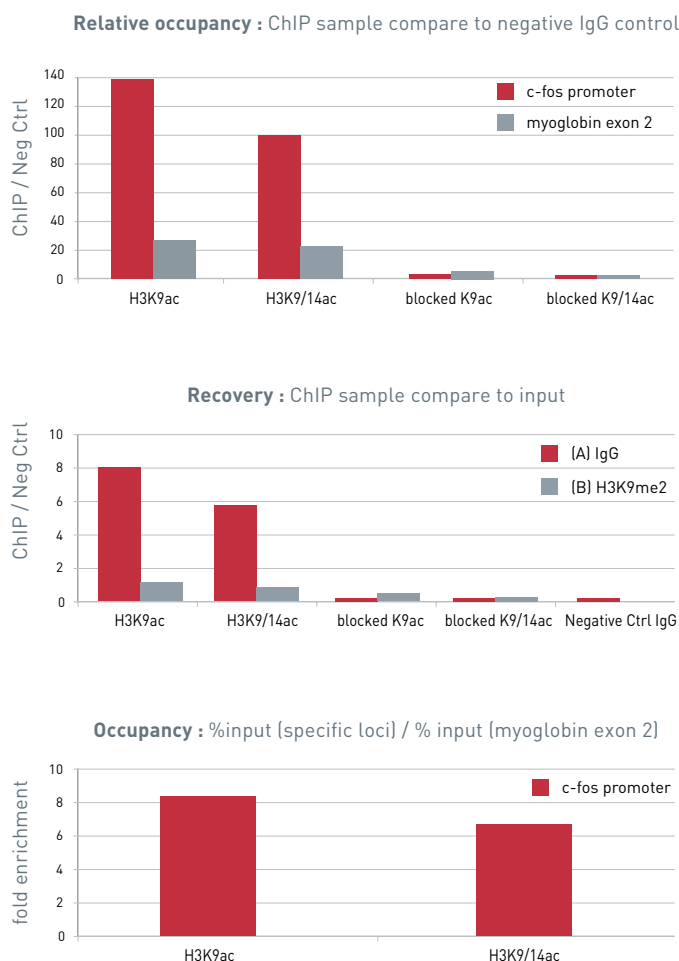
Figure 2 (OneDay ChIP results: qPCR): **OneDay ChIP kit**



ChIP results obtained with the Diagenode OneDay ChIP kit.

ChIP assays were performed using hepatoma HTC-IR cells and antibodies directed against modified histone H3. Cells were non-induced and induced with 0.1 μ M PMA for 10 and 30 minutes as indicated in the graph. Sheared chromatin was obtained with an "in house" protocol (see the "starting material" and "additional protocols" sections). Optimized qPCR primer pairs to amplify the promoter of the PMA-inducible *egr* gene were used. A negative control was included in the ChIP assay (Negative Ctrl IgG from rabbit). Data analysis was made as described in the manual (step 4- point 87.).

Figure 3 (ChIP results: qPCR): **Shearing module + Bioruptor + OneDay ChIP kit**



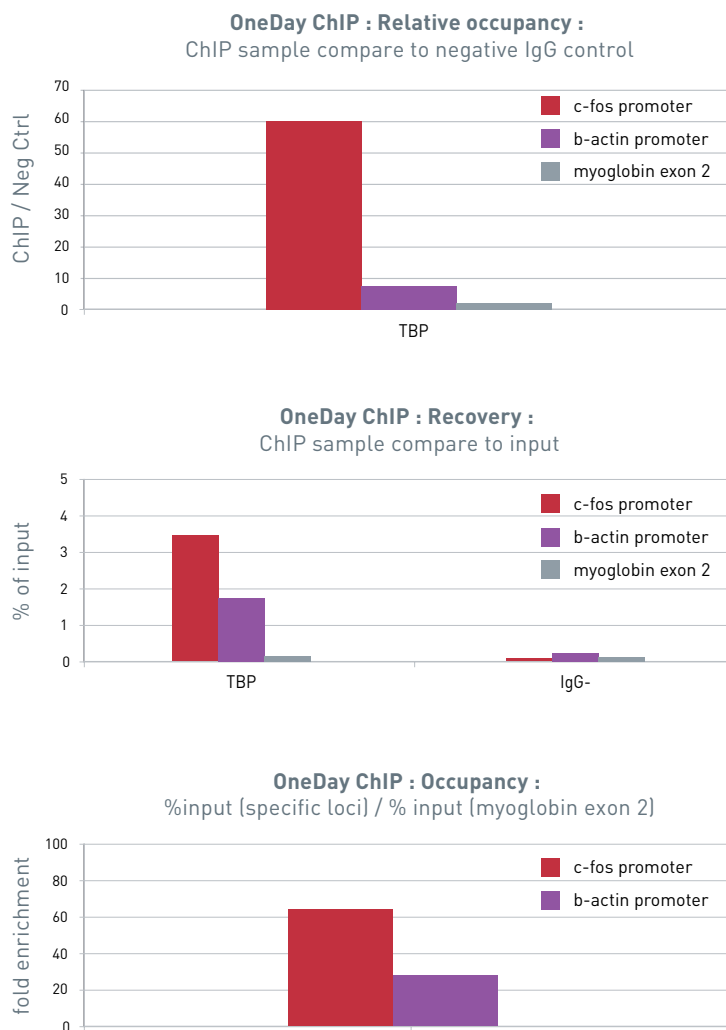
ChIP results obtained with the Diagenode OneDay ChIP kit.

ChIP assays were performed using HeLa cells, the Diagenode antibodies directed against acetylated histone H3 (H3K9ac and H3K9/14ac) and optimized PCR primer sets for qPCR.

Two Diagenode kits were used: one to prepare the sheared chromatin ready-to-ChIP and the other to ChIP (respectively: Shearing module from the Red ChIP kit and the OneDay ChIP kit). Chromatin sheared from 2x 10⁶ cells (60 µl) and 2 µg of antibody (anti-H3K9ac or anti-H3K9/14ac) were used per ChIP experiment. A negative control antibody was included in the ChIP assay (negative IgG). Other controls for IP and PCR specificity include: a/ PCR performed with the primers for myoglobin exon 2 (negative control); b/ PCR was performed with each primer pairs and the input sample (as a positive control for input and positive control for the PCR and primer pairs); and c/ IPs were performed with blocked antibodies.

Top graph: relative occupancy is given. Data analysis was performed as described in the manual ("qPCR" section: step 4- point 87.). Bottom graphs show data as % of input (recovery) and occupancy. Occupancy of the c-fos promoter is evident based on the qPCR analysis of immunoprecipitated DNA. The occupancy is greatly reduced when using blocked antibody in IP (prior to IP, antibodies were pre-incubated with the peptide used for antibody production).

Figure 4 (ChIP results: qPCR): **Shearing module + Bioruptor + OneDay ChIP kit**



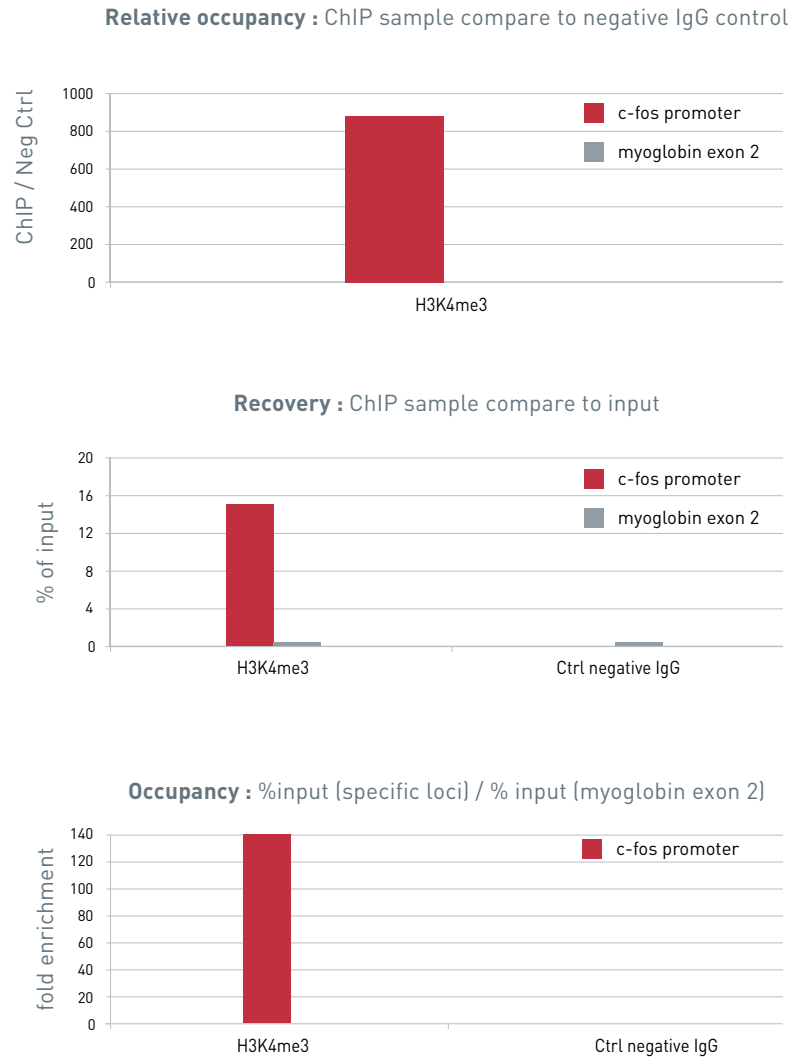
ChIP results obtained with the Diagenode OneDay ChIP kit.

ChIP assays were performed using HeLa cells, the Diagenode antibodies directed against TBP and optimized PCR primer sets for qPCR.

Two Diagenode kits were used: one to prepare the sheared chromatin ready-to-ChIP and the other to ChIP (respectively: Shearing module from the Red ChIP kit and the OneDay ChIP kit). Chromatin sheared from 2x 10⁶ cells (60 µl) and 5 µg of antibody anti-TBP were used per ChIP experiment. A negative control antibody was included in the ChIP assay (negative IgG). Other controls for IP and PCR specificity include: a/ PCR performed with the primers for myoglobin exon 2 (negative control); b/ PCR was performed with each primer pairs and the input sample (as a positive control for input and positive control for the PCR and primer pairs).

Top graph: relative occupancy is given. Data analysis was performed as described in the manual ("qPCR" section: step 4- point 87.). Bottom graphs show data as % of input (recovery) and occupancy. Occupancy of the c-fos and b-actin promoters is evident based on the qPCR analysis of immunoprecipitated DNA.

Figure 5 (ChIP results: qPCR): **Shearing-ChIP kit + Bioruptor + OneDay ChIP kit**



ChIP results obtained with the Diagenode OneDay ChIP kit.

ChIP assays were performed using HeLa cells, the Diagenode antibodies directed against histone H3K4me3 and optimized PCR primer sets for qPCR. Two Diagenode kits were used: one to prepare the sheared chromatin ready-to-ChIP and the other to ChIP (respectively: Shearing-ChIP kit and the OneDay ChIP kit). Chromatin sheared from 1.5×10^6 cells (60 μ l) and 2 μ g of antibody anti-H3K4me3 were used per ChIP experiment. A negative control antibody was included in the ChIP assay (negative IgG).

Top graph: relative occupancy is given. Data analysis was performed as described in the manual ("qPCR" section: step 4- point 87.). Bottom graphs show data as % of input (recovery) and occupancy. Occupancy of the c-fos promoter is evident based on the qPCR analysis of immunoprecipitated DNA. Controls for IP and PCR specificity include: a/ PCR performed with the primers for myoglobin exon 2 (negative control); b/ PCR was performed with each primer pairs and the input sample (as a positive control for input and positive control for the PCR and primer pairs); and c/ IP was performed with a negative control antibody.

Troubleshooting guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the fixation step for the correct period of time, at the right temperature and with the correct formaldehyde concentration. e.g: incubate for 10 minutes at room temperature with 1 % formaldehyde final concentration (weight/ volume). Also, use high quality, fresh formaldehyde.
	Cross-linking is too strong.	
	Proteins have unique ways of interacting with the DNA. Some proteins are not directly bound to the DNA but interact with other DNA-associated proteins.	Very short or very long cross-linking time can lead to DNA loss and/or elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity and efficiency of ChIP.
	Both cross-linking time and formaldehyde concentration are critical.	Cross-linking can affect both efficiency of chromatin shearing and efficiency of specific antigen immunoprecipitation. Shorter cross-linking times (5 to 10 minutes) and/or lower formaldehyde concentrations (1%, weight/ volume) may improve shearing efficiency while, for some proteins especially those that do not directly bind DNA, this might reduce the efficiency of cross-linking and thus the yield of precipitated chromatin.
	The optimal duration of cross-linking varies between cell type and protein of interest.	It is possible to optimize the fixation step by testing different incubation times: such as 10, 20 and 30 minutes. Do not cross-link for longer than 30 minutes as cross-links of more than 30 minutes can not be efficiently sheared.
	Efficient fixation of a protein to chromatin in vivo is a crucial step for ChIP. The extent of cross-linking is probably the most important parameter.	Two major problems concerning the subsequent immunoprecipitation step should be taken into account: 1/ an excess of cross-linking can result in the loss of material or reduced antigen availability in chromatin, or both. 2/ the relative sensitivity of the antigen epitopes to formaldehyde. It is essential to perform the cross-linking step with care.
	It is essential to quench the formaldehyde.	Use glycine to stop the fixation: quench formaldehyde with 125 mM glycine for 5 minutes at room temperature (add 57 µl of 1.25M glycine per 513.5 µl of sample, see STEP 2). Alternatively, wash the fixed cells properly and make sure you get rid of ALL the formaldehyde.
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol (e.g.: 1 million cells or less/ 130 µl of complete Buffer B, see STEPS 2 and 3).
	Temperature is critical.	Perform cell lysis at 4°C (cold room) or on ice. Keep the samples ice-cold at all times during the cell lysis and use ice-cold buffers see STEP 3.
	Protein degradation during lysis can occur.	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Kit protocol validation	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells) and Keratinocytes have been used to validate the OneDay ChIP kit.
Cell number necessary per ChIP	The amount of cells required for a ChIP experiment is determined by cell type, protein of interest and antibodies used.	We recommend the use of 2x 10 ⁶ cells per ChIP. For some cases, less than 2x 10 ⁶ cells could yield successful results. Also, up to 5x 10 ⁶ cells could be used per ChIP.

Chromatin shearing	Optimal shearing conditions are important for ChIP efficiency.	The conditions of shearing are to be optimized for each cell type, fixation protocol and sonicator apparatus.
	To get good shearing is critical.	You might want to optimize shearing conditions for your specific cell type and fixation protocol. Therefore, 1) to start with a small sample (3x 10e6 cells) and 2) check the shearing efficiency is advised. Also: 3) Keep samples cold. 4) Check the buffer composition. (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] prior to immunoselection. The final SDS concentration should not be higher than 0.15 to 0.20%).
	Shear the samples of chromatin using the Bioruptor™ from Diagenode (catalog # UCD-200).	Maintain temperature of the samples close to 0°C. Samples are sonicated for 10 cycles of: [30 seconds "ON" / 30 seconds "OFF"] each. Total time is 10 minutes (these conditions were tested with many mammalian cell lines and were excellent for subsequent ChIP experiments). A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.
Sheared chromatin analysis	Purify the DNA from the sheared chromatin as described in the kit protocol to analyse the shearing.	Extract total DNA from an aliquot of sheared chromatin and run on 1% agarose gel (stain with EtBr). In order to analyse the sheared chromatin on gel, take 10 µl of DNA purified from the sheared chromatin input -prepared at point 75. Some unsheared chromatin can be analysed on gel as well (purify it as done with the input sample (from points 28. to 32., then points 51. to 75.)).
	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.
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 the [P.I.-ChIP buffer 1x] prior to the immunoselection incubation. Dilute the sheared chromatin at least 3.5 fold. Adjust the ChIP buffer volume added to the chromatin accordingly.
Antibody binding beads	Beads are in suspension.	The provided beads are coated with protein A. Resuspend into a uniform suspension before each use.
	Bead centrifugation.	Do not spin the beads at high speed. Use gentle centrifugation (500 x g for 2-3 minutes) as described in the manual protocol. $g = 11.18 \times r \times (rpm/1000)^2$; knowing that r is the radius of rotation in mm. (http://www.msu.edu/~venkata1/gforce.htm). It is possible to centrifuge the 1.5 ml tubes at 1,000 – 2,000 g, for 20 seconds.
	Bead storage	Store at 4°C. Do not freeze.
	Antibody binding capacity.	pAb from rabbit, guinea pig, pig, human IgG. MAb from mouse (IgG2), human (IgG1,2 and 4); and rat (IgG2c).
Protease inhibitors	Storage.	Some inhibitors are unstable in solution. The provided P.I. mix should be kept frozen at -20°C, and thawed before use.
	P.I.-ChIP 1x buffer.	Add P.I. mix to ChIP buffer 1x, just before use. Prepare what is going to be used in the day's experiment.
Other enzyme inhibitors	Specific enzyme inhibitors are not included in the kit, such as phosphatase inhibitors.	Add phosphatase inhibitors or others to the ChIP 1x buffer if necessary, depending on your research field and protein(s) of interest to be ChIP'd.

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, see point 87.
	Do not add antibody to the IP.	No antibody is added to the sheared chromatin at step1. Therefore, at step 2, the IP incubation mix includes sheared chromatin and antibody binding beads but no antibody. Incubation with beads without antibodies could also be used as a negative ChIP control as well as non-immune IgG, see point 87.
	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.
	How many negative controls are necessary?	If multiple antibodies - of the same specie - are to be used with the same chromatin preparation then a single negative ChIP control is sufficient for all of the antibodies used.
Antibody in IP	Why is my antibody not working in ChIP?	Antibody-antigen recognition can be significantly affected by the cross-linking step resulting in loss of epitope accessibility and/or recognition.
	Which antibody should I use in ChIP?	Use ChIP-grade antibodies. If not available, it is recommended to use several antibodies directed against different epitopes of the same protein. Verify that the antibodies can work directly in IP on fresh cell extracts. Also, when testing new antibodies, include known ChIP-grade antibodies as positive control for your ChIP assay.
	How do I choose an antibody for ChIP?	Be aware of the possible cross-reactivity of antibodies. Verify by Western blot analysis the antibody specificity. Antigen affinity purification can be used to increase titer and specificity of polyclonal antibodies.
	What is the amount of antibody per ChIP to use?	It should be determined empirically for each target and antibody. For abundant proteins, like histones, use 1 to 2 µg of affinity purified or monoclonal antibody per IP. For other targets, use up to 10 µg per ChIP. To ensure efficient IP it is important to have an optimal ratio between the amount of chromatin and the amount of antibody. More antibody (or less chromatin) can be required in case of low affinity to antigen or high abundance of target protein (e.g. histones). Insufficient amount of antibody can result in low efficiency of ChIP whereas large excess of antibody might lead to lower specificity.
	Are my antibodies going to bind the protein A?	There is a significant difference in affinity of different types of immunoglobulin to protein A. For example, IgM or IgY can require a secondary antibody as a bridge to protein A.
Immuno-selection incubation	What is the best incubation time for immunoselection using the ultrasonic water bath?	To incubate the sheared chromatin with antibodies for 15 to 30 minutes works for many antibodies, however, the kinetics for reaching equilibrium of epitope-antibody binding may differ for each antibody and target. Optimization might improve the results (e.g. the incubation time may need to be increased for some antibodies).
	How does the immunoselection using the ultrasonic water bath work?	The rate-limiting step in many immunoassays is associated with the slow kinetics of binding of macro-molecular antigen to antibody. It was demonstrated that the use of ultrasonic energy to enhance mass transport across liquid/solid interfaces can dramatically accelerate antigen binding to antibodies.
	Where can I buy the ultrasonic bath?	http://www.bransonic.com/model_3510.asp : Branson cat# CPN-952-316. Fisher Scientific cat#15-337-22F.
	What are the water bath specifications?	Model MT-3510. Capacity: 5.5 liters. Size (LxWxH): 29x15x15 cm. Frequency: 42 kHz. Max power requirement: 130 W. RF-Power: 130 W
	Can I use the kit w/o an ultrasonic water bath?	Yes, then a long incubation at 4°C should be used. Depending on the antibody and target to be ChIP'd, the times of incubation range from 2 to 16 hours and should be determined empirically for each antibody.

Polymerase Chain Reaction	Primer design.	Primer length: 18 to 24 nucleotides/ Primer Tm: 60°C (+/- 3.0°C)/ % GC: 50% (+/- 4%).
	Controls: negative and positive.	Negative PCR controls: PCR with DNA from samples IP'd with non-immune antibodies (negative IgG). Alternatively, PCR using DNA from ChIP samples and primers specific for a DNA region to which, your antigen of interest is not binding. Positive PCR control: PCR using input DNA.
	No PCR signal.	Include a positive PCR control as a control for your PCR mix (your primers, dNTP and Master Mix) using the Input DNA or a DNA sample of the same origin.
	High Ct values.	Use more input chromatin.
	Ct ^{NegCtl} and Ct ^{Target} .	The ratio between target IP and negative control IP depends on the antibody used.
	Background is high.	Verify that you perform properly the following steps: Centrifugation (point 23.). Keep the antibody binding beads and DNA purifying slurry in suspension while adding to the tubes. Check by eye that equal pellets of beads and slurry are present in each tube. Washes (points 39. to 49.) are critical. Aspirate the buffer that could be in the cap (points 20., 37., 48.).
Freezing	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.
	Samples can be frozen at several steps of the protocol.	- Pellets of formaldehyde fixed cells can be stored at -80°C for at least a year. - Sheared chromatin can be stored at -80°C for months, depending on the protein of interest to be ChIP'd. - Purified DNA from ChIP and input samples can be stored at -20°C for months.
	Avoid multiple freeze/thawing.	Snap freeze and thaw on ice (e.g.: fixed cell pellets and sheared chromatin).

Additional protocols : Chromatin shearing methods

Important notes:

Each ChIP requires sheared chromatin from 1.5 to 2x 10⁶ cells; scale accordingly.

Quick overview:

Cells can be submitted to inductions and/or treatments. Then treated as well as non treated cells can be prepared and studied in ChIP. Start the protocol below when cells reach confluency.

Cross-linking and harvesting cells

1. Add formaldehyde in overlaying media and incubate.
 - Handle formaldehyde in a chemical fume hood.
 - Use final concentrations of formaldehyde of 1 to 1.5 %.
 - Incubate for 5 to 15 minutes, depending on your cells and target of interest.
2. Quench formaldehyde with glycine (125 mM final, 5 minutes at RT).
3. Scrape cells and collect by centrifugation, then wash twice with ice-cold PBS.
 - After the cross-link, keep the samples ice-cold at all times.
4. Count the cells.
5. Collect the cell pellet by centrifugation again.
 - Cell pellets can be frozen. ("STOP")
6. Add lysis buffer containing protease inhibitors to the cell pellet. Resuspend the pellet by pipetting up-and-down several times. Centrifuge and keep the pellet. (4°C)
7. Add lysis buffer containing inhibitors to the nuclear pellet, resuspend the pellet and centrifuge. Discard the supernatant. Resuspend the washed pellet in shearing buffer containing protease inhibitors. (e.g.: 1.5 to 2 million cells/ 60 µl). That is the chromatin sample.(4°C)

Chromatin shearing

8. Shear the chromatin sample (4°C). Optimal average DNA fragment size is between 200 and 600 bp.
9. Clear the lysate. Keep the supernatant. That is the sheared chromatin ready-to-ChIP.
10. Transfer an aliquot of sheared chromatin (equivalent to 0.2 million cells) to a new 1.5 ml tube. This aliquot corresponds to the input sample. From the input sample total DNA will be isolated as a control: 1) for input DNA in the ChIP assays (points 3. and 28. in the OneDay ChIP kit protocol) and 2) for shearing efficiency (see troubleshooting guide). ("Input")
11. The sheared chromatin is ready to be used in ChIP. Use the freshly prepared chromatin directly in ChIP assays. Alternatively -and depending on the protein(s) of interest to be ChIP'd - the sheared chromatin can be aliquoted and frozen at -80°C. ("STOP")

Chromatin shearing using the DIAGENODE Bioruptor and Shearing methods:

– **THE SHEARING MODULE from the Transcription ChIP Kit** catalog # kch-redmod-100

– **THE SHEARING-ChIP Kit** cat. #: kch-shchro-40

- 1/ **With the Shearing ChIP kitTM**: prepare sheared chromatin ready-to-ChIP, then ChIP with either the Transcription ChIP traditional protocol or the OneDay rapid method.
See in the “starting material” section.
- 2/ **With the Shearing Optimization kitTM**: prepare large amounts of sheared chromatin ready-to-ChIP. The Shearing-ChIP kit [cat#: kch-shchro-40] includes optimized buffers and a user-friendly protocol to produce huge amounts of sheared chromatin of high quality. The main steps of the kit protocol are described in the kit manual and below [Table 7].
Grow your cells of interest and start the protocol when cells reached confluency. Table 7 gives you detailed information about the amount and type of cells to use per chromatin shearing trial and per OneDay ChIP assay.

To prepare your sheared chromatin, use the optimized buffers provided in the Shearing-ChIP kitTM. The buffers are provided for chromatin shearing from up to 150 million cells and ready-to-ChIP chromatin to be used in 100 IPs at a maximum. The Table below indicates the volumes of buffer needed per sample size. One sample consists on 6x 10e6 cells at a minimum. Alternatively, it is possible to shear chromatin from as many as 30 million cells. If working with more cells, scale accordingly. In any case, the sheared chromatin is prepared knowing that 60 to 80 µl of sheared chromatin will be used per immunoprecipitation (IP) following our OneDay ChIP protocol (Table 7).

Table 7.

Shearing Optimization kit		
Steps and provided buffers	Quantity provided	Comments
Starting material: 150 million cells	-	Work with 5 samples
of 30 million cells each	10 ml	Vol used depends on step followed: Step1-a. or -b.
Cell fixation and collection: DTT-Buffer A	18 ml	30 million cells/ 3 ml
Lysis 1: Buffer B	18 ml	30 million cells/ 3 ml
Lysis 2: Buffer C	18 ml	30 million cells/ 3 ml
Number of OneDay ChIPs that can be performed:	75 IPs (max.)	Use 80 µl of sheared chromatin per OneDay ChIP (corresponding to 2 million cells)
	100 IPs (max.)	Use 60 µl of sheared chromatin per OneDay ChIP (corresponding to 1.5 million cells)

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