diagenode

A Hologic Company

Chromatin EasyShear Kit - Ultra Low SDS

Previous name: *Chromatin Shearing Optimization - Low SDS (iDeal Kit for Histones)*

Cat. No. C01020010

Compatible with:

iDeal ChIP-seq Kit for Histones

ChIPmentation Kit for Histones

USER GUIDE

Version 6 06_05_2020

Please read this manual carefully
before starting your experiment

Contents

Introduction

Chromatin shearing is a crucial step for the success of ChIP experiments. It is important to establish optimal conditions to shear cross-linked chromatin to get the correct fragment sizes needed for ChIP. A successful chromatin preparation relies on the optimization of **cross-linking**, **cell lysis** and **sonication** itself. All 3 steps are interconnected and need to be optimized individually for different experimental settings. Our **Chromatin EasyShear Kits (previous name: Chromatin Shearing Optimization Kits)** together with **the Bioruptor** combine **efficient cell lysis** and **chromatin shearing** leading to **consistent results**.

The Chromatin EasyShear Kits are recommended for:

- The optimization of the chromatin shearing of a new cell line/ new sample type prior to ChIP using Diagenode's ChIP kits as described at page 5
- The optimization of the chromatin shearing and/or chromatin preparation prior to ChIP for other protocols

Each Chromatin EasyShear Kit provides optimized reagents and a thoroughly validated protocol according to your specific experimental needs. SDS concentration is adapted to each workflow taking into account target-specific requirements.

Choose an appropriate kit for your specific experimental needs and get consistent results.

	Chromatin EasyShear Kit Ultra Low SDS	**Chromatin EasyShear Kit Low SDS**	**Chromatin EasyShear Kit for Plant**	**Chromatin EasyShear Kit High SDS**
Cat. No.	C01020010	C01020013	C01020014	C01020012
Sample type	Cells, tissue	Cells, tissue	Plant tissue	Cells - low amount
Target	Histones	Transcription Factors and histones	Histones	Histones
Nuclei isolation	Yes	Yes	Yes	No
SDS concentration	‹ 0.1%	0.2%	0.5%	1%
Corresponding to shearing buffers from	iDeal ChIP-seq Kit for Histones ChIPmentation Kit for Histones	iDeal ChIP-seq Kit for Transcription Factors iDeal ChIP qPCR Kit iDeal ChIP-FFPE Kit	Universal Plant ChIP-seq Kit	True MicroChIP Kit

Kit method overview

The Chromatin EasyShear Kit - Ultra Low SDS, optimized for histone proteins, is validated for the chromatin preparation from cells and tissues. Please refer to a corresponding section in the protocol.

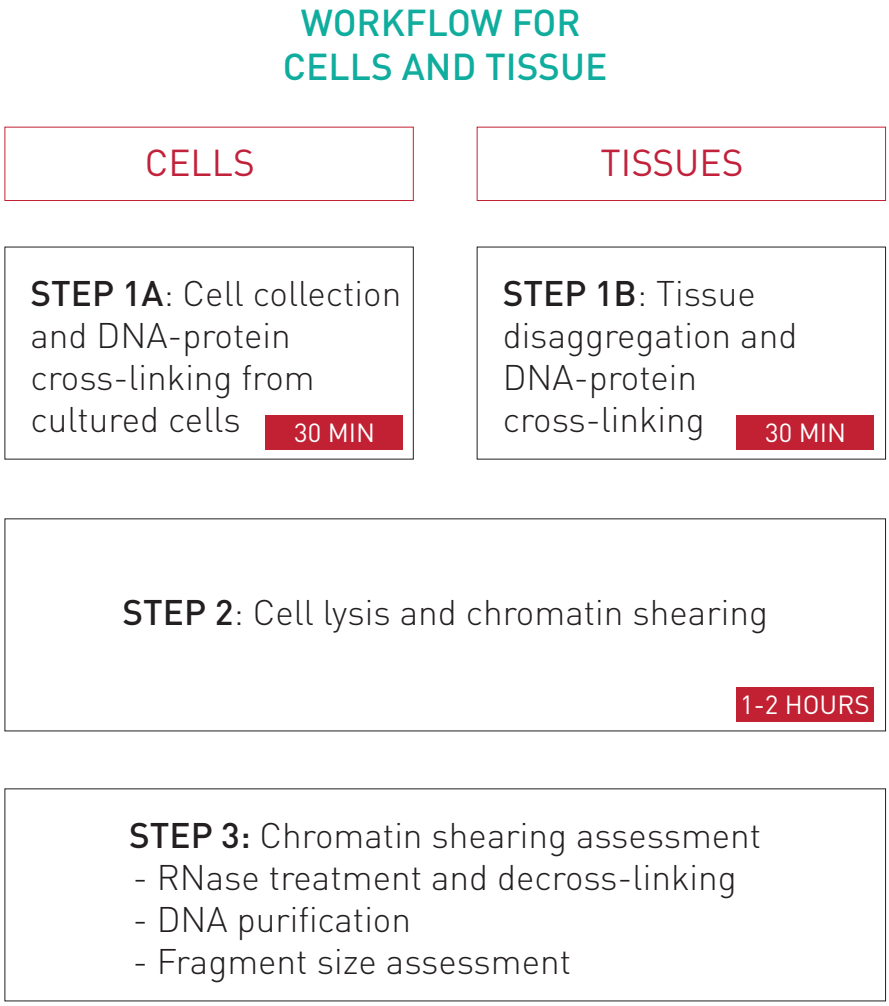


Figure 1. Kit method overview

The kit is also recommended for the optimization of chromatin preparation prior to ChIP performed with the following kits:

ChIP kit	Manual version	Automated version
iDeal ChIP-seq Kit for Histones	C01010050 C01010051 C01010059	C01010057 C01010171
ChIPmentation Kit for Histones	C01011010	C01011000

Kit materials

The kit Chromatin EasyShear Kit – Ultra Low SDS contains enough reagents to perform the number of chromatin preparations and samples as described in the Table1.

This kit **does not contain** reagents for DNA purification required for the chromatin shearing assessment. For DNA purification we highly recommend the IPure kit v2 (Diagenode, Cat. No. C03010014) or MicroChIP DiaPure columns (Diagenode, Cat. No. C03040001), which can be purchased separately.

Table 1. Number of reactions included in the Chromatin EasyShear Kit - Ultra Low SDS

Samples	Number of chromatin preparations	Material amount per one chromatin preparation	Number of samples/ sonication conditions
Cells	10	7x10e6 cells	100
Tissues	10	Up to 40 mg of tissue	100

The kit content is described in Table 2. Upon receipt, store the components at the indicated temperatures.

Table 2. Components supplied with the Chromatin EasyShear Kit - Ultra Low SDS

Description	Quantity	Storage
Glycine	580 µl	4°C
Lysis Buffer iL1	110 ml	4°C
Lysis Buffer iL2	110 ml	4°C
Shearing Buffer iS1	11 ml	4°C
Elution Buffer iE1	5 ml	4°C
Elution Buffer iE2	400 µl	4°C
TE Buffer	2 ml	4°C
DNA precipitant	1 ml	4°C
DNA co-precipitant	500 µl	-20°C
Protease inhibitor cocktail*	50 µl	-20°C

* Additional Protease inhibitor cocktail will be required for tissue protocol

The composition of the buffers is proprietary.

Required materials not provided

Materials and Reagents

- Gloves to wear at all steps
- RNase/DNase-free 1.5 ml tubes
- Formaldehyde, 37%, Molecular Grade
- Phosphate buffered saline (PBS) buffer (ice-cold, room temperature and pre-warmed at 37 °C)
- trypsin-EDTA (for adherent cells)
- culture medium (for adherent cells)

Equipment

- Bioruptor® sonication device and the associated microtubes:
  - Bioruptor Pico (Diagenode, Cat. No. B01060010) and 1.5 ml Bioruptor Microtubes with Caps (Cat. No. C30010016) or
  - Bioruptor Plus (Diagenode, Cat. No. B01020001) and 1.5 ml TPX Microtubes (Cat. No. C30010010)
- Refrigerated centrifuge for 1.5 ml tubes
- Thermomixer
- Vortex
- DiaMag Rotator (Rotating wheel) (Diagenode, Cat. No. B05000001)
- DNA sizing equipment (agarose gel electrophoresis or an automated capillary electrophoresis instrument, eg Fragment Analyzer (Agilent) and High Sensitivity NGS Fragment Analysis Kit (Agilent, DNF-473)

Reagents for DNA purification

- IPure Kit (Diagenode, Cat. No. C03010015) or
- MicroChIP DiaPure columns (Diagenode, Cat. No. C03040001) or
- Phenol-chloroform extraction (Phenol/chloroform/isoamyl alcohol (25:24:1), Chloroform/isoamyl alcohol (24:1), 100% Ethanol, 70% Ethanol
- RNase coctail (e.g. Ambion, AM2286A)

Additional supplies for tissue protocol

- Additional Protease inhibitor cocktail (Diagenode, Cat. No. C12010011 or C12010012) (50 µl per 10 chromatin preparation)
- Equipment for tissue disruption and homogenization: Dounce homogenizer with loose and tight pestles (2 ml) and TissueLyser (Qiagen) with 2 ml tubes and stainless steel beads, 5 mm (Qiagen, Cat. No.69989)
- Scalpel blades
- Petri dishes
- Qubit® Fluorometer (ThermoFisher Scientific)

Remarks before starting

A high quality chromatin sample combines two main features: a **suitable fragment size range** and the **availability of cross-linked epitopes** for ChIP. A perfect sonication profile is a profile in which the highest specific signal and the lowest background are generated.

There is no one universal size range generally applicable for ChIP. As a rule, fragments between **100-600 bp** are suitable for the majority of ChIP experiments and can be used as a starting point. This range can be fine-tuned for particular experimental requirements depending on the specificity of the target (histones or non-histone proteins) and the required downstream analysis (ChIP-qPCR or ChIP-seq). Generally, a tighter fragment distribution is recommended for ChIP-seq than for ChIP-qPCR. Note that **100-300 bp** is compatible (but not absolutely necessary) with **histone ChIP-seq** while a **broader fragment range** is more suitable for **non-histone ChIP-seq** (transcriptional factors and proteins that are not bound directly to DNA and for long-distance interacting proteins). With the recent evolution of sequencing technologies, it is common to perform ChIP-seq experiments from chromatin with a distribution in the 100–800 bp range.

Before starting the ChIP, the chromatin should be sheared to a desired size. Sonication should be optimized for each ChIP project since samples are different in their resistance to sonication. The following parameters should be considered during the chromatin shearing optimization:

1. Starting amount of material (cells, tissues or FFPE slices)
2. Fixation
3. Shearing optimization (followed by shearing assessment)

1. Starting amount of material

Cell number

The protocol describes the preparation of a batch of chromatin from approximately **7 million cells** allowing testing up to **6 sonication** conditions or prepare the chromatin for **6 immunoprecipitation** (IP) reactions, 1 input sample and 1 sample for chromatin shearing assessment. Approximately 1 million cells per sample are used in this standard protocol accordingly to the ChIP requirements for histones. Keep in mind that the Diagenode ChIP protocol described in the iDeal ChIP-seq Kit for Histones is optimized for use of 100 µl of sheared chromatin in a total volume of ChIP reaction equal 200 µl (auto kit) or 300 µl (manual kit). It is crucial to keep these volumes constant for optimal results.

Depending on the ChIP experiment requirements (the abundance of the target, the specificity of the antibody, and the amount of cells available), it may be possible to scale down to 100.000 cells as described below. Scaling up is usually not necessary for ChIP on histones but if a higher yield of IP'd DNA is required, we recommend performing separate ChIPs and pool the IP'd DNA before purification.

For using lower numbers of cells per IP:

First, determine the number of cells that you will use further per IP and the total number of IPs (keep this cell amount constant per shearing optimization trial). Fix cells as described in the standard protocol. For cell collection and lysis, scale down the volume of iL1 and iL2 buffers by 1 ml of iL1 and 1 ml of iL2 per 1 million cells. Define the volume of Shearing Buffer iS1 taking into account the following requirements:

- 100 µl of sheared chromatin (containing the desired number of cells) per IP reaction
- 1 µl of sheared chromatin per input
- 50 µl of sheared chromatin for chromatin shearing assessment
- 5% excess of iS1

> ***Note:*** *We recommend performing the calculation of the volume of Shearing Buffer iS1 accordingly to ChIP protocol to mimic exactly future ChIP, even though no ChIP experiment is planned with the chromatin samples from this optimization trial.*

Please note that cell density is a key factor for efficient shearing. Sample viscosity may hamper the cavitation process leading to less efficient shearing. Dense cell suspension will require more extensive sonication. Adapt sonication time accordingly.

Tissue amount

The protocol describes the preparation of a batch of chromatin from approximately **30-40 mg** of tissue allowing testing up to **6 sonication** conditions or **6 immunoprecipitation** (IP) reactions, 1 input sample and 1 sample for chromatin shearing assessment. Approximately 5 mg of tissue per IP reaction are used in this standard protocol. The sample volume per sonication is 100 µl. Please note that shearing efficiency depends on the sample volume and it should be kept consistent to ensure the reproducible results.

Diagenode ChIP protocol described in the iDeal ChIP-seq Kit for Histones is optimized for use of 100 µl of sheared chromatin in a total volume of ChIP reaction equal 200 µl (auto kit) or 300 µl (manual kit). It is crucial to keep these volumes constant for optimal results.

Tissue samples have to be homogenized mechanically before sonication. Soft tissues (e.g. liver or brain) can be successfully homogenized using a Dounce homogenizer while it is preferable to use the TissueLyser (Qiagen, or a similar system) for hard fibrous tissues (e.g. muscles). Please note that the TissueLyser workflow is only compatible with frozen tissues since the grinding of fresh tissue will not be efficient with this protocol.

Depending on the abundance of the target, the specificity of the antibody, and the amount of tissue available, it may be possible to scale down to 1.5 mg of tissue per IP as described below. Scaling up is usually not necessary but if a higher yield of immunoprecipitated DNA is required, we recommend performing separate ChIPs and pool the immunoprecipitated DNA before purification.

Determine the amount of tissue that you desire to use per ChIP experiment. Keep in mind that 100 µl of sheared chromatin (containing the desired number of tissue) is used per IP reaction. Keep this amount constant per shearing optimization trial.

For using lower amount of tissue per IP:

First, determine the amount that you will use per IP and the total number of IPs. Follow the standard protocol for tissue fixation, collection and lysis. Do not scale lysis buffers iL1b and iL2. Define the volume of Shearing Buffer iS1 taking into account the following requirements:

- 100 µl of sheared chromatin (containing the desired amount of tissue) per IP reaction
- 1 µl of sheared chromatin per input
- 50 µl of sheared chromatin for chromatin shearing assessment
- Add 5% excess of iS1

> ***Note:*** *We recommend performing the calculation of the volume of Shearing Buffer iS1 accordingly to ChIP protocol to mimic exactly future ChIP, even though no ChIP experiment is planned with the chromatin samples from this optimization trial.*

Resuspend the tissue in the required volume of Shearing Buffer iS1 and follow the standard protocol.

Please note that changing cell concentration in the Shearing Buffer may impact the shearing efficiency and an additional optimization of the shearing conditions may be required.

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. We recommend quantifying DNA for a rough estimation of chromatin yield obtained from tissue. The recommended amount of chromatin to be used per IP with iDeal ChIP-seq Kit for Histones protocol should be within 0.3 - 3 µg range. Perform a pilot experiment to determine the optimal amount of tissue resulting in a required amount. Once determined, it should be kept consistent between experiments.

Please note that sample density is a key factor for efficient shearing. Sample viscosity may hamper the cavitation process leading to less efficient shearing. Dense cell suspension will require more extensive sonication Adapt sonication time accordingly.

2. Fixation optimization

The described protocol uses a mild fixation sufficient for **histone proteins**. Cells should be re-suspended in PBS prior to the fixation to avoid cell clusters formation upon fixation to ensure a proper shearing efficiency using ultra low SDS concentration in the shearing buffer.

Formaldehyde is the most commonly used cross-linking reagent ideal for two molecules which interact directly. The fixation time can depend on your target of interest and might require an additional optimization (usually between 8 and 10 minutes for histones). Please note that a longer fixation may lead to chromatin resistant to sonication. Adapt sonication time accordingly.

If different fixation time will be tested, we recommend starting with a corresponding number of chromatin preparations and testing different sonication settings per each preparation.

3. Shearing optimization

The length of sonication time depends on many factors (cell type, cell density, sample volume, fixation time). Hence it is important to optimize the sonication conditions for each new ChIP project.

The protocol is used in a combination with the Bioruptor (Pico, Plus or Standard). Perform an initial sonication time course experiment to evaluate the extent of chromatin fragmentation.

Choose the protocol which is adapted to your device:

• When using the Bioruptor Pico, an initial time-course experiment of 5-10-12 sonication cycles 30'' ON/30'' OFF is recommended.

• When using the Bioruptor Plus, an initial time-course experiment of 10-20-30 sonication cycles [30 seconds "ON", 30 seconds "OFF"] at High power is recommended.

During the sonication, the mean size of DNA fragments will decline progressively approaching a lower limit of 100-150 bp (mean size of the smear). It is recommended to choose a sonication time before reaching this lower limit. **As best practice, choose the shortest sonication time**

resulting in a satisfactory shearing and ChIP efficiency (highest recovery/ lowest background). Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments, especially when non-histones proteins are to be evaluated by ChIP (Figure 2).

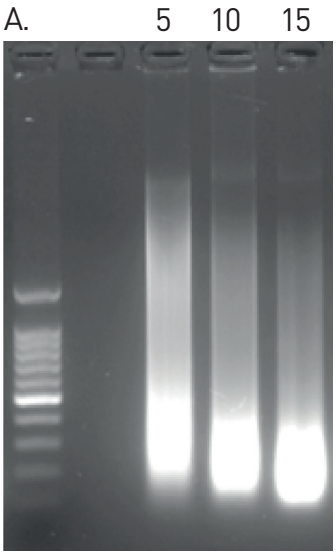
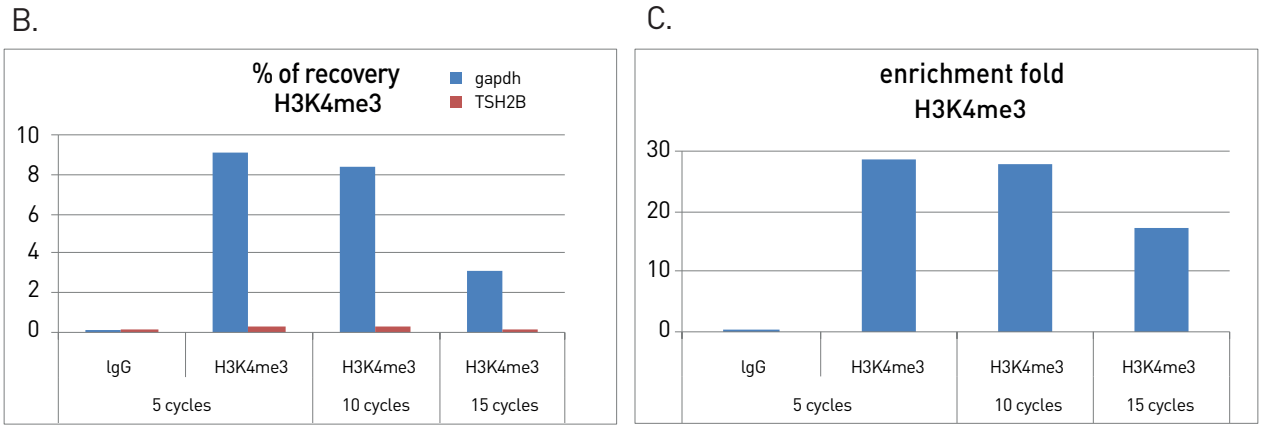


Figure 2. Optimal chromatin shearing profile.

HeLa cells were fixed with formaldehyde for 8 min and chromatin was prepared according to Diagenode's Chromatin EasyShearing Optimization Kit - Ultra Low SDS (Cat. No. C01020010). Samples were sonicated for 5-10-15 cycles of 30" ON/30" OFF as indicated with Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016) followed by de-crosslinking and DNA purification. The fragment size was assessed using agarose gel electrophoresis. A 100 bp ladder was loaded as the size standard (panel A).



Sheared chromatin has been used for immunoprecipitation with H3K4me3 and IgG (negative control) antibodies. Quantitative PCR was performed with positive (GAPDH) and negative (TSH2B) control regions. The Figure 2 shows the recovery expressed as % of input (the relative amount of immunoprecipitated DNA compared to input DNA) (panel B) and as enrichment fold of positive locus over negative (panel C).

Interpretation of the results from Figure 2.

Fragments suitable for ChIP experiments with histones marks are generated after 5 cycles (panel A). The fragment size distribution from 100-600 bp is compatible with requirements for all ChIP-seq experiments together with the best enrichment obtained for histones (panel B and C).

Chromatin sheared for 10 cycles (100- 300 bp, panel A) is compatible (but not absolutely necessary) with histone ChIP-seq since the fragment size and ChIP enrichment are suitable (panel B and C).

Chromatin after 15 cycles with fragment size distribution of 100 - 200 bp might be over-sheared: while fragment size is suitable for ChIP-seq experiment (100-200 bp, panel A), a significant drop of ChIP efficiency is observed for histone marks (panel B and C).

In some situations, it is preferable to re-shear the purified de-crosslinked DNA after immunoprecipitation rather than over-sonicate. Re-shearing enables the enrichment of fragments in the desired optimal size range suitable for next-generation sequencing. Please refer to the following protocol

www.diagenode.com/files/protocols/ChIP-re_shearing-protocol.pdf

Ensure that only the recommended tubes are used for sonication. It is important to note that sonication tubes recommended for the Bioruptor Pico are different from the tubes recommended for the Bioruptor Plus and Bioruptor Standard. Using the wrong tubes will lead to inefficient shearing. Please refer to the following guide.

www.diagenode.com/files/organigram/bioruptor-organigram-tubes.pdf

Be aware that sonication efficiency may differ depending on a type of tubes used. Switching to another type of tubes (e.g. from 1.5 ml to 15 ml tubes) will require an additional optimization.

Ensure that the sample volume per sonication is in the recommended range. Any deviations from this recommended range will lead to inefficient shearing and lack of reproducibility.

DNA purification and chromatin shearing assessment

For accurate size determination of the chromatin fragments, reverse crosslinking, including RNase treatment followed by DNA purification, is advised. Size estimation of chromatin fragments without reverse crosslinking is not precise. The presence of the crosslinks retards electrophoretic migration resulting in a misinterpretation of fragment size.

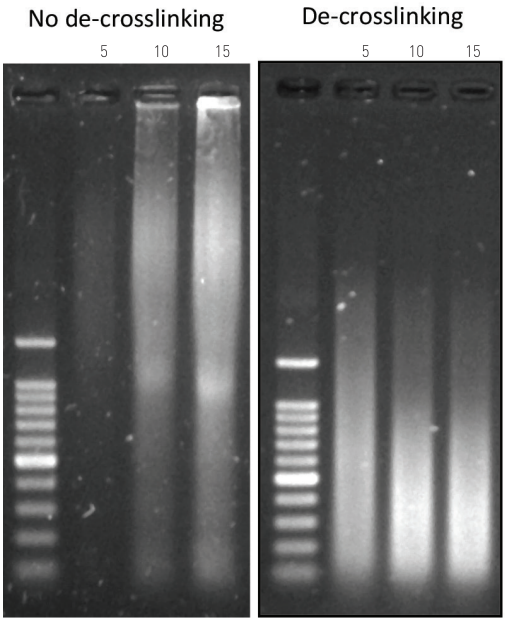


Figure 3. Reversing crosslinks is necessary for accurate size estimation.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's Chromatin Shearing Optimization Kit – Medium SDS (Cat. No. C01020011). Samples were sonicated for 5, 10 and 15 cycles of 30" ON/30" OFF as indicated with the Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (C30010016). A 100 bp ladder was loaded as size standard. Left panel: non de-crosslinked chromatin. Right panel: de-crosslinked chromatin. RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing.

RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing. The presence of degraded RNA in the sample might lead to mis-interpretation of the shearing. Smear below 100 bp is due to degraded RNA but not over-sheared DNA.

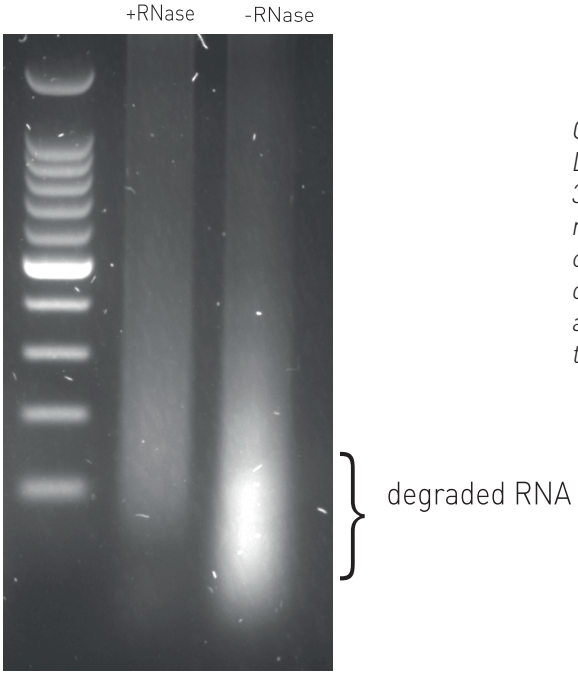


Figure 4. RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing.

Chromatin from HeLa cells was prepared according to Diagenode's protocol. Samples were sonicated for 5 cycles of 30" ON/30" OFF with Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016) followed by de-crosslinking and DNA purification in the presence or absence of RNase as indicated. The fragment size was assessed using agarose gel electrophoresis. A 100 bp ladder was loaded as the size standard

This protocol is compatible with different DNA purification methods:

- Magnetic beads purification (e.g. IPure beads from Diagenode) ,
- Columns-based DNA clean-up (e.g. MicroChIP DiaPure columns from Diagenode)
- Conventional phenol-chloroform extraction.

Please note that reagents for DNA purification are not included in the kit and should be provided by user.

DNA derived from FFPE samples should be purified using MicroChIP DiaPure columns. Eluted DNA is enough concentrated to be analysed using the Fragment Analyzer (Agilent) and High Sensitivity NGS Fragment Analysis Kit (Agilent, DNF-473). The agarose gel is not sensitive enough to visualize the low amount of DNA obtained from FFPE samples.

For the size assessment of sheared chromatin we recommend using an agarose gel analysis or the Fragment Analyzer (Agilent).

If using an **agarose gel**, the sheared chromatin should be analyzed on a 1.2 - 1.8% agarose gel. The optimal DNA amount from sheared chromatin is around 300 ng per lane. A serial dilution from 100 ng to 500 ng could be run. Do not overload the gel as the migration of large quantities of chromatin on an agarose gel can lead to poor quality pictures which do not reflect the real DNA fragmentation. The minimum amount of sheared chromatin that can be visualized in an agarose gel corresponds to 60,000 cells equivalent. Both the pre- and post-staining of the agarose gel with ethidium bromide or SybrSafe dye can be used for visualization of sheared fragments. Some slight differences might be observed between post- and pre-stained gels. Post-staining eliminates any possibility that the dye interferes with the migration and ensures an even background noise. However, pictures are usually less clear and bright with some background noise. If pre-stained agarose gels are used, it is advised that the electrophoresis buffer contains the stain in the same concentration as in the gel. If the stain is present in the gel but not in the buffer, the gel will result in uneven staining because the free dye migrates towards the top of the gel leaving the bottom part with no stain. Therefore, the background noise becomes non-uniform.

Using **Fragment Analyzer** (Agilent), please follow the manufacturer's instruction.

If using the Agilent BioAnalyzer, please keep in mind that traces are log-based, so a large distribution of higher molecular weight fragments are compacted into a much smaller area of the trace as compared to the smaller size fragments leading to a visual misinterpretation of fragment distribution.

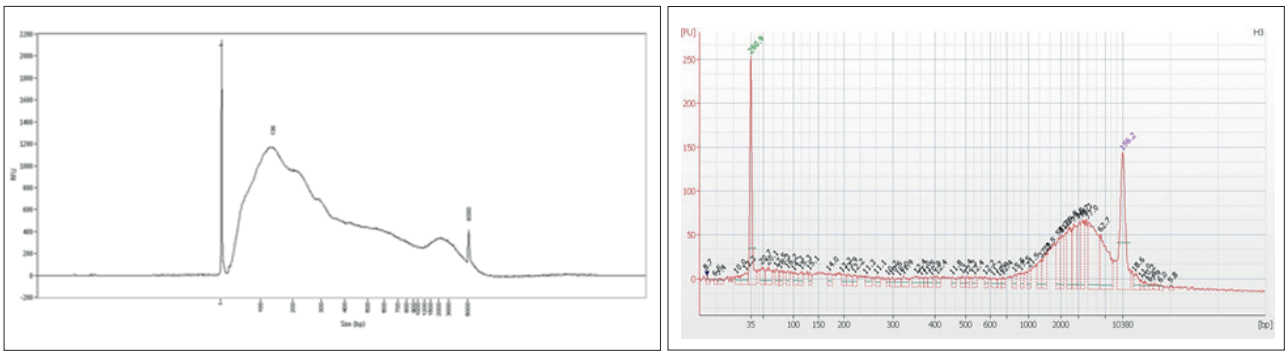


Figure 5. An appropriate method should be used for the shearing assessment

HeLa cells were fixed with formaldehyde for 8 min and chromatin was prepared according to Diagenode's Chromatin EasyShear Kit - Ultra SDS (Cat. No. C01020010). Samples were sonicated for 10 cycles of 30" ON/30" OFF with Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016) followed by de-crosslinking and DNA purification. The fragment size was assessed using Fragment Analyzer (left) and BioAnalyzer (right). The BioAnalyzer trace is biased towards the high molecular weight fragments.

If high molecular weight fragments are present, it is recommended estimating a molar ratio between fragments in a desired range and higher molecular weight fraction. The molarity allows estimating a number of molecules in a particular range. The presence of high molecular weight fragments up to 15% - 20% (molar ratio) is acceptable (Figure 6).

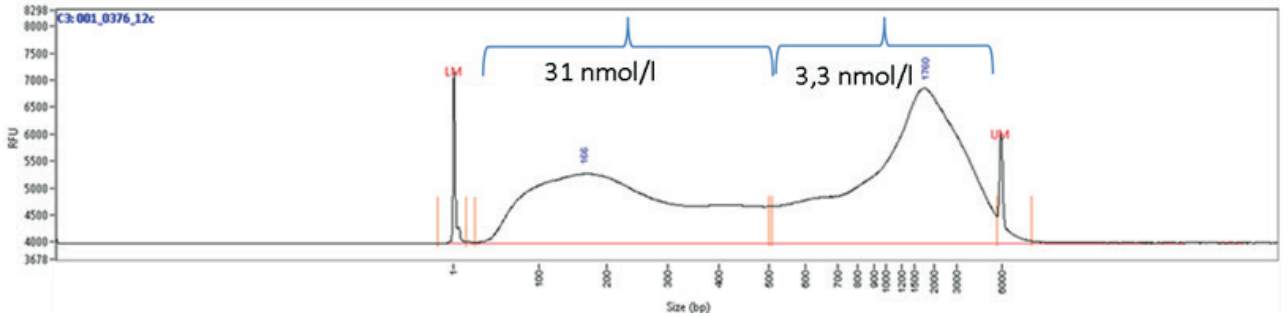


Figure 6. A molar ratio between desired fragments range and high molecular weight fraction should be estimated.

HeLa cells were fixed with formaldehyde for 10 min and chromatin was prepared according to Diagenode's Chromatin EasyShear Kit - Low SDS (Cat. No. C01020013). Samples were sonicated for 12 cycles of 30" ON/30" OFF with Bioruptor Pico using 1.5 ml Bioruptor microtubes with caps (Cat. No. C30010016) followed by de- crosslinking and DNA purification. The fragment size was assessed using Fragment Analyzer. The molar content of fragments in the range 100-500 bp and 500-5.000 bp was estimated showing that large fragment do not exceed 15%.

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PROTOCOL FOR CHROMATIN PREPARATION

STEP 1A

Cell collection and DNA-protein cross-linking

Day 1 | 30 minutes

FOR CULTURED CELLS

NOTE: *PBS at different temperatures (ice-cold, room temperature and pre-warmed at 37 °C) will be required at this step.*

For adherent cells:

1.1. Pre-warm PBS, culture medium and trypsin-EDTA at 37°C.

1.2. Remove the medium and rinse the cells with **pre-warmed PBS** (10ml for a 75 cm^2 culture flask). Gently shake the flask for 2 minutes.

1.3. Remove the PBS and add sterile **trypsin-EDTA** to the culture flask to detach adherent cells from the bottom. Add enough trypsin-EDTA to completely cover the cells (e.g. 1 ml for a 75 cm^2 culture flask). Gently shake the culture flask for 1-2 minutes or until the cells start to detach.

NOTE*: The time needed may depend on the cell type. Do not continue trypsin treatment longer than necessary as prolonged treatment may damage the cells. Regularly check if the cells start to detach.*

1.4. Immediately add fresh culture medium to the cells when they are detached. For correct inactivation, the volume of medium must be 2x the volume of trypsin-EDTA (e.g. 2 ml for a 75 cm^2 culture flask). Transfer cell suspension to a 15 ml tube.

1.5. Rinse the flask by adding **10ml** of **warm PBS**. Add this volume to your 15 ml tubes containing cells from above point. Proceed immediately with step 1.6.

For suspension cells:

Collect suspension cells in a 50 ml tube and go directly to point 1.6 of the protocol.

1.6. Centrifuge for 5 minutes at 500 x g at room temperature and remove the supernatant.

1.7. Resuspend the cells in **20 ml** of **warm PBS** and count them. Collect the cells (approximately 7 millions) by centrifugation for 5 minutes at 500 x g at room temperature.

1.8. Resuspend the cells (approximately 7 millions) in **500 µl** of **PBS** (room temperature) and add **13.5 µl** of **37% formaldehyde** (under a fume hood) to each tube. Mix by gentle vortexing and incubate 8 minutes at room temperature to allow fixation to take place.

NOTE: *The fixation time might require an additional optimization. Please refer to the "Remarks before starting"*

1.9. Add **57 µl** of **Glycine** to the cells to stop the fixation. Incubate for 5 minutes at room temperature with gentle shaking. Keep everything at 4°C or on ice from now on.

1.10. Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet.

1.11. Wash the cells twice with **1 ml** of **cold PBS**: resuspend the cell pellet in 1 ml of cold PBS. Collect the cells by centrifugation at 500 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

NOTE: *We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 1B

Tissue disaggregation and DNA-protein cross-linking

Day 1 *30 minutes*

FOR TISSUES

NOTE: *PBS at different temperatures (ice-cold and room temperature) will be required at this step*

1.1. Weigh **30-40 mg** of fresh or frozen **tissue** in a petri dish. Keep samples on ice at all times and minimize the time of manipulation to prevent sample degradation.

1.2. Chop tissue into small pieces (between 1-3 mm^3) using a scalpel blade. Disaggregate sample using a Dounce homogenizer or TissueLyzer.

For Dounce homogenizer:

Add 5 µl of protease inhibitors cocktail to 1 ml of ice-cold PBS and resuspend the tissue pieces. Transfer it to the Douce homogenizer and disaggregate using a loose pestle to get a homogeneous suspension.

Transfer the tissue suspension into a 1.5 ml tube and centrifuge at 850 x g for 5 minutes at 4°C. Gently discard the supernatant and keep the pellet. Proceed with the fixation (step 1.3)

For TissueLyzer:

Transfer tissue pieces to 2 ml tubes, supplemented with 2 stainless

steel beads (5 mm) and immerse in liquid nitrogen for a few minutes. Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 8 hours at -80°c) and operate the TissueLyser for 2-4 minutes at 25-30 Hz. Proceed with the fixation on the grinded sample (step 1.3).

1.3. Add **27 µl of 37% formaldehyde** (under a fume hood) to **1 ml of PBS** (room temperature) , add 1 ml of the cross-linking solution directly to the tissue lysate and resuspend the tissue suspension in diluted formaldehyde.

1.4. Mix by vortexing and incubate for 8 minutes at room temperature with gentle rotation on a DiaMag Rotator to allow fixation to take place.

***NOTE**: The fixation time might require an additional optimization. Please refer to the "Remarks before starting".*

1.5. Add **100 µl of Glycine** to the tissue suspension to stop the fixation. Incubate for 5 minutes at room temperature with gentle mixing on a DiaMag rotator.

1.6. Centrifuge samples at 850 x g for 5 minutes at 4°C. Discard the supernatant without disturbing the pellet. Keep everything at 4°C or on ice from now on.

1.7. Wash the pellet twice with **1 ml of cold PBS**: resuspend the cell pellet in **1 ml** of **cold PBS**. Collect the cells by centrifugation at 850 x g for 5 minutes and 4°C. Discard the supernatant without disturbing the cell pellet. Repeat one time.

***NOTE**: We strongly recommend using freshly fixed cells for preparation of sheared chromatin prior to ChIP. If not possible, the pellets of fixed cells can be stored at -80°C for up to 4 months.*

STEP 2

Cell lysis and chromatin shearing

1 *Day 1* *1-2 hours*

FOR CELLS AND TISSUES

2.1. Add **1 ml** of **ice-cold Lysis Buffer iL1** to the 1.5 ml tube containing cells or tissue suspension. Resuspend the samples by pipetting up and down several times and transfer them to a 15 ml tube. Add **6 ml** of **Lysis Buffer iL1** and incubate for 10 minutes at 4°C with gentle mixing.

2.2. Pellet samples by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.3. Resuspend the cell pellet in **1 ml** of **ice-cold Lysis Buffer iL2** by pipetting up and down several times. Add another **6 ml** of **Lysis Buffer iL2** and incubate for 10 minutes at 4°C with gentle mixing.

2.4. Pellet the cells again by centrifugation at 500 x g (cells) or 850 x g (tissue suspension) at 4°C for 5 minutes and discard the supernatant.

2.5. Prepare **complete Shearing Buffer** by adding **3.5 µl** of **200x protease inhibitor cocktail** to **700 µl** of **Shearing Buffer iS1**. Keep on ice.

2.6. Add **700 µl of complete Shearing Buffer iS1** to the cells (7 million cells) or tissue sample (30-40 mg of tissue). The final cell concentration in the Shearing Buffer should be 1 million of cells or ~5 mg tissue per 100 µl of iS1. Resuspend by pipetting up and down several times and incubate for 10 minutes in ice.

NOTE - *optional, for tissue samples:*

If the suspension is not homogeneous after pipetting, an additional homogenization using a Dounce homogeniser or TissueLyser could be performed.

*For **Dounce homogeniser**, transfer the suspension to the homogeniser, perform several strokes to get a homogeneous suspension and proceed with the sonication as described below.*

*For **TissueLyser**, transfer the suspension to 2 ml tubes, supplemented with 2 stainless steel beads (5 mm). Insert 2 ml tubes into pre-cooled TissueLyser Adaptors (at least 2 hours at 4°c) and operate the TissueLyser for 2-4 minutes at 25 Hz. Proceed with the sonication as described below.*

2.7. Shear the chromatin by sonication using the Bioruptor. Choose the protocol which is adapted to your device:

- When using the Bioruptor Pico shear for 5 to 15 cycles [30 seconds "ON", 30 seconds "OFF"].
- When using the Bioruptor Plus, shear for 10 to 30 cycles [30 seconds "ON", 30 seconds "OFF"] each at High power setting.

2.8. Briefly spin down the liquid in the samples for 15 seconds. Transfer samples to new 1.5 ml tubes and centrifuge at 16,000 x g at 4°C for 10 minutes. Pool the supernatants which contain the sheared chromatin. Use the chromatin for immunoprecipitation (Step 3 Magnetic immunoprecipitation) or store it at -80°C for up to 2 months.

2.9. Take an aliquot of **50 µl** of **sheared chromatin** for the shearing assessment. The protocol is described in the "Additional Protocols" section.

STEP 3

Chromatin shearing assessement

FOR CELLS AND TISSUES

Workflow for analysis of sheared chromatin:

- RNAse treatment (1h, optional but highly recommended for an accurate size assessment)
- Reverse **cross-linking** (4h or overnight)
- DNA purification
- Fragment size assessment (agarose gel or Fragment Analyzer) (1h)

RNAse treatment

***NOTE**: RNase cocktail (e.g. Ambion, AM2286A) is not supplied with this kit.*

1. Take **50 µl of sheared chromatin** (step 2.10 for cells or step 2.15 for tissues in the protocol) and transfer to a 1.5 ml microtube.
2. Dilute **1 µl of RNase cocktail** (e.g. Ambion, AM2286A) in **150 µl of ChIP-seq grade water**.
3. Add **2 µl of diluted RNase cocktail** to the aliquot of sheared chromatin.
4. Incubate for 1 hour at 37°C.

Reverse cross-linking

5. Add **50 µl of Elution Buffer iE1** and **4 µl** of **Elution Buffer iE2** to the sample, mix thoroughly.
6. Incubate samples at 65°C for 4 hours (or overnight) with shaking.

7. Purify DNA using a method of choice (reagents not included in the kit):
   - Option A: IPure kit (Diagenode, Cat. No. C03010014)
   - Option B: MicroChIP DiaPure columns (Diagenode, Cat. No. C03040001)
   - Option C: phenol-chloroform extraction

Option A - DNA purification using the IPure Kit v2

***NOTE**: Before the first use of the kit, prepare Wash Buffer 1 and Wash Buffer 2 by adding an equal volume of isopropanol. Wash Buffers 1 and 2 should be stored at 4°C. Never leave the bottle open during storage to avoid evaporation. This option is not recommended for FFPE samples.*

8. Add **2 µl of carrier** to the sample.

9. Add **108 µl of 100% isopropanol** to the samples.

   ***NOTE:** Following the addition of isopropanol the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.*

10. Resuspend the **IPure beads v2** by vortexing and add **20 µl** to the sample.

11. Incubate samples for 10 minutes at room temperature on the DiaMag Rotator.

12. Briefly spin the tubes, place in the DiaMag1.5 magnetic rack, wait 1 minute and discard the buffer.

13. Add **100 µl of Wash Buffer 1** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes, place in the DiaMag1.5 magnetic rack, wait 1 minute and discard the buffer.

14. Add **100 µl of Wash Buffer 2** per tube. Close the tubes and vortex well until the beads pellet is completely resuspended. Incubate for 30 seconds at room temperature. Briefly spin the tubes and place them into the DiaMag 1.5 magnetic rack, wait 1 minute and discard the buffer.

15. Spin the tubes again and place them on the DiaMag1.5. Discard the remaining Wash Buffer 2 if necessary. Resuspend the beads pellet in

25 µl of Buffer C. Incubate at room temperature for 15 minutes on the DiaMag Rotator.

16. Spin the tubes and place them into the DiaMag 1.5, wait 1 minute and transfer the supernatants into a new 1.5 ml tube. Discard the beads and keep the supernatant containing purified DNA.

17. Analyze the purified DNA on a 1.5% agarose gel. Load around 300 ng of DNA for an optimal separation and visualization. Alternatively, you can use a Fragment Analyzer (Standard Sensitivity NGS Fragment Analysis Kit (DNF-473)). Chose the shortest sonication time resulting in a satisfactory shearing profile. Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments, especially for non-histone proteins.

Option B - DNA purification using MicroChIP DiaPure columns

8. Proceed to DNA purification using MicroChIP DiaPure columns. Add 0.5 ml of ChIP DNA Binding Buffer to each sample and mix briefly.

9. Transfer the mixture to a provided spin column in a collection tube and centrifuge at ≥10,000 x g for 30 seconds. Discard the flow-through.

10. Add **200 µl** of **DNA Wash Buffer** to the column. Centrifuge at ≤10,000 x g for 30 seconds.

11. Add **200 µl** of **DNA Wash Buffer** to the column. Centrifuge at ≤10,000 x g for 30 seconds.

12. Add **50 µl** of **DNA Elution Buffer** directly to the column matrix. Transfer the column to a new 1.5 ml microcentrifuge tube and centrifuge at ≤10,000 x g for 30 seconds to elute the DNA.

    NOTE: *Use 6 µl of DNA elution buffer in a case of ChIP-FFPE workflow in order to ensure the sufficient DNA concentration per analysis.*

13. Analyze the purified DNA using an agarose gel electrophoresis or Fragment Analyzer (Standard Sensitivity NGS Fragment Analysis Kit (DNF-473). Chose the shortest sonication time resulting in a satisfactory shearing profile. Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments, especially for non-histone proteins.

NOTE: *Fragment Analyzer and High Sensitivity NGS Fragment Analysis Kit (DNF-474) should be used for FFPE samples.*

Option C - DNA purification using phenol-chloroform extraction

8. Under a fume hood, add an **equal volume** of **phenol/chloroform/isoamyl alcohol (25:24:1)** to the sample. Vortex thoroughly for 1 minute.
9. Centrifuge for 2 minutes at 16,000 x g at room temperature. Transfer the top aqueous phase into a new 1.5 ml tube.
10. Add an **equal volume** of **chloroform/isoamyl alcohol (24:1)** to the sample. Vortex thoroughly for 1 minute.
11. Centrifuge for **2 minutes** at 16,000 g at room temperature. Transfer the top aqueous phase into a new 1.5 ml tube.
12. Add **10 µl** of **DNA precipitant**, **5 µl** of **co-precipitant**, and **500 µl** of cold **100% ethanol** to the sample. Vortex thoroughly for 1 minute and incubate at -80 °C for 30 minutes (or at -20°C for 1 hour).
13. Centrifuge for 25 minutes at 16,000 x g at 4°C. Carefully remove the supernatant and add **500 µl** of ice-cold **70% ethanol** to the pellet.
14. Centrifuge for 10 minutes at 16,000 x g at 4°C. Carefully remove the supernatant and let the sample dry at room temperature for 2-5 minutes.
15. Re-suspend the pellet in **20 µl** of **TE Buffer**.

NOTE: *In case of FFPE sample re-suspend the pellet in 6µl of TE Buffer.*

16. Analyze the purified DNA using an agarose gel electrophoresis or Fragment Analyzer (High Sensitivity NGS Fragment Analysis Kit (DNF-473).
17. Chose the shortest sonication time resulting in a satisfactory shearing profile. Avoid over-sonication, as it may lead to a drop of efficiency in ChIP experiments, especially for non-histone proteins.

Related products

Product	Cat. No.
Chromatin EasyShear Kit – Low SDS	C01020013
Chromatin EasyShear Kit - Ultra Low SDS	C01020010
Chromatin shearing optimization kit – High SDS	C01020013
Chromatin shearing optimization kit – for Plant	C01020014
IPure Kit v2	C03010014
MicroChIP DiaPure columns	C03040001
iDeal ChIP-seq kit for Histones	C01010055
ChIPmentation Kit for Histones	C01010080
Bioruptor Pico	B01060010
Bioruptor Plus	B01020001

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