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Innovating Epigenetic Solutions

Chromatin shearing optimization kit

Low SDS

(for Transcription Factors)

Cat. No. **C01020013**

Version 2 | 04.16

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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.

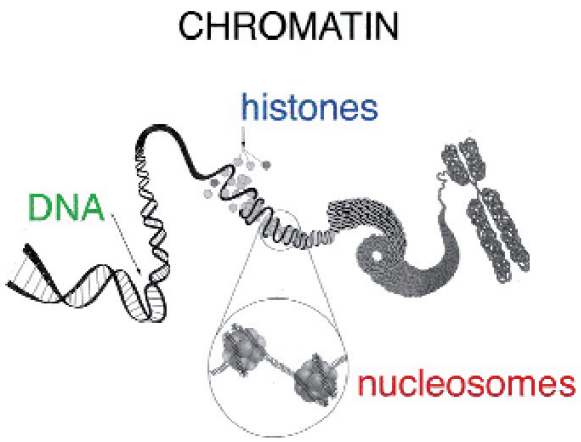

Figure 1

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

This kit contains enough reagents for the shearing of chromatin from 100 million cells. These components are identical to the ones included in the iDeal ChIP-seq kit for Transcription Factors (Cat. No. C01010055).

Description	Quantity	Storage
Glycine	10 ml	4°C
Shearing Buffer iS1b	7.5 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
Fixation buffer	6.4 ml	4°C
Lysis Buffer iL1b	115 ml	4°C
Lysis Buffer iL2	68 ml	4°C
DNA co-precipitant	125 µl	-20°C
Protease inhibitor cocktail	38 µl	-20°C

Required Materials Not Provided

Reagents

- Formaldehyde (37% stock)
- Ice-cold PBS buffer
- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 100% Ethanol
- 70% Ethanol
- Agarose and TAE buffer
- DNA molecular weight marker
- Diagenode ChIP cross-link Gold (Cat N° C01019021) (Optional)
- Additional Protease Inhibitor Mix (C12010012) for tissue

Equipment

- Cell scraper
- Centrifuges (at 4°C) for 1.5 ml tubes and 15/50 ml tubes
- Bioruptor from Diagenode
- Agarose gel apparatus
- 37°C/65°C incubator or water bath

- Dounce homogenizer with loose pestle 2 mL (for tissues)
- Scalpel blades (for tissues)
- Petri dishes (for tissues)

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.

When working with tissue, please note that this protocol has been optimized for ChIP from fresh or snap-frozen mammalian tissues. The chromatin for one IP is prepared from 40-50 mg of tissue. However, the exact amount of tissue needed may vary depending on protein abundance, antibody etc. and should be determined for each tissue type. When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. Usually, the amount of chromatin to be used per IP is within 3-10 µg. We recommend performing a pilot experiment to determine an optimal amount if tissue. Once determined, it should be kept constant between experiments.

The first step of the chromatin preparation is the cross-linking in order to fix protein-DNA interactions. Formaldehyde is the most commonly used cross-linking reagent. However, formaldehyde is usually not effective to cross-link proteins that are not directly bound to the DNA. For example, chromatin interactions with inducible transcription factors or with cofactors that interact with DNA through protein-protein interactions are not well preserve with formaldehyde. When studying this kind of factors, we recommend the use of the Diagenode **ChIP cross-link Gold (Cat N° C01019021)**. This reagent is to use in combination with formaldehyde. The protocol involves a sequential fixation. A first protein-protein fixation by the ChIP cross-link Gold followed by protein-DNA fixation by formaldehyde.

Protocol

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

Note: When studying inducible transcription factors or cofactors, it is recommended to perform the fixation using the **ChIP cross-link Gold (C01019021)** in addition to the formaldehyde fixation

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

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

STEP 1b. Tissue disaggregation and DNA-protein cross-linking (derived from fresh or snap-frozen tissue samples)

5. Prepare cross-linking solution in fume hood by adding formaldehyde to **Fixation buffer** to a final concentration 1%. Use 2 ml of Fixation buffer for up to 200 mg of tissue. Scale accordingly.
   Keep at room temperature.
6. Weigh fresh or frozen tissue in a petri dish. Use about 50 mg per one IP. Up to 200 mg can be processed in the same preparation. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.
7. Chop tissue into small pieces (between 1-3 mm3) using a scalpel blade.
8. Transfer the tissue into a dounce homogenizer and add 1 ml of diluted formaldehyde.
9. Immediately disaggregate tissue using a dounce homogenizer (loose fitting pestle) to get a homogeneous suspension.
10. Transfer the tissue suspension into 15 ml tube and add additional 1 ml of formaldehyde diluted in **Fixation buffer** (from step 1) and rotate the tube for 15 min at room temperature.
    The fixation time might require an additional optimization. In general, the fixation time from 10 to 20 min is optimal for transcriptional factors. Please note that stronger fixation may lead to a chromatin resistant to sonication.
11. Stop the cross-linking reaction by adding 200 µl of glycine per 2 ml of Fixation buffer. Continue to rotate at room temperature for 5 min.

12. STEP 2a. Cell lysis and chromatin shearing (for cultured cells)

For adherent cells:

12a. Remove the medium and wash the cells once with 20 ml of PBS. Keep everything at 4°C from now on.

13a. Add 5 ml of cold Lysis buffer iL1b to the plate and collect the cells by scraping.

14a. Add an additional volume of Lysis buffer iL1b to rinse the flask and add this to the collected cells. The total volume of Lysis buffer iL1b should be about 10 ml per 107 cells (e.g. for a T175 culture flask (~25 milllion cells), rinse with an additional 20 ml of buffer iL1b.

15a. Incubate at 4°C for 20 minutes.

For suspension cells:

12b. Pellet the cells by centrifugation at 1,600 rpm and 4°C for 5 minutes. Discard the cell culture medium.

13b. Wash the cells once with PBS. Resuspend the cells in 20 ml of PBS, centrifuge at 1,600 rpm and 4°C for 5 minutes and discard the supernatant. Keep everything at 4°C from now on.

14b. Add 1 ml ice-cold lysis buffer iL1b to the cell pellet and resuspend the cells by pipetting up and down several times. Add an additional amount of buffer iL1b to obtain a total volume of about 10 ml per 107 cells (e.g. for a T175 culture flask (~25 milllion cells), add an additional 24 ml of buffer iL1b.

15b. Incubate at 4°C for 20 minutes.

16. Pellet the cells by centrifugation at 1,600 rpm for 5 minutes and 4°C and discard the supernatant.

17. Add 1 ml ice-cold Lysis buffer iL2 to the cell pellet and resuspend the cells by pipetting up and down several times. Add an additional amount of buffer iL2 and incubate for 10 minutes at 4°C with gentle mixing. For 25 million cells the total amount of iL2 should be 15 ml.

18. Pellet the cells again by centrifugation for 5 minutes at 1,600 rpm (500 x g) and 4°C and discard supernatant.

19. Add 200x protease inhibitor cocktail to Shearing buffer iS1b. Prepare 1 ml of complete shearing buffer per tube of 15 million cells. Keep on ice

20. Add 1 ml of complete Shearing buffer iS1b to 15 Million cells. Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1.5 Million cells per 100 µl buffer iS1b. Split into aliquots of 100 to 300 µl and transfer the cell suspension to 1.5 ml TPX microtubes (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.

21. 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.

22. Centrifuge at 13,000 rpm (16,000 x g) for 10 minutes and collect the supernatant which contains the sheared chromatin. Use the chromatin immediately in immunoprecipitation or store it at -80°C for up to 2 months. The chromatin shearing efficiency can be analysed at this step.

STEP 2b. Cell lysis and chromatin shearing (derived from tissue sample)

23. Centrifuge samples at low speed (3000 rpm) at 4°C.
24. Gently discard the supernatant and keep the pellet.
25. Wash the pellet with 10 ml of ice-cold PBS.
26. Centrifuge samples at low speed (3000 rpm) at 4°C.
27. Gently discard the supernatant and keep the pellet.
28. Add 200x protease inhibitor cocktail to ice-cold lysis buffer iL1b. Prepare 10 ml of complete lysis buffer iL1b per each sample of 200 mg of tissue. Keep on ice.
29. Add 1 ml ice-cold lysis buffer iL1b to the pellet and resuspend by pipetting up and down several times. Add the remaining amount of buffer iL1b.
30. Incubate at 4°C for 20 minutes.
31. Pellet the cells by centrifugation at 3.000 rpm for 5 minutes and 4°C and discard the supernatant.
32. Add 200x protease inhibitor cocktail to ice-cold lysis buffer iL2. Prepare 10 ml of complete lysis buffer iL2 per each sample of 200 mg of tissue. Add 1 ml ice-cold Lysis buffer iL2 to the cell pellet and resuspend the cells by pipetting up and down several times. Add the remaining amount of buffer iL1b. Incubate at 4°C for 10 minutes.
33. Pellet the cells again by centrifugation for 5 minutes at 3.000 rpm and 4°C and discard supernatant.
34. Add 200x protease inhibitor cocktail to Shearing buffer iS1b. Prepare 300 µl of complete shearing buffer per one sample (30-50 mg of tissue). Scale accordingly. Keep on ice.
35. Add the required volume of complete Shearing buffer iS1b to the pellet. Resuspend the cells by pipetting up and down several times. Split into aliquots of to 300 µl and transfer the cell suspension to 1.5 ml TPX microtubes (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.
36. 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.
37. Transfer samples to new standard 1.5 ml tubes and centrifuge at 13,000 rpm (16,000 x g) for 10 minutes. 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. The chromatin shearing efficiency can be analysed at this step.

STEP 3. Sheared chromatin analysis

38. Take an aliquot of 50 µl of sheared chromatin and spin the chromatin at 12,000 rpm for 10 min at 4°C. Transfer the supernatant to a new tube for chromatin analysis.
39. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of ChIP-seq grade water).
40. Add 2 µl of diluted RNase cocktail.
41. Incubate 1h at 37°C.
42. Add 50 µl of elution buffer iE1.
43. Add 4 µl of elution buffer iE2, mix thoroughly.
44. Incubate samples at 65°C for 4h (or overnight).

45. 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.
46. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
47. Add 1 volume of chloroform/isoamyl alcohol (24:1). Incubate the sample at RT for 10 min on a rotating wheel.
48. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
49. 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.
50. 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.
51. 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.
52. Re-suspended the pellet in 20 µl of TE buffer.
53. Run samples (20 µl of DNA + 4 µl of 6x loading dye) in a 1.5% agarose gel.

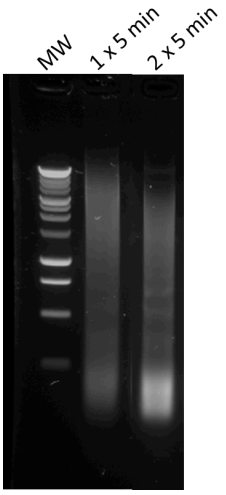

Figure 2. Chromatin shearing from K562 cells.

K562 cells (myelogenous leukemia cell line) are fixed with 1% formaldehyde (for 15 min at RT). Nuclei isolation of 4,5 millions fresh cells are performed using buffers of Diagenode's Chromatin Shearing Optimization kit - Low SDS (for iDeal ChIP-seq kit for TFs) and are then resuspended in 300 µl of Shearing Buffer iS1b prior to Chromatin shearing.

Samples are sheared during 1 or 2 rounds of 5 cycles of 30 sec ON/ 30 sec OFF with the Bioruptor Pico. All samples were treated with RNase and reverse cross-linked. 10 µl of DNA (equivalent to 300 ng) are analyzed on a 1.5% agarose gel. This example clearly shows that "difficult cells" such as K562 can readily be sheared using the Bioruptor®.

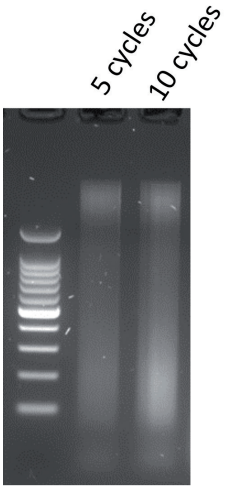

Figure 3. Chromatin shearing from mouse liver using Diagenode Chromatin shearing optimization kit – Low SDS (for Transcriptional Factors).

Chromatin from mouse liver was sheared using the Bioruptor® Pico for 5 and 10 cycles (30'' On/30'' Off). 100 bp ladder is loaded as size standard.

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