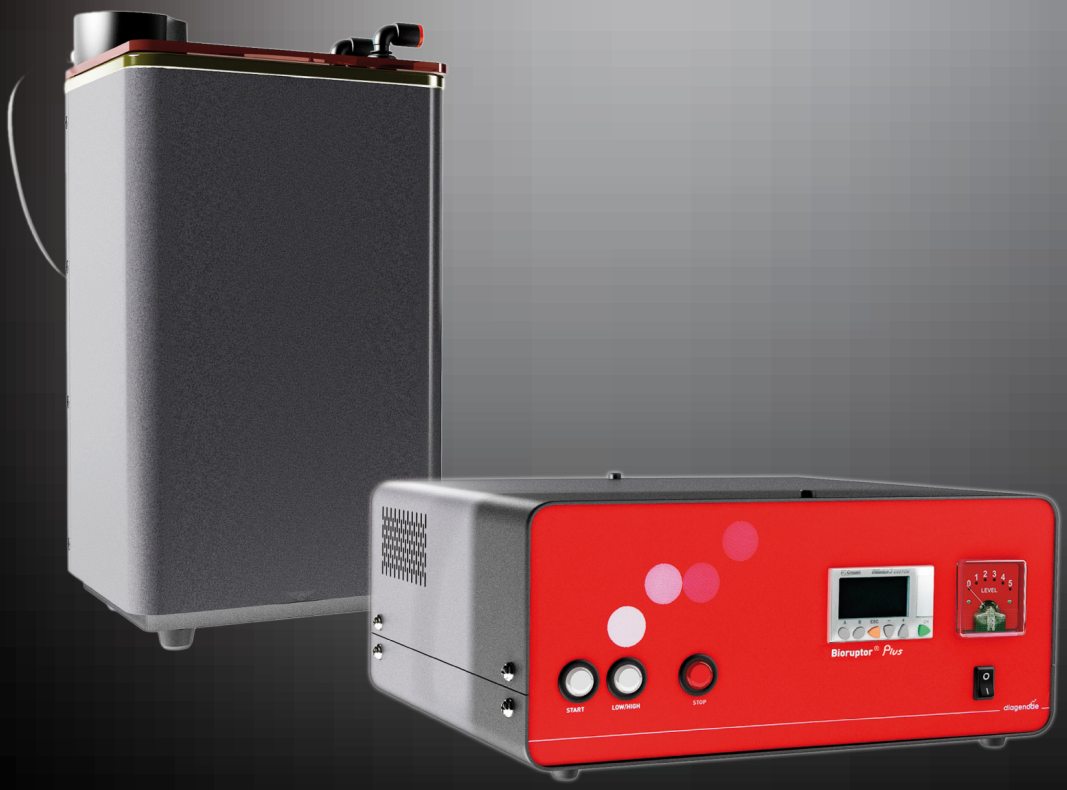
diagenode
Innovating Epigenetic Solutions

The Ultimate Guide for Chromatin Shearing Optimization with Bioruptor® Standard and Plus

USER'S GUIDE



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TRADEMARKS

Content

Critical points for chromatin shearing!

- Chromatin shearing should be tested and optimized when one is starting a new ChIP project.
- Given that every cell type may behave differently, it is highly recommended to optimize fixation and sonication conditions for each cell type before processing large quantities of cells or samples! One should perform an initial sonication time course experiment; evalution of the tests may be required to determine optimal shearing conditions.
- Sample viscosity may have a major impact on sonication results. Careful homogenization of chromatin sample is strongly recommended before and during sonication processing. For optimal results, samples should be gently vortexed before and after performing 5 or 10 sonication cycles (depending on the protocol), followed by a short centrifugation at 4°C to recover sample volume at the bottom of the tube. When working with large cell numbers careful pipetting can help ensure better homogenization but it will not replace the vortexing step mentioned before.
- Always use the highest available power setting (e.g. H: High Power on the Bioruptor® Plus) for all your chromatin shearing experiments.
- After shearing, it is essential to produce chromatin fragments of a size suitable for ChIP and subsequent analysis of the immunoprecipitated DNA. Although chromatin fragments from 100 - 1000 bp are recommended for ChIP PCR or ChIP qPCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 100 and 600 bp. Larger chromatin fragments can negatively influence ChIP-seq data quality.

Tube holders & tubes for Bioruptor® chromatin shearing

For chromatin shearing, depending on the final shearing volume, we recommend the following tube holders:

- From 100 – 300 µl: 1.5 ml tube holder for Bioruptor® Standard & Bioruptor® Plus (Cat. No. UCD-pack 1.5) with 1.5 ml TPX microtubes for Chromatin shearing (Cat. No. M-50001) (Figure 1).
- From 50 – 100 µl: 0.5/0.65 ml tube holder for Bioruptor® Standard, Bioruptor® Plus and the corresponding shearing tubes (Cat. No. WA-005-0500) (Figure 2).

To use the tube holder, remove the lower part by turning counterclockwise. Then place microtubes in the unit. Attach the lower part to the upper part of the adaptor. To guarantee homogeneity of chromatin shearing, the tube holders should always be completely filled with tubes. Never leave empty spaces in the tube holder. Fill the empty spaces with tubes containing the same volume of distilled water.

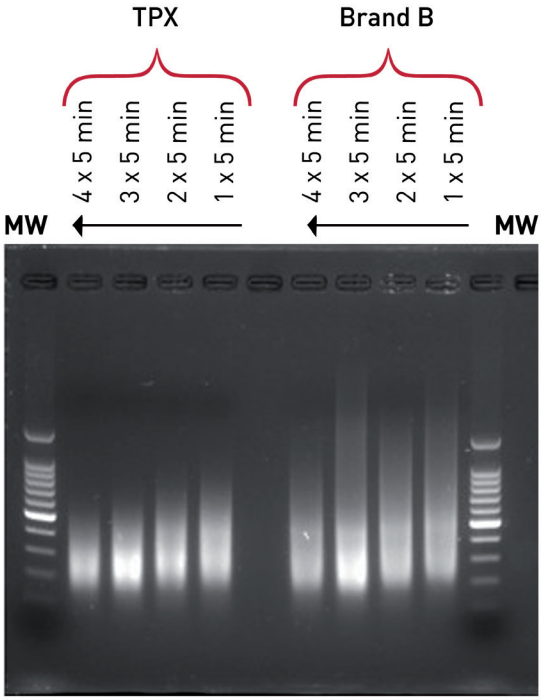


Figure 1: Superior chromatin shearing results obtained with Diagenode's TPX 1.5 ml tubes compared to standard 1.5 ml tubes

HeLa cells are fixed with 1% formaldehyde (for 8 min at room temperature (RT)). 100,000 cells are resuspended in 130 µl of buffer B from Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) prior to chromatin shearing. Samples are sheared during 1, 2, 3, or 4 rounds of 5 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) using 1.5 ml TPX microtubes (Cat. No. M-50001) or 1.5 ml standard tubes (Brand B).

The sheared chromatin is decross-linked prior to agarose gel electrophoresis. 10 µl of DNA are analyzed on a 1.5% agarose gel. The left and right lanes show the 100 bp DNA molecular weight marker (MW).

As shown on the left part of the image, the optimal shearing conditions correspond to 3 rounds of 5 cycles (30 sec ON/30 sec OFF; means 1 min ON & OFF time) (fragments between 100 and 600 bp) using TPX 1.5 ml tubes.

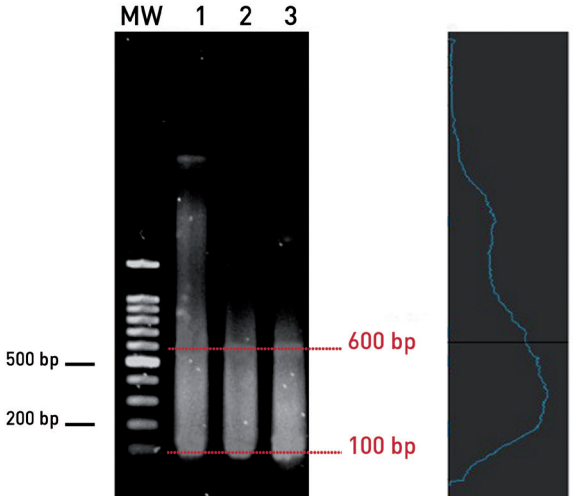


Figure 2: Efficient chromatin shearing with the Bioruptor® PLUS using small sonication volumes (50 µl)

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). 500,000 cells (lanes 1, 2, and 3) are resuspended in 50 µl of buffer B from Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) prior to chromatin shearing. Samples are sheared for several rounds of sonication 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) using shearing tubes (Cat. No. WA-005-0500). Between each sonication round, the samples are vortexed gently and then shortly centrifuged at 4°C to recover sample volume at the bottom of the tube.

The sheared chromatin is decross-linked prior to agarose gel electrophoresis. 10 µl of DNA (equivalent to 500 ng as measured with Qubit) are analyzed on a 1.5% agarose gel.

Left panel: The left lane shows the 100 bp DNA molecular weight marker (MW). Lane 1: 2x 5 cycles (each 30 sec ON/30 sec OFF); Lane 2: 3x 5 cycles (each of 30 sec ON/30 sec OFF); Lane 3: 4x 5 cycles (each 30 sec ON/30 sec OFF).

Right panel: Gel image was captured with Gel Logic 1500 Imaging System (KODAK). Lane 1 was scanned using Kodak software (V.4.0.5) to estimate DNA size distribution.

Using shearing volume as low as **50 µl**, optimal DNA size range for ChIP and ChIP-seq analysis can be easily obtained using Bioruptor® shearing tubes (Cat. No. WA-005-0500) (see lane 3).

Important additional comments about chromatin shearing

1. Cross-linking duration

- The extent of cross-linking is critical for the efficient disruption of fixed cells and also affects DNA yield and average size of chromatin fragments. Over-cross-linked chromatin will not produce small fragments, even by prolonged sonication and should be avoided.
- Fix cells for 8 min (default time) and up to 10 min at room temperature (RT), always stop the reaction by glycine and perform thorough washes to remove all traces of formaldehyde. Over fixation may have numerous negative effects on ChIP and/or ChIP-seq experiments and should be carefully monitored. First, longer fixation times (e.g. 10 - 30 min) may cause cells to form aggregates that do not sonicate efficiently. Second, for some antibodies, longer cross-linking time may increase background by increasing non-specific binding of epitopes to antibody. Finally, over fixation has been shown to generate artefacts such as sonication resistant 1.5 - 1.7 kb fragments.

2. Sonication time

- Extensive sonication may alter antigenic properties and lead to loss of antigenecity during the immunoprecipitation step. Ideally, the least time course conditions that gives satisfactory results should be used (Figure 3).
- For optimal fragmentation assessment, a serial dilution of sheared chromatin from 100 ng to 500 ng should be run on a 1.2 - 1.5% agarose gel (ideally the gel should be pre-stained). If only one unique sample has to be tested, we strongly recommend using de-cross-linked chromatin DNA to avoid any misleading size assessment using agarose gel electrophoresis (e.g. from 400,000 - 500,000 cells) (Figure 4).

3. Impact of cells on chromatin shearing

- It is best to prepare samples of sheared chromatin from **fresh cells** and directly use them in ChIP, especially when doing ChIP-seq. However, it is also possible to freeze cells and shear chromatin another day. Pellets of frozen cells are by far more stable than sheared frozen chromatin samples (fresh cells > frozen cells >> frozen chromatin). Given that frozen cells may behave differently than fresh ones, it is recommended to optimize sonication conditions for each cell condition (Figure 5).

  Some cell types such as primary cells (e.g. mouse splenocytes) or cells growing in a suspension culture (e.g. blood-derived cells (monocytes, macrophages, T or B lymphocytes) are more difficult to shear (the so-called «difficult cells»)). In general, "difficult" cells require more cycles to obtain the same shearing as cultured cells but, in general, we recommend the following to optimize your shearing (Figure 6):
  - o whenever it is possible, start with an abundant source of cells
  - o fix them according to Diagenode's ChIP protocols
  - o to ensure the best results, Diagenode strongly recommends to use fresh cells (compared to frozen cells) (Figure 5)

  Note: In case of longer sonication time, it may also be helpful to assess the integrity of your epitope during the shearing process. To do so, remove 5 µl of your sample at each time point. Add 25 µl of H_20 and 2 µl 5M NaCl. Incubate overnight in hybridization oven or PCR machine at 65˚ C. Run 10 µl on SDS¬PAGE gel and Western Blot with an antibody to your protein. Please contact Diagenode for Western blot grade antibodies

4. Importance of RNase treatment

- Treating samples with RNase prior to agarose gel analysis can also significantly improve visual assessment of shearing (Figure 7). Also, it has to be noted that the dye used in the High Sensitivity DNA chips (e.g. for Bioanalyzer 2100) while being specific for DNA can also stain RNA, especially at higher RNA concentrations. Therefore, it is better to include RNase cocktail treatment in order to drastically reduce backgrounds caused by RNA.

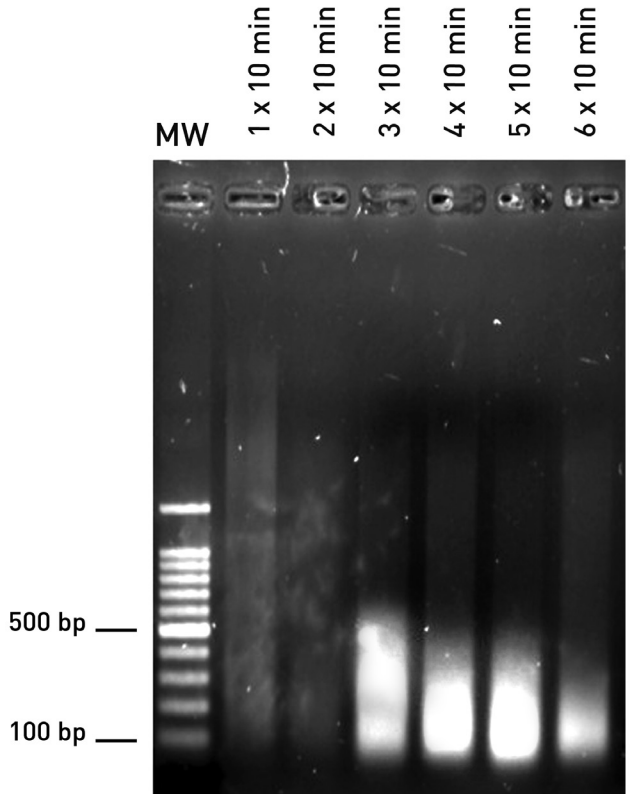


Figure 3: Time course sonication experiment with the Bioruptor® PLUS using the buffers and protocol from Diagenode's Chromatin Shearing Optimization kit - Medium SDS

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 5 million cells are performed using buffers from Diagenode's chromatin Shearing Optimization kit - Medium SDS (Cat. No. AA-002-0100) 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 least time course condition corresponds to 3 rounds of 10 cycles (30 sec ON/30 sec OFF).

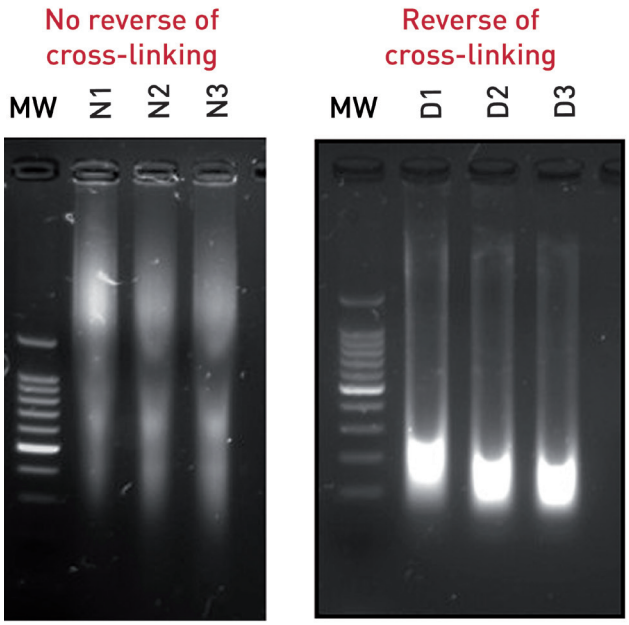


Figure 4: Comparison of shearing assessment with agarose gel using non decross-linked versus decross-linked chromatin

HeLa cells were fixed using the buffers and protocol from Diagenode's Chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-001-0100).

Samples were sheared during 3 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 analysis on a 1.5% agarose gel, samples were decross-linked (D1, D2, D3) or were directly loaded on the gel without any other treatment.

MW: Molecular Weight Marker

Lane N1, N2, N3 – non decross-linked samples: volume loaded equivalent to 250,000 cells

Lane D1, D2, D3 – decross-linked samples: volume loaded equivalent to 500,000 cells

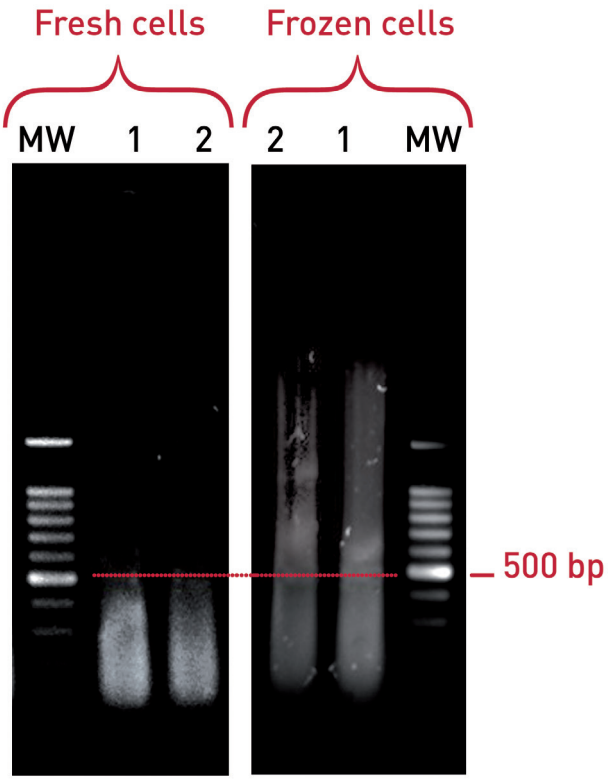


Figure 5: Superior chromatin shearing results obtained with fresh compared to frozen cells

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 3x10e6 cells are performed using buffers from Diagenode's chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-001-0100) and are then resuspended in 100 µl of Shearing Buffer iS1 prior to chromatin shearing. Samples are either immediately processed (Fresh cells) or frozen at -80°C (Frozen Cells). Each experiment is done in duplicate.

Samples are sheared during 3 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). Samples are gently vortexed before and after performing each sonication round (10 cycles per round), followed by a short centrifugation at 4°C to recover sample volume at the bottom of the tube. Prior to decross-linking, samples are treated with RNase cocktail mixture at 37°C during 1 h. The sheared chromatin is then decross-linked as described in the kit manual and analyzed by agarose gel electrophoresis. 10 µl of DNA are analyzed on a 1.5% agarose gel.

Left panel: Sheared chromatin obtained from **Fresh cells** (Lane 1 and 2). (MW corresponds to the 100 bp DNA molecular weight marker).

Right panel: Sheared chromatin obtained from **Frozen cells** (Lane 1 and 2) (MW corresponds to the 100 bp DNA molecular weight marker).

This example clearly shows that the best results are obtained using fresh cells (controlled DNA size range between 100 - 500 bp. Please note that when using frozen cells, a high molecular weight fraction of DNA (→ 2000 bp) might persist even after 30 min of sonication indicating that freezing the sample might alter the DNA's susceptibility to shearing by sonication.

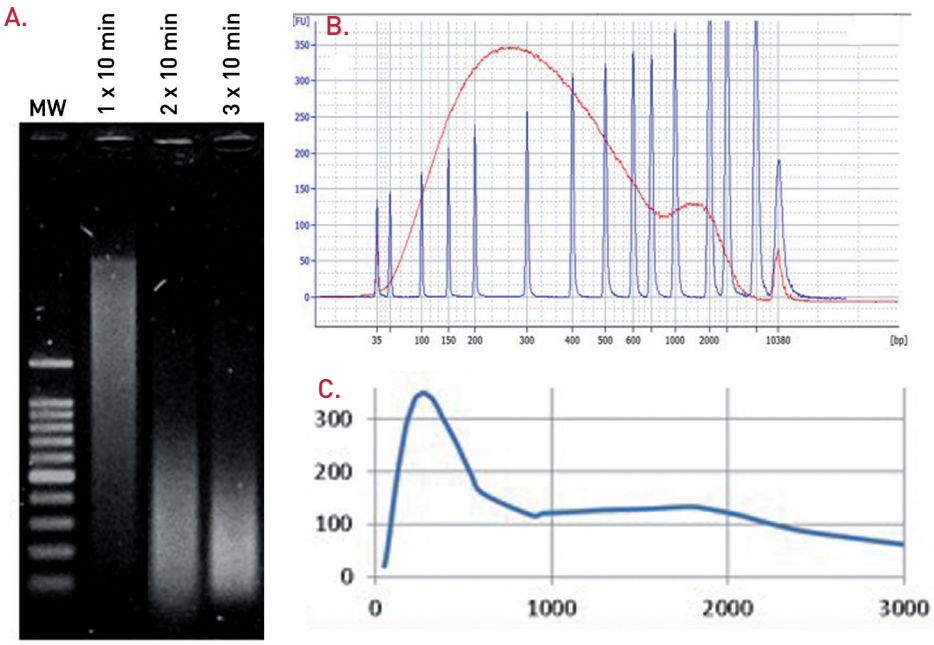


Figure 6: Successful chromatin shearing of chromatin from «difficult cells» (K562)

K562 cells (myelogenous leukemia cell line) are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 1 million fresh cells are performed using buffers from Diagenode's Chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-001-0100) and are then resuspended in 100 µl of Shearing Buffer iS1 prior to chromatin shearing.

Samples are sheared during 1, 2 or 3 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). For optimal results, samples are vortexed before and after performing 10 sonication cycles, followed by a short centrifugation at 4°C. All samples were treated with RNase and reverse cross-linked.

Panel A: 10 µl of DNA (equivalent to 300 ng) are analyzed on a 1.5% agarose gel.

Panel B and C: Third sample of panel A (3x 10 min) was analyzed on Bioanalyzer 2100 using DNA High Sensitivity chip. The default log scaled Bioanalyzer output can be seen in Panel A, while Panel C represents their linear transformation for better vizualisation. Out of range fragments were not shown on this graph.

This example clearly shows that «difficult cells» such as K562 can readily be sheared using the Bioruptor®.

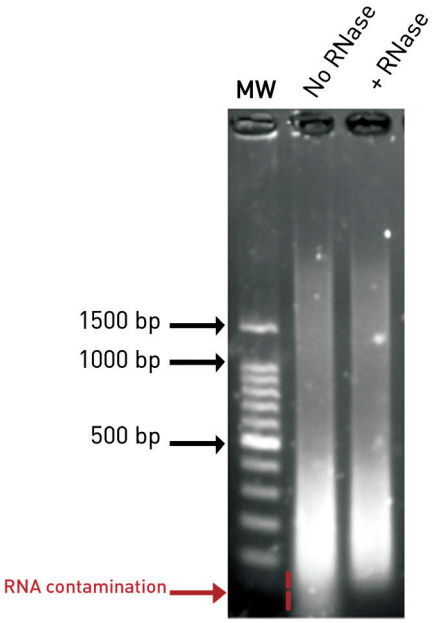


Figure 7: Improved agarose gel chromatin visual assessment with RNase treatment

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 5 million cells are performed using buffers from Diagenode's Chromatin Shearing Optimization kit - Medium SDS (Cat. No. AA-002-0100) and are then resuspended in 200 µl of Shearing Buffer S1 prior to chromatin shearing.

Samples are sheared during 3 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). One sample (right lane) was treated with RNase (and further reverse cross-linked). Equivalent to 300 ng are analyzed on a 1.5% agarose gel.

This example clearly shows that better chromatin visual assessment is obtained after RNase treatment.

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

	Chromatin Shearing Optimization kit Low SDS	Chromatin Shearing Optimization kit Medium SDS	Chromatin Shearing Optimization kit High SDS
SDS concentration in the sonication buffer	<0.1%	0.5%	1%
Nuclei isolation	yes	yes	no
Cell number per sonication sample	1 million cells	1-10 million cells	100.000 -1 million cells
Cell number per IP	1 million cells (1 sonication sample = 1 IP)	1-10 million cells (1 sonication sample = 1 IP)	10.000-100.000 cells (1 sonication sample = 10 IP)
Allows for shearing of ... cells	100 million cells	100 million cells	100 million cells
Compatible with	**iDeal ChIP-seq kit** AB-001-0024	**HighCell# ChIP kit** kch-mahigh-A16 kch-mahigh-G16	**LowCell# ChIP kit** kch-maglow-A16 kch-maglow-A48 kch-maglow-G16 kch-maglow-G48

Our Chromatin Shearing Optimization kits are used in combination with the Bioruptor® in order to ensure a highly reproducible chromatin shearing and to 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.

Before starting:

The maximum volume for chromatin shearing with the Bioruptor® is 300 µl/1.5 ml microtube. We recommend using TPX tubes (Cat. No. M-50050 or M-50001) as it has been shown that the shearing is more efficient and reproducible using these tubes.

Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation. Note that an equivalent of at least 100.000 cells is required for the analysis of chromatin shearing.

Once optimal conditions are established, use the same settings for all assays to assure reproducibility.

Standard protocol for chromatin shearing with Shearing Buffers from Chromatin Shearing Optimization kit-Low SDS

This protocol is based on Diagenode's Chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-001-0100) manual. Check the corresponding manual for more information (e.g. about buffer types and names).

A. Cell collection and DNA-protein cross-linking

ChIP and ChIP-seq assays work best when cells are healthy and in exponential growth. Do not grow suspension cells to maximum density or too high acidity, and for adherent cells, do not grow cells to confluency prior to cross-linking.

Cell density may also affect sonication efficiency. Do not use too dense cell suspension. Using Diagenode's Chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-001-0100) shearing buffers, the optimal cell density is about 1x10e6 cells per 100 µl of sonication buffer.

The protocol below is intended for adherent cells. Collect suspension cells by centrifugation and continue with the protocol starting from step 6.

1. Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.
2. Remove the medium and rinse the cells with pre-warmed PBS (10 ml for a 75 cm^2 culture flask). Gently shake the flask for 2 min.
3. Remove the PBS and add sterile trypsin-EDTA to the culture flask to detach adherent cells from the bottom. Table 1 shows the required amount of trypsin for different numbers of cells. Gently shake the culture flask for 1 - 2 min or until the cells start to detach. The time needed may depend on the cell type but do not continue trypsin treatment longer than necessary as prolonged treatment with trypsin may damage the cells. Regularly check if the cells start to detach.

Table 1			
No. of cells	3x10e6 cells	10e7 cells	5x10e7 cells
Trypsin-EDTA	1 ml	3 ml	15 ml

4. Immediately add fresh culture medium to the cells when they are detached (Table 2). This will inactivate trypsin.

Table 2			
No. of cells	3x10e6 cells	10e7 cells	5x10e7cells
Culture medium	2 ml	6 ml	30 ml

5. Collect the cells by adding 10 ml of PBS to the culture flask. Transfer the PBS with the cells to a 50 ml centrifugation tube. Rinse the flask once with another 10 ml of PBS and add it to the 50 ml tube.
6. Centrifuge for 5 min at 1,600 rpm and 4°C and remove the supernatant.
7. Resuspend the cells in 20 ml of PBS and **count** them. Collect the cells by centrifugation for 5 min at 1,600 rpm and 4°C.
8. Resuspend the cells in PBS to obtain a concentration of up to 10 million cells per 500 µl of PBS. If desired, the cell concentration can be decreased down to 1 million per 500 µl. Label 1.5 ml tubes and aliquot 500 µl of cell suspension in each tube.
9. Add 13.5 µl of formaldehyde 36.5% to each tube containing 500 µl of cell suspension (final concentration should be ~1%). Mix by gentle vortexing and incubate for 8 min at RT to allow fixation to take place.

   NOTE: It is highly recommended to **always** use fresh formaldehyde
10. Add 57 µl of glycine to the cells to stop the fixation. Mix by gentle vortexing and incubate for 5 min at RT. Keep the cells on ice from this point onwards.
11. Collect the cells by centrifugation at 1,600 rpm for 5 min and 4°C. Discard the supernatant without disturbing the cell pellet.
12. Wash the cells twice with 1 ml of cold PBS.

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.

B. Cell lysis and chromatin shearing

This section describes cell lysis and chromatin shearing with the Bioruptor®. At this stage, it is essential to produce chromatin fragments of a size suitable for ChIP and subsequent analysis of the immunoprecipitated DNA.

Although chromatin fragments from 100 - 1000 bp are recommended for ChIP PCR or ChIP qPCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 100 and 600 bp. Larger chromatin fragments can negatively influence ChIP-seq data quality.

13. Add 1 ml of ice-cold Lysis Buffer iL1 to the 1.5 ml tube containing 10 million cells. Resuspend the cells by pipetting up and down several times and transfer them to a 15 ml tube. Add 9 ml of Buffer iL1 and incubate for 10 min at 4°C with gentle mixing. If the starting amount of cells was less than 10 million, scale down accordingly (e.g. use a total of 5 ml buffer iL1 for 5 million cells).
14. Pellet the cells by centrifugation at 1,600 rpm for 5 min and 4°C and discard the supernatant.
15. Add 1 ml of ice-cold Lysis Buffer iL2 and resuspend the cells by pipetting up and down several times. Add another 9 ml of Buffer iL2 and incubate for 10 min at 4°C with gentle mixing. Scale down accordingly when using less than 10 million cells.
16. Pellet the cells again by centrifugation for 5 min at 1,600 rpm (500 x g) and 4°C and discard supernatant.
17. Add 200x protease inhibitor cocktail (Cat. No. kch-107-020, kch-502-100 or kch-502-300) to Shearing Buffer iS1. Prepare 1 ml of complete shearing buffer per tube of 10 million cells. Keep on ice.
18. Add 1 ml of Complete Shearing Buffer iS1 to 10 million cells (if less than 10 millions cells are used, scale accordingly). Resuspend the cells by pipetting up and down several times. The final cell concentration should be 1 million cells per 100 µl buffer iS1. Split into aliquots of 100 - 300 µl and transfer the cell suspension to 1.5 ml TPX microtubes (Cat. No. M-50001). Incubate on ice for 10 min at 4°C. Vortex and spin down the samples.
19. Shear the chromatin by sonication using the Bioruptor® at high power setting.

    Carry out a time course shearing experiment to optimize the chromatin shearing parameters specific for your cell line. For the initial experiment, we suggest a time course of 1, 2 to 3 runs of 10 cycles [30 sec "ON", 30 sec "OFF"] at high power setting. Briefly vortex and spin down between each run.
20. Centrifuge at 13,000 rpm (16,000 x g) for 10 min at 4°C and collect the supernatant which contains the sheared chromatin. Take an aliquot of 50 µl (an equivalent of 500.000 cells) for assessment of chromatin shearing (step C). The remaining chromatin might be stored at -80°C for up to 2 months for further use in the immunoprecipitation.
    Important: Do not freeze/thaw.
21. Proceed to Sheared chromatin analysis.

C. Sheared chromatin analysis

This protocol refers to Diagenode's Elution Module (Cat. No. mc-magme-002) that can be ordered separately.

Reagents not supplied:

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 100% Ethanol
- 70% Ethanol

22. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of water).
23. Add 2 µl of diluted RNase cocktail to 50 µl of chromatin sample.

24. Incubate 1h at 37°C.

25. Prepare the Complete Elution Buffer by mixing thoroughly Buffers D, E and F as follows:

Reagents	Volume
Buffer D	96 µl
Buffer E	10 µl
Buffer F	4 µl
Total volume	110 µl*

* enough volume for two chromatin samples

26. Add 54 µl of the Complete Elution Buffer to each chromatin sample.

27. Mix thoroughly before incubating sample at 65°C for 4h (or overnight).

28. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol. Incubate the sample at RT for 10 min on a rotating wheel.

29. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

30. Add 1 volume of chloroform/isoamyl alcohol [24:1]. Incubate the sample at RT for 10 min on a rotating wheel.

31. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

32. Precipitate the DNA by adding 5 µl of meDNA coprecipitant, 10 µl of meDNA-IP precipitant and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.

33. Centrifuge for 25 min at 13,000 rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.

34. 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.

35. Re-suspended the pellet in 20 µl of TE buffer.

36. Run samples (20 µl of DNA + 4 µl of 6x loading dye) on a 1.5% agarose gel. As an alternative run 1 µl on a microfluidic chip (e.g. Bioanalyzer High Sensitivity DNA chip).

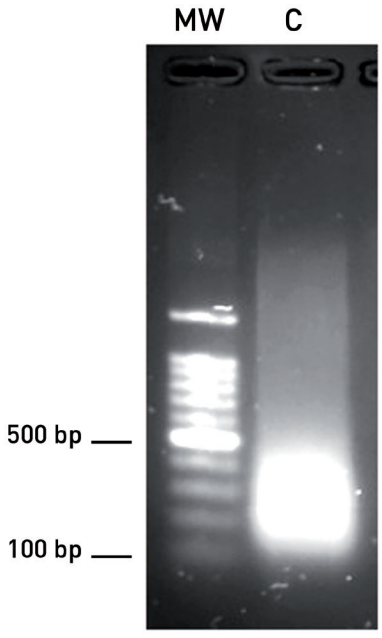


Figure 8: Superior chromatin shearing efficiency with the Bioruptor® PLUS using Diagenode's Chromatin Shearing Optimization kit - Low SDS

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 7.5 million cells are performed using buffers from Diagenode's Chromatin Shearing Optimization kit - Low SDS (Cat. No. AA-003-0100) and are then resuspended in 500 µl prior to chromatin shearing.

Samples are sheared during 3 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). The sheared chromatin is then decross-linked and treated with RNase prior to agarose gel electrophoresis. Equivalent of 500,000 cells are analyzed on a 1.5% agarose gel. The left lane shows the 100 bp DNA molecular weight marker (MW). Lane C: sample of sheared chromatin.

This example shows that 3 rounds of 10 cycles (30 sec ON/30 sec OFF) using the Bioruptor® generates fragments between 100 and 400 bp compatible with ChIP-seq.

Standard protocol for chromatin shearing with Shearing Buffers from Chromatin Shearing Optimization kit - Medium SDS

This protocol is based on Diagenode's Chromatin Shearing Optimization kit - Medium SDS (Cat. No. AA-002-0100) manual. Check the corresponding manual for more information (e.g. about buffer types and names).

A. Cell collection and DNA-protein cross-linking

ChIP and ChIP-seq assays work best when cells are healthy and in exponential growth. Do not grow suspension cells to maximum density or too high acidity, and for adherent cells, do not grow cells to confluency prior to cross-linking.

Cell density may also affect sonication efficiency. Do not use too dense cell suspension.

Using Diagenode's Chromatin Shearing Optimization kit -Medium SDS (Cat # AA-002-0100) shearing buffers, a time course experiment using 1 - 10 million cells per sonication is recommended.

The protocol below is intended for adherent cells. Collect suspension cells by centrifugation and continue with the protocol starting from step 6.

1. Pre-warm PBS, culture media and trypsin-EDTA.
2. Remove old media and rinse cells with pre-warmed PBS. Shake dishes for 2 min. Remove the PBS.
3. Add sterile trypsin-EDTA to tissue culture flask or dish containing adherent cells. Brief treatment with trypsin-EDTA removes adherent cells from the bottom of the tissue culture flask.

   NOTE: Each cell line will differ in the degree of adherence it has, this will be described in the literature that accompanies it. If necessary, place flask back into incubator for about 1 - 2 min.
4. Check after one min 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, immediately add culture medium to the cells. The addition of medium will inactivate the trypsin.
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 tube before centrifugation.
7. Wash the cell suspension two times with PBS.
8. Label new 1.5 ml tube(s), count the cells and put 1 to 10 million cells in 500 µl of PBS at RT.
   - In order to preserve 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%).

   NOTE: It is highly recommended to **always** use fresh formaldehyde
10. Mix by gentle vortexing. Incubate for 8 min at RT to allow fixation to take place.
11. Add 57 µl of glycine to the sample.
12. Mix by gentle vortexing. Incubate for 5 min at RT. This is to stop the fixation.
13. Centrifuge at 1,500 rpm (300 x g) for 5 min at 4°C. We recommend the use of a swing-out rotor with soft settings for deceleration.
14. Aspirate the supernatant. Take care to not remove the cells. Aspirate slowly and leave approximately 30 µl of the solution behind.

- These are your cross-linked cells ready for chromatin shearing!
- Do not disturb the pellet.

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.

B. Cell lysis and chromatin shearing

This section describes cell lysis and chromatin shearing with the Bioruptor®. At this stage, it is essential to produce chromatin fragments of a size suitable for ChIP and subsequent analysis of the immunoprecipitated DNA.

Although chromatin fragments from 100 - 1000 bp are recommended for ChIP PCR or ChIP qPCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 100 and 600 bp. Larger chromatin fragments can negatively influence ChIP-seq data quality.

15. 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).

NOTE: Work on ice unless otherwise stated.

16. Add the ice-cold PBS solution (or any inhibitor containing PBS solution, e.g. NaBu-PBS solution), gently vortex and centrifuge at 470 x g (in a swing-out rotor with soft settings for deceleration) for 10 min at 4°C.

17. 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.

18. After the last wash, aspirate the supernatant. Leave about 10 - 20 µl behind.

19. Avoid taking out too much as that could lead to material loss.

20. 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 min at 4°C with gentle mixing.

21. Centrifuge for 5 min at 1,600 rpm (500 x g) at 4°C. Discard the supernatant, keep the pellet.

22. 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 min at 4°C with gentle mixing.

23. Pellet again by centrifugation for 5 min at 1,600 rpm (500 x g) at 4°C, and discard supernatant.

24. Add 200 x protease inhibitor Cocktail (Cat. No. kch-107-020, kch-502-100 or kch-502-300) to the Shearing Buffer S1 (RT). This is the complete Shearing Buffer S1 for sonication.

25. Keep the buffer at RT until use; discard what is not used during the day.

26. Add 200 µl of complete Shearing Buffer S1 (RT) to the cells. Vortex until resuspention. Incubate for 10 min on ice. (Different cell concentrations from 1 to 10 million cells per 200 µl of S1 buffer may be tested.)

27. Shear the chromatin by sonication using the Bioruptor® at high power setting.
Carry out a time course shearing experiment to optimize the chromatin shearing parameters specific for your cell line. For the initial experiment, we suggest a time course of 1, 2 to 3 runs of 10 cycles [30 sec "ON", 30 sec "OFF"] at high power setting. Briefly vortex and spin down samples between each run.

28. Centrifuge at 13,000 rpm (16,000 x g) for 10 min at 4°C and collect the supernatant which contains the sheared chromatin.

29. Take an aliquot of chromatin equivalent to 100.000 - 500.000 cells for assessment of chromatin shearing (step C). The remaining chromatin might be stored at -80°C for up to 2 months for further use in immunoprecipitation experiments. **Important: Do not freeze/thaw.**

30. Proceed to Sheared chromatin analysis.

C. Sheared chromatin analysis

This protocol refers to Diagenode's Elution Module (Cat. No. mc-magme-002) that can be ordered separately.

Reagents not supplied:

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 100% Ethanol
- 70% Ethanol

31. If the sample volume is less than 50 µl, adjust it with S1 shearing buffer to reach 50 µl.

32. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of water).

33. Add 2 µl of diluted RNase cocktail to the chromatin sample.

34. Incubate 1h at 37°C.

35. Prepare the Complete Elution Buffer by mixing thoroughly Buffers D, E and F as follows:

Reagents	Volume
Buffer D	48 µl
Buffer E	5 µl
Buffer F	2 µl
Total volume	55 µl*

* One volume of Complete Elution Buffer is required per sample. Scale accordingly.

36. Add one volume of the Complete Elution Buffer to each chromatin sample.

37. Mix thoroughly before incubating sample at 65°C for 4h (or overnight).

38. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol. Incubate the sample at RT for 10 min on a rotating wheel.

39. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

40. Add 1 volume of chloroform/isoamyl alcohol [24:1]. Incubate the sample at RT for 10 min on a rotating wheel.

41. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.

42. Precipitate the DNA by adding 5 µl of meDNA coprecipitant, 10 µl of meDNA-IP precipitant and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.

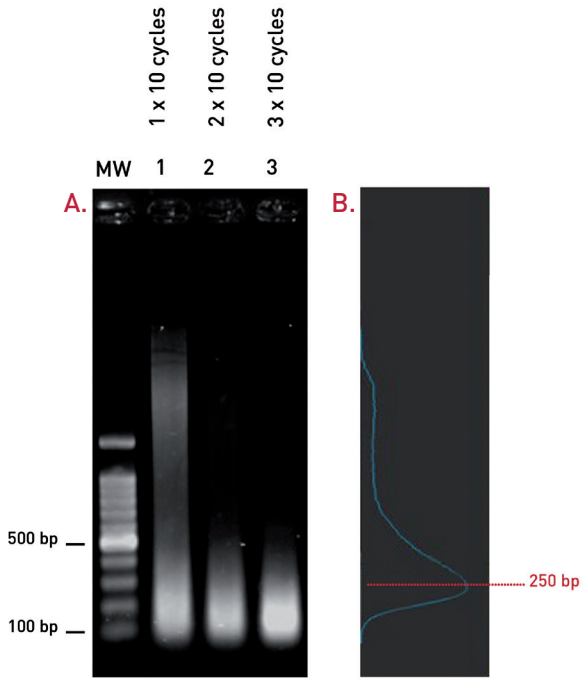
43. Centrifuge for 25 min at 13,000 rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice cold 70% ethanol to the pellet.

44. 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.

45. Re-suspended the pellet in 20 µl of TE buffer.

46. Run samples (20 µl of DNA + 4 µl of 6x loading dye) on a 1.5% agarose gel. As an alternative run 1 µl on a microfluidic chip (e.g. Bioanalyzer High Sensitivity DNA chip).

Figure 9: Agarose gel analysis of chromatin sheared with the Bioruptor® PLUS using the buffers and protocol from Diagenode's Chromatin Shearing Optimization kit - Medium SDS



HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). Nuclei isolation of 1 million cells are performed using buffers from Diagenode's Chromatin Shearing Optimization kit - Medium SDS (Cat. No. AA-002-0100) and are then resuspended in 100 µl of Shearing Buffer S1 prior to chromatin shearing.

Samples are sheared during 1, 2 or 3 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).

Panel A: The sheared chromatin is then decross-linked prior to agarose gel electrophoresis. 10 µl of DNA are analyzed on a 1.5% agarose gel. The left lane shows the 100 bp DNA molecular weight marker (MW). Lane 1: 1x 10 cycles (each of 30 sec ON/30 sec OFF); Lane 2: 2x 10 cycles (each of 30 sec ON/30 sec OFF); Lane 3: 3x 10 cycles (each 30 sec ON/30 sec OFF).

Panel B: Gel image was captured with Gel Logic 1500 Imaging System (Kodak). Lane 2 was scanned using Kodak Molecular Imaging Software (v 4.0.5) to estimate DNA size distribution and concentration.

In this example, the optimal shearing condition corresponds to 2 rounds of 10 cycles (30 sec ON/30 sec OFF).

Standard protocol for chromatin shearing with Shearing Buffers from the Chromatin Shearing Optimization kit - High SDS

This protocol is based on Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) manual. Check the corresponding manual for more information (e.g. about buffer types and names).

A. Cell collection and DNA-protein cross-linking

ChIP and ChIP-seq assays work best when cells are healthy and in exponential growth. Do not grow suspension cells to maximum density or too high acidity, and for adherent cells, do not grow cells to confluency prior to cross-linking.

Cell density may also affect sonication efficiency. Do not use too dense cell suspension. Using Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) shearing buffers, the optimal cell density is about 100.000 - 1 million cells per sonication sample. Please note that each sonication sample which will be used per IP sould be diluted 10x to decrease the SDS concentration. Please refer to the table below to choose the optimal cell number per sonication sample.

		Number of cells per IP					
Number of cells per shearing		100,000	50,000	20,000	10,000	5,000	1,000
	1 million	Buffer B (130 µl) IP Buffer (870 µl) **10 IP's**	Buffer B (130 µl) IP Buffer (1,870 µl) **20 IP's**	Buffer B (130 µl) IP Buffer (4,870 µl) **50 IP's**	Buffer B (130 µl) IP Buffer (9,870 µl) **100 IP's**	-	-
	500,000	Buffer B (50 µl) IP Buffer (450 µl) **5 IP's**	Buffer B (50 µl) IP Buffer (870 µl) **10 IP's**	Buffer B (50 µl) IP Buffer (2,370 µl) **25 IP's**	Buffer B (50 µl) IP Buffer (4,870 µl) **50 IP's**	Buffer B (130 µl) IP Buffer (9,870 µl) **100 IP's**	-
	100,000	-	-	Buffer B (50 µl) IP Buffer (450 µl) **5 IP's**	Buffer B (50 µl) IP Buffer (450 µl) **10 IP's**	Buffer B (50 µl) IP Buffer (450 µl) **20 IP's**	Buffer B (130 µl) IP Buffer (9,870 µl) **100 IP's**

Please note that the dilution with the IP buffer is required only for the IP step, but not for shearing. This buffer is provided with the LowCell# ChIP kit (Cat. No. kch-maglow-A16 or A48 and kch-maglow-G16 or G48) but not with the Chromatin Shearing Optimization kit-High SDS (Cat. No. AA-003-0100).

1. Prepare and harvest cells as follows:
   - Immediately before cell harvesting, if necessary, add any desired inhibitors (e.g. protease inhibitors, HDAC inhibitors such as 20 mM NaBu available separately; Cat. No. kch-817-001) to the culture medium and mix gently. If adding sodium butyrate (NaBu) from a 1 M stock solution, dilute to reach a final concentration of 20 mM. The complete PBS buffer mentioned below refers to PBS with inhibitor(s).
   - Place PBS at RT.
   - If using adherent cells, discard medium to remove dead cells. Wash cells by adding 10 ml PBS. Harvest cells by trypsinization using trypsin with inhibitor(s). Transfer cells in a tube containing 10 ml PBS (RT), and centrifuge 5 min at 1,300 rpm. Keep the cell pellet and discard the supernatant. Optionally, before transferring the trypsin it can be inactivated by adding serum and inhibitors (if necessary). Then wash the cells in complete PBS and discard medium.
   - If using suspension cells, centrifuge for 5 min at 1,600 rpm. Keep the cell pellet and discard the supernatant. Wash cells by adding 10 ml complete PBS (RT) containing inhibitor(s).
2. Count the cells (e.g. determine the number of cells in about 200 µl of your sample).
3. Label new 1.5 ml tube(s). Add PBS to a final volume of 500 µl after the cells have been added. If necessary add inhibitors. Then, transfer cells and wash the pipette tip thoroughly in the sample.
   - 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.

4. Add 13.5 µl of 36.5% fresh formaldehyde per 500 µl of sample (final concentration should be ~1%).

   NOTE: It is highly recommended to **always** use fresh formaldehyde

5. Mix by gentle vortexing. Incubate for 8 min at RT to enable fixation. Optimization of fixation time may be required depending on cell type; it could be 8 - 10 min.

6. Add 57 µl of 1.25 M glycine to the sample.

7. Mix by gentle vortexing. Incubate for 5 min at RT to stop the fixation. Work on ice from this point onwards.

8. Centrifuge at 470 x g for 10 min at 4°C.
   - We recommend the use of a swing-out rotor with soft settings for deceleration.

9. Aspirate the supernatant slowly and leave approximately 30 µl of the solution. Do not disturb the pellet. Take care to not remove these cross-linked cells.

10. Wash the cross-linked cells twice with 0.5 ml of ice cold PBS with inhibitor(s).
    - Add the PBS solution, gently vortex and centrifuge at 470 x g (in a swing-out rotor with soft settings for deceleration) for 10 min at 4°C.
    - For 100,000 cells or more you might need to resuspend with a pipette to ensure cells are thoroughly washed.
    - Smaller cell numbers are more easily washed and resuspended by vortexing. Make sure that cells are in suspension before proceeding to the next point.

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.

B. Cell lysis and chromatin shearing

This section describes cell lysis and chromatin shearing with the Bioruptor®. At this stage, it is essential to produce chromatin fragments of a size suitable for ChIP and subsequent analysis of the immunoprecipitated DNA.

Although chromatin fragments from 100 - 1000 bp are recommended for ChIP PCR or ChIP qPCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 100 and 600 bp. Larger chromatin fragments can negatively influence ChIP-seq data quality.

11. Place the Buffer B at RT before use. After the last wash, aspirate the supernatant, and leave 10 - 20 µl behind to avoid material loss.
    Note: Work on ice unless otherwise stated.

12. Prepare Buffer B. Add 200x protease inhibitor cocktail (Cat. No. kch-107-020, kch-502-100 or kch-502-300) (1x final concentration) and if necessary, NaBu (20 mM final concentration) to Buffer B (RT). This is the complete Buffer B. Keep the buffer at RT until use. Discard what is not used within a day.

    Attention: Make sure that there are no crystals in the Buffer B. Gently heat and mix until crystals disappear.

13. Add complete Buffer B to cells.

    Vortex until resuspended and transfer to the sonication tubes. Use 1.5 TPX microtubes (M-50050) for 130 µl samples *or 0.5 ml DNA shearing microtubes (WA-004-0500) for 50 µl samples.* Incubate for 5 min on ice.

14. Shear the chromatin by sonication using the Bioruptor® at high power setting.

    Carry out a time course shearing experiment to optimize the chromatin shearing parameters specific for your cell line. For the initial experiment, we suggest a time course of 1, 2 to 3 runs of 10 cycles [30 sec "ON", 30 sec "OFF"] at high power setting. Briefly vortex and spin down samples between each run.
    The different cell concentrations from 100.000 to 1 million cells per sample may be tested.

15. Take an aliquot of chromatin equivalent to 100.000 - 500.000 cells for assessment of chromatin shearing (step C). The remaining chromatin might be stored at -80°C for up to 2 months for further use in immunoprecipitation experiments. **Important: Do not freeze/thaw.**
16. Proceed to Sheared chromatin analysis.

C. Sheared chromatin analysis

This protocol refers to Diagenode's Elution Module (Cat. No. mc-magme-002) that can be ordered separately.

Reagents not supplied:

- RNase cocktail (e.g. Ambion, AM 2286 A)
- Phenol/chloroform/isoamyl alcohol (25:24:1)
- Chloroform/isoamyl alcohol (24:1)
- 100% Ethanol
- 70% Ethanol

17. Prepare RNase cocktail dilution (e.g. Ambion, AM 2286 A: dilute 1µl of cocktail in 150 µl of water).
18. Add 2 µl of diluted RNase cocktail to the chromatin sample.
19. Incubate 1h at 37°C.
20. Prepare the Complete Elution Buffer by mixing thoroughly Buffers D, E and F as follows:

Reagents	Volume
Buffer D	48 µl
Buffer E	5 µl
Buffer F	2 µl
Total volume	55 µl*

* One volume of Complete Elution Buffer is required per sample. Scale accordingly.

21. Add one volume of the Complete Elution Buffer to each chromatin sample.
22. Mix thoroughly before incubating sample at 65°C for 4h (or overnight).
23. Extract DNA once with an equal volume of phenol/chloroform/isoamyl alcohol. Incubate the sample at RT for 10 min on a rotating wheel.
24. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
25. Add 1 volume of chloroform/isoamyl alcohol [24:1]. Incubate the sample at RT for 10 min on a rotating wheel.
26. Centrifuge for 2 min at 13,000 rpm at RT. Transfer the top aqueous phase into a new 1.5 ml tube.
27. Precipitate the DNA by adding 5 µl of meDNA coprecipitant, 10 µl of meDNA-IP precipitant and 500 µl of cold 100% ethanol to the sample. Incubate at -80 °C for 30 min.
28. Centrifuge for 25 min at 13,000 rpm at 4°C. Carefully remove the supernatant and add 500 µl of ice-cold 70% ethanol to the pellet.
29. 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.
30. Re-suspended the pellet in 20 µl of TE buffer.
31. Run samples (20 µl of DNA + 4 µl of 6x loading dye) on a 1.5% agarose gel. As an alternative run 1 µl on a microfluidic chip (e.g. Bioanalyzer High Sensitivity DNA chip).

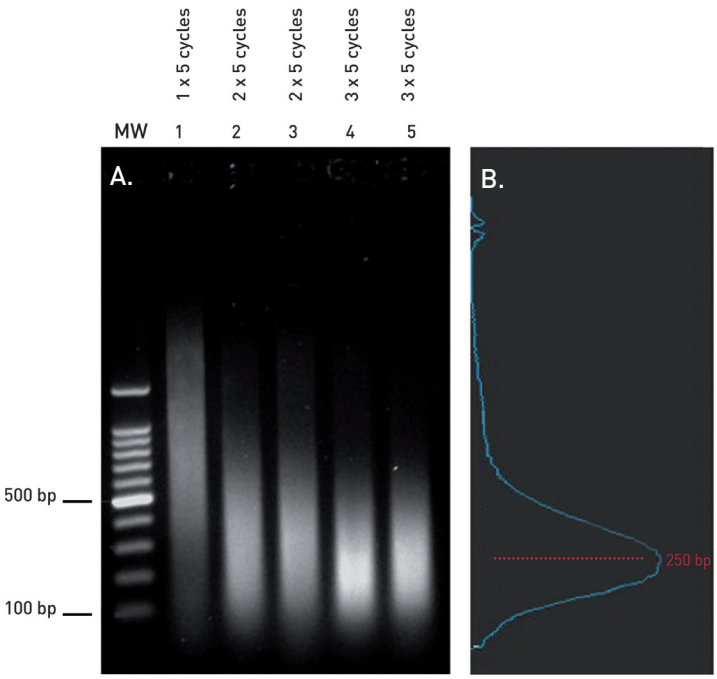


Figure 10: Efficient chromatin shearing with the Bioruptor® PLUS using the buffers and protocol from Diagenode's Chromatin Shearing Optimization kit - High SDS

HeLa cells are fixed with 1% formaldehyde (for 8 min at RT). 1 million cells are resuspended in 130 µl of buffer B from the Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) prior to chromatin shearing. Samples are sheared during 1, 2 or 3 rounds of 5 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) using 1.5 ml TPX microtubes (Cat No. M-50001).

Panel A: The sheared chromatin is then decross-linked prior to agarose gel electrophoresis. 10 µl of DNA are analyzed on a 1.5% agarose gel. The left lane shows the 100 bp DNA molecular weight marker (MW). Lane 1: 1x 5 cycles (each 30 sec ON/30 sec OFF); Lane 2 and 3 (duplicate): 2x 5 cycles (each of 30 sec ON/30 sec OFF); Lane 4 and 5 (duplicate): 3x 5 cycles (each 30 sec ON/30 sec OFF).

Panel B: Gel image was captured with Gel Logic 1500 Imaging System (Kodak). Lane 4 was scanned using Kodak Molecular Imaging Software (v 4.0.5) to estimate the DNA size distribution and concentration.

In the given example, the optimal shearing condition corresponds to 3 rounds of 5 cycles (30 sec ON/30 sec OFF) corresponding 100 - 400 bp.

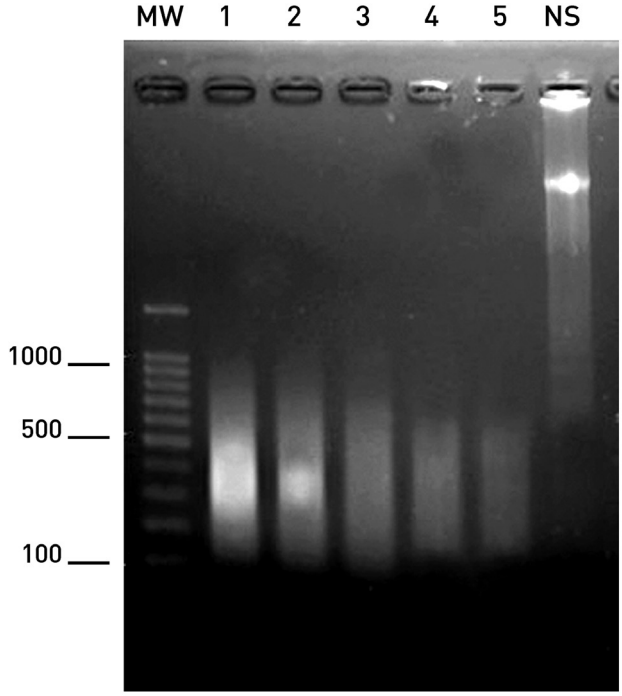


Figure 11: Reproducible Chromatin shearing using different cell numbers with the Bioruptor® PLUS using Diagenode's Chromatin Shearing Optimization kit - High SDS

MCF7 cells (human breast adenomacarcinoma cells) are fixed with 1% formaldehyde (for 8 min at RT). From 1 million to 100,000 cells are resuspended in 130 µl of Buffer B from the Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100) prior to chromatin shearing. Samples are sheared for 3 rounds of 4 sonication 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) using 1.5 ml TPX microtubes (Cat No. M-50001). The sheared chromatin is decross-linked prior to agarose gel electrophoresis. 10 µl of DNA are analyzed on a 1.5% agarose gel. The left lane shows the 100 bp DNA molecular weight marker (MW) and the right lane shows unsheared DNA (NS).

The amounts of sheared chromatin are 1 million cells equivalent (lane 1), 750,000 cells equivalent (lane 2), 500,000 cells equivalent (lane 3), 100,000 cells equivalent (lane 4 and 5). This figure shows that a reproducible DNA size range (100 - 600 bp) can be obtained with the Bioruptor® using Diagenode's Chromatin Shearing Optimization kit - High SDS (Cat. No. AA-003-0100).

Frequenytly Asked Questions about chromatin shearing optimization with Bioruptor®

Fixation

What is the recommended formaldehyde concentration?

1% formaldehyde in PBS (pay attention to always use **fresh** formaldehyde).

Always use methanol-free formaldehyde since methanol can generate artefacts.

The formaldehyde concentration used for fixation is critical. The usual concentration is 1%. Formaldehyde is the most common cross-linking agent used for ChIP. Since efficiency of cross-linking depends on the nature of the protein to cross-link, it is important to consider the nature of cross-linker and duration of cross-linking. Formaldehyde has a relatively short cross-linking spacer arm and cross-links nuclear components located within 2 Å of each other, thus it can be ineffective when analyzing proteins indirectly bound to DNA. To capture indirect associations between DNA and transcriptional cofactors (e.g. G9A factor), one could consider longer range cross-linkers, such as dimethyl apidimidate (DMA) to cross-link proteins not in direct contact with DNA, or contrary one might consider cisplatin that cross-links proteins to DNA but not proteins to proteins.

How long is the fixation step?

The recommended default fixation time is 8 min. However, performing a time course (e.g. from 5 - 10 min) is strongly recommended when studying proteins with unknown binding strength to chromatin.

It is possible to fix for as little as 5 min (e.g. when studying strong binding proteins) and as long as 10 min (e.g. when studying complex chromatin protein complexes). To capture weak or transient protein–DNA interactions, an even longer incubation time with formaldehyde may be required. However, increasing the cross-linking time can make fragmentation of the chromatin more difficult.

What is the temperature to use for fixation?

Fix at room temperature.

If you want to use another fixation temperature (e.g. 4°C or 37°C), always refer to the corresponding protocol or article. Make sure you perform the fixation step at the right temperature.

Are the washes after fixation important?

Wash the fixed cells properly. Make sure you get rid of **all** the formaldehyde. Use glycine to stop the fixation.

Cell lysis

How can I achieve complete cell disruption?

Do not use too many cells in the cell lysis buffer.

Careful homogenization of chromatin samples is strongly recommended before and during sonication processing. For optimal results, samples should be gently vortexed before and after performing 5 or 10 sonication cycles (depending on the protocol), followed by a short centrifugation at 4°C to recover sample volume at the bottom of the tube. When working with large cell numbers (e.g. careful pipetting can help to ensure better homogenization but it will not replace the vortexing step mentioned before).

Number of cells/shearing buffer volume

What amount of cells should be used per shearing trial?

- 1x10e6 – 10x10e6cells/ 300 µl

Do not use a cell concentration that is too high.

Shearing conditions

How long is the shearing?

Perform a time course for chromatin shearing.

It is possible to shear from 5 - 30 min. If 30 min, interrupt sonication after every 10 min and centrifuge tubes briefly before proceeding with the remaining time.

What is the optimal cycle?

30 sec ON/30 sec OFF

What is the best temperature for shearing?

4°C. Make sure the water bath is kept cool (e.g. by using Bioruptor® Water Cooler; Cat. No. BioAcc-Cool). Once optimal conditions are reached, use for all assays to assure reproducibility.

What is the best volume/tube for shearing?

- 2 ml per 15 ml tube
- 300 µl per 1.5 ml tube
- 100 µl per 0.5 ml tube

Do not use a sample volume that is too large.

Checking for high-quality shearing on agarose gel and microfluidic chip

What kind of gel should I use to determine size accuracy?

Check disrupted material on a 1.2 - 1.5% agarose gel. Run the gel slowly. As an alternative run 1 µl on a DNA microfluidic chip (e.g. Bioanalyzer High Sensitivity DNA chip).

Reverse cross-link from DNA before loading on gel.

What do smears indicate?

Gel electrophoresis of cross-linked samples often give a smear on the gel. Also, take several pictures of the gel to assure image quality. To obtain clearer image with accurate fragment size, reversion of the cross-linking (decross-linking) is strongly advised. (see chapter C. Chromatin shearing analysis)

How much DNA should I load and is RNase treatment necessary?

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. (see chapter C. Chromatin shearing analysis)

In some cases, you might need to dilute your sheared chromatin sample before running it to get an idea of the actual size of your starting material. Never overload your microfluidic chip (e.g. Bioanalyzer High Sensitity DNA chips) since it will bias your peak profile results (e.g. biased correlation between sample concentration and observed size of the fragments). Overloading should also be avoided when using agarose gel electrophoresis but its impact is less critical than for microfluidics.

Various contaminants such as chemicals (e.g. methanol) or proteins that would still remain after de-cross-linking and purification step can also generate aberrant migration during electrophoresis especially when using microfluidic devices. So, great care should therefore be taken when purifying sheared chromatin before size assessment.

What should my running buffer concentration be?

1x TAE or TBE is preferred to 0.5x TAE which can lead to smears on the gel.

What is the optimal size range for ChIP or ChIP-seq?

For ChIP PCR or ChIP qPCR assays it is essential to produce chromatin fragments of a size suitable for ChIP and subsequent analysis of the immunoprecipitated DNA.

Although chromatin fragments from 100 - 1000 bp are recommended for ChIP PCR or ChIP qPCR assays, the optimal size range of chromatin for ChIP-seq analysis should be between 100 and 600 bp. Larger chromatin fragments can negatively influence ChIP-seq data quality.

For more information about the optimal fragment size range for your specific ChIP-seq application, please **contact us**: techsupport@diagenode.com.

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