

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

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Abstract

Chromatin immunoprecipitation coupled with next-generation sequencing technology (ChIP-seq) has enabled researchers 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 chromatin.¹ 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} 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 (DMA, DSG, and EGS) was used prior to carrying out the Covaris truChIP® Chromatin sample preparation protocol for high-throughput sequencing.

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. ChIP requires optimization of fixation 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 target epitope. Relative to a standard single fixation method, our results indicate that the Covaris-developed protocol enhanced signal over background at known binding sites for members of the BAF complex including the ATPase subunit, BRG1.⁴ 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 cells lines and conditions.

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, and the cells were rocked in a 5 mL solution of 3 mM EGS, DSG, and DMA crosslinkers for 5 min at room temperature.
- This solution was aspirated, the cells were washed twice with ice-cold 1X PBS, and 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 with two 5 mL aliquots of 1X PBS and were transferred to a 15 mL conical tube.
- The cells were then pelleted at room temperature centrifugation for 5 min at 200 × g, washed twice in 5 mL of ice-cold 1X PBS, pelleted by centrifugation placed on ice before proceeding to nuclei preparation.

Single Fixation Protocol

- A standard single-fixation protocol was performed as described in the Covaris truChIP chromatin shearing kit protocol.

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, and rotated at 4 °C for 10 min followed by centrifugation at 1,700 × g for 5 min.
- The lysis buffer was discarded and the cells were washed twice in 1 mL of Wash Buffer C. 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

- Nuclear pellet was suspended in 1 mL of 1X Shearing Buffer D3, and transferred to a prechilled Covaris milliTUBE with AFA Fiber (PN 520130).
- Shearing was performed using a Covaris E220 Focused-ultrasonicator with the recommended settings of 140 PIP, 5% duty factor, and 200 CPB for a time course of 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, and replaced with 60 µL of Shearing Buffer D3.

Chromatin Fragment Sizing

- 1 µL of 10 mg/mL RNase A was added to 25 µL of each sheared chromatin sample and incubated at 37 °C for 30 min.
- 1 µL of 10 mg/mL proteinase K was then added to the samples followed by an overnight incubation at 65 °C 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 60 °C 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 120V for 1 hour.
- 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 4 °C, 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

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**).

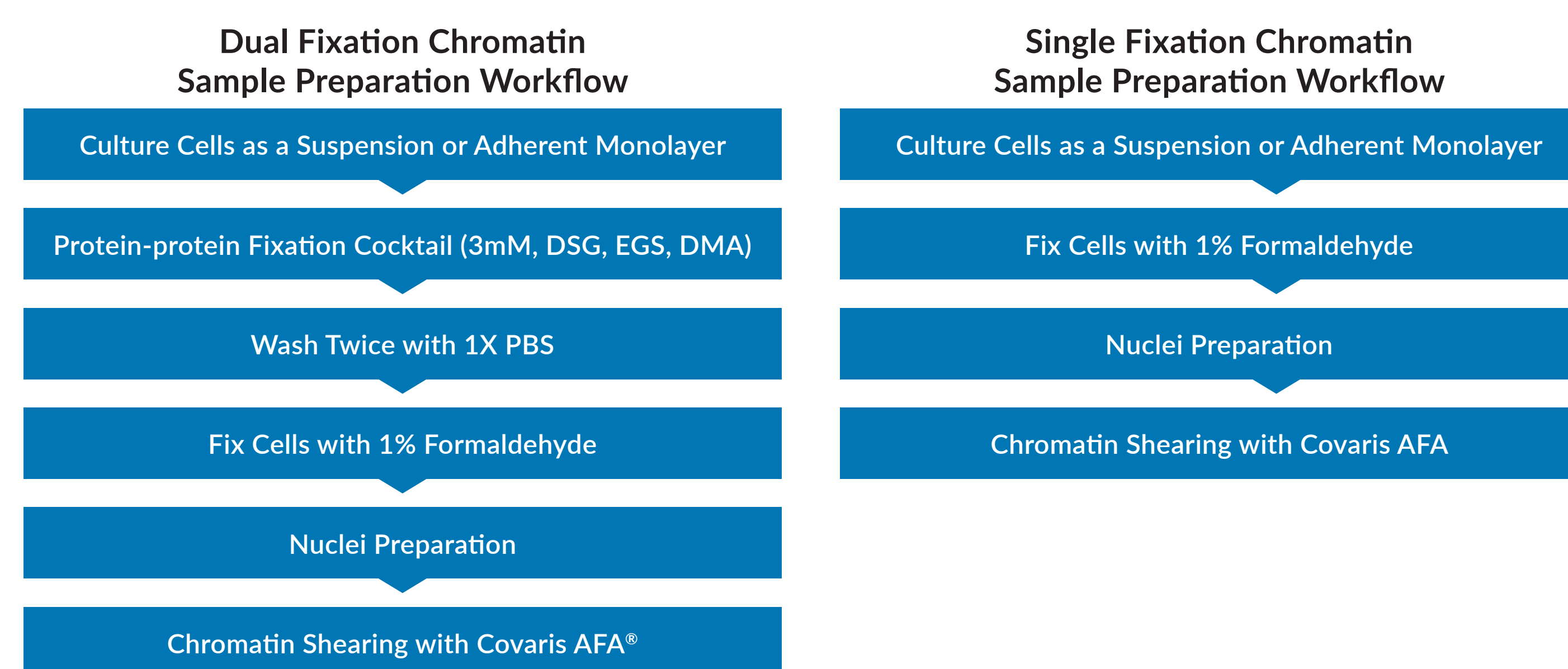


Figure 1. Comparison of dual-fixation and single-fixation workflows.

Shearing Time Course Evaluation

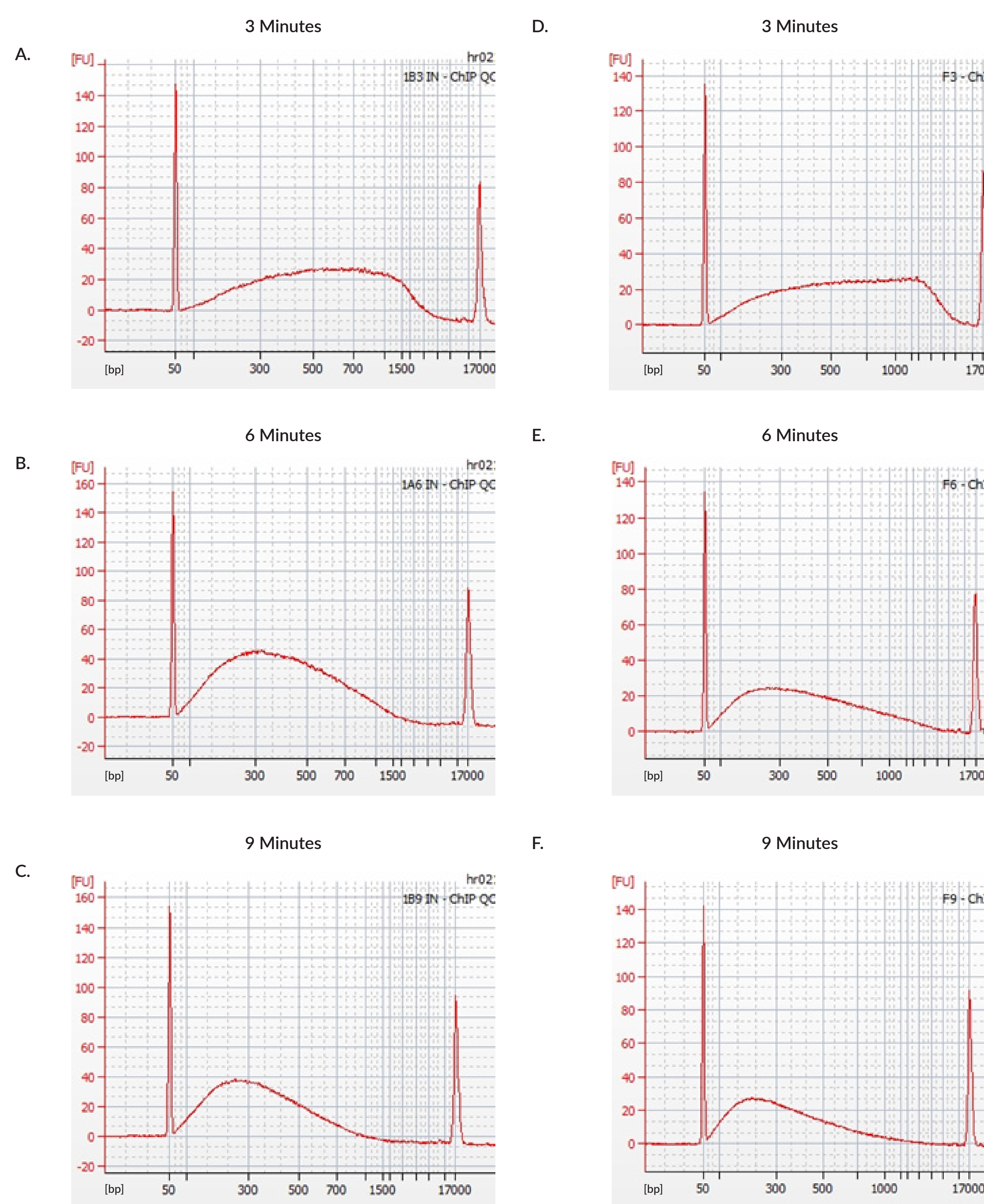


Figure 2. A-C: electropherogram trace files for samples processed using the dual fixation method and sheared for the indicated time. D-F: electropherogram trace files for samples processed using the single fixation method and sheared for the indicated time.

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**).

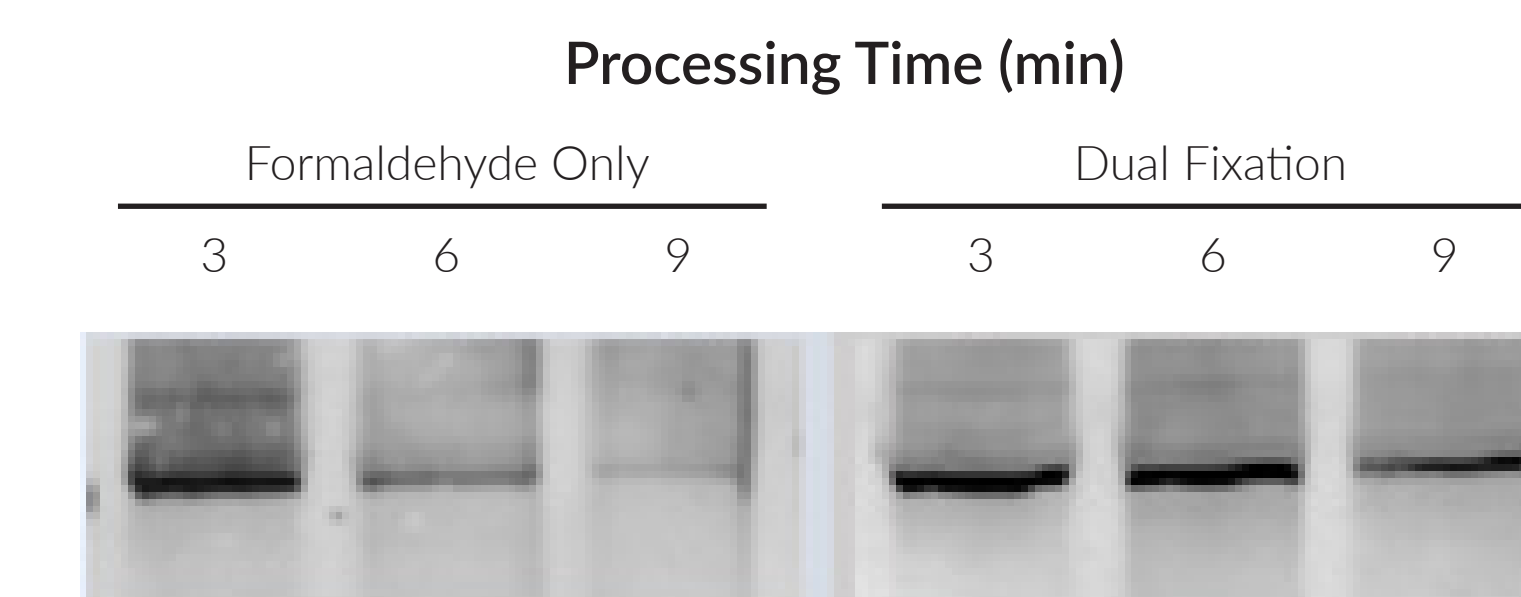


Figure 2. BRG1 (250 kDa) epitope integrity after shearing. Western blot results demonstrate that epitope integrity was maintained through 6 minutes of shearing in both samples, and through 9 minutes with the dual fixation method.

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**).

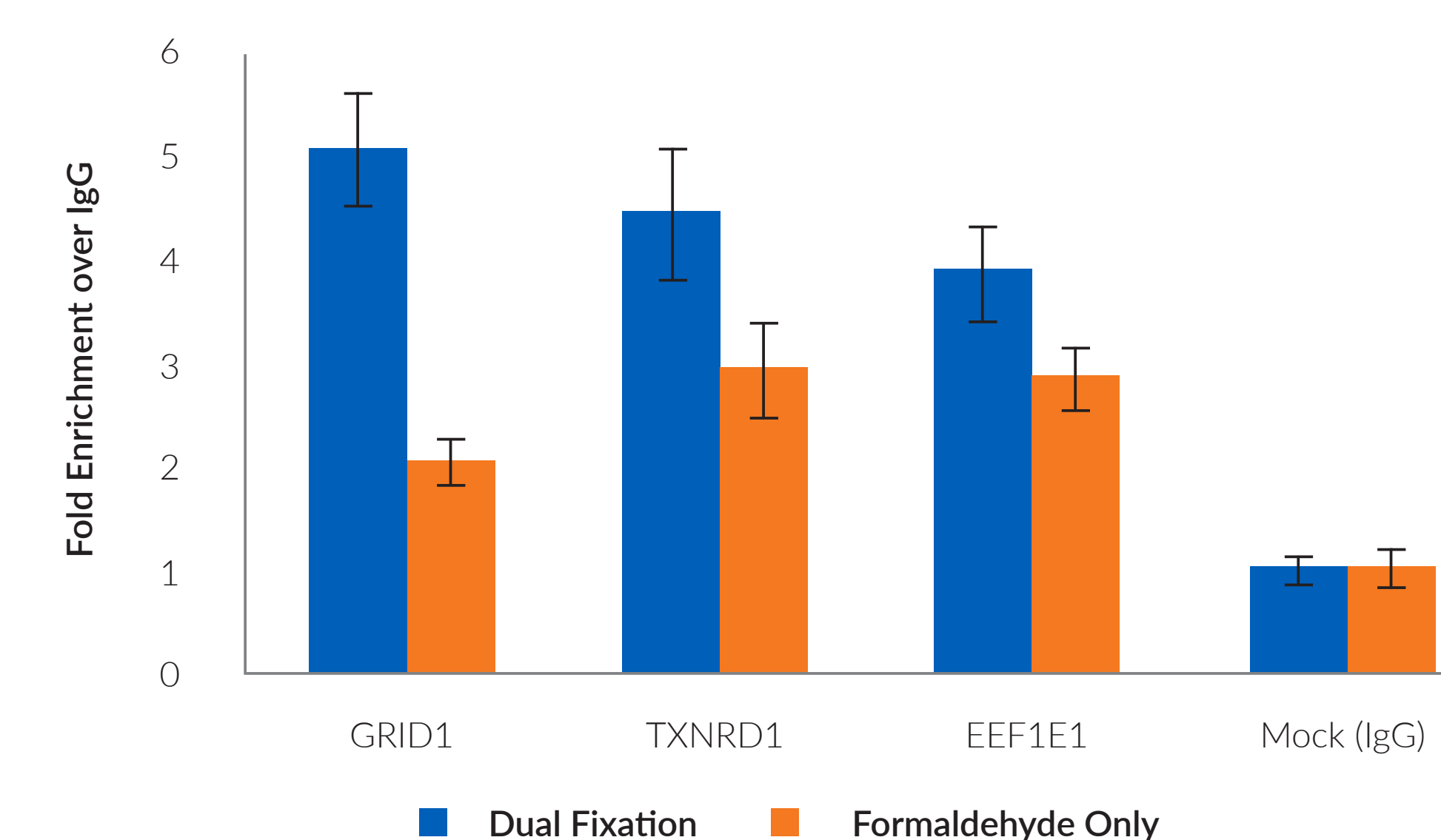


Figure 3. ChIP-qPCR signal over background at three enriched genes and mocks (IgG). ChIP-qPCR analysis of BRG1 enrichment at three promoter regions enriched for the BAF complex in Caki2 cells. Mock immunoprecipitations (IP) are plotted for both the single and dual fixation methods to assess the background 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 cells lines and conditions.

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