Optimizing Sample Fixation and Chromatin Shearing for Improved Sensitivity and Reproducibility of Chromatin Immunoprecipitation

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Abstract

Chromatin shearing is the first critical step to provide sensitive and reproducible results in chromatin immunoprecipitation (ChIP) experiments. Adaptive Focused Acoustics® (AFA®) acoustic technology provides precise control over mechanical shearing and thermal control during processing to deliver high quality chromatin for the most sensitive results, in a highly reproducible manner. Optimizing the sample preparation and chromatin shearing provides more chromatin available for the immunoprecipitation, preserves protein epitopes, protein-DNA interactions, and DNA quality.

Introduction

Chromatin Immunoprecipitation (ChIP) is a powerful technique for evaluating the interactions of proteins with specific regions of genomic DNA, helping to better understand the mechanisms of epigenetics, gene regulation, DNA replication, repair and recombination (Das et al., 2004). The ChIP technique utilizes formaldehyde to cross-link proteins to DNA in vivo followed by sonication or nuclease treatment to obtain small DNA fragments. Immunoprecipitation is then carried out using specific antibodies to the DNA-binding protein of interest. The optimal DNA fragment size is dependent on the intended downstream analytical methods to be employed, typically targeting fragments from 200 to 700 base pairs. The immuno-enriched DNA is purified, and analyzed by one of several methods for example, quantitative PCR (ChIP-qPCR), microarray chip (ChIP-Chip), or Next-Gen Sequencing (ChIP-Seq).

The main impediments to successful ChIP assays are low sensitivity and reproducibility of the results. The sensitivity and reproducibility of ChIP experiments can be significantly influenced by several factors during sample preparation including, variability in the formaldehyde crosslinking, chromatin shearing efficiency, the quality and specificity of the antibodies employed, and Protein-A/G beads used in the IP. Only stringent control overall experimental conditions will reduce variability and assure the reproducibility of results, as well as the quality and sensitivity of the data (Haring et al., 2007). To optimize ChIP experiments, three critical steps have to be optimized: 1) sample fixation and preparation, 2) chromatin shearing, 3) the amount of input material and antibody used.

Endeavoring to develop more sensitive and reproducible ChIP assays, much attention has been given on optimizing the downstream sample preparation including optimizing sample input, antibody, and Protein A/G concentrations, as well as optimizing the analytical methods employed. However, little attention has been given to optimizing the preparation of the input sample material. The results and information presented here define an optimized sample preparation and chromatin shearing method, including optimal mechanical shearing technology for the best preserved and most reproducibly sheared input chromatin. Use of this optimized method will deliver the most sensitive and reproducible ChIP results.

Materials and Methods

Chromatin Immunoprecipitation Assay

Covaris truChIP® Tissue Chromatin Shearing Kit with SDS Shearing Buffer and truChIP Low Cell Chromatin Shearing Kit with SDS Shearing Buffer reagents and protocols were used for all the Chromatin sample preparation steps. For a detailed protocol please refer to the truChIP Chromatin Shearing Kit manuals (http://covarisinc.com/resources/protocols/).

Formaldehyde Fixation

Six aliquots of $1.4x10^7$ cells were each cross-linked for the indicated times in Covaris Buffer A containing freshly prepared 1% formaldehyde to conduct a time course of fixation times. Where indicated, the 1% formaldehyde solution was spiked with methanol to a final concentration of 1.5% methanol. Fixation was quenched by the addition of Covaris Buffer E and continued incubation for an additional 5 minutes. The quenched fixation solution was removed and the cells were washed with cold PBS.

Nuclei Preparation

Covaris Buffer B supplemented with protease inhibitors was added to the fixed cells and incubated for 20 minutes at 4 °C to lyse the cellular membrane. The nuclei were collected by centrifugation at 500 g, 4 °C. The nuclei pellets were then gently resuspended and washed twice in Covaris Buffer C supplemented with protease inhibitors. After collecting again by centrifugation, the washed nuclei were resuspended in Covaris Shearing Buffer D2 and 130 µl aliquots of the nuclei suspension were divided into 6 separate microTUBEs to conduct a shearing time course on each fixation time point. The resuspended nuclei were incubated in shearing buffer on ice for 10 minutes to equilibrate, with occasional vortexing to thoroughly mix the samples, before proceeding with the chromatin shearing.

Chromatin Shearing

The nuclei samples were then processed for the indicated times in a Covaris E210 Focused-ultrasonicator using a setting of 2% Duty Cycle, 3 Intensity (105 W PIP), and 200 Cycles per Burst for 2, 4, 6, 8, 10, and 12 minutes to complete the 6 point time course.

Immunoprecipitation

Aliquots of the sheared chromatin were processed with the Millipore Magna ChIP kit using the ubiquityl-histone H2B or Suz12 antibody (Millipore, Billerica, MA) following the manufacturers recommended protocol. Equal aliquots of each sample were processed in parallel with a non-specific mouse IgG as a negative control.

DNA Purification and Chromatin Shearing Efficiency Analysis

The immunoprecipitated and negative control samples were transferred to a microcentrifuge tube, RNAse and proteinase K treated, and the cross-links were reversed by overnight incubation at 65 °C. The DNA was purified with Qiagen QIAquick PCR Purification Kit following the manufacturer's protocol.

Chromatin Shearing Efficiency Analysis

Aliquots of the sheared chromatin samples were RNase and proteinase K treated, the cross-links reversed, and DNA purified as stated above. The purified DNA was then analyzed by agarose gel electrophoresis and BioAnalyzer DNA 12000 Kit (Agilent, Santa Clara, CA) to determine the DNA size range of the sheared chromatin.

qPCR Analysis

The resultant DNA samples were normalized for concentration, and qPCR was carried out using GAPDH, and Hox1A promoter primers. Fold enrichment of Ubiquityl-Histone H2B and Suz12 proteins at the GAPDH and Hox1A promoters were then empirically determined by qPCR analysis.

Results and Discussion

Isothermal Processing Through Acoustic Field Control

AFA is Covaris' patented acoustic technology empowering Focused-ultrasonicators to mechanically process samples. AFA employs highly controlled bursts of focused high-frequency acoustic energy to efficiently and reproducibly process samples in a temperature controlled, non-contact, and closed environment. The very high frequency ultrasound utilized in AFA results in a wavelength of only a few millimeters, enabling the acoustic energy to be focused into a discrete zone within a sample vessel (***Figure 1***). This focused and efficient delivery method requires a minimal amount of energy avoiding the adverse effects of excess energy such as damaging heat, experimental variability, and sample over-processing typical of ordinary sonicators.

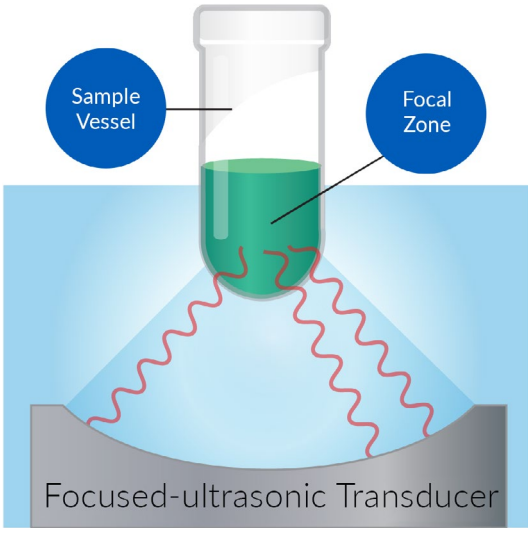


Figure 1: Isothermal processing through acoustic field control.

Sample processing with AFA ultrasonic energy is accomplished by controlling the creation and collapse of millions of cavitation bubbles within the closed sample vessel. Acoustic energy passing through an aqueous medium causes localized pressure fluctuations which forms small cavities (or bubbles) in the regions of relative low pressure. The cavitation bubbles oscillate or grow to a critical size and then collapse. The oscillation and collapse of the cavitation bubbles generates acoustic microstreaming, which creates hydrodynamic shear stress in the sample. Focused-ultrasonicators provide exquisite control of the acoustic bursts delivered to a sample. The tuning of peak incident power, duration, and duty factor, controls the microstreaming, and in turn the generation of shearing forces.

Probe and bath sonicators have traditionally been used for chromatin shearing; however, these acoustic methods are prone to uncontrolled heating which can cause thermal damage to the protein and DNA, lead to thermal shearing bias, and reverse formaldehyde cross-links. When mechanically shearing chromatin with acoustic energy, any excess energy delivered to the system is converted to heat, thereby raising the temperature of the system. The uncontrolled, unfocused nature of probe and bath sonicators results in less control and therefore requires higher acoustic energy input to create cavitation. The excess energy of probe and bath sonicators compared with Focused-ultrasonicators results in uncontrollable heating of samples.

Excess heat in a sample has deleterious effects causing thermal damage to protein epitopes and DNA, as well as reversing formaldehyde cross-links. Taken together these effects drastically decrease the amount of protein-DNA complexes available for immunoprecipitation thereby decreasing assay sensitivity. Additionally, as the thermal effects are uncontrolled, they are variable and have a further effect on assay reproducibility. Covaris' AFA Focused-ultrasonicators provide an efficient means of shearing chromatin isothermally, protecting the protein epitopes, DNA, and formaldehyde cross-links to increase sensitivity and reproducibility.

Formaldehyde Fixation Affects Chromatin Shearing Efficiency

As the first step in ChIP analysis, formaldehyde fixation has far-reaching effects on the sensitivity and variability of the method. Formaldehyde is used to cross-link protein and DNA to preserve the protein-DNA interactions during shearing and downstream processing. Prolonged fixation time can lead to creation of large molecular weight complexes that are resistant to shearing. The effects of fixation time on chromatin fragmentation is determined by analyzing the chromatin shearing efficiency at given fixation times (***Figure 2***). Longer fixation times lead to an increase in large molecular weight chromatin fragments (greater than 2 kb) that are resistant to shearing, even at processing times exceeding 10 minutes (***Figure 2C through E***). With the MS4221 lymphoblast cells used in this example, fixation times as short as 10 minutes led to an increase in the shearing resistant 2 kb fragments (***Figure 2C***). Longer fixation times, 20 or 30 minutes, result in the majority of chromatin being resistant to shearing, even with shearing times greater than 12 minutes (***Figure 2C through E***). Different cell types exhibit different responses to formaldehyde fixation; therefore, optimal fixation times should be independently determined for each cell type.

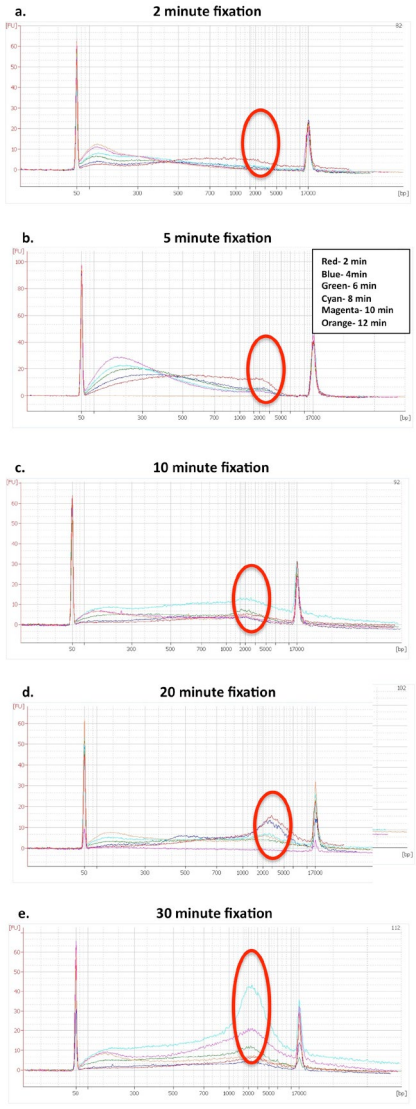
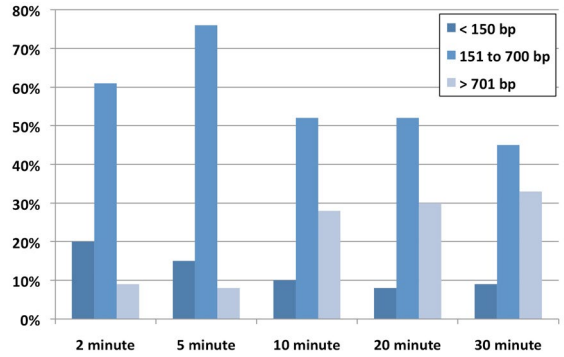


Figure 2: Effects of Formaldehyde Fixation on Chromatin Shearing Efficiency. Evaluation of the effects of sample fixation and AFA processing time on chromatin shearing efficiency. Traces from Bioanalyzer DNA 12000 Kit for chromatin sheared from MS4221 lymphoblast (2 x 10^6) cells for, 2 min (red), 4 min (blue), 6 min (green), 8 min (cyan), 10 min (magenta), and 12 min (orange). The cells were fixed for 2 min (a), 5 min (b), 10 min (c), 20 min (d), or 30 min (e).

a.



b.

Fixation time	< 150 bp	151 to 700 bp	> 701 bp
2 min	20%	61%	9%
5 min	15%	76%	8%
10 min	10%	52%	28%
20 min	8%	52%	30%
30 min	9%	45%	33%

Figure 3: Effect of Cross Linking Time on Cell Based Chromatin Shearing Efficiency. Graph depicting the fragment size distribution, for fragments <150 bp, 151 to 700 bp, or >700 bp, as determined by the Agilent Bioanalyzer (Figure 2), for indicated fixation times sheared in S220 Focused-ultrasonicator for 8 minutes (a). The percent of the total DNA for each size range is provided in the table (b).

It is possible to quantitate the effect of fixation time on shearing efficiency by analyzing the percentage of fragments in the target range of 151 to 700 bp (***Figure 3***). With a fixation time of 5 min and shearing chromatin for 6 min following the truChIP protocol, 76% of the DNA from the sheared chromatin is in the target range. Shorter fixation times allows the chromatin to be more easily sheared, resulting in a higher percentage of short fragments less than 150 bp and a concomitant decrease in the optimal 151 to 700 bp sized fragments. In this manner, too short fixation time leads to overshearing and a decrease in ChIP sensitivity. Insufficient cross linking time also might not preserve the chromatin structure and protein-DNA interactions as well, causing false negative results.

Likewise, fixation times longer than 10 minutes results in an increase in the fragments greater than 700 bp, with a concomitant decrease in the targeted sized fragments. Increases in the size of the fragments above 700 bp decreases the resolution of the determined binding site, increases occurrence of falsepositives, and increases the background signal, which decreases sensitivity.

Many formaldehyde solutions contain methanol as a preservative to prevent the oxidation and polymerization of formaldehyde. Methanol can affect the permeability of cell membranes as well as act as a fixative in its own right. To test whether the presence of methanol in the fixative can have an effect on chromatin shearing efficiency, methanol was spiked into fresh formaldehyde to a final concentration of 1.5%. Expectedly, the presence of methanol leads to the presence of large molecular weight chromatin resistant to shearing with fixation times as short 5 min (***Figure 4***). Variables such as age of the formaldehyde solution and the temperature of the samples during fixation also affect the fixation process (data not shown). To reduce the effects of these variables on your ChIP experiments it is advisable to always use fresh single aliquot vials of formaldehyde (e.g., 16% Formaldehyde, methanol free from Thermo Scientific (Pierce) PN 28908, 1 ml ampules) and always use the same temperature during fixation. The work conducted here was done at room temperature; fixation at higher temperatures (e.g., 37 °C) tends to increase the rate of the reaction. In our experience, it is easier and more reproducible to use a room temperature fixation; if higher temperatures are required, shorter fixation times should be anticipated and care should be taken to match the fixation time for each sample.

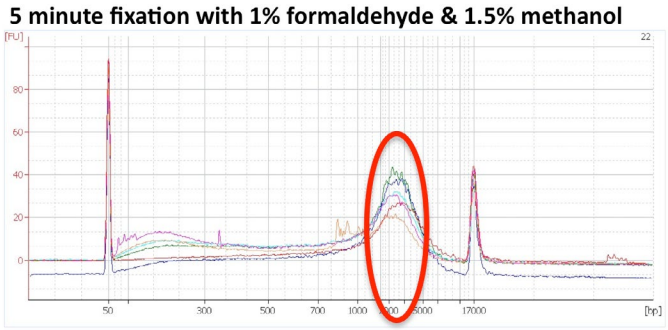


Figure 4: Effects of methanol on shearing efficiency. Evaluation of sample fixation and AFA processing time on chromatin shearing efficiency of 2 x 106 MS4221 lymphoblast cells. (a-f) Traces from Bioanalyzer DNA 12000 Kit for chromatin sheared for 2 min (Red), 4 min (blue), 6 min (green), 8 min (cyan), 10 min (magenta), and 12 min (orange). The cells were fixed for 5 min in 1% formaldehyde supplemented with 1.5% methanol.

Inefficient Chromatin Shearing Increases Background Signal

Inefficient chromatin shearing can increase the background signal, which leads to a decrease in the signal to noise ratio of an experiment ultimately decreasing the sensitivity of the assay. The prolonged fixation times discussed above and other factors, such as the overall performance of the shearing method can negatively affect shearing efficiency. To test the effect of formaldehyde fixation on background signal samples were fixed, sheared, and processed as in a ChIP experiment, but without the inclusion of a target specific antibody for a mock immunoprecipitations (mock IP).

The qPCR results from a primer set specific to the GAPDH promoter on the mock IP'd sample indicates a significant increase in the fold enrichment for the samples fixed for 20 minutes or more (***Figure 5***).

This demonstrates the importance of efficient chromatin shearing for sensitive ChIP results and how factors such as formaldehyde fixation can adversely affect results by increasing background signal.

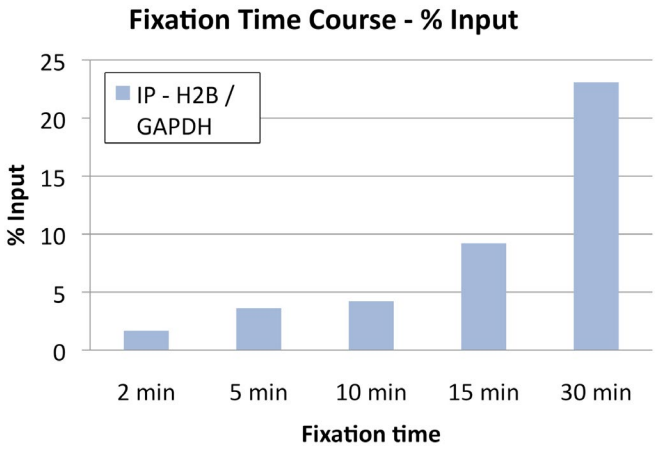
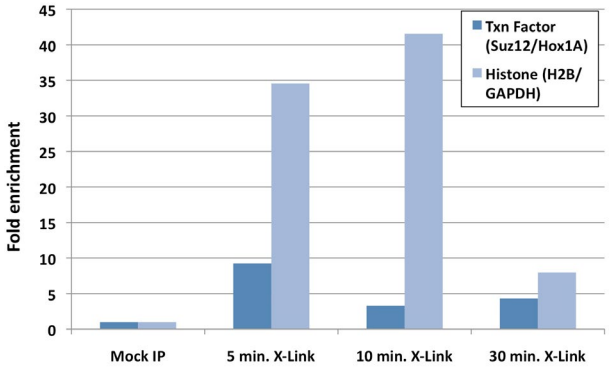


Figure 5: Inefficient chromatin shearing increases background signal. Evaluation of the effects of chromatin shearing efficiency on background signal. MS4221 Lymphoblast cells were fixed for the indicated times, chromatin from 5 x 10^5 cells were used IP without a target specific antibody (mock IP) and qPCR was conducted on 5 ng of purified DNA to determine the fold enrichment compared to the input (a). The fold enrichment over input for each fixation time and enriched target is provided (b).

Improved ChIP Results Through Optimized Shearing

There are a variety of ChIP protocols for assessing histone modification and transcription factor association with chromatin. Unfortunately, many of the protocols developed with older technologies for histones and highly expressed transcription factors using probe and bath sonicators do not work well with rare histone modifications and low abundance transcription factors. This can require the need for multiple protocols and different samples of sheared chromatin to be used for different targets. Using separate chromatin for each target increases the required bench work and more importantly decreases the fidelity of the intra-experiment comparisons of binding site between multiple factors. The truChIP optimized protocols work equally well for low abundant transcription factors as they do with histones. Using optimized fixation and shearing conditions of 5 minutes fixation and 8 minutes of shearing in a Focused-ultrasonicator provided robust signal for both the rare transcription factor Suz12 at the Hox1A promoter, and ubiquityl-Histone H2B at the GAPDH promoter were easily obtained from as little as 5x10^5 cells (***Figure 6***). Fixation times of ten minutes or more decreases the fold enrichment of the rare transcription factor Suz12 by half. A fixation time of 30 minutes drastically decreases the fold enrichment of both Suz12 and ubiquityl- Histone H2B. The focused acoustics, thermally controlled processing, and optimized energy input with AFA Focused-ultrasonicators enables highly efficient chromatin shearing of minimally fixed chromatin, vastly improving assay sensitivity.

a.



b.

	Txn Factor (Suz12/Hox1A)	Histone (H2B/ GAPDH)
Mock IP	1	1
5 min. X-Link	9.25	34.57
10 min. X-Link	3.29	41.57
30 min. X-Link	4.3	7.98

Figure 6: Improved ChIP results through optimized shearing. Evaluation of Suz12 and ubiquityl-Histone H2B enrichment as determined by qPCR, at the Hox1A and GAPDH promoters respectively. MS4221 Lymphoblast cells were fixed for the indicated times, chromatin from 5 x 10^5 cells were used for IP and qPCR was conducted on 5 ng of purified DNA to determine the fold enrichment compared to the input (a). The fold enrichment over input for each fixation time and enriched target is provided (b).

References

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