diagenode

A Hologic Company

Chromatin shearing optimization kit

Medium SDS

(for HighCell# ChIP kit)

Cat. No. **C01020011**

Version 4 | 04.16

Content

MANUAL

Introduction

The first critical step of a successful Chromatin Immunoprecipitation (ChIP) experiment is the preparation of sheared chromatin. We therefore suggest the use one of our optimized Shearing ChIP kits.

Chromatin Shearing Optimization kit - **Low** SDS (for Histones) (Cat. No.C01020010)

Chromatin Shearing Optimization kit – **Low** SDS (for Transcription Factors) (Cat. No. C01020013)

Chromatin Shearing Optimization kit - **Medium** SDS (Cat. No.C01020011)

Chromatin Shearing Optimization kit - **High** SDS (Cat. No.C01020012)

Our Chromatin Shearing Optimization kits are used in combination with the Bioruptor® in order to ensure a highly reproducible chromatin shearing and make sure you obtain the right fragment size needed for your experiment. Establish optimal conditions required for shearing cross-linked chromatin (protein-DNA) is usually laborious; the protocol of the Chromatin Shearing Optimization kits is fast, easy-to-use and optimized to get the best results possible.

The ChIP assay offers great potential to improve knowledge about the regulation of gene expression. This technique is now used in a variety of life science disciplines including cellular differentiation, tumor suppressor gene silencing, and the effect of histone modifications on gene expression.

Isolation and shearing of the chromatin after formaldehyde fixation is the first step in the ChIP method. The following two steps are: 1/ specific immunoprecipitation of chromatin fragments using antibody against the protein of interest and 2/ analysis of the immunoprecipitated DNA fragment. With the present kit, the first step of the ChIP method is guaranteed, providing you with the best results.

Using standard protocols and kits leads 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.

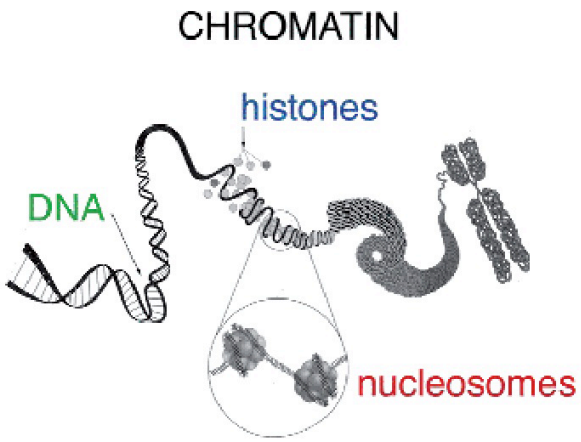

Figure 1

Representation of the chromatin

Chromatin is that portion of the cell nucleus which contains all the DNA of the nucleus in animal or plant cells. DNA in chromatin is organised in arrays of nucleosomes. Two copies of histone proteins are assembled into an octamer that has 145-147 base pairs (bp) of DNA wrapped around it to form a nucleosome core. The nucleosome, in its role as the principal packaging element of DNA within the nucleus, is the primary determinant of DNA accessibility.

(Luger et al. 1997)

Kit method overview & time table

Table 1 : Protocol overview

Step		Time needed
1	Cell collection and DNA-protein crosslinking	1 to 2 hours
2	Cell lysis and Chromatin shearing	1 to 2 hours
3	Sheared chromatin analysis	1 day

Kit Content

Table 2: Kit content and storage

This kit contains enough reagents for the shearing of chromatin from 100 million cells. These components are identical to the ones included in the HighCell# ChIP kit (Cat. No. C01010060, C01010062, C01010061 and C01010063).

Description	quantity	storage
Glycine	1.5 ml	4°C
Lysis Buffer L1	25 ml	4°C
Lysis Buffer L2	25 ml	4°C
Shearing Buffer S1	5 ml	4°C
Elution Buffer iE1	1.25 ml	4°C
Elution Buffer iE2	100 µl	4°C
TE buffer	500 µl	4°C
DNA precipitant	250 µl	4°C
DNA co-precipitant	125 µl	-20°C
Protease inhibitor cocktail	25 µl	-20°C

Required Materials Not Provided

Reagents

- Formaldehyde (37% stock)
- Ice-cold PBS buffer
- Agarose and TAE buffer
- DNA molecular weight marker
- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 100% Ethanol

- 70% Ethanol

Equipment

- Cell scraper
- Centrifuges (at 4°C) for 1.5 ml tubes and 15 ml tubes
- Bioruptor® from Diagenode (with recommended consumables)
- Agarose gel apparatus
- 30°C incubator or water bath

Remarks before starting

Before starting the ChIP, the chromatin should be sheared to fragments in the 100 to 600 bp range. Our kits and protocols are optimized for Chromatin shearing using the Bioruptor® (Pico, Plus and Standard). The maximum volume for shearing with the Bioruptor® is 300 µl per 1.5 ml microtube. We recommend using TPX tubes (Cat. No.C30010010-1000) for Bioruptor Plus and Standard and 1.5 ml Bioruptor Microtubes with caps (C30010016) for Bioruptor Pico as shearing has been shown to be more efficient and reproducible using these tubes. The shearing conditions mentioned in the protocol are adequate for a variety of cell types.

However, given that cell types are different, we recommend optimizing sonication conditions for each cell type before processing large quantities of cells or samples. Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation.

Short protocol for experienced users

STEP 1. Cell collection and DNA-protein crosslinking

1. Use a trypsinisation method to collect the cells. Wash two times the cell suspension with PBS
2. Label new 1.5 ml tube(s), count the cells and put 1 million to 10 million cells in 500 µl of PBS.
3. Add 13.5 µl of 36.5% formaldehyde per 500 µl of sample (final concentration has to be approximatively 1%).
4. Mix by gentle vortexing. Incubate for 8 minutes at room temperature to allow fixation to take place.
5. Add 57 µl of 1.25 M Glycine to the sample.
6. Mix by gentle vortexing. Incubate for 5 minutes at room temperature. This is to stop the fixation.
   - Work on ice from this point onwards
7. Centrifuge at 1,500 rpm (300 x g) for 5 minutes at 4°C.
   - We recommend the use of a swing-out rotor with soft settings for deceleration.
8. Aspirate the supernatant. Take care to not remove the cells. Aspirate slowly and leave approximately 30 µl of the solution behind.

STEP 2. Cell lysis and chromatin shearing

9. Wash the cross-linked cells twice with 1 ml of ice-cold PBS.
10. After the last wash, aspirate the supernatant. Leave about 10 to 20 µl behind.
    - These are the cross-linked cells ready for chromatin shearing.
11. Add 1 ml of ice-cold Lysis Buffer L1 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
12. Centrifuge for 5 minutes at 1,600 rpm (500 x g) at 4°C. Discard the supernatant, keep the pellet.
13. Add 1 ml of ice-cold Lysis Buffer L2 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
14. Pellet again by centrifugation for 5 minutes at 1,600 rpm (500 x g) at 4°C, and discard supernatant.
15. Add protease inhibitor to the Shearing Buffer S1 (RT). This is the complete shearing Buffer S1 for sonication. Keep the buffer at room temperature until use; discard what is not used during the day.
16. Add 200 µl of complete Shearing Buffer S1 (RT) to the cells. Vortex until resuspention. Incubate for 10 minutes on ice and and transfer the cell suspension to 1.5 ml TPX microtubes (Diagenode cat. No. M-50001) when using the Bioruptor Standard or Plus or to 1.5 ml Bioruptor® Microtubes with Caps (Cat No C30010016) optimized for chromatin shearing with the Bioruptor® Pico.
17. Shear the chromatin by sonication using the Bioruptor®. When using the Bioruptor Standard or Plus, shear for 1 to 3 runs of 10 cycles [30 seconds "ON", 30 seconds "OFF"] each at high power setting. Briefly vortex and spin between each run. Shear for 8-10 cycles [30 seconds "ON", 30 seconds "OFF"] when using the Bioruptor Pico. These shearing conditions will work excellent for many cell types. However, depending on the cell type and Bioruptor® system used, optimisation may be required.

STEP 3. Sheared chromatin analysis

18. Spin 50 µl of the sheared chromatin at 12,000 rpm for 10 min at 4°C. Use the supernatant for chromatin analysis.
19. Add 2 µl of diluted RNase cocktail and incubate 1h at 37°C.
20. Add 50 µl of elution buffer iE1 and 4 µl of elution buffer iE2, mix thoroughly.
21. Incubate samples at 65°C for 4h (or overnight).
22. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Incubate the sample at RT for 10 min on a rotating wheel.
23. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
24. Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the sample at RT for 10 min on a rotating wheel.
25. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
26. Precipitate the DNA by adding 10 µl DNA precipitant, 5 µl of co-precipitant, and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.
27. Centrifuge for 25 min at 13,000rpm at 4°C. Add 500 µl of ice-cold 70% ethanol to the pellet.
28. Centrifuge for 10 min at 13,000 rpm at 4°C. Air-dry the pellet.
29. Re-suspended the pellet in 20 µl of TE buffer.
30. Run samples in a 1.5% agarose gel.

Detailed protocol

STEP 1. Cell collection and DNA-protein crosslinking

1. Pre-warm PBS, culture media and trypsin-EDTA.
2. Remove old media and rinse cells with pre-warmed PBS (Table 3). Shake dishes for 2 minutes. Remove the PBS.

Table 3			
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 4). 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 4			
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 5). The addition of medium will inactivate the trypsin.

Table 5			
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. Wash two times the cell suspension with PBS.
8. Label new 1.5 ml tube(s), count the cells and put 1 million to 10 million cells in 500 µl of PBS.
   - In order to preserve the cells, use either a 1000 µl pipette tip or a smaller tip that has been cut in order to increase the opening.
9. Add 13.5 µl of 36.5% formaldehyde per 500 µl of sample (final concentration has to be approximatively 1%).
10. Mix by gentle vortexing. Incubate for 8 minutes at room temperature to allow fixation to take place.
11. Add 57 µl of 1.25 M Glycine to the sample.
12. Mix by gentle vortexing. Incubate for 5 minutes at room temperature. This is to stop the fixation.
13. Preferably work on a pre-chilled DiaMag1.5 magnetic rack or work on ice from this point onwards.
14. Centrifuge at 1,500 rpm (300 x g) for 5 minutes at 4°C.

15. We recommend the use of a swing-out rotor with soft settings for deceleration.
16. Aspirate the supernatant. Take care to not remove the cells. Aspirate slowly and leave approximately 30 µl of the solution behind.
    - These are the cross-linked cells ready for chromatin shearing.
    - Do not disturb the pellet.

STEP 2. Cell lysis and chromatin shearing

This section describes cell lysis and Bioruptor® chromatin shearing. At this stage, it is essential to produce fragments of size suitable for ChIP and subsequent PCR analysis of the immunoprecipitated DNA. The average size is of 500 base pairs (bp) (range: 200 - 1,000 bp).

Work on ice unless otherwise stated.

17. Wash the cross-linked cells twice with 1 ml of ice-cold PBS (adding NaBu (20mM final concentration) and/or any other inhibitor of choice).
    - Add the NaBu-PBS solution, gently vortex and centrifuge at 470 x g (in a swing-out rotor with soft settings for deceleration) for 10 minutes at 4 °C.
    - Resuspend with a pipette to ensure cells are thoroughly washed.
    - In any case make sure that cells are in suspension before proceeding to the next point.
17. After the last wash, aspirate the supernatant. Leave about 10 to 20 µl behind.
    - These are the cross-linked cells ready for chromatin shearing.
    - Avoid taking out too much as that could lead to material loss.
18. Add 1 ml of ice-cold Lysis Buffer L1 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
19. Centrifuge for 5 minutes at 1,600 rpm (500 x g) at 4°C. Discard the supernatant, keep the pellet.
20. Add 1 ml of ice-cold Lysis Buffer L2 per pellet of cells. Resuspend cells by pipetting up and down several times and incubate for 10 minutes at 4°C with gentle mixing.
21. Pellet again by centrifugation for 5 minutes at 1,600 rpm (500 x g) at 4°C, and discard supernatant.
22. Add protease inhibitor to the Shearing Buffer S1 (RT). This is the complete shearing Buffer S1 for sonication. Keep the buffer at room temperature until use; discard what is not used during the day.
23. Add 200 µl of complete Shearing Buffer S1 (RT) to the cells. Vortex until resuspention. Incubate for 10 minutes on ice and transfer the cell suspension to 1.5 ml TPX microtubes (Diagenode cat. No. C30010010-1000) when using the Bioruptor Standard or Plus or to 1.5 ml Bioruptor® Microtubes with Caps (Cat No C30010016) optimized for chromatin shearing with the Bioruptor® Pico.
24. Shear the chromatin by sonication using the Bioruptor®. When using the Bioruptor Standard or Plus, shear for 1 to 3 runs of 10 cycles [30 seconds "ON", 30 seconds "OFF"] each at high power setting. Briefly vortex and spin between each run. Shear for 5-15 cycles [30 seconds "ON", 30 seconds "OFF"] when using the Bioruptor Pico
25. These shearing conditions will work excellent for many cell types. However, depending on the cell type and Bioruptor® system used, optimisation may be required.
26. Transfere sample to new 1.5 ml standard tubes and centrifuge for 10 min at 12.000 rpm. Collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. Take an aliquot of 50 µl of sheared chromatin for sheraing analysis.

STEP 3. Sheared chromatin analysis

27. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of ChIP-seq grade water).
28. Add 2 µl of diluted RNase cocktail.
29. Incubate 1h at 37°C.
30. Add 50 µl of elution buffer iE1.
31. Add 4 µl of elution buffer iE2, mix thoroughly.
32. Incubate samples at 65°C for 4h (or overnight).
33. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol (25:24:1). Incubate the sample at RT for 10 min on a rotating wheel.
34. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
35. Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the sample at RT for 10 min on a rotating wheel.
36. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
37. Precipitate the DNA by adding 10 µl DNA precipitant, 5 µl of co-precipitant, and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.
38. Centrifuge for 25 min at 13,000rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.
39. Centrifuge for 10 min at 13,000 rpm at 4°C. Carefully remove the supernatant, leave tubes open for 30 min at RT to evaporate the remaining ethanol.
40. Re-suspended the pellet in 20 µl of TE buffer.
41. Run samples (20 µl of DNA + 4 µl of 6x loading dye) in a 1.5% agarose gel.

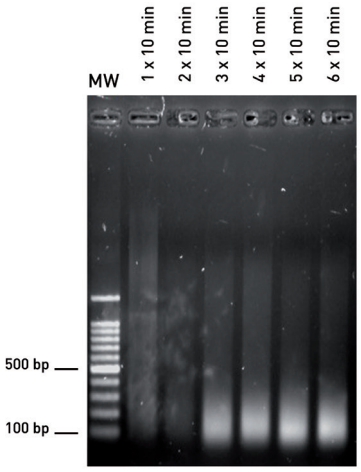

Figure 2

Time course sonication experiment with the Bioruptor® PLUS using buffers and protocol of Diagenode's Chromatin Shearing Optimization kit - Medium SDS

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nucleus/Nuclei isolation of 5 million cells are performed using buffers of Diagenode's Chromatin Shearing Optimization kit - Medium SDS (Cat. No. C01020011) and are then resuspended in 200 µl of Shearing Buffer S1 prior to Chromatin shearing.

Samples are sheared during 1, 2, 3, 4, 5 or 6 rounds of 10 cycles of 30 sec ON/30 sec OFF with the Bioruptor® PLUS combined with the Bioruptor® Water cooler (Cat. No. BioAcc-cool) & Single Cycle Valve (Cat. No. VB-100-0001) at HIGH power setting (position H). All samples were treated with RNase prior to agarose gel analysis. 300 ng of each sample was analyzed on a 1.5% agarose gel (except for lanes 2 and 3).

In this example, the optimal shearing condition (the least time course condition) corresponds to 3 rounds of 10 cycles (30 sec ON/30 sec OFF). Troubleshooting guide.

Troubleshooting guide

Process	Protocol step	Issues and resolutions
Crosslinking and fixation	Optimize crosslinking time	Poor crosslinking causes DNA loss, elevated background, and/or reduced antigen availability in chromatin. Emperically determine optimal crosslinking time for maximal specificity and efficiency of ChIP. The optimal duration of cross-linking varies between cell type and protein of interest. Short crosslinking time (5-10 minutes) may improve shearing efficiency. Crosslinking duration should not exceed 30 minutes or shearing will be inefficient.
	Assure proper fixation time with formaldehyde	Crosslinking may be too weak or too strong without proper fixation time. Optimize fixation step e.g: incubate for 8 minutes at room temperature with high-quality, fresh 1% formaldehyde final concentration (weight/volume).
	Optimize formaldehyde concentration	Lower formaldehyde concentrations (1%weight/volume) may improve shearing efficiency. For some proteins, however, especially those that do not directly bind DNA, this might reduce crosslinking efficiency and thus the yield of precipitated chromatin. Empirically determine the formaldehyde concentration as some antigen epitopes may be more sensitive to formaldehyde.
Cell lysis	Make sure cells disrupt completely	Do not use too many cells per amount of lysis buffer (w/v) so that cells can be completely disrupted. Follow the instructions in the protocol (e.g.: 1 million cells or less/130 µl of complete Buffer B). See steps 2 and 3.
	Maintain cold temperature during lysis	Perform cell lysis at 4°C (cold room) or on ice. Always keep the samples ice cold during cell lysis and use cold buffers as in Step 3.
	Prevent protein degradation	Add the protease inhibitors to the lysis buffer immediately before use.
Cell type	Determine which cell types have previously been validated with the kit	HeLa, NCCIT 293T, Chondrocytes, P19, ASC (adipose stem cells), U2OS and kerationocytes have been used to validate this magnetic ChIP protocol.
Number of cells required	Determine number of cells for ChIP	The number of cells for ChIP is determined by cell type, protein of interest, and antibodies used. Use chromatin from 1,000 to 10,000 cells per ChIP. (In some cases, chromatin from up to 100,000 cells may be needed). You may need to empirically determine the optimal number.
Chromatin shearing	Maintain 4°C temperature during shearing	Keep samples cold at 4°C before sonication to maintain sample integrity.
	Maintain 0ÐC temperature during sonication	Maintain temperature of the samples at 4°C to maintain sample integrity.
	Optimize SDS concentration	High % SDS favours better sonication but inhibits immunoselection (optimal range: 0.1% to 1%). Final SDS concentration should not be higher than 0.15 to 0.20% (e.g. If the shearing buffer contains 0.75% SDS, the sheared chromatin is diluted 3.5 to 4.0 fold in the ChIP buffer.
	Determine amount of sheared chromatin needed for ChIP	Most of the sheared chromatin will be used for ChIP and the input control. A small amount will be checked on agarose gel.
	Dilute the sheared chromatin in ChIP buffer for Immunoselection incubation	The sheared chromatin is diluted in complete Buffer A prior to the immunoselection incubation (see Step 3). Dilute the sheared chromatin at least 7-fold. Adjust the ChIP buffer volume added to the chromatin accordingly.

Sonication tips	Determine cell number	Start with 1x10e6 cells or less.
	Sonication conditions with the Bioruptor®	Shear the samples of chromatin using the Bioruptor® for 10-12 cycles of: [30 seconds "ON" / 30 seconds "OFF"] each. These conditions were tested with many mammalian cell lines and were excellent for subsequent ChIP experiments. A troubleshooting guide for Bioruptor-chromatin shearing is available.
	Chromatin shearing with Diagenode modules	You can also use the other Chromatin Shearing Optimization kits which differ by their SDS concentration (Cat. No. AA-001-0100 ; AA-002-0100)
Gel analysis of sheared chromatin	Load enough DNA on gel	Chromatin equivalent to at least 100,000 cells can be be visualized on a gel. Do not use an excessive amount or it will obscure the visualization. The DNA amount to load depends on well size and on the gel size.
	Use correct agarose concentration	Use a 1-1.5% agarose gel.
	Use correct running buffer concentration and run time	1x TAE or TBE is preferred to 0.5x TAE (which can lead to smears). Run slowly.

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A Hologic Company

DIAGENODE HEADQUARTERS

DIAGENODE S.A.
BELGIUM | EUROPE
LIEGE SCIENCE PARK
Rue Bois Saint-Jean, 3
4102 Seraing - Belgium
Tel: +32 4 364 20 50
Fax: +32 4 364 20 51
orders@diagenode.com
info@diagenode.com

DIAGENODE INC.
USA | NORTH AMERICA
400 Morris Avenue, Suite #101
Denville, NJ 07834
Tel: +1 862 209-4680
Fax: +1 862 209-4681
orders.na@diagenode.com
info.na@diagenode.com

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