Simplifying ChIP from Primary Cells and Clinical Samples for High-throughput Epigenetic Applications

Pairing Covaris truChIP® Chromatin Shearing Kit with Chromatrap® ChIP technology

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Abstract

Large-scale analysis of histone marks and transcription factor interactions requires high-quality chromatin, use of validated Chromatin Immunoprecipitation (ChIP)-grade antibodies, and optimized reagents to produce meaningful and reproducible results. In this application note, we demonstrate:

i. Isolation of nuclei using Covaris Adaptive Focused Acoustics® (AFA®) technology followed by purification using a sucrose ultracentrifugation step standardizes ChIP workflows and enables scientists to process-hard-to-lyse samples and reduce input requirements, which is frequently encountered when working with clinical samples.

ii. Pairing Covaris truChIP Kits with Chromatrap bead-free immunoprecipitation (IP) technology provides a simple, sensitive, and scalable workflow for downstream applications, such as ChIP-qPCR and ChIP-seq.

We validated our ChIP protocol, which can be performed within 2 working days, using the well-characterized ubiquitous H3K4me3 histone mark associated with active transcription. Our results indicate that the integration of a highly efficient nuclei isolation step into the Covaris AFA-based sample preparation workflow allows for generating chromatin with a high degree of sensitivity and reproducibility even when working with challenging samples, such as primary human hepatocytes. Combining this improved sample preparation with Chromatrap IP technology results in a highly specific enrichment in ChIP samples shown by a very good signal-to-noise ratio in qPCR as well as ChIP-seq. Furthermore, we show that our protocol is suitable for higher diluted samples from scarce input material as it allows for the usage of higher concentrations of Covaris shearing buffer in the IP step.

Introduction

DNA is a long polymer that if stretched out, would span over two meters long. In order for DNA to fit in the nucleus, the polymer wraps itself around histone octamers to form tight structures called chromatin. The real time changes during compaction of chromatin in eukaryotic cells create a highly dynamic environment that influences gene function.

In recent years, an increasing number of studies have shown that an aberrant epigenome contributes to the onset of many complex diseases such as haematological malignancies (1), behavioural disorders (2), and autoimmune diseases (3). As a result, mapping of histone modifications and transcription factor binding sites using ChIP-Seq has significantly improved our understanding of diseases on an epigenetic level.

Addressing these changes during the onset of disease is technically challenging due to the lack of reliability, speed, ease-of-use, and scalability of traditional workflows. Therefore, we decided to provide a solution for chromatin preparation and shearing of hard-to-lyse primary samples and further evaluate the compatibility of the truChIP chromatin sample preparation workflow with the IP workflow developed by Chromatrap.

This evaluation proved that combining two highly efficient and complimentary workflows allows ChIP assays to be performed within two working days, obtaining high-levels of enrichment with minimal non-specific binding. The workflow allows users to reduce input and can be scaled up for high throughput analysis using the 96-well microplates.

Workflow

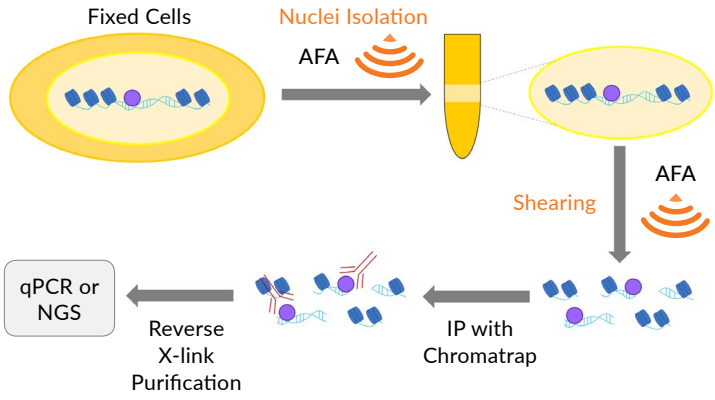


Figure 1: Schematic representation of the ChIP workflow partly adopted from Ling et al. (4) and Arrigoni et al. (5). Nuclei are isolated from fixed cells using AFA and purified using sucrose ultracentrifugation. The pure nuclei fraction is subjected to AFA for nuclei lysis and chromatin shearing. From the sheared chromatin IP is performed using an antibody raised against the protein of interest. Following reverse cross-linking, purified DNA is analyzed for factor/mark enrichment using qPCR or Next Generation Sequencing (NGS).

Materials and Methods

Required Material

Covaris

- M220 Focused-ultrasonicator (PN 500295)
- M220 Holder XTU Insert milliTUBE 1mL (PN 500422)
- M220 Holder XTU (PN 500414)
- milliTUBE-1 mL with AFA Fiber (PN 520130)
- truChIP Chromatin Shearing Kit with Formaldehyde (PN 520154)

Other Suppliers

- Chromatrap ChIP seq Protein G (Product Code: 500190)
- H3K4me3 antibody (Cell Signaling, PN 9751)
- Rabbit IgG (Cell Signaling, PN 2729)
- EpiTect GAPDH primer assay (SABiosciences, PN GPH110001C(+)01A)
- EpiTect MYC primer assay (SABiosciences, PN GPH1012813(-)01A
- EpiTect CYP3A4 primer assay (SABiosciences, PN GPH1025703(+)02A)
- EpiTect IGX1A primer assay (SABiosciences, PN GPH100001C(-)01A)
- RNAse A (Thermo Fisher Scientific, PN EN0531)
- Proteinase K (Qiagen, PN 19131)
- Ultracentrifuge Tubes: Ultra-Clear Centrifuge Tubes, Cat#: 344060 (buffer volumes mentioned in this AppNote are optimized for these consumables)
- Agilent 2100 Bioanalyzer
- Sucrose Buffer for isolation of nuclei (4): 10 mM HEPES-KOH (pH8), 25 mM KCL, 1 mM EDTA, 2M sucrose, 10% glycerol, 0.15 mM spermine, 0.5 mM spermidine, 10 mM NaF, 1 mM orthovanadate, 1 mM PMSF, 0.5 mM DTT, 1x protease inhibitor cocktail

This buffer is prepared and stored at 4 °C as 10 mM HEPES pH 7.9, 25 mM KCl, 1 mM EDTA, 2 M sucrose, and 10% glycerol. The other buffer components: spermine, spermidine, NaF, orthovanadate, PMSF, DTT, and protease inhibitor cocktail are prepared separately as concentrated stock solutions (100 X for NaF, orthovanadate (both in water), PMSF (in isopropanol), and protease inhibitor cocktail (in DMSO); 2000 X for spermine, spermidine, and DTT (all in water)), and stored in aliquots at −20 °C. The concentrated components are then added to the buffer just before tissue dissection and homogenization.

- 1x PBS
- Nuclease-free water
- Agilent High Sensitivity DNA Kit (Agilent Technologies PN 5067-4626)
- PowerUp™ SYBR® Green Master Mix (Thermo Fisher Scientific, PN A25780)QIAquick PCR Purification Kit (Qiagen, PN 28106)
- Sodium Acetate Solution (3 M), pH 5.2 (Thermo Fisher Scientific, PN R1181)
- Spectrophotometer/fluorometer for DNA quantification
- End to end rotator
- Microcentrifuge (4 °C)

Cells

Human induced pluripotent stem cells (Takara Bio, ChiPSC22) were cultured as an adherent monolayer in Essential8 Medium (E8) on vitronectin-coated 100 mm culture dishes. Cells were grown for 72 hours to approximately 80% confluency (approx. 10M cells) at 37 °C, 5% CO_2 in a humidified atmosphere. Dishes were washed by rinsing once with 5 ml cold, sterile 1x PBS before fixation.
2 vials of primary human hepatocytes (PhoenixSongsBiologicals, LHum16114, 12M cells each) were thawed from liquid nitrogen and resuspended in room temperature PBS. Cells were centrifuged at 300xg for 5 min at ambient temperature and washed one time with ice cold PBS before fixation.

Formaldehyde Fixation

10 M hiPSCs/12M PHHs were fixed for 2.5 minutes and 5 minutes respectively using fresh 1% methanol-free formaldehyde in Fixing Buffer A at room temperature on a shaking platform. The fixation was quenched using Buffer E according to manufacturer's instructions (truChIP Chromatin Shearing kit, Covaris, Woburn, MA).

Chromatin Shearing

To perform efficient chromatin shearing without epitope disruption, we used the truChIP Chromatin Shearing Kit. In order to ensure complete lysis of human-induced pluripotent stem cells as well as primary human hepatocytes without retention of cytosolic contaminants, we made minor modifications to the standard Covaris workflow. The original Covaris workflow is modified at the nuclei preparation step (Section B). No modifications were introduced into the cell preparation and cross-linking steps (Section A). In section B, steps 3, 4, and 5 were replaced by the following modifications: (1) Enhanced lysis of cellular membrane by treating hiPSCs/PHHs, resuspended in lysis buffer, in the M220 Focused-ultrasonicator at 75W Peak Power, 5% Duty Cycle and 200 Cycles/Burst for 30/60 seconds (hiPSCs) and 5 minutes (PHHs), respectively. This AFA-supported lysis step replaces steps 3 and 4 of the original workflow and ensures an efficient and robust lysis of cell membrane even in difficult-to-lyse samples. (2) Introduced a cleaning step of isolated nuclei by centrifuging the lysate obtained from (1) at 4 °C, 1700xg for 5 minutes, resuspending in 1 ml supplemented 2 M sucrose buffer and overlaying on top of 6 ml supplemented 2 M sucrose buffer. The overlaid lysate was subsequently centrifuged at 4 °C, 90,000 xg for 60 minutes. Following ultracentrifugation, any cell debris floating on the top was removed prior to continuation with the following steps. This step is critical as any remaining cytosolic contaminations may result in increased non-specific binding in subsequent IP steps. Remaining sucrose buffer was discarded by simply decanting the ultracentrifugation tube. Tubes were placed upside down on ice and any material adhering to the wall was removed by wiping the wall of the tube with a tissue paper soaked in PBS and wrapped around a spatula. This step was repeated with a clean tissue paper to wipe dry the tube wall. This nuclei purification step replaces step 5 of the original protocol. At this point, samples were re-introduced into the original workflow at step 6 (section B) by washing purified nuclei by resuspension in Wash Buffer C. As the nuclei pellet is transparent and sticking to the tube wall, great attention should be paid in subsequent resuspension and centrifugation steps. To minimize sticking of nuclei to the tube wall, it may be helpful to use a centrifuge with a swinging bucket rotor instead of a conventional microcentrifuge (which uses fixed angle rotors) for subsequent centrifugation steps. Optimal chromatin shearing conditions were determined by performing a time course for the two fixation times (see above) and processing hiPSCs/PHHs for 2, 4, 8, 12, 15, and 20 minutes using the Covaris M220 Focused-ultrasonicator following the high cell protocol. 25 µl of sheared chromatin were reverse cross-linked in 100 mM NaHCO3 and 200 mM NaCl at 65 °C overnight. The next day, samples were treated with 2 mg/ml RNAse A at 37 °C for 30 minutes followed by 2 mg/ml Proteinase K at 37 °C for 60 minutes. DNA was purified using QiaQuick PCR Purification Columns. Shearing analysis was carried out on an Agilent 2100 Bioanalyzer using High Sensitivity DNA Chips. The following treatment settings were found to give best IP results in hiPSCs/PHHs: 2.5 minutes fixation/12 minutes shearing (hiPSCs) and 2.5min fixation/20min shearing (PHHs). After effective nuclei isolation chromatin shearing experiments were carried out with identical power settings for all cell types (75W peak Power, 10% Duty Cycle, 200 Cycles/Burst).

Immunoprecipitation and qPCR

To test for flexibility regarding the amount of input material at the IP step when pairing Covaris and Chromatrap workflows, we wanted to check whether we can increase the concentration of lysis buffer D3 which would allow to subject more than the recommended maximal amount of chromatin (100 µl) into the IP. Using 100 µl sheared chromatin each, we carried out IPs against H3K4me3 with increasing total amounts of lysis buffer D3 (100 µl, 500 µl, 1000 µl). Additionally, IPs against normal rabbit IgG with corresponding amounts of antibody and lysis buffer D3 were carried out. 6.14 µg H3K4me3 / 6.14 µg normal rabbit IgG antibodies and 2 µl PIC were added to the IP slurries. The same amount of sheared chromatin was saved as input. All slurries were incubated at 4 °C for 1 hour on an overhead rotator and afterwards immunoprecipitation was performed by passing slurries through a pre-conditioned protein G Chromatrap spin column. The wash and elution steps were subsequently performed according to manufacturer's instructions (Chromatrap, UK). Reverse cross-linking was carried out in 100 mM NaHCO3 and 200 mM NaCl at 65 °C overnight. The next day, samples were treated with 2 mg/ml RNAse A at 37 °C for 30 minutes followed by 2 mg/ml Proteinase K at 37 °C for 60 minutes. DNA purification was carried out using QiaQuick PCR Purification kit according to manufacturer's instructions (Qiagen, Germany).

To assess ChIP efficiency, ChIP-qPCR was performed using the ChIP and undiluted input samples. We used EpiTect primer assays to amplify the GAPDH promoter (housekeeping positive locus for H3K4me3 in human cell types), MYC promoter (specific positive locus for H3K4me3 in hiPSCs), IGX1A (housekeeping negative locus in human cell types) and CYP3A4 (specific negative locus in hiPSCs). The results were plotted as % input precipitated within the ChIP experiments.

ChIP-Seq and Analysis

ChIP for the active histone mark H3K4me3 was performed for two replicates of hiPSCs as well as human hepatocytes. After a quality check of the ChIP experiment with qPCR for respective positive and negative control loci samples were subjected to library preparation using Diagenode MicroPlex Library Preparation Kit v2 (Cat#: C05010012) using 500 pg ChIP'ed DNA as starting material and sequencing was performed on Illumina HiSeq2500.

Alignment was performed by applying Bowtie2, version: 2.3.2 and peaks were called using MACS2, version: 2.1.1.20160309 with filtered read counts (mapping quality >20) and duplicates were removed for further analysis.

Important Notes

- When pelleting the nuclei using the ultracentrifuge it is essential to use swinging bucket rotors in order to pellet nuclei efficiently and avoid loss of material due to nuclei sticking to the wall of the tube when choosing fixed angle rotors. G-force and time have been optimized for the rotor/tube combination highlighted in the material section and might have to be optimized for other consumables. It is essential to use Ultra-Clear Tubes to visualize the transparent nuclei pellet.
- The use of fresh methanol-free formaldehyde is required to achieve reproducible results.
- The methanol-free formaldehyde provided in the truChIP Chromatin Shearing Kit is for one-time use only. Storage for later use is not recommended.
- Please refer to Appendix A of truChIP Chromatin Shearing Kit protocol if using another instrument than the Covaris M220. https://covaris.com/wp-content/uploads/pn_010179.pdf

Results and Discussion

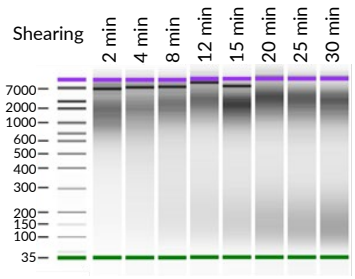
Optimized Nuclei Lysis and Isolation for Reproducible ChIP from Hard-to-Lyse Primary Cell Types

Having observed that primary samples such as primary human hepatocytes are often difficult input material for ChIP, we aimed to develop a simple and reproducible sample preparation workflow to allow reproducible ChIP for such sample types. Since we were not able to observe good chromatin shearing with these hard-to-lyse cell types using standard conditions, we first tried to enhance nuclei extraction by applying AFA to support lysis of the cell membrane. This improvement helped to isolate the nuclei in primary human hepatocytes, but resulted in a fraction of contaminating material that co-sediment with nuclei when centrifuging at 1700xg for 5 min in lysis buffer D3 (***Figure 2A and C***). Such contaminating material even remained after performing an AFA time course during nuclei lysis (2.5, 5, 7.5, and 10 min; data not shown) thereby disturbing the sonication as well as the immunoprecipitation process. Together, these issues during sample preparation end up in transient ChIP results in primary human hepatocytes.

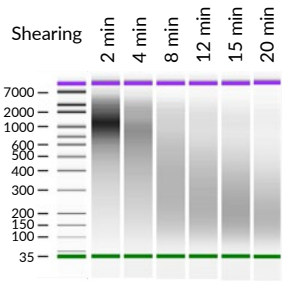
Therefore, we decided to separate the nuclei isolated with AFA from the contaminating material via sucrose ultracentrifugation (***Figure 1***). When shearing these purified nuclei using truChIP kit and AFA, we observed very reproducible shearing results for primary human hepatocytes (***Figure 2B and C***) and a range of other difficult-to-process samples (data not shown) and realized that independent of the cell type, the same settings can be used in the chromatin shearing step when supplying isolated nuclei.

Strikingly, the reproducibility of ChIP enrichments was dramatically improved by this chromatin preparation method suggesting that such a workflow ensures reproducible ChIP when starting with very difficult-to-lyse cell types (***Figure 2D***).

2A. 5 min AFA lysis w/o UC



2B. 5 min AFA lysis with UC



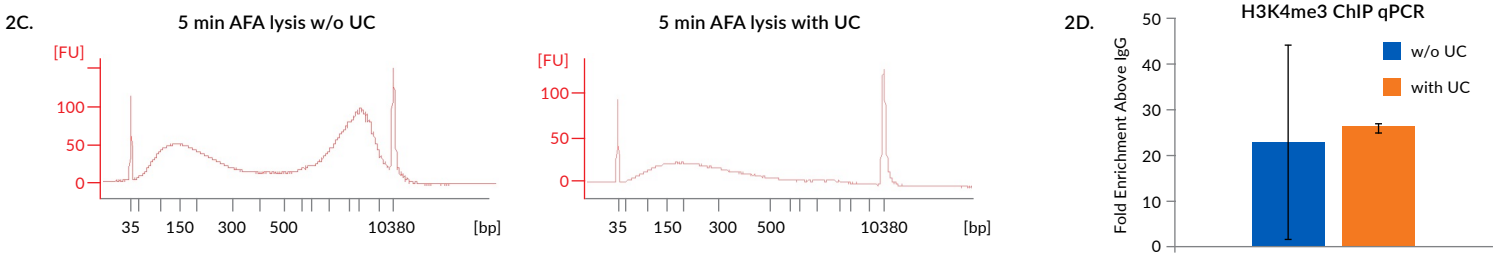


Figure 2: Supporting nuclei extraction with AFA followed by nuclei purification provides a streamlined and reproducible ChIP workflow. A. Nuclei of primary human hepatocytes were isolated using 5 min of AFA and subsequently chromatin was sheared performing a shearing time course (2 to 30 min). B. Same as in A, but nuclei were purified using sucrose ultracentrifugation (UC) and shearing time course was only performed until 20 minutes. C. Electropherograms of maximum shearing time applied in A (left panel) and B (right panel). D: qPCR results for H3K4me3 enrichment at the GAPDH promoter with and without sucrose ultracentrifugation (UC). Fold enrichment above IgG is plotted on the y-axis and error bars reflect SD of three replicates.

Compatibility of Covaris and Chromatrap Workflow

Having developed a streamlined chromatin preparation workflow, which allows comparable results for even hardest to lyse input sample types, we next wanted to test whether we could adopt an easy, reproducible and scalable downstream workflow for ChIP. Therefore, we tested the compatibility of the Chromatrap IP workflow with the upstream Covaris sample preparation workflow using fixed chromatin of adherent monolayers of human induced pluripotent stem cells sheared with optimised conditions using AFA technology. Herein, our results indicated that a fixation time of 2.5 minutes, 30 sec of AFA-supported nuclear extraction, and 12 minutes of acoustic shearing (75W peak Power, 10% Duty Cycle, 200 Cycles/Burst) of chromatin provided the best results and these parameters were then chosen for further experiments (***Figure 3A and B***).

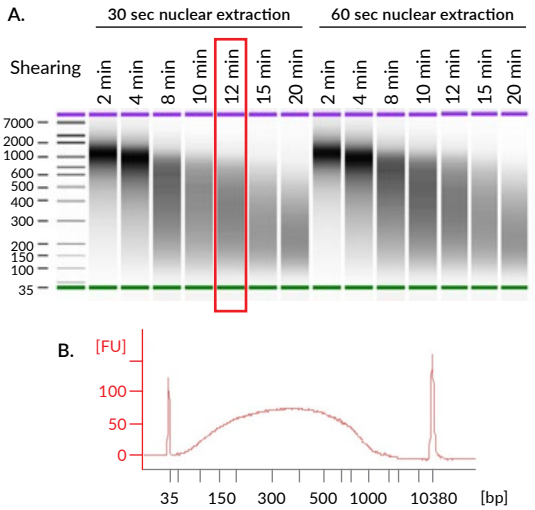


Figure 3: Optimization of chromatin preparation and shearing from hiPSCs. A. 10 M hiPSCs were fixed with methanol-free formaldehyde for 2.5 minutes and subjected to AFA for lysis of cell membrane for 30 sec and 60 sec, respectively. After nuclei purification by sucrose ultracentrifugation, chromatin was prepared using truChIP kit and a sonication time course was performed by applying AFA for 2, 4, 8, 10, 12, 15, and 20 minutes each. After reversal of the crosslinks, purified DNA was resolved by an Agilent 2100 Bioanalyzer using High Sensitivity DNA Chips. B. Electropherogram of the condition highlighted by red box in A which was chosen for later experiments.

Immunoprecipitation was then performed using the H3K4me3 antibody as well as the same amount of rabbit IgG to correct for background enrichment. This experiment revealed that the Covaris truChIP kit paired with the AFA technology for chromatin preparation and shearing integrate well in the Chromatrap ChIP workflow, displaying reproducible and strong enrichment for H3K4me3 at active promoters (***Figure 4***).

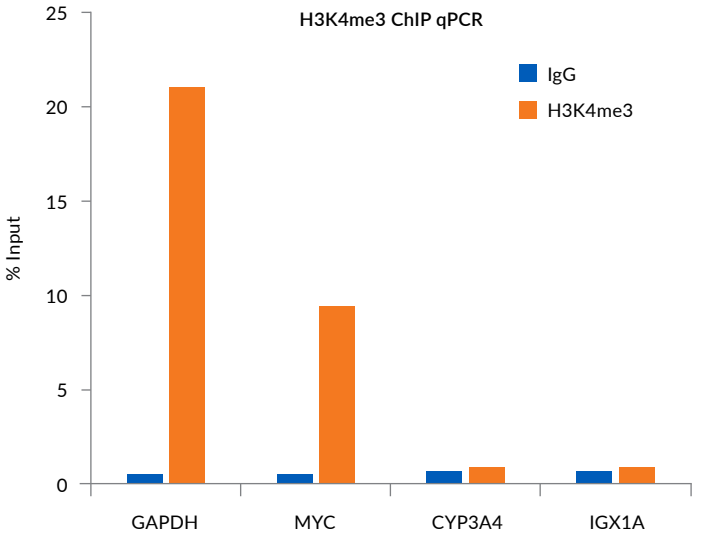


Figure 4: Shearing with Covaris truChIP kit and AFA technology integrates well in the Chromatrap ChIP workflow. Cells were fixed for 2.5 minutes and lysis of cell membrane via AFA was applied for 30 sec. Chromatin was sheared for 12 minutes and IP was carried out for H3K4me3 and IgG control. The enrichment was calculated as percentage of input and plotted on the y-axis. qPCR was performed for two open, active promoters (GAPDH and MYC) as positive controls and two closed, inactive loci (CYP3A4 and IGX1A) as negative controls.

Determing the Maximum Concentration of Shearing Buffer in IP when Pairing Covaris Shearing with Chromatrap Workflow

After observing that Covaris shearing pairs seamlessly with the Chromatrap workflow, we wanted to determine whether a volume larger than 100 µl of sheared chromatin in D3 buffer could be subjected to IP. This will be of relevance when working with highly diluted chromatin e.g. when starting from very limited amount of input material such as primary tissue or scarce cell populations. Therefore, we tested how well different ratios of Covaris D3 shearing buffer and Chromatrap Column Conditioning buffer perform in H3K4me3 IP. To investigate this, we mixed 100 µl of sheared chromatin in D3 buffer with (a) 900 µl Conditioning Buffer, (b) 400 µl D3 buffer and 500 µl Conditioning Buffer or (c) 900µl D3 buffer (***Figure 5***).

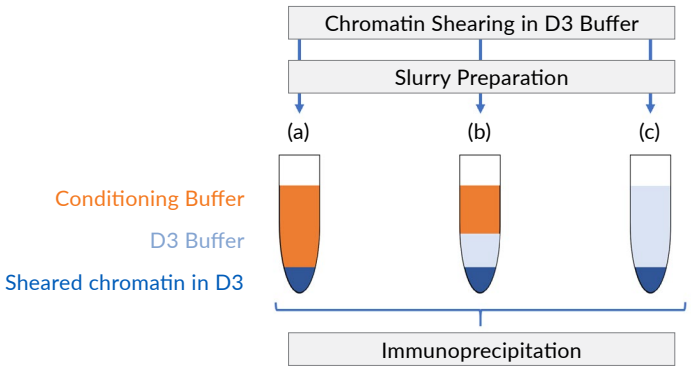


Figure 5: Schematic representation of IP slurry preparation. Chromatin derived from human-induced pluripotent stem cells was sheared in a milliTUBE - 1 ml using the M220 Focused-ultrasonicator. 100 µl of each sample were distributed in three different tubes. To the 100 µl sheared chromatin in D3 buffer, the following was added to the IP slurry to obtain a 1000 µl volume (a) 900 µl of Chromatrap Conditioning Buffer (b) 400 µl Buffer D3 and 500 µl Chromatrap Column Conditioning Buffer or (c) 900 µl of buffer D3. Using these three different slurries, prepared from the same sheared chromatin as starting material, an IP against H3K4me3 was performed followed by qPCR.

Interestingly, input chromatin can be diluted 1:1 with conditioning buffer without losing enrichment in the IP (***(b) Figure 6***). This suggests that without risking any loss in performance, measured by ChIP-qPCR, for low concentrated chromatin samples up to 500 µl sheared chromatin in D3 buffer can be used as input material for the IP. This allows for increased flexibility regarding the amount of input material when working with low input material for ChIP including primary tissues or scarce sample types that involve long protocols for generation. However, as visible from condition (c), dilution of chromatin sheared in D3 buffer is required before subjection to IP and we recommend at least a 1:1 dilution with Chromatrap Column Conditioning Buffer (***Figure 6***).

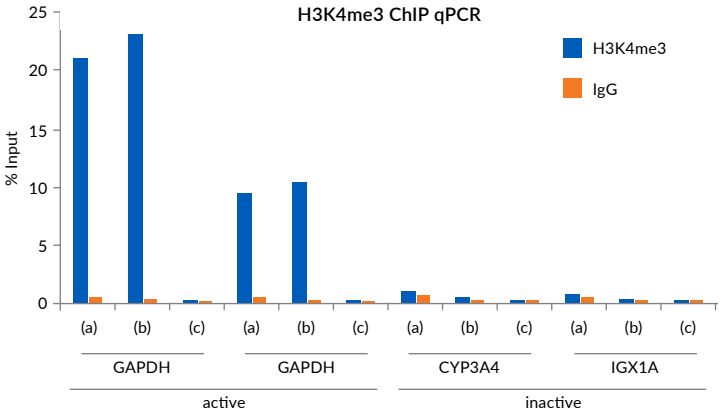


Figure 6: Up to 500 µl sheared chromatin in D3 buffer gives good IP results using Chromatrap workflow. Using the slurries prepared as depicted in Figure 5, IP for H3K4me3 and respective IgG control was conducted, and qPCR was performed for two open, active promoters (GAPDH and MYC) as positive controls and two closed, inactive loci (CYP3A4 and IGX1A) as negative controls. Percentage of input was plotted on the y-axis.

Chromatin Prepared with this Workflow Represents a Highly Suitable Input for ChIP-Seq Experiments

Having observed that chromatin prepared with our optimized workflow gives very good enrichment above background upon ChIP-qPCR, we next asked whether such experiments could deliver good ChIP-Seq data. Therefore we subjected respectively prepared chromatin from iPSCs as well as human hepatocytes to H3K4me3 ChIP followed by next generation sequencing. Visual inspection of multiple genomic loci revealed overall high quality of these data with good signal-to-noise ratio. Furthermore, we observed specific enrichments of the active histone modification H3K4me3 at promoters of highly expressed housekeeping genes such as GAPDH (***Figure 7A and B***). Likewise, tissue-specifically expressed genes such as Nanog for iPSCs and Serpina1 for human hepatocytes displayed high H3K4me3 enrichment while at promoters of tissue-specifically silenced genes this active histone mark was absent (***Figure 7A and B***). Both replicates display very comparable enrichment patterns highlighting the high degree of reproducibility achieved in the workflow.

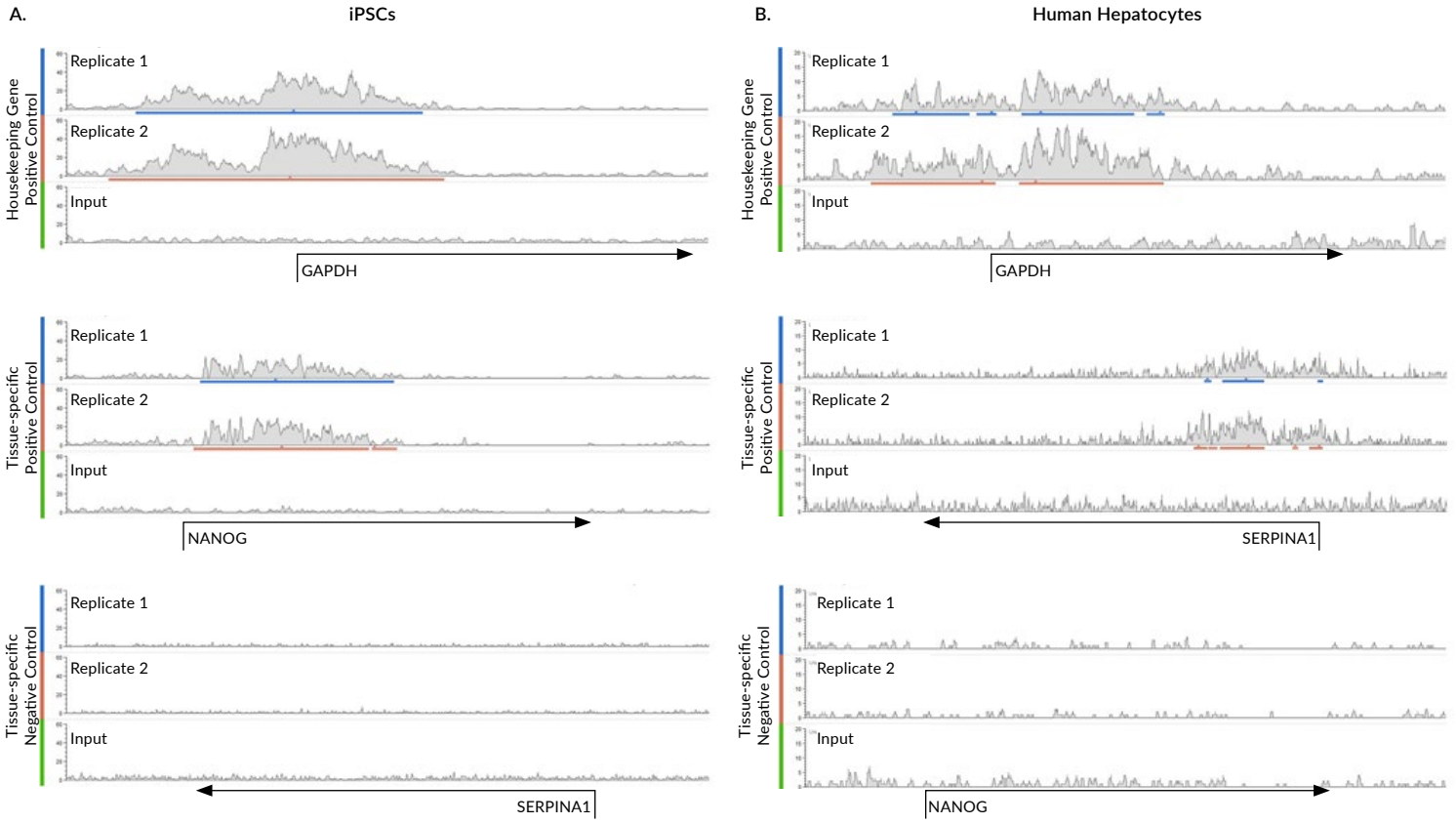


Figure 7: High quality sequencing data are derived from chromatin prepared according to the described workflow. ChIP for the active histone mark H3K4me3 was performed from iPSCs and human hepatocytes. Representative genome browser tracks are shown for two biological replicates as well as input. Tracks are shown for a highly expressed housekeeping gene (HKG) (GAPDH) as positive control (PC), a tissue specifically expressed gene for each cell type (iPSCs: Nanog, human hepatocytes: Serpina1) as positive control and a tissue specifically silenced gene (iPSCs: Serpina1, human hepatocytes: Nanog) as negative control (NC). Arrow direction indicates the direction of expression of the respective gene.

Conclusion

A major challenge of monitoring dynamics of histone modifications and transcription factor binding events on a large scale (e.g. in patient cohorts of different subgroups or during disease progression) has been the ChIP workflow scalability and reproducibility. Especially when working with primary cells, sample preparation is often not straight-forward and hampers the acquisition of reliable data due to non-reproducible chromatin extraction and shearing. For such sample types we found that supporting nuclei extraction with AFA, followed by purification via sucrose ultracentrifugation provides a reliable nuclei preparation workflow resulting in highly reproducible ChIP-Seq enrichments.

Furthermore, the combination of Covaris truChIP sample preparation with the Chromatrap IP workflow provides a desired simple, easy to use method for reproducible ChIP-Seq experiments. The combined workflows remove many of the difficult steps associated with traditional bead-based methods, such as sample loss with bead handling, extended incubation times, wash buffer optimization, and hands-on time required. In summary, our results confirm that Covaris truChIP and AFA technology for chromatin preparation integrates efficiently into the Chromatrap IP workflow. When combined with highly efficient nuclei purification via sucrose ultracentrifugation, the two workflows provide an easy, scalable, and reliable method to prepare even hard to lyse primary mammalian cells for chromatin-based applications.

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