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Application Note

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Optimized protocol for robust chromatin shearing and immunoprecipitation of human pancreatic islets using the Covaris® Focused-ultrasonicator

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ABSTRACT

Epigenomic profiling of human islet (HI) cells allows investigators to understand the molecular mechanisms governing tissue-specific regulatory functions and disease. Here, we developed a chromatin shearing and immunoprecipitation (IP) protocol of fixed isolated human pancreatic islets. Demonstrated here, we show how the Covaris S220 Focused-ultrasonicator provides highly reproducible chromatin shearing, resulting in improved signal-to-noise ratios and sensitivity for ChIP-Seq or ChIP-qPCR.

INTRODUCTION

Why are we studying HI?

Profiling human primary tissues is necessary to gain insight into the molecular mechanisms that control organ development and disease. In the case of human pancreatic islets, the motivation is driven by the worldwide growing incidence of diabetes mellitus, which currently affects more than 400 million people globally[1]. As a result, Human Islet (HI) research is critical to improve our understanding of its biology, however, the availability of pancreatic islets from donors are very limited and considered a precious resource to the research community.

What are the challenges of studying HI?

Traditional ChIP sample preparation protocols are optimized for use with cultured cell lines, which are easy to disperse into single cells and lyse. In contrast, processing primary tissue is more technically challenging and requires optimization using precious and limited human material. HI of Langerhans are small clusters of endocrine cells composed of several cell types including: insulin-secreting β-cells, glucagon-secreting α-cells, and somatostatin-secreting δ-cells. The size and purity of these clusters can vary greatly between donors and isolation centers, which pose technical challenges to their processing and downstream applications.

In this application note, we present an optimized protocol to process human pancreatic islets using the Covaris S220. Acoustic shearing provides fragments usable for highly sensitive downstream applications such as ChIP-seq and ChIP-qPCR.

Comparison between Covaris Focused-ultrasonicator and Bioruptor and probe sonicators

Compared to standard probe sonicators, where the metal probe comes in direct contact with the human sample, chromatin shearing using the Covaris S220 Focused-ultrasonicator provides a non-contact robust shearing protocol. Furthermore, unpredictable foaming induced by metal probe sonicators is avoided to minimize sequencing bias.

Unlike traditional water bath sonicators, chromatin shearing is isothermal–the external chiller controls the water bath temperature, which eliminates the need to frequently stop the run due to excessive heating, or to manually add ice chips to the tank between cycles.

The assessment of chromatin shearing patterns using the Agilent High Sensitivity DNA chip revealed that this protocol allows for the enrichment of fragments in the 200 to 700 bp range. In addition, the protocol also reduces the amount of high molecular weight chromatin which is commonly found after sonicating compact HI. More importantly, improved shearing provides a higher recovery of immunoprecipitated chromatin as shown in the RT-qPCR results.

This protocol is easy to follow, provides reproducible chromatin shearing across valuable human samples, and preserves protein-DNA interactions. We have applied this protocol to create ChIP-Seq libraries and map the binding sites of several histone modification marks, transcription factors and co-factors.

MATERIALS AND METHODS

Instrument and Consumables

- S220 Focused-ultrasonicator (Covaris, 500217)
- microTUBE AFA Fiber Pre-Slit Snap-Cap 6x16mm (Covaris, 520045)

Reagents

- 16% Paraformaldehyde (Agar Scientific, R1026)
- 1.25 M Glycine
- Protease inhibitor cocktail (Sigma-Aldrich, 4693132001)
- Proteinase K 20 mg/ml (Roche, #03 115 852 001)
- RNAse A 10 mg/ml (Qiagen, #19101)

Buffers and Solutions

- **Lysis Buffer:** 2% Triton X-100, 1% SDS, 100 mM NaCl, 10 mM Tris-HCl pH 8, 1 mM EDTA pH 8.0, 1x protease inhibitor cocktail (to be added just before use).
- **Sonication Buffer:** 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 20 Mm Tris-HCl pH 8, 2 mM EDTA pH 8.0,
- **ChIP Dilution Buffer:** 0.75% Triton X-100, 0.1% Na-deoxycholate,140 mM NaCl, 50 mM HEPES pH8.0, 1 mM EDTA, 1x protease inhibitor cocktail (to be added just before use).
- **Low Salt Wash Buffer:** 1% Triton X-100, 0.1% SDS, 150 mM NaCl, 20mM Tris-HCl, pH 8.0, 2mM EDTA pH 8.0.
- **High Salt Wash Buffer:** 1% Triton X-100, 0.1 % SDS, 500 mM NaCl,20 mM Tris-HCl pH 8.0, 2 mM EDTA pH 8.0.
- **LiCl Wash Buffer:** 0.25 M LiCl, 1% NP40, 1% deoxycholate sodium, 10 mM Tris-HCl pH 8.0, 1 mM EDTA pH 8.0.
- **DNA Elution Buffer:** 1% SDS, 50 mM Tris-HCl pH 8, 10mM EDTA pH 8.0.

Human islet culture and fixation

De-identified isolated human pancreatic islets were received from several European Isolation Centers in accordance to local and national regulations. HI were placed on medium upon arrival and cultured for 72h. Next, the samples were manually picked up under a stereomicroscope and fixed with 1% Paraformaldehyde (PFA) at RT for 10 minutes. For all purposes, we assumed that each islet contained approximately 1000 cells. PFA was quenched with 10mM Glycine for 5 min at RT and cells pelleted at 4C for 10 minutes at 500 x g. After washing the sample twice with PBS supplemented with protease inhibitors, the samples were snap frozen and stored at -80C until further usage.

Chromatin shearing

Fixed human islets were thawed on ice and subsequently lysed using ice-cold Lysis Buffer for 15 to 20 minutes while frequently forcing the sample through a 1 ml syringe attached to a 25G to 30G needle with care. Lysed cells were pelleted for 5 minutes at 500 x g at 4C and re-suspended in 130 µL of Sonication Buffer (for co-factor ChIPs) or Lysis Buffer (for subsequent histone or TF ChIPs). The sample was transferred to microTUBE-130 and sheared using the following settings: Duty Factor (DF): 2%, Peak Incident Power (PIP): 105W, Cycles per Bust (CPB): 200, treatment time: 16 minutes.

Sheared chromatin was centrifuged at full speed for 15 minutes at 4C to remove debris and insoluble chromatin. Supernatant was transferred to a fresh low-binding tube to proceed with the ChIP assay and 5% of the lysate was stored at -20C to be used as the input sample.

DNA isolation and assessment of shearing efficiency

In order to optimize and assess the size distribution after chromatin shearing, the equivalent of at least 100,000 cells were de-crosslinked and analyzed using an Agilent High Sensitivity DNA chip. Briefly, 1.5 µl of RNase A was added to the sheared chromatin and incubated at 37C for 30 min. Then, 5 µl of Proteinase K was added followed by de-crosslinking at 65C overnight. Next, DNA was purified using phenol-chloroform and precipitated with sodium acetate and ethanol. Between 5 and 10 ng of purified the DNA was used to assess fragment size distribution.

ChIP

The ChIP protocol was adapted from Pasquali *et al.* and Kagey *et al.* Sheared chromatin was diluted 4 times with ChIP Dilution Buffer (for histones or TF ChIPs) or Sonication Buffer (for co-factor ChIPs). Then, 30 µl of pre-blocked magnetic beads were added to preclear chromatin by rotation for 1h at 4C. Between 1 and 3 µg of the antibody of interest was added to the sample and incubated overnight at 4C while rotating. 50 µl of magnetic beads were added to the sample and rotated at 4C for 2h. Beads were subsequently washed at 4C using a Low Salt Wash Buffer, High Salt Wash Buffer, LiCl Wash Buffer, and TE. Beads were eluted in 300 µL of DNA elution buffer at 65C for 40 min with agitation. Beads were placed on a magnet and supernatant was transferred to a fresh tube and de-crosslinked together with input sample following DNA isolation protocol above.

ChIP-qPCR and ChIP-Seq

RT-qPCR was used to evaluate specific enrichment in at least two positive and two negative genomic regions where the protein of interest is known to be bound. Once the conditions were optimized, immunoprecipitated DNA was quantified and sequenced on the Illumina HiSeq 2500. High quality reads were mapped to hg19 genome and clonal reads were removed. MACS2[3] was used to determine regions of statistically significant enrichment over input. UCSC Browser was used to visualize the data.

RESULTS AND DISCUSSION

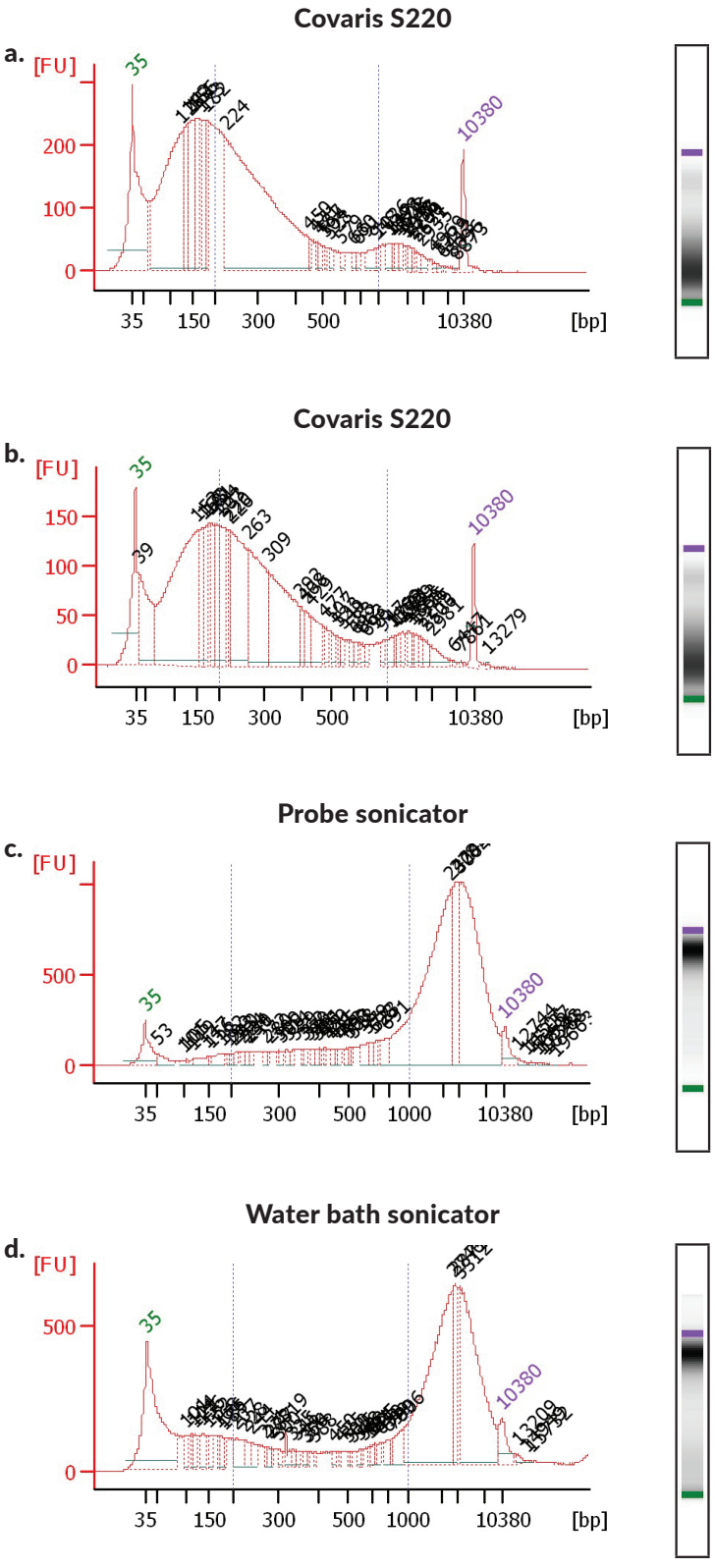

Figure 1. Chromatin shearing in human islets is greatly affected by fragmentation technique. Representative electropherograms of human islets fixed for 10 min at RT and sheared using Covaris S220 (***a***, ***b***), a metal probe sonicator (***c***) or a water bath sonicator (***d***). The X axis measures DNA fragment size in base pairs. The Y axis labels fluorescent units.

Human pancreatic islet samples were processed and fixed using the same conditions and their chromatin sheared using three different mechanical shearing methods available to date. A distribution of fragments between 200 to 1000 bp with minimum amounts of high molecular weight DNA was considered optimal.

Chromatin shearing using the Covaris S220 provided a distribution of fragment sizes that are mostly enriched in the 200 to 600 bp range, which is suitable for successful sequencing (***Fig 1a, b***). In our experience with HI, the presence of high molecular weight fragments is difficult to avoid when using fixation times that are compatible with probe and water bath sonicators. However, the amount of under sheared chromatin when using the Covaris Focused-ultrasonicator is considerably lower when compared to the relative proportion of under sheared chromatin obtained using a metal probe sonicator (***Fig 1c***) or water bath sonicator (***Fig 1d***).

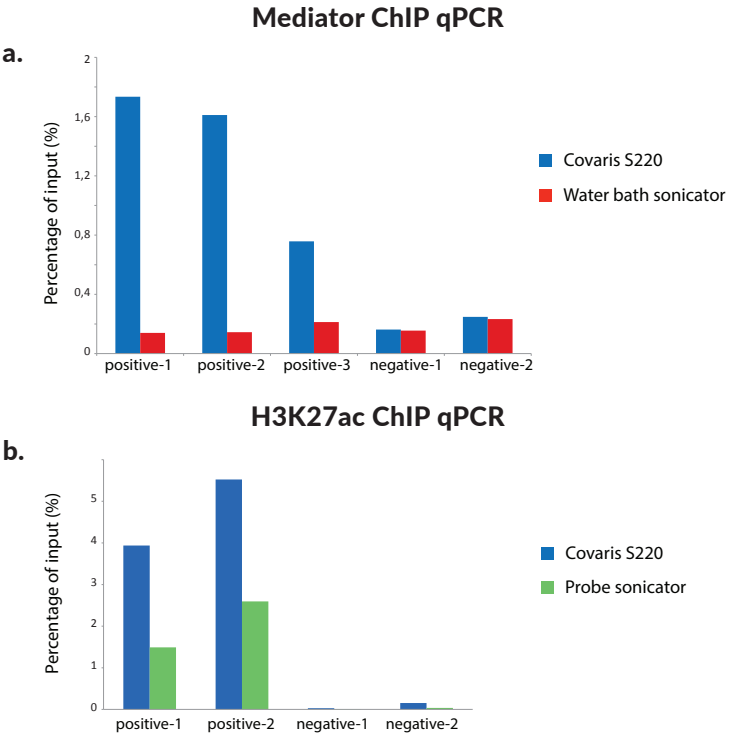

Figure 2. ChIP qPCR results using human islets and several commercially available sonicators. Enrichment of Mediator binding (***a***) and H3K27ac mark (***b***) are represented as percentage of input in several genomic regions where the factors are known to be binding (positive1-3) and 2 negative regions (negative1-2).

In order to assess the quality of the sheared chromatin to be used for efficient ChIP reactions, we proceeded to enrich a protein complex that is loosely bound to the DNA (the co-factor Mediator). To start, HI were fixed and split in two equal parts. One sample was processed using the optimized protocol with Covaris S220 while the other sample was processed using a water bath sonicator. qPCR was performed using the enriched DNA revealed a higher signal to noise ratio in the reactions performed using the Covaris S220. No enrichment in positive regions was observed when using a regular water bath sonicator and equal ChIP conditions suggesting that the

sonication conditions were either inefficient, or led to the destruction of epitope or their release from the DNA (***Fig 2a***). Similarly, superior results were obtained after processing a different human islet sample using the Covaris S220 when compared to a standard probe sonicator using the H3K27ac antibody (***Fig 2b***).

Several Mediator and Cohesin ChIP-Seq libraries produced following this protocol were sequenced. A representative screenshot of PDX1 locus is shown in ***Figure 3***, which depicts a robust and reproducible signal between human donors and expected co-occupancy patterns with other histone modification marks previously mapped.

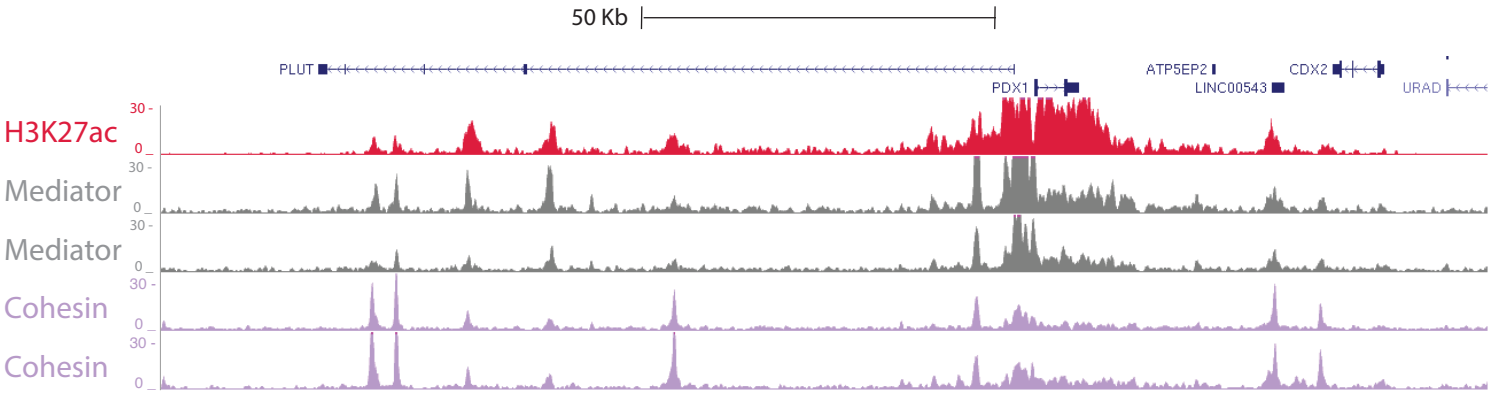

Figure 3. UCSC Browser screenshot of several human islet ChIP-Seq samples on PDX1 locus. A region of chromosome 13 shows specific enrichment of Mediator and Cohesin binding co-factors. Each track represents a different human pancreatic sample donor. Please note reproducibility between donors assayed for the same protein. H3K27ac ChIP-Seq track is added for reference.

CONCLUSION

The main challenge of working with primary human tissue is its inherent variability. The variation may be from the origin of the tissue, isolation center, or cause of death; this will invariably affect the reproducibility of downstream applications such as ChIP-Seq. This sample preparation protocol optimized with the Covaris S220 Focused-ultrasonicator provides consistent chromatin shearing which is suitable for ChIP applications.

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