Instruction Manual Version 3 - 01.14

Shearing Optimization kit

Cat. No. **C01020022** (kch-shchro-040)

diagenode

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Content

MANUAL

Introduction

From Diagenode, the Shearing Optimization kit is now available to prepare sheared chromatin ready-to-ChIP. The Shearing Optimization kit provides you with an easy and highly reproducible chromatin shearing method.
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 Optimization Kit is fast, easy-to-use and optimized to get the best results possible.

The ChIP method is used to study in vivo protein-DNA interactions. It has been applied to the dynamics of chromatin structure, regulation by transcription factors and coregulators and other epigenetic changes (Kuo and Allis, 1999). 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.

The Diagenode Shearing Optimization kit is therefore the first key for a successful ChIP. With this kit, find the best way to prepare sheared chromatin readyto-ChIP. It is fast, optimized and user-friendly. Then, ChIP with your "in house" protocol or ChIP with our rapid ChIP method using our OneDay ChIP kits.

Using standard protocols and kits lead to a significant decrease in potential variables that can occur from shearing to ChIP, which make interpretation and analysis from experiment to experiment much more accessible.

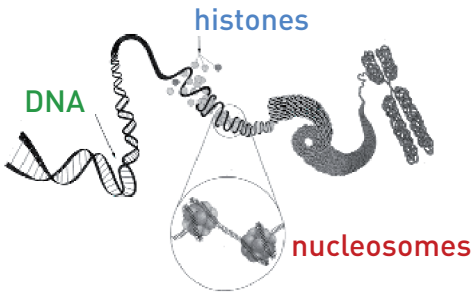

Fig 1: Representation of the chromatin
Chromatin is that portion of the cell nucleus which contains all the DNA of the nucleus in animal or plant cells. DNA in chromatin is organised in arrays of nucleosomes. Two copies of histone proteins are assembled into an octamer that has 145-147 base pairs (bp) 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

This Diagenode novel Shearing Optimization kit includes optimized buffers, and a userfriendly protocol to produce sheared chromatin of high quality. The 4 steps of the protocol and the main goal of the kit method are described here below.

→ **Protocol main steps**:

Step 1: Cell fixation and collection of your own cells: using buffer A.
- Add DTT to buffer A before use.

Step 2: Cell lysis: using buffer B, buffer C and buffer D.
- Add Protease inhibitor mix to buffer D before use.

Step 3: Chromatin shearing: using the Bioruptor from Diagenode.

Step 4: Analysis of the sheared chromatin: on agarose gel.

→ **Kit goal**:

Use the optimized buffers provided in the Shearing Optimization kit with your own cells to prepare sheared chromatin ready-to-ChIP.

With the Shearing Optimization kit

Prepare large amounts of sheared chromatin ready-to-ChIP. (Cat. No. kch-shchro-40) Buffers provided in the Shearing Optimization kit allow the researcher to perform chromatin shearing trials with his own treated and/or induced cells. Also, this second kit is a "large scale" chromatin shearing kit compared to the control kit. The first step of the protocol consists on cell culturing and fixation (step 1 of the protocol, see above). The buffers are provided for chromatin shearing from up to 150 million cells. The sheared chromatin is ready-to-ChIP. Then, use your own ChIP protocol or use our OneDay ChIP kit.

With our OneDay ChIP Kit

"The Rapid ChIP": save time and analyse many samples in a short period of time. (Cat. No. kch-oneDIP-060, -180) The kit is designed to perform ChIP assays in abundance: ChIP in 2 hours and recover the DNA ready-to-PCR in 2 hours. With this new method, it takes one day from cell collection to PCR results. Two kits format are available: 60 IPs or 180 IPs per kit.

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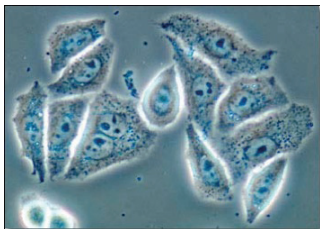
Shearing Optimization kit to use prior to Chromatin IP:

→ S**tarting material**: Cell culture

→ **Kit protocol step 1** – Cell fixation and collection



→ **Kit protocol step 2** – Cell lysis

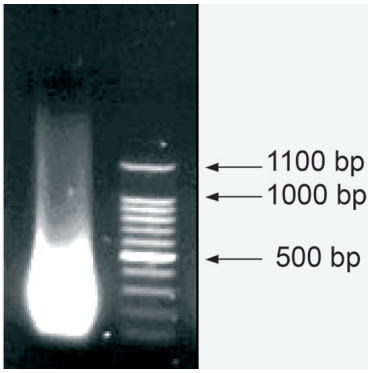
→ **Kit protocol step 3** – Bioruptor-Chromatin Shearing

→ **Kit protocol step 4** – Sheared Chromatin analysis

Analysis on an agarose gel of the sheared chromatin obtained with the Bioruptor® and the Shearing Optimization Kit from Diagenode.

Cells are fixed with 1% formaldehyde (10 minutes at RT). Three million cells are resuspended in 120 µl of lysis buffer prior to chromatin shearing. Samples are sonicated for 10 cycles of: [30 seconds: "ON"/ 30 seconds: "OFF"] in 1.5 ml Eppendorf tubes with the Bioruptor® from Diagenode (cat.#: UCD-200).

The sheared chromatin is analyzed on a 1% agarose gel. Chromatin shearing efficiency is analyzed alongside the DNA molecular weight marker in the right lane.



OneDay ChIP kit (or your "in house" ChIP protocol)

Fig 2: Representation of the kit method, step by step.
Use the ChIP procedure to study transcription factor binding and alterations in chromatin structure. The first step of the ChIP method is the isolation and shearing of the chromatin after formaldehyde fixation. Prior to your immunoprecipitation (IP), ensure the first step of a successful ChIP by using the Diagenode Shearing Optimization Kit (kit protocol: step 1 to 4 above). The Shearing Optimization Kit allows you to prepare large amounts of high quality sheared chromatin ready-to-ChIP. Then perform ChIP using our OneDay ChIP kit, our rapid ChIP protocol.

Kit Materials

Kit content

The kit content is described in the following Table 1. Upon receipt, store the components at the temperatures indicated in Table 1.

Kit component

Table 1. Kit content and storage

SHEARING MODULE (steps 1 to 3)			
Component	Description	Quantity	Storage
Buffer A (Cell collection)	-	18 ml	4°C
Buffer B (Lysis 1)	Detergent and ion chelator mix included	18 ml	4°C
Buffer C (Lysis 2)	Salt and ion chelator mix included	18 ml	4°C
Buffer D (Chromatin shearing)	Detergent mix and ion chelator included	18 ml	4°C
1M DTT	100x stock solution	230 µl	-20°C
Protease Inhibitor Mix (P.I.)	25x stock solution (see kit protocol: at Step 2 pt. 17., dissolve the tablet in 2 ml H2O and store at -20°C.)	1 tablet	-20°C

Required Materials Not Provided

Reagents

- Formaldehyde (37% stock)
- Ice-cold PBS buffer
- Agarose and TAE buffer
- DNA molecular weight marker
- 1.25 M glycine (optional, see kit protocol)

Equipment

- Cell scraper
- Centrifuges (at 4°C) for 1.5 ml tubes and 15 ml tubes
- Bioruptor® from Diagenode, cat # UCD-200, website: http://www.diagenode.com/
- Agarose gel apparatus
- 30°C incubator or water bath

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Kit assay protocol

Starting material- Cells

Use your adherent mammalian cells and start the protocol below when cells reached confluency. Follow the instructions of the kit protocol from step 1 to 4 to get sheared chromatin ready-to-ChIP.

IMPORTANT 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 cm^2 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 6 million cells at a minimum and 30 million cells at a maximum (Table 2).
- Number of IPs: With a sample of about 6 million cells, 4 IPs can be performed (Table 2). With a sample of about 30 million cells, 20 IPs can be performed (Table 2).

STEP 1. Cell fixation and collection

1. Add formaldehyde 37% dropwise directly to cell culture medium to reach a final concentration of 1%.
   - For example: add 666 µl of formaldehyde 37% per 24 ml of culture media.
   - Handle formaldehyde in a chemical fume hood.

2. Roll the cell culture dish to mix the formaldehyde. Incubate at room temperature for 10 minutes. That is the fixation step.

3. Add to the medium 1/10th of volume of 1.25 M glycine, mix and incubate for 5 minutes at room temperature. That is for quenching the cross-linking reaction.
   - For example add 2.5 ml of 1.25 M glycine to 25 ml [formaldehyde-culture medium] mix.
   - Alternatively: Carefully get rid of all the solution covering the cells as much as possible and proceed to the next point. Do not leave traces of formaldehyde.

4. Remove the solution from the plate and wash cells twice with 10 ml ice-cold PBS.

5. Add 5 ml of ice-cold PBS to the cells. Collect the cells with a cell scraper. Transfer the cells into a 15 ml tube.

6. Count the cells to determine how many cells you have per sample.
   - Samples of 6 million cells are small size samples.
   - Samples of 30 million cells are big size samples.
   - Aliquot your samples based on the total of cells you work with and need for ChIP.

7. Centrifuge at 3,000 x g at 4°C for 5 minutes to pellet the cells. Discard the supernatant.
   - Pellets can be frozen and stored at -80°C for several months.

8. During the centrifugation, add DTT to buffer A. Add 10 µl of 1 M DTT per ml of buffer A (to reach a 10 mM final concentration of DTT).
   - One ml of freshly prepared DTT-Buffer A is needed per 10 million cells.

9. Add the freshly prepared DTT-Buffer A to the pellet. Resuspend the pellet in the buffer.
   - Volume of buffer to add is indicated in Table 2. Add 1 ml per 10 million cells.
   - Use freshly prepared buffer and discard what is not used on the day.

10. Incubate for 15 minutes on ice, then for 15 minutes at 30°C.

11. Vortex briefly and centrifuge at 3,000 x g at 4°C for 5 minutes. Keep the pellet of formaldehyde fixed cells and discard the supernatant.
    - At this stage the cell pellet can be frozen at -80°C.
    - One pellet should be of 6 million cells at a minimum.
    - Pellets of frozen cell pellets are by far much more stable than sheared chromatin samples.

STEP 2. Cell lysis

12. If frozen, thaw on ice the pellet of formaldehyde fixed cells that you prepared in step 1.
    - By starting with frozen pellets of fixed cells, you save more than one hour of your time on the day you shear and ChIP.

13. Resuspend gently the cell pellet in buffer B.
    Add 1 ml per 10 million cells.

14. Centrifuge at 3,000 x g at 4°C for 5 minutes. Discard gently the supernatant.

15. Resuspend gently the pellet in 1 ml of buffer C.
    Add 1 ml per 10 million cells.

16. Centrifuge at 3,000 x g at 4°C for 5 minutes. Discard the supernatant.

17. During the centrifugation, dissolve the protease inhibitor mix (P.I.) tablet in 2 ml of water. It is then a 25x stock solution. After use, aliquot and store at -20°C (it is stable for over 4 months).

18. Add Protease inhibitor mix (P.I.) to buffer D before use. Add 16 µl of P.I. per 400 µl of buffer D.
    - One volume of 400 µl of P.I.-buffer D is needed per 10 million cells.

19. Add freshly prepared P.I.-buffer D to the cells.

20. Resuspend the cell pellet in the buffer. That corresponds to the chromatin sample ready to be sheared in the next step. Prior to shearing, keep unsheared chromatin in a new tube (e.g.: 10 µl).
    - Transfer the chromatin sample to be sheared in a 1.5 ml tube or 15 ml tube.
    - The unsheared chromatin can be analysed on gel, see the step 4.

STEP 3. Bioruptor chromatin shearing

This protocol is very successful for a large variety of mammalian cells. Optimize shearing conditions for your specific cell type and fixation protocol, starting with a small sample (3x 10e6 cells). Once shearing has been optimized for your cells and conditions, this Shearing Optimization kit will allow you to prepare large amounts of sheared chromatin ready-to-ChIP.

21. Shear each chromatin sample with the Bioruptor® (power setting on "High", 4°C) for 1 to 3 runs of 5 to 10 cycles (30 seconds "ON", 30 seconds "OFF"). Briefly vortex and spin between each run.
- The above shearing conditions are given for samples of 300 µl in 1.5 ml tubes. These conditions were tested with many mammalian cell lines and were excellent for subsequent ChIP experiments.
- 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. A troubleshooting guide for Bioruptor-chromatin shearing is available at Diagenode.

22. After shearing, centrifuge at 10,000 x g at 4°C for 5 minutes. Discard the pellet (insoluble material). Keep the supernatant (that is the sample of sheared chromatin).

STEP 4. Sheared chromatin analysis

Reversion of the cross-linking is advised. 15 µl of the sheared chromatin sample is mixed with 400 µl of buffer D and 5 µl of proteinase K (stock:10 mg/ml). Incubate at least for 4 hours at 65°C. (4 hours minimun / overnight maximum).

23. Prepare a 1% agarose gel.

24. Analyse on the gel 10 µl of sample per lane. Analyse also on the same gel the corresponding unsheared chromatin (obtained at the end of the step 2). Size of the sheared chromatin should be around 200-600 bp.

Sheared Chromatin Ready-to-ChIP

The buffers in the Shearing Optimization kit are provided for chromatin shearing of over 100 million cells. The sheared chromatin is ready-to-ChIP. Following shearing, use your own ChIP protocol or use our OneDay ChIP kit.

25. Per IP, use a sample of sheared chromatin obtained from 1.5-2 x million cells (60-80 µl). The sheared chromatin used in IP will be diluted in the final volume of IP incubation mix as described bellow.
Add to the sheared chromatin the following:
- antibody binding beads (blocked in a free-detergent buffer and corresponding to 1:10 of the total volume of IP incubation mix)
- antibody
- BSA and/or other blocking reagent (if you wish to avoid unspecific binding, and decrease possible background signal)
- P.I. mix (protease Inhibitors)
- IP buffer, which is detergent-free

And reach a final volume of the IP incubation mix of about 280 µl.

IMPORTANT NOTES

- It is best to freshly prepare samples of sheared chromatin and directly use them in ChIP (e.g.: OneDay ChIP kit).
- However, it is also possible to freeze the sheared chromatin and perform the ChIP another day. The chromatin can be stored in liquid nitrogen for several months or no more than a few weeks depending on your ChIP target.
- You can use frozen sheared chromatin in ChIP but usually fresh chromatin gives the best results: e.g: to study histone modifications. If the sample of sheared chromatin gets older than 1 or 2 months (stored at -80°C) the quality goes down.
- To study transcriptions factors the stability of the sheared chromatin is further reduced. For GR-ChIP assays the sheared chromatin can be kept in liquid nitrogen (not -80°C) for 1 to 2 weeks. For TBP-ChIP assays, the sheared chromatin can be kept for as long as 6 months.

Troubleshooting Guide

Critical steps	Troubles, solutions and comments	
Cross-linking	Cross-linking is too weak.	Make sure you perform the 10 minutes fixation step at the right temperature and with the correct formaldehyde concentration. Use high quality formaldehyde.
	Cross-linking is too strong.	
	Proteins have unique ways of interacting with the DNA. Some proteins are not directly bound to the DNA but interact with other DNAassociated proteins.	Longer cross-linking time was described. Very long cross-linking can lead to elevated background, therefore the optimal cross-linking time should be found empirically as maximal specificity accompanied with significant efficiency of ChIP.
	Efficient fixation of a protein to chromatin in vivo is a crucial step for ChIP. The extent of cross-linking is probably the most important parameter.	Two major problems concerning the subsequent immunoprecipitation step should be taken into account:1/ an excess of cross-linking can result in the loss of material or reduced antigen availability in chromatin, or both. 2/ the relative sensitivity of the antigen epitopes to formaldehyde. It is essential to perform the cross-linking step with care.
Cell lysis	Cells are not completely disrupted.	Do not use too many cells per amount of lysis buffer (W/V). Follow the instructions in the protocol.
Cell number	The amount of cells required for a ChIP experiment is determined by cell type, protein of interest and antibodies used.	We recommend to use 1.5 x 10e6 cells per ChIP. For some cases, one million cells or less could give successful results. .Also, up to 5 x10e6 cells could be used per ChIP.
Chromatin shearing	Buffer composition.	Use the buffers provided in the kit. They are optimized containing essential key components. Keep samples cold.
High quality picture of the sheared DNA	For accurate size determination of the DNA fragments	Reverse the cross-linking and precipitate the DNA after phenol/ chloroform extraction.
	Gel electrophoresis of cross-linked samples could give smears.	Reversion of the cross-linking is advised. 15 µl of the sheared chromatin is mixed with 400 µl of buffer D and 5 µl of proteinase K (10 mg/ml). Incubate at least 4 hours at 65°C. (minimum 4 hours and maximum O.N.).
	The migration of large quantities of DNA on agarose gel can lead to poor quality pictures which do not reflect the real DNA fragmentation.	Do not load too much on a gel. Do not load more than 5 µg/lane. Also treat the sample with RNAse.
	Agarose concentration.	Do not use more than 1% agarose gel and run slowly
	Running buffer concentration.	1X TAE or TBE is preferred to 0.5X TAE which can-lead to smears on gel..
rpm/ x g?	Centrifugations	g = 11.18 x r x (rpm/1000)^2; knowing that r is the radius of rotation in mm or:http://www.msu.edu/~venkata1/gforce.htm

References

- Kuo M.H. and Allis C.D. 1999 *Methods* (3):425-33.
- Luger K., Mader A.W., Richmond R.K., Sargent D.F. and Richmond T.J. 1997 *Nature* 389(6648):251-60.

MANUAL

Bringing it all together: Diagenode's ChIP-seq workflow

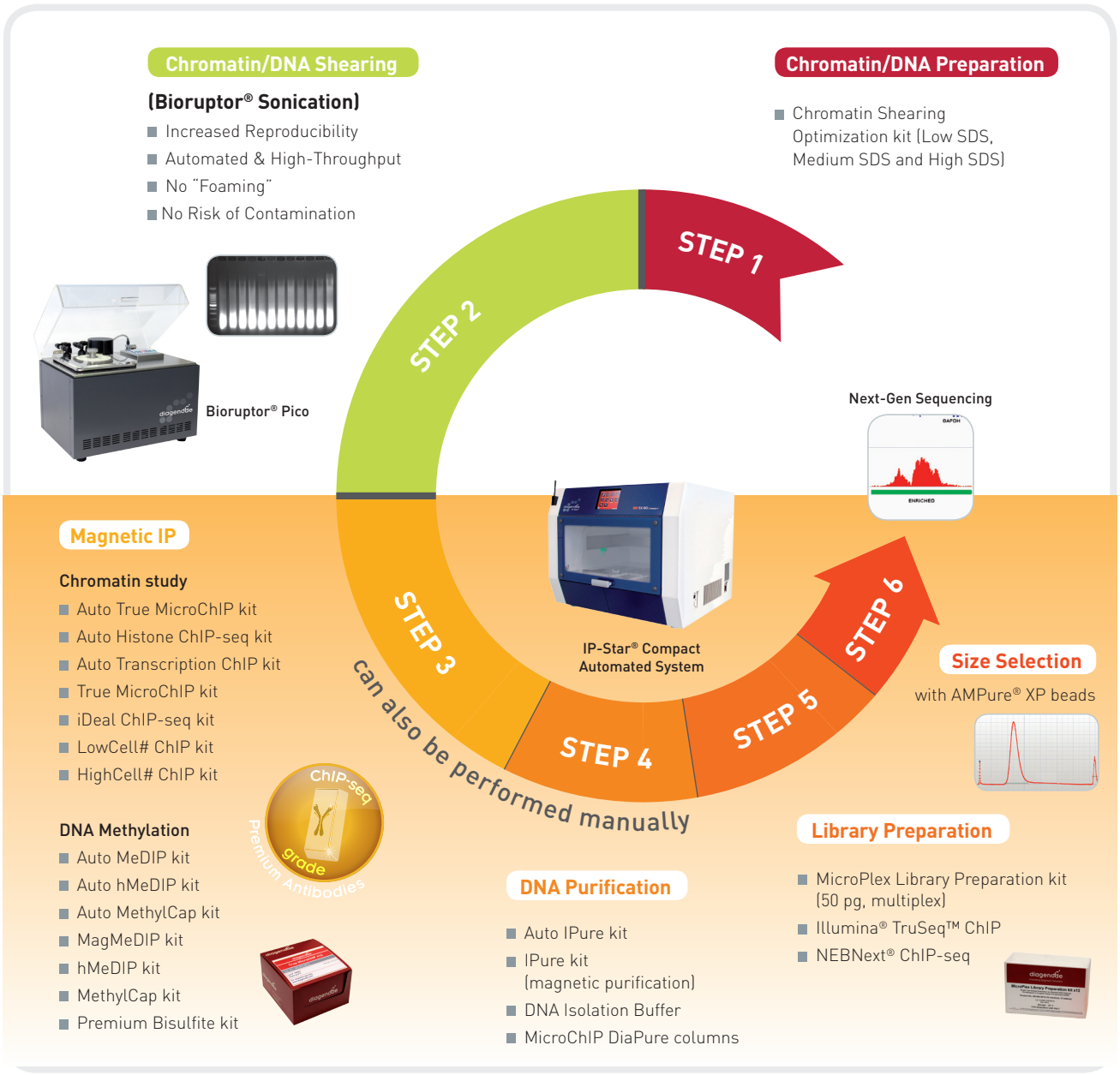

Figure 1. Diagenode provides a full suite of manual and automated solutions for ChIP experiments.

For Step 1, we offer products to isolate nuclei and chromatin. Step 2 describes reproducible sample shearing with the Bioruptor® product line. In Step 3 and Step 4, the Diagenode IP-Star® Compact provides error-free, walk-away automation for all your immunoprecipitation and antibody capture needs.

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