Covaris *tru*ChIP– the Next Generation Technology for Chromatin Shearing

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Abstract

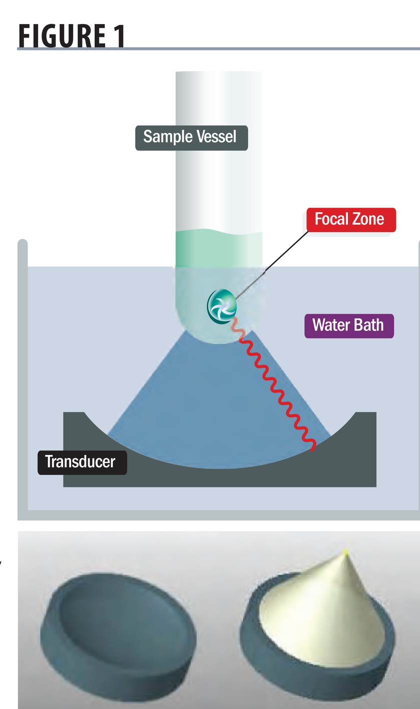
ChIP used in conjunction with NGS is becoming an even more powerful application for studying in vivo transcription factor association with regulatory elements in the genome. Unfortunately, to date the process of shearing chromatin to manageable fragments for ChIP-Seq has been low tech at best requiring the brute force of high energy, unfocused sonicators. In a sonicator-based process, sample components can be subject to thermal damage, significantly reducing the information content available for use in highly sensitive NGS systems. Since thermal and acoustic events cannot be separated in probe and bath based sonicators, researchers have historically resorted to over-crosslinking to overcome the degree of thermal damage to the chromatin. Over-crosslinked chromatin is resistant to shearing, resulting in a loss of material available to downstream analysis processes. In order to offset the loss of information from these samples, a greater amount of starting material is required to ensure sufficient coverage of the possible epitopes. This constrains the use of current protocols to samples available in relative large quantities.

We will present data illustrating the isothermal, and highly controllable Covaris Adaptive Focused Acoustics™ (AFA) technology, long considered the gold standard for NGS DNA shearing, for use in ChIP based applications. Our data will show:

1. Acoustic energy/thermal mapping of the Covaris focused acoustics on a sample tube, its content and immediate vicinity, as compared to the bath, and probe sonicators.
2. Samples processed using the Covaris *tru*ChIP™ protocol and with AFA -based reagents require significantly reduced formaldehyde fixation time while retaining epitope integrity.
3. Since the controlled, isothermal AFA processing does not destroy delicate DNA/protein epitopes, starting material requirements are reduced for Covaris *tru*ChIP.
4. Covaris AFA's unprecedented control over the size range and distribution of the sheared chromatin allow use of the sheared chromatin in all NGS platforms' library preparation.

Overview of Adaptive Focused Acoustics (AFA) Technology

FIGURE 1

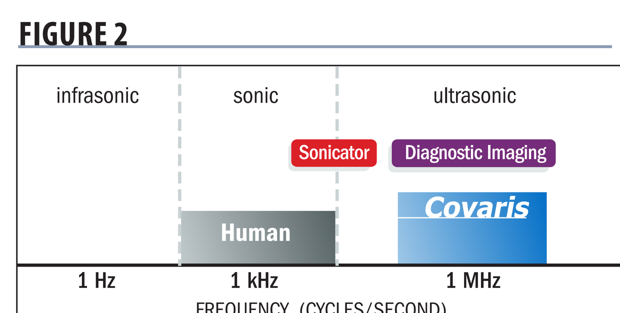


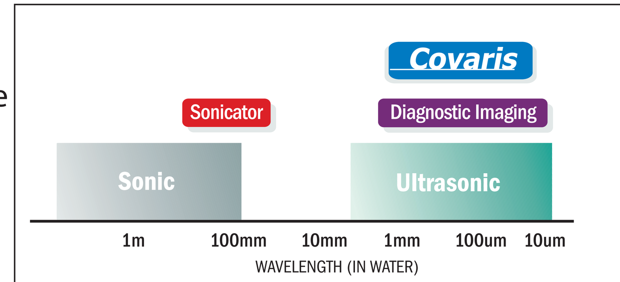
Covaris' patented Adaptive Focused Acoustics (AFA) technology evolved from highly developed therapeutic lithotripsy (such as kidney stone treatment) and diagnostic imaging.

AFA works by sending high frequency acoustic energy waves from a dish-shaped transducer. These converge to a small localized area creating intense mixing (Figure 1). Also, unlike regular sonicators, which have lower frequency (around 20kHz) and longer wavelength, the Covaris acoustic transducer operates at 500khz with a wavelength of ~1mm, unlike conventional sonics which have a wavelength of ~100mm (Figure 2). This enables the acoustics energy to be focused on samples in glass vials or tubes in a non-contact and isothermal mode, avoiding contamination and sample degradation cause by heat.

With these unique design features, a much more precise and reproducible control can be obtained with the Covaris technology, rather than other technology, such as probe and waterbath sonicators.

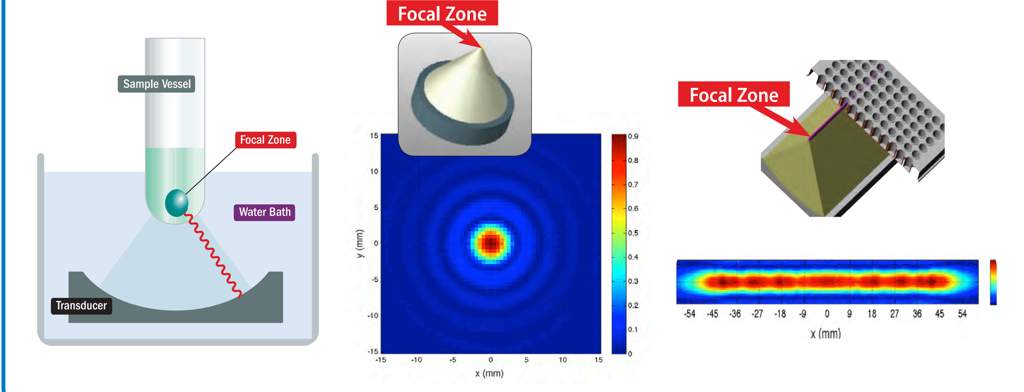
FIGURE 2





TRANSDUCER TECHNOLOGY— *Non-contact, high energy density*

- 500 kHz Transducer technology
- Circular transducer
- Serial processing
- Linear Transducer
- Parallel processing



TECHNOLOGY ADVANTAGE

COVARIS AFA

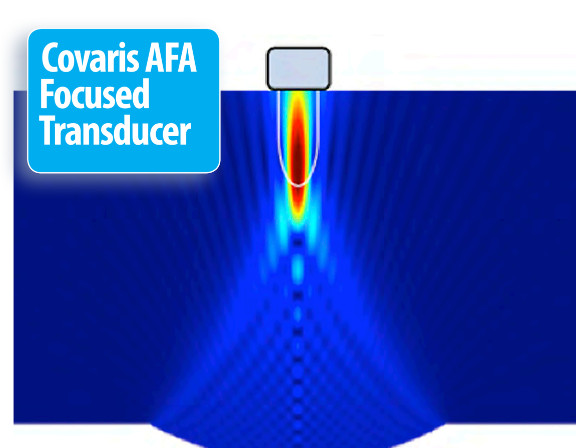
- Isothermal sample processing due to precise control and targeting of AFA energy
- Reproducible processing from sample to sample, day to day, and scientist to scientist
- Easy to automate

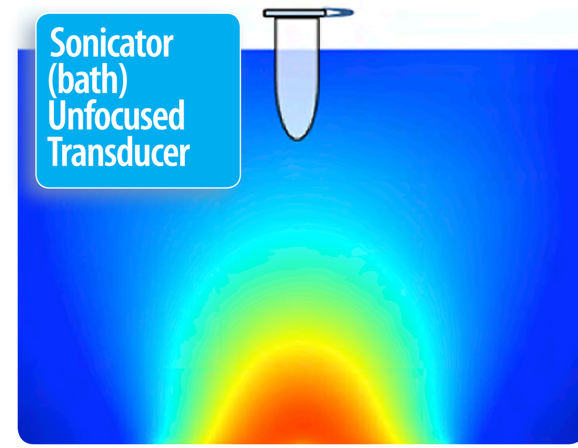
BATH SONICATORS

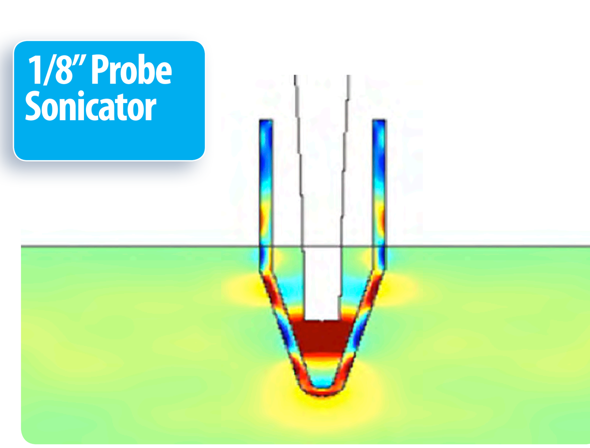
- Lack of thermal control
- Up to 150X more energy (heat) applied to the experiment
- Inconsistent sample processing
- Low throughput processing

PROBE SONICATOR

- Up to 150X more energy (heat) applied to the experiment
- Lack of thermal control
- Inconsistent sample processing
- Cross contamination risk
- Low throughput processing







Materials and Methods

Covaris *tru*ChIP Tissue Chromatin Shearing Kit with SDS Shearing Buffer, and *tru*ChIP 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 *tru*ChIP Chromatin shearing Kit manuals.

Fixation

Tissue: Five aliquots of 120mg of flash frozen mouse liver tissue were thawed on ice, washed with cold PBS, and cross linked for 0, 5, 10, 15, and 20 minutes in Covaris Buffer A containing freshly prepared 1% formaldehyde. The fixation was quenched using Covaris Buffer E, and the tissue sample washed with cold PBS. The fixed tissues were then transferred to a Covaris TT05XT tissue tube for pulverization using the Covaris CryoPrep.

Cultured Cells: Six aliquots of $1.4x10^7$ cells were cross linked for 0, 2, 5, 10, 20, and 30 minutes in Covaris Buffer A containing freshly prepared 1% formaldehyde. One aliquot was fixed with 1% formaldehyde spiked with 1.5% methanol. The fixation was quenched using Covaris Buffer E, and the cells washed with cold PBS

Tissue Pulverization

The fixed tissue samples were submerged in liquid Nitrogen for 45 seconds, and pulverized on a Covaris CryoPrep using a setting of 4. The pulverized tissues were transferred to a Covaris TC12 tube, and placed on dry ice.

Nuclei Preparation

Tissues: Covaris Buffer B supplemented with protease inhibitors was added to the pulverized tissues and incubated for 20 minutes at 4° C for lysis. The samples were then incubated in Covaris Buffer C supplemented with protease inhibitors and the nuclei washed twice with the same buffer. After centrifugation, the nuclei preparations were resuspended in sufficient Covaris Shearing Buffer D2 to accommodate six 130µl microTUBEs and incubated on ice for 10 minutes with occasional vortexing.

Cells: Covaris Buffer B supplemented with protease inhibitors was added to the fixed cells and incubated for 20 minutes at 4° C for lysis. The samples were then incubated in Covaris Buffer C supplemented with protease inhibitors and the nuclei washed twice with the same buffer. After centrifugation, the nuclei preparations were resuspended in sufficient Covaris Shearing Buffer D to accommodate six 130µl microTUBEs.

Chromatin shearing

The nuclei preparations were aliquoted into 6 Covaris snap-cap microTUBEs each containing ~20mg of tissue equivalent nuclei, or $2x10^6$ cells, for a 6 time point course of chromatin shearing of 2, 4, 6, 8, 10, and 12 minutes. The samples were then processed on a Covaris E210 instrument using a setting of 2% Duty Cycle, 3 Intensity (105 PIP), and 200 Cycles per Burst.

Chromatin Shearing Efficiency Analysis

Aliquots of each of the lysate were transferred to a microcentrifuge tubes, RNAse, and proteinase K treated, and cross-links reversed overnight at 65° C. The reverse cross-linked lysates where then processed with Qiagen QIAquick Kit for DNA isolation and purification. Aliquots of the samples where then analyzed on an agarose gel, and on a BioAnalyzer for shearing size range determination.

Epitope Integrity determination

15 µl aliquots of the lysate were loaded on an SDS-PAGE, and transferred to a PVDF membrane using a semi-dry blotter. The membranes were then probed with antibodies against the transcription factor β-catenin, GAPDH, and ubiquityl-histone H2B protein.

Immunoprecipitation and qPCR for Fold Enrichment Analysis

Aliquots of the sheared chromatin were processed using Millipore Magna ChIP kit using the ubiquityl-histone H2B and/or Suz12 antibody following the manufacturers recommended protocol. Equal Aliquots of each sample were processed with mouse IgG as mock for the IP. The immunoprecipitated materials were treated with RNase and proteinase K, and reverse cross-linked at 65 °C, and the DNA isolated using a Qiagen QIAquick kit. 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 at the GAPDH and Hox1A promoters were then empirically determined.

Discussion

Historically over cross-linking of samples for ChIP experiments has been closely tied to the use of uncontrolled bath and probe sonicators which use brute high energy to shear chromatin. Over cross-linking samples leads the chromatin to become resistant to shearing (Figures 3 and 9). This process thereby reduces the amount of chromatin available for sensitive ChIP analysis, such as ChIP-Seq library preparation (Figures 4 and 10), and necessitates an increase in the amount of starting material to compensate.

The focused energy of the Covaris AFA technology allows for the isothermal, highly reproducible, and non-contact shearing of chromatin at ~150 fold less energy than bath and probe sonicators (Technology Advantage Section). Used in conjunction with the optimized Covaris *tru*ChIP Chromatin Shearing Kits and protocol, universal chromatin shearing is achieved from mammalian cells and tissues without spending months optimizing the fixation, lysis, and shearing conditions (Figures 7, 12, and 13) for different cell lines and tissue types. The highly tunable process also allows for control over the size range and distribution of fragments for use with the library preparation protocols of all available NGS systems (Figure 6).

Currently, there are a variety of protocols available for assessing histone modification, and transcription factor association with chromatin using ChIP. Unfortunately, protocols developed for histones and highly expressed transcription factors do not necessarily work with rare histone modifications and transcription factors. Therefore, analyzing multiple histones, modifications, and transcription factors typically require the need for multiple protocols. However, the Covaris *tru*ChIP optimized protocols work equally well with histones and low abundant transcription factors from both tissues and cultured cells. In our lab, we routinely use as few as $5x10^5$ cells, and 5 mg of tissue in ChIP experiments (Figures 8 and 14) without the need for multiple protocols.

Conclusion

Efficient, isothermal, and closed-vessel, chromatin shearing is possible without over cross-linking or depending on large quantities of cells or tissue as starting material. The focused shorter acoustic wavelength generated by the Covaris AFA technology delivers a high degree of control to the shearing of cross-linked chromatin, maintaining epitopes, and retaining the temperature sensitive cross-linked chromatin during shearing. In contrast, probe and water-bath based sonicators, due to their inherit lack of thermal control, can destroy epitopes completely, or unintentionally reverse the cross-links used to preserve the chromatin protein-protein and protein-DNA complexes for subsequent IP, significantly reducing their availability for subsequent downstream processing. The highly controlled Covaris AFA, in conjunction with the optimized *tru*ChIP Chromatin Shearing Kits and protocols, is a new generation of chromatin shearing technology and methodology. The advantages of AFA and *tru*ChIP Chromatin Shearing are in line with the high sensitivity and throughput demands of ChIP-Seq applications utilizing the currently available NGS platforms.

Tissue Based ChIP

Figure 3: Reduced Sample Fixation time provides increased availability of chromatin for shearing

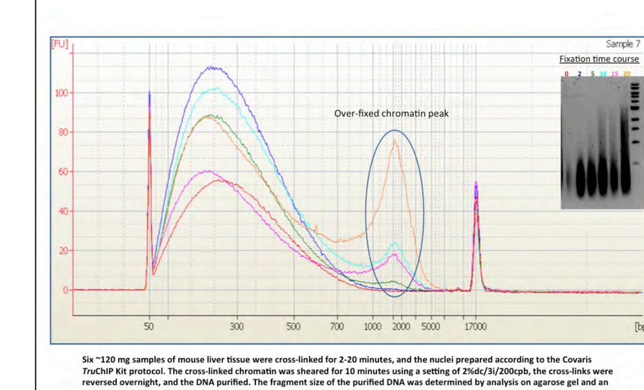


Figure 4: Effect of Cross Linking Time on tissue Chromatin Shearing Efficiency

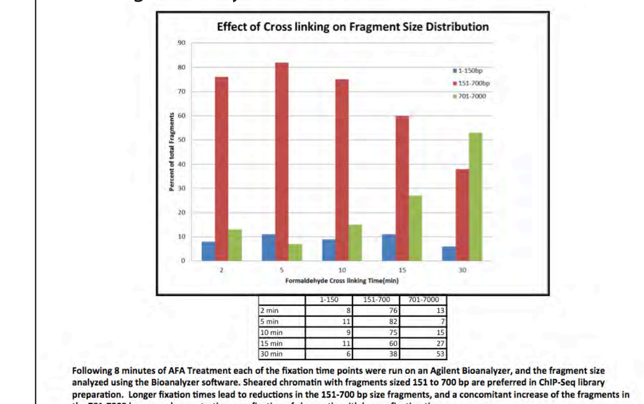


Figure 5: Unprecedented Control over Chromatin Shearing Size and Distribution

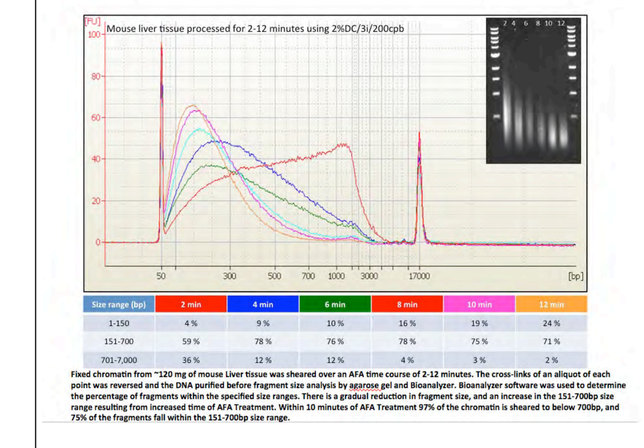


Figure 6: Fine Control of Chromatin Shearing Size Range to Accommodate all NGS platforms for ChIP-Seq

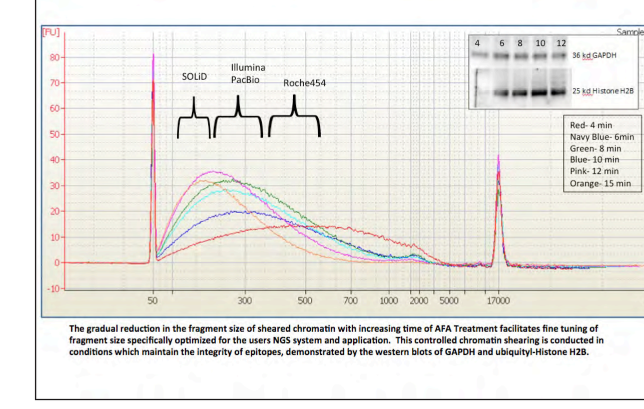


Figure 7: Consistent Chromatin Shearing Reproducibility Across Tissue Types

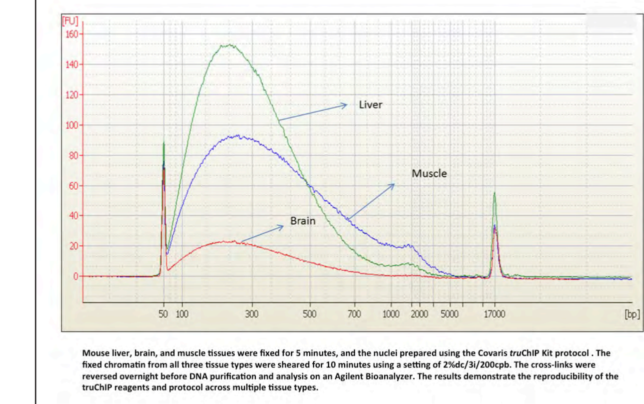
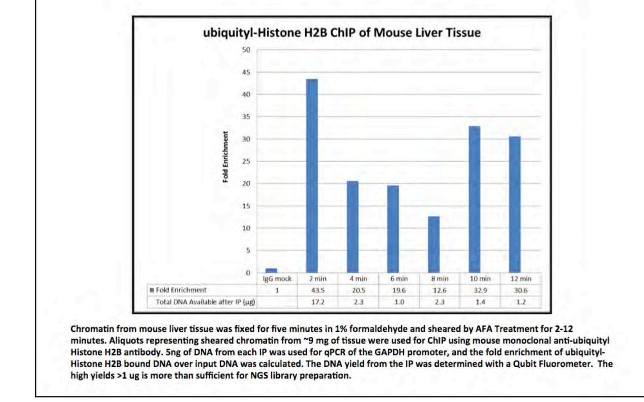


Figure 8: *tru*ChIP Validated Protocol Generates Sufficient DNA for ChIP-Seq Library Preparation from milligrams of tissue



Cell Based ChIP

Figure 9: Reduced Sample Fixation time provides increased availability of chromatin for shearing

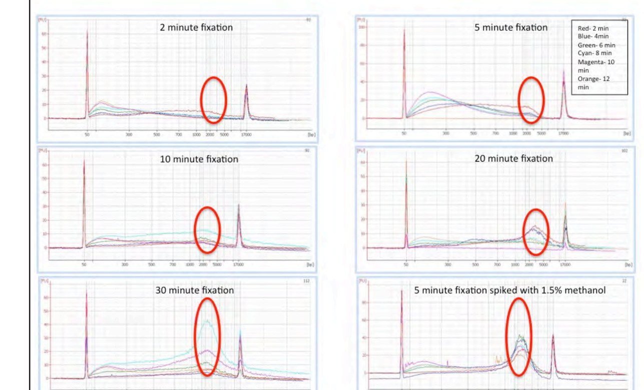


Figure 10: Effect of Cross Linking Time on Cell Based Chromatin Shearing Efficiency

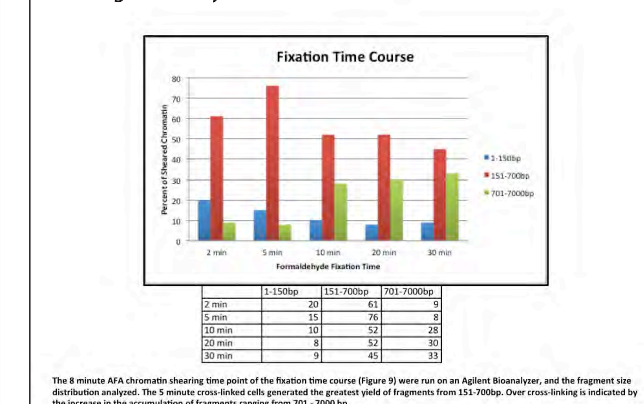


Figure 11: Effect of Methanol on Fixation Efficiency

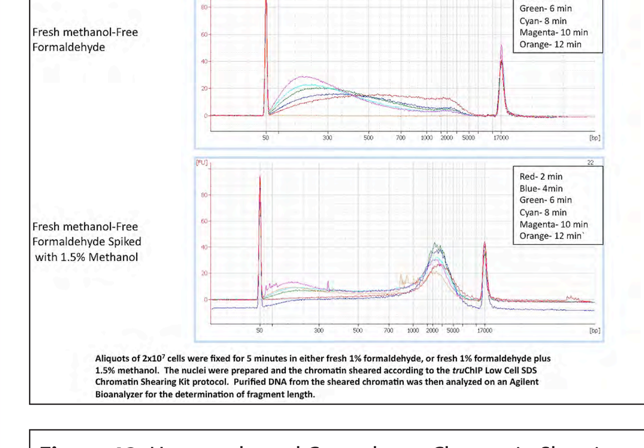


Figure 12: Unprecedented Control over Chromatin Shearing Size and Distribution

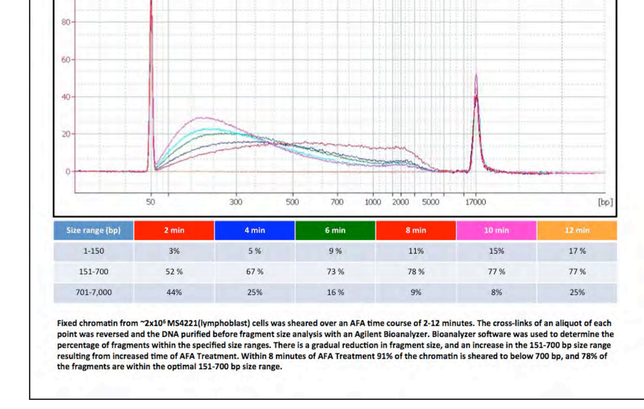


Figure 13: Universal Protocol and Reagents Optimized for use with all Mammalian cell Lines

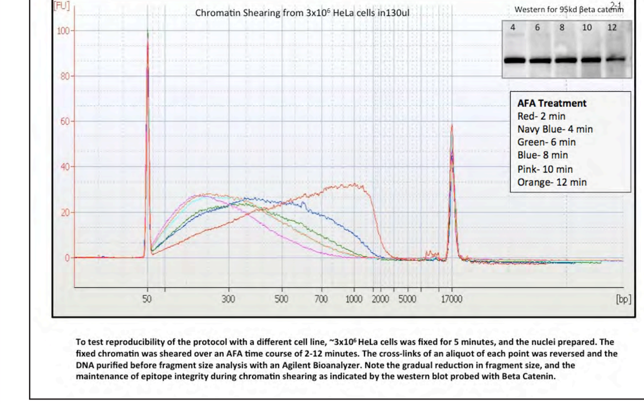
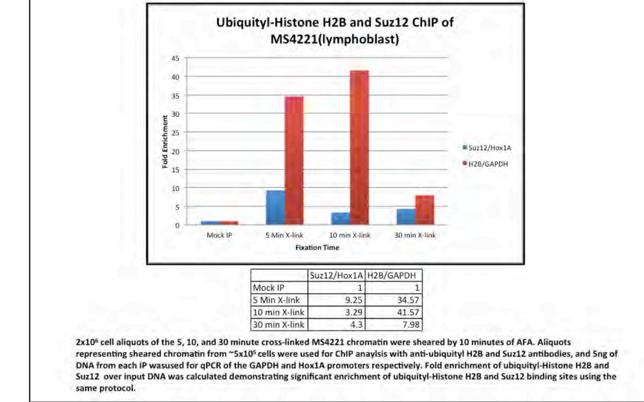


Figure 14: ChIP for Modified Histones and Rare Transcription Factors Using the Same Shearing Protocol



References and Acknowledgments

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Ralph M Bernstein, Ph.D. and Frederick C. Mills, Ph.D., Laboratory of Immunology, Division of Therapeutic Proteins, CDER, FDA,NIH Campus, Bethesda, MD. We very much appreciate their contribution to the shearing buffer SDS concentration titration experiment, formaldehyde fixation reduction time, and initial evaluation of our protocols and reagents.

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