



A Hologic Company

Guide for successful chromatin preparation using the Bioruptor[®] Pico



SONICATION

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A check-list for successful chromatin preparation

Chromatin immunoprecipitation (ChIP) is a common tool used to study protein-DNA interactions in cells and tissues. The assay can be cumbersome and the success is highly dependent on the quality of chromatin. The first critical step of a successful ChIP experiment is the preparation of sheared chromatin which is representative of the biological scenario of interest.

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 100-500 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 down-stream 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-histones (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.

Successful shearing requires optimization but is easy with right tools. We suggest using the Bioruptor Pico for best results. The Bioruptor Pico delivers high performance ultrasound in a precise and uniform manner allowing optimal shearing efficiency. More than 1000 peer-reviewed scientific papers cite the Bioruptor.

Important Note: The Bioruptor Pico is a more powerful system compared to the previous generation of Bioruptors (Standard and Plus). Consequently, the sonication protocol should be re-adjusted to the Bioruptor Pico by decreasing a total sonication time. DO NOT use a sonication protocol established for your Bioruptor Standard (UCD-200) or Bioruptor Plus (UCD 300) on the Bioruptor Pico!

A successful chromatin preparation relies on the optimization of cross-linking, cells lysis and sonication itself. All 3 steps are interconnected and need to be optimized individually for different experimental settings. The following check-list should be considered for chromatin preparation using the Bioruptor Pico:

1. Optimize cross-linking
2. Choose an appropriate lysis strategy
3. Optimize the sonication parameters:
 - a. Optimize the sonication time for different experimental conditions and for each new sample type
 - b. Use only the recommended tubes for sonication
 - c. Use the recommended sample volume
 - d. Control the temperature during the sonication process
 - e. Use purified water according to specifications
4. Use an appropriate protocol for the analysis of sheared chromatin
5. Adapt primer design to fragment size
6. Consider complementary experimental approaches to improve the sensitivity and resolution of ChIP-seq experiments

1. Optimize cross-linking

Cross-linking is typically achieved by using formaldehyde which forms reversible DNA-protein links. Formaldehyde rapidly permeates the cell membranes and enables a fast cross-linking of closely associated proteins in intact cells. Formaldehyde cross-linking is ideal for two molecules which interact directly. However, for higher order and/or dynamic interactions, other cross-linkers should be considered for efficient protein-protein stabilization such as Diagenode ChIP Cross-link Gold, an innovative dual cross-linking ChIP fixation reagent (C01019021).

Adopt the fixation strategy that is best suited to studying your particular proteins. Some epigenetic marks may be more elusive than others. When studying weak or rare protein-DNA binding events fixation should be done promptly and directly in medium. When studying histone marks, cells can be put in suspension by trypsinization before fixation. Generally, a shorter fixation is required for histones marks (8-10 min) than for transcriptional factors (10-20 min) using a standard formaldehyde single step fixation protocol.

Use fresh formaldehyde. The use of high quality and fresh formaldehyde is crucial while using methanol-free reagent is not mandatory. Replace your stock every month. This will ensure high inter-assay reproducibility between ChIP experiments.

Always carry out a fixation time course for your cell line to empirically determine the optimal fixation time for your cell line and epitope of interest. Cell lines and epitopes differ widely in their fixation efficiency and sensitivity to fixation.

Be precise with the fixation time and temperature. The formaldehyde fixation is a time and temperature –depending process. A stronger cross-linking will be achieved at a higher temperature and at longer duration. Whether you use RT or 37°C, 5 or 15 min (depending on your specific target and cell types), make sure that the temperature and time are consistent.

Target	Fixator	Formaldehyde	ChIP Cross-link Gold C01019021
Histones		Yes (8-10 min)	No need
Transcriptional factors directly bound to DNA		Yes (10-20 min)	No need
Indirect higher order and/or dynamic interactions		Yes (10-15 min)	Yes (30-45 min)

2. Choose an appropriate lysis strategy

The lysis step is required to liberate cellular components by dissolving the cell membrane with detergent based solutions and to extract the crosslinked protein-DNA complexes from cells or tissue into solution. Sonication is required to complete the cell lysis. Generally, the stronger the fixation, the harsher the conditions that should be used for cell lysis and sonication.

It is possible to use a one-step lysis to lyse cells directly with an SDS-containing buffer or a two-step lysis to isolate nuclei using a buffer with non-ionic detergents in which cellular membranes are first lysed followed by treatment of the isolated nuclei with an SDS-containing buffer. Two-step lysis allows the removal of most of the soluble cytosolic proteins. This can improve the sonication efficiency as well reduce background and increase sensitivity of the ChIP assay.

One-step lysis is most appropriate when starting with a limited number of cells (less than 1,000,000) while two-step lysis with nuclei isolation is preferable when working with a high number of cells (more than 1,000,000), strongly fixed cells and “difficult” cells (see step 3).

3. Optimize the sonication parameters

a. Optimize the sonication time for different experimental conditions and for each new sample type

The length of sonication time depends on many factors, like cell type, cell density, sample volume, fixation time, concentration of detergent in the shearing buffer, etc. Hence it is important to optimize the sonication conditions for each new experiment.

An initial time-course experiment of **5-10-15 sonication** cycles 30'' ON/30'' OFF is recommended when starting a new ChIP project using the Bioruptor Pico. In some experimental conditions, even shorter sonication time of 2-3 cycles might result in a satisfactory chromatin shearing.

As best practice, **choose the shortest sonication** time resulting in a satisfactory 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.

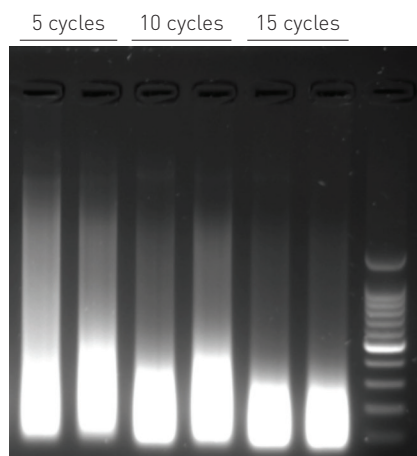


Figure 1: Bioruptor® Pico generates fragments suitable for ChIP experiments.

HeLa cells were fixed with formaldehyde and chromatin was prepared according to Diagenode's Chromatin EasyShear Kit - 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). A 100 bp ladder was loaded as the size standard.

Sonication should be optimized for each new cell type. Cells are different in their resistance to sonication. Some cell lines are more difficult to shear than others. Generally, primary cells culture, lymphocyte-related cells, fibroblasts are more difficult to shear. Two-step lysis (including nuclei isolation) is recommended when working with "difficult" cells.

Please refer to the following list of difficult cells. Note this list is not exhaustive and only includes some of the most common models.

- Peripheral blood mononuclear cell (PBMC)
- Naive CD4+ T cells
- Monocyte derived macrophage
- Primary splenocytes
- Primary lymphocytes
- Jurkat cells (immortalized line of T lymphocytes)
- K562 (human immortalised myelogenous leukaemia line)
- Raji (lymphoblastoid cell's derived from a Burkitt's lymphoma)
- U937 (histiocytic lymphoma)
- HEL (erythroleukemia cell line)
- MOLT-3 (human acute lymphoblastic leukemia cells)
- MT-4 (Human T cell leukaemia)

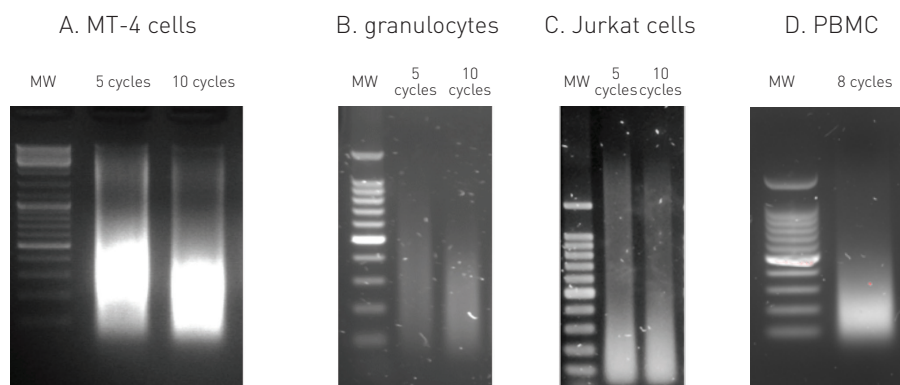


Figure 2. Successful chromatin shearing from “difficult” cells using the Bioruptor® Pico.

MT- 4 (panel A) , granulocytes (panel B) Jurkats cells (panel C) and PBMC cells (panel D) were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode’s Chromatin EasyShear Kit - Low SDS (Cat. No. C01020010). Samples were sonicated for 5-10 cycles of 30” ON/30” OFF as indicated with Bioruptor® Pico using 1.5 ml Bioruptor® microtubes with caps (Cat. No. C30010016). A 100 bp ladder was loaded as size standard.

Use fresh cells for chromatin preparation whenever possible. Freezing fixed cells alter DNA susceptibility to sonication and may result in a less reproducible shearing with a persistent high molecular weight fraction.

Adapt sonication time to cell density. 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. If cell density is too high, this will decrease the efficiency of sonication. If the shearing results are not satisfactory, consider reducing cell density. Generally, cell density **1,000,000 -3,000,000 cells per 100 µl** (scale accordingly to final volume) results in good shearing.

Use detergents in a shearing buffer, preferably SDS. SDS-containing buffers increase sonication efficiency and chromatin yield. Moreover, it may improve epitope availability leading to a better specific signal. However, high SDS concentration may result in a partial loss of signal for proteins not directly bound to DNA. Altogether, an optimal SDS concentration in a shearing buffer should be chosen depending on experimental settings (cell type, target proteins, and downstream analysis) (fig. 3).

Diagenode recommends using our validated Chromatin Shearing Optimization kits with varying SDS concentrations.

	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

GUIDE FOR SUCCESSFUL CHROMATIN PREPARATION USING THE BIORUPTOR® PICO

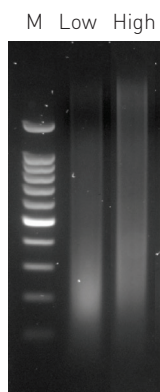


Figure 3: The presence of SDS in a sonication buffer improves the sonication efficiency.

HeLa cells were fixed with formaldehyde for 10 minutes. Nuclei were extracted using a buffer with non-ionic detergent and resuspended in a sonication buffer with 1% SDS (High SDS) or \leftarrow 0.1% SDS (Low SDS). Samples were sonicated for 10 cycles of 30" ON/30" OFF with the Bioruptor® Pico using 1.5 ml Bioruptor® microtubes with caps (Cat. No. C30010016). A 100 bp ladder was loaded as size standard.

b. Use only the recommended tubes for sonication

Ensure that only the recommended tubes are used for sonication. Bioruptor Pico tubes were developed specifically for use with the Bioruptor Pico. They ensure a maximum energy delivery to samples with a minimal attenuation of ultrasound intensity. Please check the table below and choose the appropriate tubes for sonication on the Bioruptor Pico.



IMPORTANT NOTE: Sonication tubes recommended for the Bioruptor® Pico are different from the tubes recommended for the Bioruptor® Standard and Bioruptor® Plus. Using the wrong tubes on the Bioruptor® Pico will lead to inefficient shearing!

WHICH TUBES FOR BIORUPTOR® PICO?



Catalog number	C30010020	C30010011	C30010016	C30010017	C01020031
Recommended sample volume	20 µl - 100 µl	100 µl	100 µl - 300 µl	500 µl - 2 ml	500 µl - 2 ml
APPLICATIONS	DNA shearing	✓	✓	✓	
	Chromatin shearing	✓	✓	✓	✓
	RNA shearing	✓	✓		
	FFPE Extraction			✓	
	Cell lysis	✓	✓	✓	✓
	Tissue disruption for protein extraction			✓*	✓*

✓ = validated tubes

* Protein extraction beads (C20000021) must be used

Be aware that sonication efficiency may differ depending on a type of tubes used. Diagenode 0.65 ml tubes (Cat. No. C30010011) and 1.5 ml microtubes with caps (Cat. No. C30010016)) show a similar efficiency while 15 ml tubes (Cat. No. C01020031) usually require a longer sonication to get a comparable fragment distribution.

Note that 15 ml tubes should be supplemented with sonication beads for successful chromatin shearing. The 15 ml tubes (Cat. No. C01020031) are suitable for chromatin shearing experiments requiring a broad size distribution (e. g. ChIP for non-histone proteins).

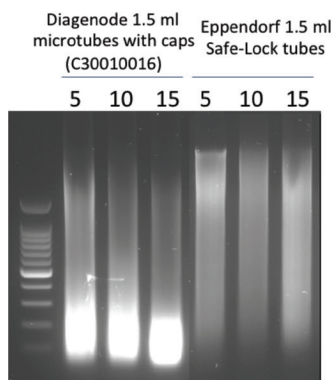


Figure 4 : Diagenode sonication tubes ensure improved sonication efficiency.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's Chromatin EasyShear Kit - Low SDS (Cat. No. C01020010). 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 (Cat. No. C30010016) or 1.5 ml Eppendorf Safe-Lock tubes. A 100 bp ladder was loaded as size standard.

c. Use the recommended sample volume

It is important to use a recommended sample volume for efficient and reproducible shearing (check the table below). Any deviation from this recommended range will lead to inefficient shearing and lack of reproducibility.

Please note that shearing efficiency increases as volume of sample decreases. For example, with the same energy applied for a 100 µl or a 300 µl sample, more efficient shearing would be expected for the 100 µl volume.

Recommended sample volume per sonication tube

Description	Reference	Maximum volume per tube	Minimum volume per tube
0.2 ml Bioruptor® Microtubes	C30010020	100 µl	20 µl
0.65 ml Bioruptor® Microtubes	C30010011	100 µl	100 µl
1.5 ml Bioruptor® Microtubes with Caps	C30010016	300 µl	100 µl
15 ml Bioruptor® Tubes & sonication beads	C01020031	2 ml	500 µl

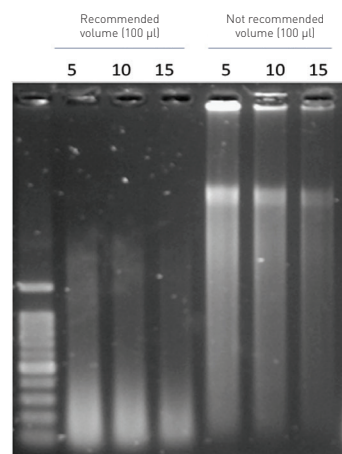


Figure 5 : The efficiency of chromatin shearing relies on using a recommended volume.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's Chromatin EasyShear Kit - Low SDS (Cat. No. C01020010). Samples were sonicated for 5, 10 and 15 cycles of 30" ON/30" OFF as indicated with the Bioruptor® Pico using 0.65 ml Bioruptor® microtubes (Cat. No. C30010011) with 100 µl (recommended volume) or 200 µl (not recommended range). A 100 bp ladder was loaded as size standard.

d. Control the temperature during a sonication process

The Bioruptor Pico ensures epitope preservation due to isothermal controls via the built-in cooling system and controlled delivery of ultrasonic energy to biological samples.

The Bioruptor’s water bath ensures optimal energy transfer, reducing the chance of overheating samples. Moreover, the built-in cooling system additionally ensures high precision temperature control during the entire sonication process, resulting in higher quality samples. The Single Cycle Valve for the Bioruptor® controls the water flow between the Bioruptor® Pico and the water cooler. Cold water cycles into the Bioruptor® sonication bath only during the off cycle (no sonication) and is stopped during the on cycle (sonication). This reduces any water flow disturbance in the tank and allows for more efficient sonication of samples.

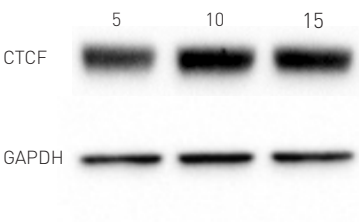


Figure 6 : Thermal control during chromatin shearing preserves protein epitopes.

Western blot of proteins after shearing and reverse of cross-linking. HeLa cells were fixed with formaldehyde, and chromatin was prepared accordingly to Diagenode’s Chromatin EasyShear Kit - Low SDS (Cat. No. C01020010). Samples were sonicated for 5, 10 and 15 cycles of 30" ON/30" OFF as indicated with Bioruptor® Pico using 1.5 ml ml Bioruptor® microtubes (Cat. No. C30010016). An aliquot of 10 µl was mixed with 10 µl of 2x Laemli buffer, boiled for 15 min and loaded on SDS-PAGE. Western blots were immunostained using CTCF (Diagenode, ref. C15410210) and GAPDH (Cell Signaling, ref. 5174B) antibodies.

e. Use purified water according to specification

The type of water used in the sonication bath and in the cooler is important for optimal ultrasound propagation and the life span of the equipments. Any foreign materials (eg. algae, particulates) and reagents (e.g. water protective agents) may alter the ultrasound beam affecting the sonication efficiency.

Always use distilled or demineralized (also known as deionized or DI) water. Both show an appropriated conductivity and resistivity.

We **do not recommend** using ultrapure water (eg. Milli-Q) which may accelerate the corrosion of metallic parts of the system (sonication tank and cooler) and could alter the efficiency of sonication. We also **do not recommend** using tap water which may contain large and potentially variable amounts of contaminants.

Please refer to the table below showing the specification for water.

	Grade of Water	Compatibility with the Bioruptor® sonication
Ultrapure water	Type 1 or Type 1+	No
Demineralized (deionized, DI) water	Type 2+ or Type 2	Yes
Distilled water	Type 3	Yes
Tap water	N/A	No

4. Use an appropriate protocol for analysis of sheared chromatin

Verify each input chromatin batch even if you are carrying out the same process repeatedly. It is crucial to check the quality of your chromatin before each experiment.

For accurate size determination of the chromatin fragments, the reversing of the crosslinks, including RNase treatment followed by DNA purification, is advised. Measuring the size of chromatin fragments is not accurate without first removing the crosslinks. The presence of the crosslinks retards electrophoretic migration resulting in a misinterpretation of fragment size (fig. 7).

RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing (fig. 8).

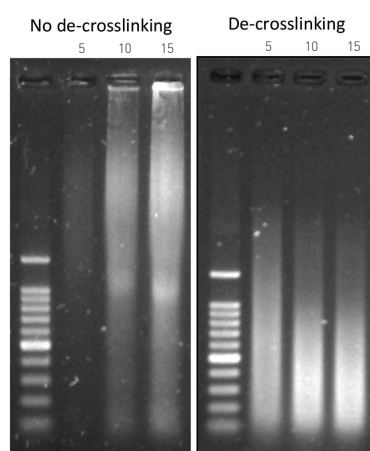


Figure 7 : Reversing crosslinks is necessary for accurate size estimation.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's Chromatin EasyShear 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.

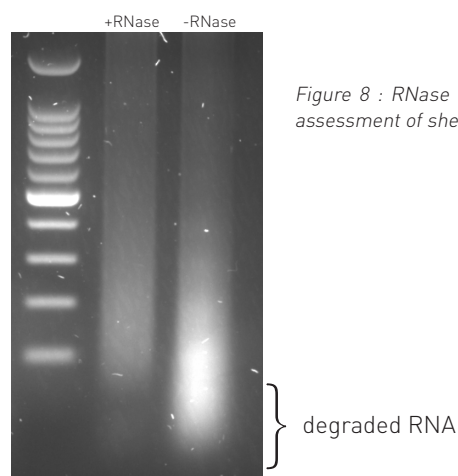


Figure 8 : RNase treatment significantly reduces background caused by degraded RNA and improves visual assessment of shearing.

For accurate fragmentation assessment, 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.

Although the microfluidics technology (Agilent BioAnalyzer 2100, Agilent 2200 TapeStation System, Perkin Elmer Caliper LabChip GX Touch, Fragment Analyzer from Agilent) is widely used for size assessment of DNA fragments before library preparation for NGS, this technology is less optimal for sheared chromatin. For example, some inconsistencies between agarose gel and BioAnalyzer 2100 profiles have been documented. It may be linked to a higher sensitivity of microfluidics chips to residual contaminants (ions, SDS, proteins, carrier used for DNA precipitation etc), overloading and to conformation/spacial structure of DNA molecules which might be affected by fixation and not fully relieved by de-crosslinking.

Moreover, BioAnalyzer 2100 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.

Another key point is about quantitation of BioAnalyzer 2100 peaks. If each region is calculated using molarity which represent number of molecules in a particular range, a significantly higher level of molecules are found in low molecular weight region (fig. 9).

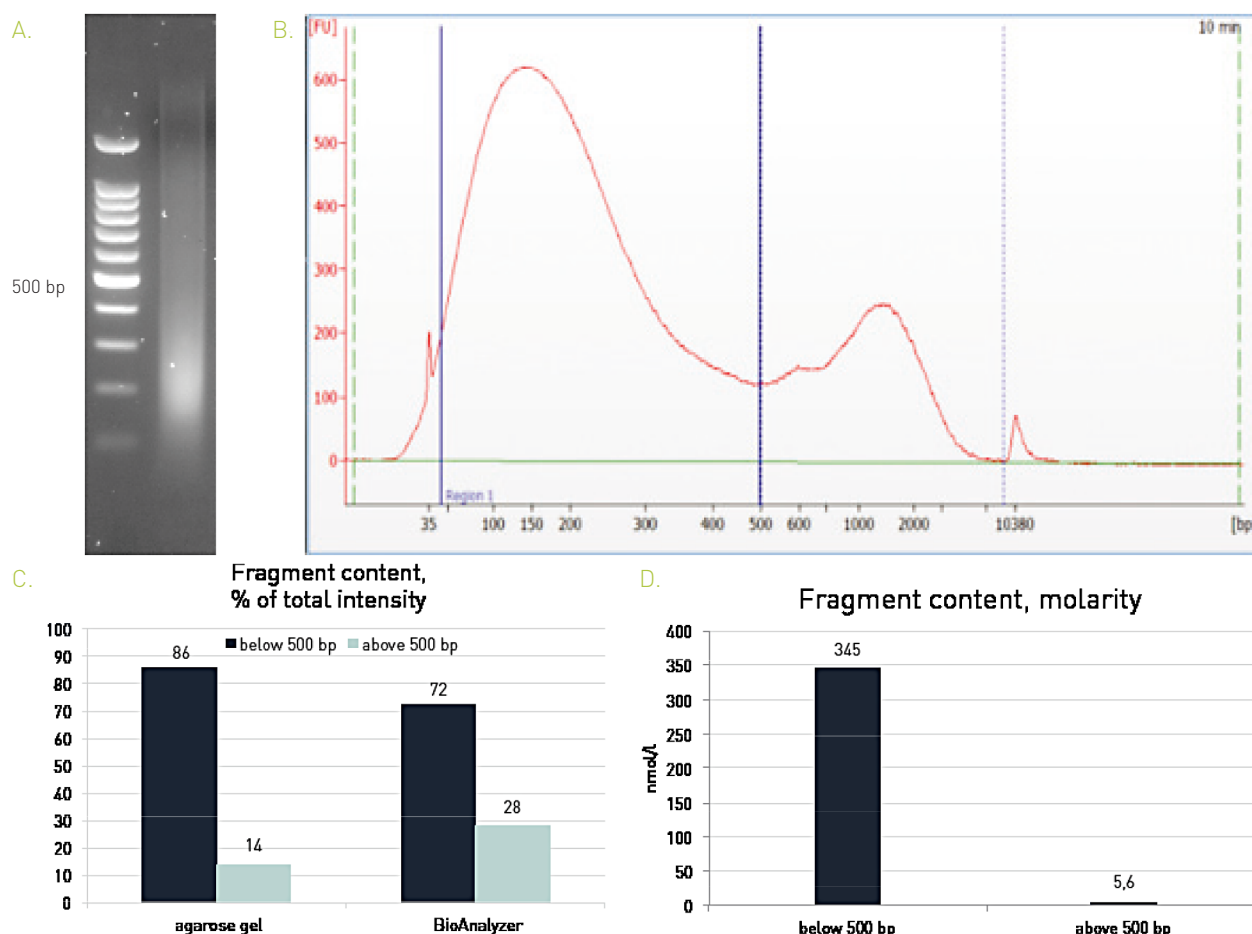


Figure 9 : Size assessment of sheared chromatin using an agarose gel and the BioAnalyzer.

HeLa cells were fixed with formaldehyde and chromatin was prepared accordingly to Diagenode's Chromatin EasyShear Kit -High SDS [Cat. No. C01020012]. Samples were sonicated for 10 cycles of 30" ON/30" OFF with Bioruptor® Pico using 1.5 ml Bioruptor® microtubes with caps [C30010016] and analyzed by agarose gel [panel A] or by BioAnalyzer, High Sensitivity Agilent DNA Kit [panel B]. Fragment content below and above 500 bp was calculated as percentage of total surface [panel C]. Panel D shows fragment content calculated as molarity (BioAnalyzer trace only).

5. Adapt primer design to fragment size

Success in obtaining high-quality ChIP data is critically dependent on good primer design. Ideally, a PCR product amplified by primers for ChIP-qPCR should be located around a binding site to allow detection/quantification of the genomic fragments bound to a target protein. Amplicons less than 150 bp should be used to provide a good sensitivity of qPCR. A longer PCR product will exclude short fragments from the analysis resulting in a reduced sensitivity of qPCR. Moreover, the efficiency of amplification of long amplicons (\rightarrow 150 bp) is substantially lower. An amplicon size should not exceed the average size of chromatin fragments. For example, if chromatin is fragmented in a range 100-400 bp, an amplicon size should not be longer than 150 bp. Primers should be 20 to 30 bases long with a T_m between 55° and 60°C. Ideally, all primer pairs should have a similar T_m to be analyzed in the same qPCR run.

In some situations, it might be difficult to design primers flanking a target site that fulfills all the recommended requirements. In this case, the location of PCR product can be shifted relative to a binding site. The size of amplified PCR product and the location relative to a potential binding site (BS) should be coupled with the average size of chromatin fragments to provide the best balance between sensitivity and resolution. Shorter chromatin fragments will require an amplicon closer to a binding site - in addition, a shorter amplicon should be preferred (fig. 10).

6. Consider complementary experimental approaches to improve the sensitivity and resolution of ChIP-seq experiments

Complementary experimental approaches like re-shearing and ChIP-exo methodology can be used to increase the sensitivity and/or resolution of standard ChIP-seq experiments.

The first approach consists of the re-shearing of purified and de-crosslinked DNA after immunoprecipitation. This enables to enrich additional fragments in a desired optimal size range suitable for next-generation sequencing. This approach potentially allows the reduction of starting material. Moreover, it may improve specific signal-over-noise at least for inactive histone marks (e.g. H3K27me3). Nevertheless, a re-shearing strategy should not be considered as a universal approach since improved ChIP-seq results are mark-specific.

When a high resolution mapping of transcriptional factors binding site is desired, a ChIP-exo protocol can be used. This new technique employs the use of exonucleases to degrade strands of the protein-bound DNA in the 5'-3' direction to within a small number of nucleotides of the protein binding site and significantly enhances the resolution of transcription factor binding sites to single nucleotide levels.

Please contact Diagenode for more detailed information about these approaches.

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