

Optimizing a Dual Fixation Protocol to Study Protein Complexes Binding to Chromatin *in vivo*

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Introduction

Chromatin immunoprecipitation coupled with next-generation sequencing technology (ChIP-seq) has enabled investigators to generate high-resolution maps of genomic binding sites for transcription factors and other members of the chromatin-associated machinery.

Many epigenetic factors that associate with chromatin do so as components of large multi-subunit complexes in which only a subset of the members directly bind to chromatin¹. For example, members the mammalian SWI/SNF chromatin remodeling complex BAF, which promotes proper gene expression and chromatin dynamics, commonly associate with chromatin in assemblies of over ten subunits^{2,3}. The use of ChIP-seq to map the genomic binding of specific members of such complexes often requires stabilization of the complex via protein-protein crosslinking treatments.

ChIP requires optimization of fixing conditions for each tissue or cell line and for each protein complex being studied. Importantly, optimization is required for the subsequent shearing of the chromatin to obtain fragments within the distribution size required for sequencing while simultaneously preserving the integrity of the epitope.

Here, we tested a Covaris-developed chromatin sample preparation protocol to effectively study the interactions of protein complexes associated with chromatin. An initial crosslinking cocktail was used prior to carrying out the Covaris truChIP® Chromatin Shearing Protocol. Relative to a standard single fixation method, this Covaris-developed protocol enhanced signal over background at known binding sites for members of the BAF complex including the ATPase subunit, BRG1⁴. In this application note, we provide shearing profiles, evaluate epitope integrity at each shearing time, and ChIP-qPCR results obtained using the single step formaldehyde fixation and the dual crosslinking protocol.

Materials and Methods

Instrument and Consumables

- E220 Focused-ultrasonicator (Covaris, [PN 500239](#))
- Rack 24 Place milliTUBE 1 mL (Covaris, [PN 500368](#))
- milliTUBE-1mL with AFA fiber (Covaris, [PN 520130](#))

Reagents

- truChIP Chromatin Shearing Kit (Covaris, [PN 520154](#))
- DMA (Thermo Fisher, PN 20660)
- DSG (CovaChem, PN 13301)
- EGS (CovaChem, PN 13308)

Dual Fixation Protocol

Approximately 30 million Caki2 kidney clear cell carcinoma cells in 15 cm plates were washed once with 5 mL of ice-cold 1X PBS. This wash was aspirated, and the cells were rocked in a 5 mL solution of 3 mM EGS, DSG, and DMA crosslinkers in 1X PBS for 5 min at room temperature. This solution was aspirated, and the cells were washed twice with ice-cold 1X PBS. A second crosslinking step was performed by rocking the cells in 5 mL of 1% formaldehyde in 1X Fixing Buffer A at room temperature for 1 min. 300 µL of Quenching Buffer E was added, and the cells were rocked for an additional 5 min. The cells were scraped from the 15 cm plates using a plastic scraper and two 5 mL 1X PBS washes and were transferred to a 15 mL conical vial. The cells were then pelleted by room temperature centrifugation for 5 min at 200 x g. Two washes were performed by suspending the cells in 5 mL of ice-cold 1X PBS followed by centrifugation. The final wash was aspirated completely, and the cells were placed on ice before proceeding to nuclei preparation.

Single Fixation Protocol

A standard single-fixation protocol was performed as a comparative control for the dual fixation method described above. After collection and washing of the Caki2 cells, crosslinking was performed by rocking the cells in 5 mL of 1% formaldehyde in 1X Fixing Buffer A at room temperature for 5 min. 300 µL of Quenching Buffer E was added, and the cells were rocked for an

additional 5 min. The cells were scraped from the 15 cm plates using a plastic scraper and two 5 mL PBS washes and were transferred to a 15 mL conical vial. The cells were then pelleted by room temperature centrifugation for 5 min at 200 x g. Two washes were performed by suspending the cells in 5 mL of ice-cold 1X PBS followed by centrifugation. The final wash was aspirated completely, and the cells were placed on ice before proceeding to nuclei preparation.

Nuclei Preparation

Cell lysis and isolation of nuclei were performed as directed in the Covaris truChIP protocol. In short, cells were suspended in 1 mL of Lysis Buffer B, and the suspension was transferred to a 1.5 mL microcentrifuge tube. The sample was rotated at 4C for 10 min followed by centrifugation at 1,700 x g for 5 min. The lysis buffer was discarded, and the cell pellet was suspended in 1 mL of Wash Buffer C followed by an additional rotation and centrifugation with the same parameters. The wash buffer was removed, and the inner surfaces of the tube were washed with 1 mL of Shearing Buffer D3. The sample was centrifuged, and the shearing buffer was discarded. This wash step was repeated one additional time.

Chromatin Shearing

The supernatant was aspirated completely and the nuclear pellet was suspended in 1 mL of 1X Shearing Buffer D3. This suspension was transferred to a prechilled Covaris milliTUBE 1ml AFA Fiber (PN 520130). Shearing was performed using a Covaris E220 Focused-ultrasonicator with the recommended settings: 140 PIP, 5% duty factor, and 200 CPB. A time course was performed using 3, 6, and 9 minutes as the pre-defined processing intervals. 60 µL of sample was recovered at each time point for sizing and epitope integrity analysis. 60 µL of Shearing Buffer D3 was added to replace the volume removed at each time point.

Chromatin Fragment Sizing

Chromatin shearing was analyzed by preparing an input sample and determining the size distribution for the chromatin fragments using an Agilent bioanalyzer. To prepare the input samples, 1 µL of 10 mg/mL RNase A was added to 25 µL of each sheared chromatin sample. The samples were incubated at 37C for 30 min to degrade the RNA. 1 µL of 10 mg/mL proteinase K was then added to the samples followed by an overnight incubation at 65C on a thermal cycler to reverse the crosslinking. The next day, the DNA was purified using a QIAquick PCR Purification Kit (Qiagen). DNA fragment size distributions were then determined for the resulting purified input DNA samples using the DNA 12000 chip (Agilent Technologies).

Evaluation of Epitope Integrity

Epitope integrity was assayed via Western blot. In short, 10 µL of each sheared chromatin sample was combined with an equal volume of 2X Laemmli buffer (4% SDS, 20% glycerol, 120 mM Tris pH 6.8, bromophenol blue) and heated at 60C for 10 min on a thermal cycler. The samples were then loaded on a NuPAGE precast gradient gel (Thermo Fisher Scientific) and run at a constant current of 40 mA per gel for 1h 15 min. Proteins were transferred to an Immobilon-FL membrane in Towbin buffer (25 mM Tris, 129 mM glycine, 10% methanol, pH adjusted to 8.3) at 120 V for 1h. Western blotting was performed by blocking the membrane with 5% BSA for 1h, incubating with a 1:10,000 dilution of the primary antibody overnight at 4C, washing the membrane 3X with PBS-T, and rocking the membrane in a 1:10,000 dilution of LI-COR IRDye secondary antibody for 1h. Bands were visualized using a LI-COR Odyssey fluorescence imager.

Results and Discussion

Results

Caki2 renal carcinoma cells were harvested and fixed using either the dual crosslinking method or the standard formaldehyde-only method (**Figure 1**). Cell lysis, nuclear isolation, and chromatin purification steps were performed according to the truChIP Chromatin Shearing Protocol. Electropherogram trace files were generated from the shearing time course performed (**Figure 2**). Input samples were prepared, and DNA fragment size distributions were calculated for over sheared (41 to 150 bp) optimally sheared (151 to 700 bp), and under sheared (701 to 7,000 bp) size ranges (**Table 1**).

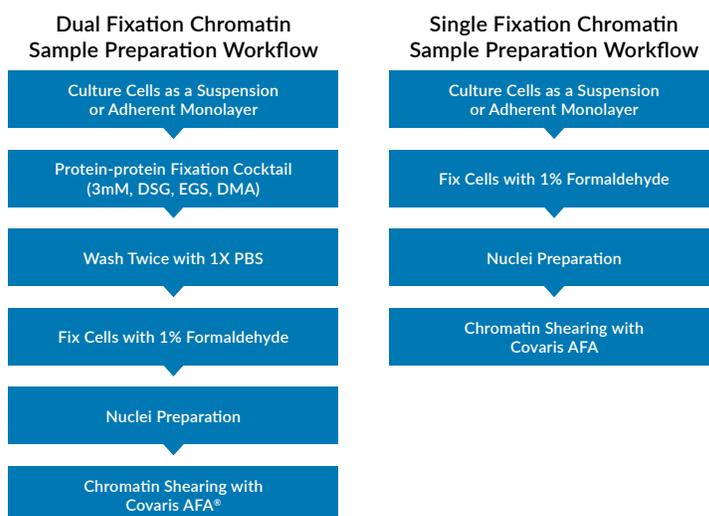


Figure 1. Comparison of dual-fixation and single-fixation workflows.

Shearing Time Course Evaluation

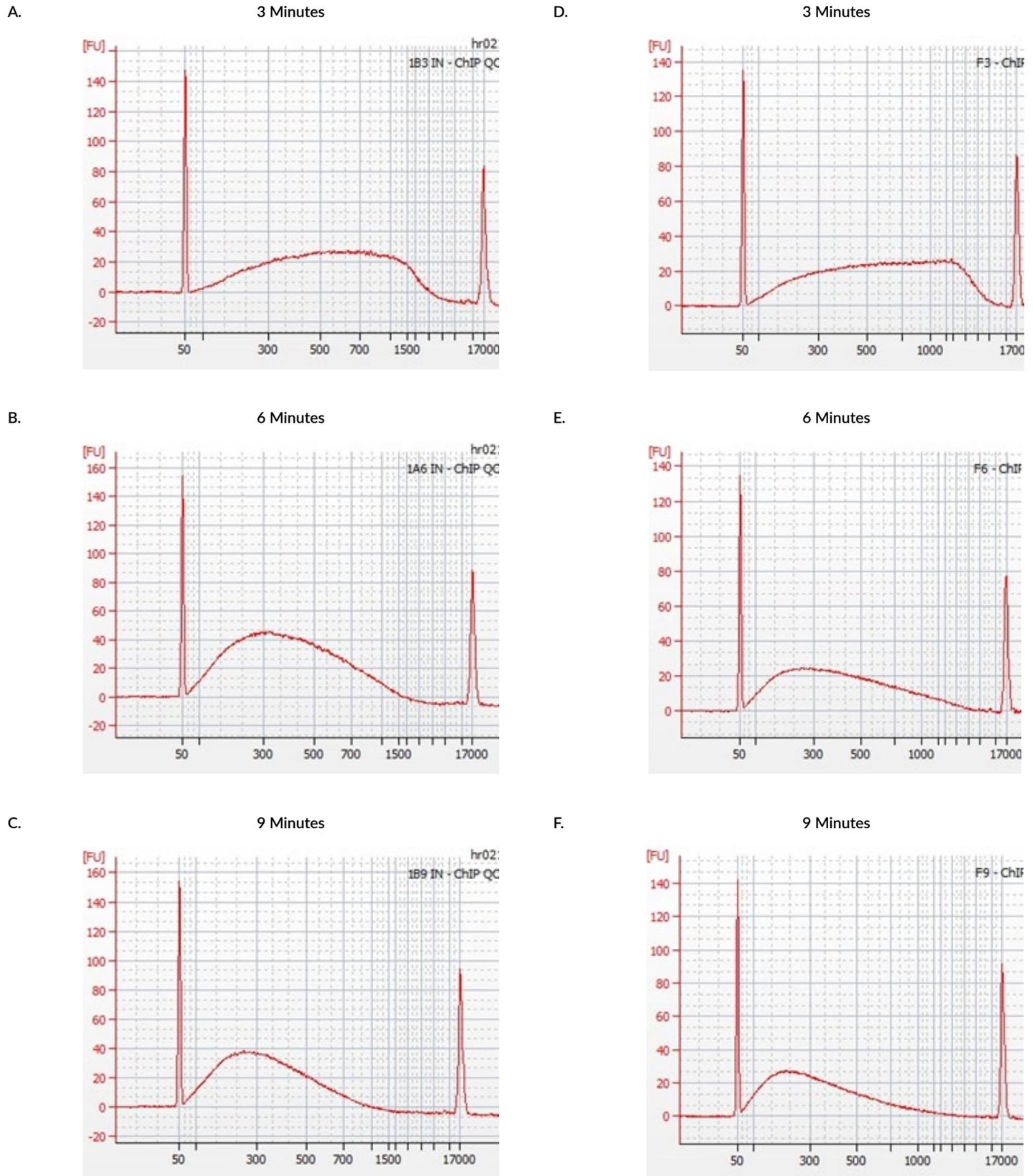


Figure 2. A-C: electropherogram trace files for samples processed using the dual fixation method and sheared for the indicated time. D-F: electropherogram trace files for samples processed using the single fixation method and sheared for the indicated time.

Fragment Size Range	Formaldehyde Only			Dual Fixation		
	3 min	6 min	9 min	3 min	6 min	9 min
41 to 150 bp	6%	9%	12%	3%	7%	11%
151 to 700 bp	79%	83%	81%	54%	72%	78%
701 to 7,000 bp	13%	5%	3%	41%	18%	9%

Table 1. Size distribution of chromatin fragments. Using the regional table function in the Bioanalyzer software, the percentage of fragments within the ranges were calculated for the two fixation methods defined.

Both the dual fixation and formaldehyde-only methods yielded good fragment size distributions at 6 and 9 minutes of shearing. To evaluate epitope integrity under these conditions, we performed immunoblots of BRG1, a large BAF complex subunit that migrates over 250 kDa, using an anti-BRG1 primary antibody. We observed that epitope integrity was maintained through 6 minutes of shearing in both samples, and through 9 minutes of shearing with the dual fixation method (**Figure 2**).

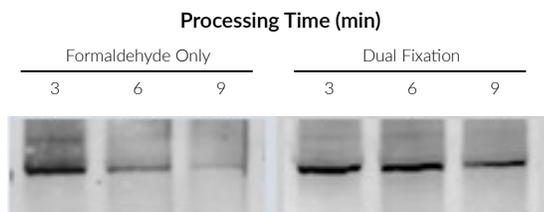


Figure 2. BRG1 (250 kDa) epitope integrity after shearing. Western blot results demonstrate that epitope integrity was maintained through 6 minutes of shearing in both samples, and through 9 minutes with the dual fixation method.

Based on the DNA fragment size distributions and the epitope integrity blot, we proceeded with a 6 minute shearing time for the formaldehyde-only method and a 9 minute shearing time for the dual fixation method. Using these parameters, we performed ChIP-qPCR using an anti-BRG1 antibody and primers that amplified putative BRG1 peaks based on ChIP-seq datasets from similar cell lines. We found that the dual fixation method resulted in increased enrichment over background for the three peak regions relative to the standard single fixation method (**Figure 3**).

References

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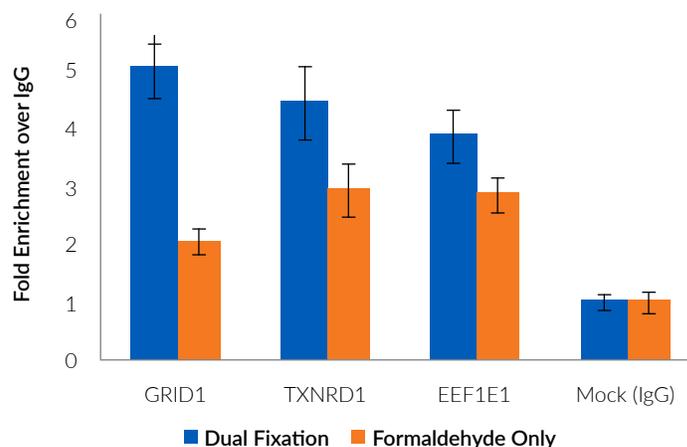


Figure 3. ChIP-qPCR signal over background at three enriched genes and mocks (IgG). ChIP-qPCR analysis of BRG1 enrichment at three promoter regions enriched for the BAF complex in Caki2 cells. Mock immunoprecipitations (IP) are plotted for both the single and dual fixation methods to assess the background non-specific binding. Fold enrichment is displayed relative to the IgG mock ChIP treatment. Error bars indicate one standard deviation between two biological replicates.

Discussion

In this study, we tested the dual fixation protocol for ChIP-qPCR and ChIP-Seq analysis of BAF complex subunits in Caki2 cells. Whereas formaldehyde is typically used to study interactions in very close proximity, the chemical crosslinking agents used in the dual fixation protocol exhibit a variety of crosslinking distances. This enhances stabilization of protein complexes relative to the single fixation method. Examination of chromatin shearing profile of dual fixed samples using Agilent bioanalyzer indicated improvement in fragment size distribution as compared to formaldehyde only fixed samples. Western blot analysis of the sheared chromatin also indicated greater epitope integrity for dual fixed samples when probed with the same antibody used for ChIP. Compared to a standard formaldehyde-only fixation method, the dual fixation protocol enhanced signal over background up to twofold for ChIP-qPCR of BRG1. Taken together, the results indicate that the dual fixation method improves the detection of chromatin-associated machinery in ChIP assays. With optimization of fixation and shearing times, this protocol is suitable for use in other cell lines and conditions.