Video Article

A Filtration-based Method of Preparing High-quality Nuclei from Cross-linked Skeletal Muscle for Chromatin Immunoprecipitation

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Abstract

Chromatin immunoprecipitation (ChIP) is a powerful method to determine protein binding to chromatin DNA. Fiber-rich skeletal muscle, however, has been a challenge for ChIP due to technical difficulty in isolation of high-quality nuclei with minimal contamination of myofibrils. Previous protocols have attempted to purify nuclei before cross-linking, which incurs the risk of altered DNA-protein interaction during the prolonged nuclei preparation process. In the current protocol, we first cross-linked the skeletal muscle tissue collected from mice, and the tissues were minced and sonicated. Since we found that ultracentrifugation was not able to separate nuclei from myofibrils using cross-linked muscle tissue, we devised a sequential filtration procedure to obtain high-quality nuclei devoid of significant myofibril contamination. We subsequently prepared chromatin by using an ultrasonicator, and ChIP assays with anti-BMAL1 antibody revealed robust circadian binding pattern of BMAL1 to target gene promoters. This filtration protocol constitutes an easily applicable method to isolate high-quality nuclei from cross-linked skeletal muscle tissue, allowing consistent sample processing for circadian and other time-sensitive studies. In combination with next-generation sequencing (NGS), our method can be deployed for various mechanistic and genomic studies focusing on skeletal muscle function.

Video Link

The video component of this article can be found at https://www.jove.com/video/56013/

Introduction

Skeletal muscle plays important roles in physiology and behavior. The multi-nucleated muscle fiber consists of myofibrils where actin and myosin form functional units called sarcomeres to generate contractile force. Skeletal muscle is also the largest metabolic organ in the body, accounting for >80% postprandial glucose intake and regulating insulin response and metabolic homeostasis[1,2]. Muscle physiology and metabolism are closely regulated by the circadian clock, an intrinsic biological timer[3,4,5,6]. For example, the skeletal muscle-specific deletion of *Bmal1*, one of the core circadian clock components, resulted in insulin resistance and diminished glucose uptake in skeletal muscle, and the animals were found to develop type 2 diabetes[7]. In addition, skeletal muscle is also increasingly being appreciated as an endocrine organ[8], secreting myokines to regulate systemic metabolism and physiology. Mechanistic studies are required to fully understand these regulatory functions in skeletal muscle.

ChIP is a powerful approach to delineate promoter recruitment of DNA binding proteins. ChIP was initially developed to identify nucleosome organization on chromatin DNA[9,10]. A variety of methods have since been reported to cross-link proteins and chromatin DNA using formaldehyde, dimethyl sulfate or ultraviolet irradiation (UV)[11,12]. Formaldehyde cross-linking is the most commonly used, preserving chromatin structure and DNA-protein interactions[9,13,14]. Cross-linked chromatin is shredded by sonication and immunoprecipitated with antibody against the particular DNA binding protein of interest[15,16]. In recent years, ChIP-sequencing (ChIP-seq), a method combining ChIP with NGS, has been developed to interrogate genome-wide transcription factor binding[17], and in some cases to monitor dynamic changes over a time course[18,19,20]. For example, circadian ChIP-seq studies have revealed a highly orchestrated sequence of genomic binding of circadian clock components and histone markers, which drives temporally precise gene expression throughout the ~ 24 h circadian cycle[18].

Most available ChIP protocols are designed for soft tissues (*e.g.* liver, brain, *etc.*), and very few have been published for hard tissues including skeletal muscle. It is technically challenging to homogenize fiber-rich skeletal muscle and isolate high-quality nuclei[21], especially for ChIP experiments which require cross-linking. In a recent muscle ChIP study[22], satellite cells were separated from myofibers, and nuclei were prepared from both cell types through a prolonged procedure involving tissue digestion. The entire process took approximately three hours to complete before formaldehyde cross-linking was performed on isolated nuclei. While this procedure avoided cross-linking muscle fiber, which makes muscle tissue even more refractory to efficient homogenization, and was able to produce high-quality nuclei, the significant time-lag from tissue collection to nuclei cross-linking incurs the risk of altered DNA-protein interaction. In contrast, most studies performed cross-linking immediately after experimental treatment or tissue collection in order to preserve the real-time DNA-protein binding[12]. A second drawback of nuclei isolation before cross-linking is that it precludes time-sensitive applications such as circadian sample collection which typically occurs at 3

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- 4 h intervals. Without cross-linking the nuclei, the isolation needs to proceed immediately after dissection, whereas cross-linked samples can be processed together after the entire time course is completed, thus ensuring greater experimental consistency.

Other protocols for nuclei isolation from uncrosslinked skeletal muscle have also been reported. Two studies described the use of gradient ultracentrifugation to separate nuclei from remaining myofibrils and cell debris[23,24]. While sucrose or colloidal gradient ultracentrifugation is effective with uncrosslinked muscle tissues, our experiments revealed that after crosslinking, gradient ultracentrifugation failed to separate nuclei from cell debris on the gradient.

We therefore developed a procedure to isolate high-quality nuclei using cross-linked mouse skeletal muscle tissues. Rather than gradient ultracentrifugation, we devised a serial filtration method to effectively separate nuclei from debris. Following ultrasonication, the chromatin samples were successfully applied for ChIP studies which showed a circadian pattern of BMAL1 protein binding to target promoters. Our method can be broadly applicable to various mechanistic studies of muscle tissues.

Protocol

Animal care was performed under Institutional Animal Care and Use Committee (IACUC) guidelines, and the procedures were conducted according to an animal protocol approved by the University of Texas Health Science Center at Houston.

1. Nuclei Isolation from Cross-linked Skeletal Muscle

1. Weigh and mince hind limb skeletal muscle, isolated from approximately 20-week old C57BL/6 male mice, in ice-cold phosphate buffered saline (PBS) as described in detail previously[22].
   NOTE: We usually obtain 1.0 - 1.5 g of skeletal muscle from one mouse.
   1. Place minced skeletal muscle tissue in a 50-mL conical tube containing 10 mL PBS on ice.
2. Centrifuge at 300 x g at 4 °C for 5 min.
3. Carefully remove PBS supernatant by aspiration.
4. Estimate pellet volume in each 50-mL tube by using reference tubes with 1, 2, 3 and 4 mL of water.
5. Add 7 volumes of ice-cold 1% formaldehyde in PBS and homogenize the samples on ice using a probe tissue homogenizer (see **Table of Materials**).
   NOTE: Optional: The homogenization procedure can be conducted in a cold room.
6. Cross-link the sample by incubating at room temperature for 10 min.
7. Quench the cross-linking reaction by the addition of 1 M glycine to a final concentration of 0.125 M and incubate for 5 min at room temperature.
8. Centrifuge the samples at 3,000 x g for 5 min at 4 °C. Remove the supernatant via aspiration.
9. Rinse with 10 mL of ice-cold base buffer (10 mM HEPES-KOH at pH 7.3, 10 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT) containing freshly added protease inhibitors (0.2 mM phenylmethylsulfonyl fluoride (PMSF), and 10 µg/mL leupeptin). Centrifuge at 3,000 g for 5 min at 4 °C and carefully remove the supernatant by aspiration.
10. Resuspend the pellet in 6 mL of lysis buffer (10 mM HEPES-KOH at pH 7.3, 10 mM EDTA, 10 mM KCl, 5 mM $MgCl_2$, 0.5 mM DTT, 1% Triton X-100) containing freshly added protease inhibitors (0.2 mM PMSF, and 10 µg/mL leupeptin).
11. Transfer the sample to a prechilled 15 mL Dounce homogenizer and incubate for 10 min on ice.Dounce homogenize each sample on ice with 15 slow strokes using loose pestle followed by 15 strokes with tight pestle to release the nuclei.
12. Filter the homogenate (6 mL) through cell strainer (pore size: 100 µm) and rinse with 4 mL of lysis buffer. Centrifuge the samples at 1,000 x g for 10 min at 4 °C. Resuspend into 5 mL of ice-cold base buffer and dounce 10 times with tight pestle to release nuclei.
    1. Remove an aliquot of 20 µL for DNA concentration measurement with a spectrophotometer (OD260/OD280) and microscopic observation with trypan blue if necessary (see below) (**Figure 1A**).
13. Filter the suspension through a cell strainer (Pore size: 70 µm), rinse the tube and filter again with 2 mL base buffer as above.
14. Repeat the filtration as in step 1.13 with cell strainers of gradually reduced pore size (40 µm, 30 µm, 20 µm and 10 µm).
    NOTE: In total, strainers of 6 pore sizes were used in steps 1.12-1.14.
15. Centrifuge at 1,000 g at 4 °C for 10 min. Remove supernatant and resuspend the pellet in 500 µL base buffer.
16. Measure DNA concentration as above.
    NOTE: Approximately 200 µg of nucleic DNA was obtained from combining both hind limbs from one animal. Optionally, save 20 µL for microscopic observation with trypan blue staining (stock concentration 0.4%, 1:5 dilution); nuclei will stain blue (**Figure 1B**).
17. Centrifuge at 1,000 g at 4 °C for 10 min, and discard the supernatant. Store pellet at -80 °C.

2. Sonication

1. Thaw samples in 500 µL of SDS lysis buffer and resuspend with gentle pipetting.
2. Transfer the nuclei DNA suspension to a glass vial on ice.
3. Run the sonication with focused bursts of ultrasonic acoustic energy from a dish-shaped transducer. See **Table of Materials** for equipment details. Use the following setting: cycles per burst: 200; intensity: 5; duty cycle: 20; temperature 4 - 6 °C; 30 s on/off.
   NOTE: An example for evaluating sonication efficacy is shown in **Figure 2**.
4. Transfer the sonicated chromatin to a 1.5 mL tube. Centrifuge at 12,000 x g for 15 min and transfer the supernatant to a new tube. Transfer 50 µL of sonicated samples (~ 7 - 10 µg/muscle sample) into a new 1.5 mL tube for reverse crosslinking overnight at 65 °C. Freeze the remaining samples at -80 °C.

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3. Evaluation of Sonication and Quantitation

1. Add 1.0 µL of RNase A (500 U/mL RNase A: 20,000 U/mL RNase T1) to the saved 50 µL sonicated samples and incubate for 30 min at 37 °C.
2. Add 8 µL of protease K (10 mg/mL) and incubate 30 min at 55 °C.
3. Harvest the DNA by using a DNA extraction kit.
    1. Add 5 volumes of binding buffer, apply it to spin column, and centrifuge at 4,000 x g, 10 min.
    2. Apply the pass-through onto the same column and centrifuge again.
    3. Wash with washing buffer two times at 13,000 x g, 1 min.
    4. Centrifuge again at 13,000 x g, 1 min to dry the column.
    5. Add 50 µL of H_2O and centrifuge at 13,000 x g, 1 min to elute DNA.
    6. Apply the pass-through onto the same column and centrifuge again.
4. Quantify the amount of DNA with a spectrophotometer (OD260/OD280). Run samples on 0.8% gels to evaluate the size and amount (1 µg) of the sonicated products (**Figure 2**).
   NOTE: The average chromatin yield is approximately ~ 20%.

4. ChIP

1. Thaw the previously saved chromatin at -80 °C and aliquot the chromatin to ~ 100 - 120 µg per tube. Dilute 1:10 with radioimmunoprecipitation assay (RIPA) buffer (20mM Tris-HCl (pH 7.4), 150mM NaCl, 1mM EDTA, 1mM NaF, 1mM Na3VO4, 1mM PMSF, 1x Protease inhibitor cocktail (PIC), 0.1% Na-deoxycholate (w/v), 1% Triton X-100).
    1. If there are multiple samples in each group, combine equal amounts of chromatin DNA to the final amount of ~ 100 - 120 µg/sample. Save 10% as input.
2. Add 40 µL (50% v/v slurry in RIPA buffer) of BSA-pre-blocked (~ 2 h, 4 °C) Protein A/G agarose (non-Chicken antibody) or IgY beads (Chicken antibody) and incubate on a rotator for 3 h at 4 °C.
3. Centrifuge at 1,000 x g, 10 min and carefully transfer the supernatant to new tubes.
4. Add antibodies at 1 µg antibody per ~ 25 - 100 µg chromatin DNA.
5. Rotate gently at 4 °C overnight at approximately 15 rpm.
6. Add 10 µL of BSA-pre-blocked (~2 h, 4 °C) Protein A/G agarose (non-Chicken antibody) or IgY beads (Chicken antibody) mix and rotate in the cold room for 2 h.
7. Wash beads as follows. Wash with 1 mL of RIPA buffer for 3 min twice, wash with 1 mL of high salt buffer (20mM Tris-HCl (pH 7.4), 500mM NaCl, 2mM EDTA, 1mM PMSF, 1% Triton X-100) for 3 min twice, wash with 1 mL of LiCl buffer (20mM Tris-HCl (pH 7.4), 250mM LiCl, 2mM EDTA, 1mM PMSF, 1% Na-deoxycholate, 0.5% NP40) for 3 min twice.
    1. Wash with 1 mL of Tris-EDTA (TE) buffer (10mM Tris-HCl (pH 7.4), 1mM EDTA) for 3 min once. Then elute DNA with elution buffer (20 mM Tris-HCl, 5 mM EDTA and 0.5% SDS).
8. Centrifuge at 1,000 g, 1 min and carefully remove the supernatant.
9. Resuspend the bead with 50 µL of elution buffer.
10. Incubate for 10 min at 65 °C.
11. Centrifuge at 12,000 g for 5 min.
12. Transfer the supernatant to a new tube, add another 50 µL and centrifuge once again at 12,000 g for 5 min. The final elution volume will be 100 µL.
13. Reverse crosslinking and DNA elution.
    1. Incubate the eluted chromatin overnight at 65 °C.
    2. Add 1.0 µL of RNase A and incubate for 30 min at 37 °C.
    3. Add 8 µL of protease K (10 mg/mL) and incubate 30 min at 55 °C.
    4. Harvest the DNA by using a PCR cleanup kit with elution volume at 50 µL according to the manufacturer's protocol.
14. Analyze the profiles by Real-time qPCR as previously described[25,26].
    NOTE: The primer sequences are listed in **Figure 3 Legend**. Data are presented as mean ± SEM (**Figure 3**).

Representative Results

Here we performed formaldehyde cross-linking immediately after tissue collection to preserve real-time DNA-protein interaction. However, we found that sucrose or colloidal gradient, commonly used for nuclei isolation[23,24], was not effective in separating nuclei from myofibrils (data not shown). The reason may be that cross-linking conferred similar gravity for nuclei and myofibrils. Therefore, we developed a serial filtration process to effectively remove large myofibrils and other debris from the nuclei fraction. After 100 µm filtration, there were still large tissue debris and myofibrils (**Figure 1A**). In comparison, at the end of the sequential filtration, the majority of large tissue debris, intact cells and large myofibrils were successfully removed (**Figure 1B**).

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Those nuclei were used for dish-shaped ultrasonication. Nuclei stored at -80 °C were suspended in SDS lysis buffer and immediately subjected to sonication. Although incubation of nuclei in SDS lysis buffer on ice or room temperature may improve sonication for some tissue such as liver[27] or mouse embryonic fibroblast (MEF)[28], in our experience it interfered with sonication of skeletal muscle nuclei and resulted in broad smearing, indicating ineffective sonication. Using the current protocol with immediate sonication after suspension in SDS lysis buffer we observed efficient sonication with chromatin gradually shredded in a cycle-dependent manner and eventually producing ~ 500 bp DNA fragments (**Figure 2**). Pre-incubation in the lysis buffer detectably compromised the sonication efficiency.

To confirm the quality of chromatin preparation for ChIP, we examined DNA binding of the circadian transcription factor BMAL1, which showed binding peak and trough at Zeitgeber time (ZT)6 and ZT18, respectively[18,29]. Skeletal muscle samples from C57B/6J mice were collected at ZT6 and ZT18, and chromatin samples were prepared as above. Briefly, after sonication, shredded chromatin samples were pre-cleared with IgY beads that have been pre-blocked with BSA followed by incubation with anti-BMAL1 antibody at 4 °C overnight. After elution and purification of chromatin, we performed RT-qPCR to detect BMAL1 binding on E-Box elements of two target genes, *Nr1d1* and *Dbp*[30]. We detected robust binding of BMAL1 to the E-box elements at ZT6 and minimal binding at ZT18 (*Nr1d1*: 0.452 ± 0.022 vs. 0.039 ± 0.002, *Dbp* -0.4: 0.627 ± 0.013 vs. 0.062 ± 0.009, *Dbp* +0.8: 0.176 ± 0.013 vs. 0.008 ± 0.001, *Dbp* +2.4: 0.466 ± 0.010 vs. 0.122 ± 0.014; all values are mean ± SEM) (**Figure 3**), validating the protocol for time-sensitive transcription factor binding analysis in skeletal muscle.

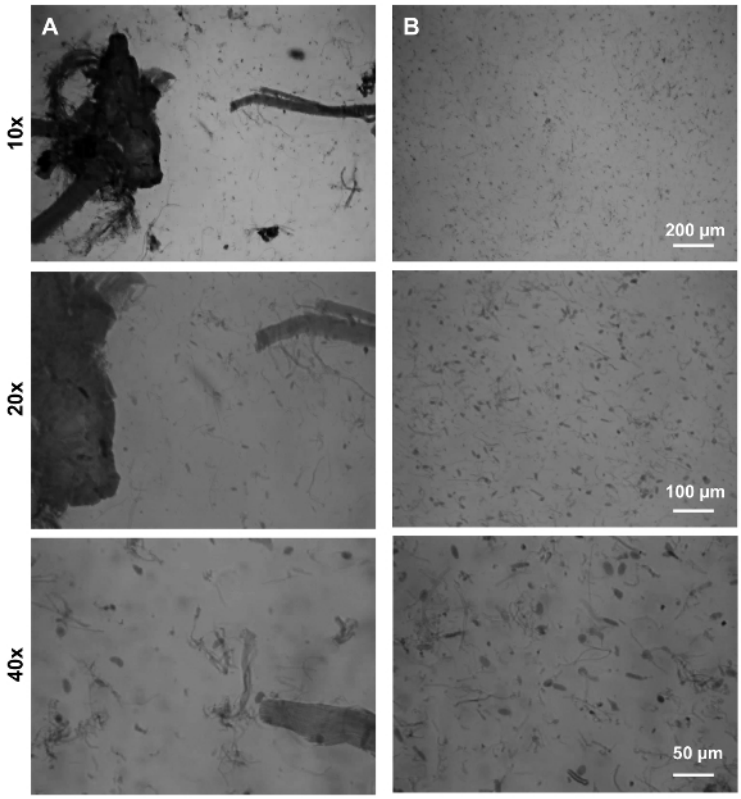

Figure 1: Sequential Filtration Effectively Removed Tissue Debris. (A) Representative images showing samples after 100 µm filtration. Large tissue and fiber debris are observed. **(B)** Representative images showing samples after serial filtration. Large fiber debris were cleared. Only isolated nuclei and small myofibril fragments are observed. Pictures were taken by using a light microscope at 10X, 20X and 40X magnifications. Scale bars are shown on the right hand side panels. Please click here to view a larger version of this figure.

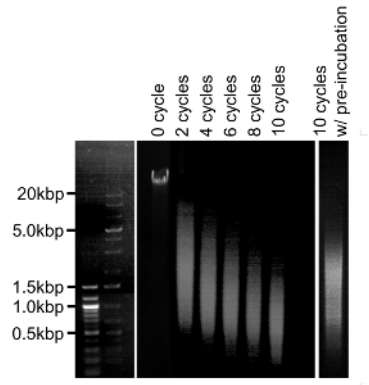

Figure 2: Progressive Chromatin Shredding Through 10 Cycles of Sonication. Ten cycles of sonication with digested chromatin DNA to ~ 500 bp, as revealed in a 0.8 % agarose gel, run at 150 V for 60 min. The right panel indicates a lower sonication efficiency after pre-incubation in ice-cold SDS lysis buffer for 1 h. Please click here to view a larger version of this figure.

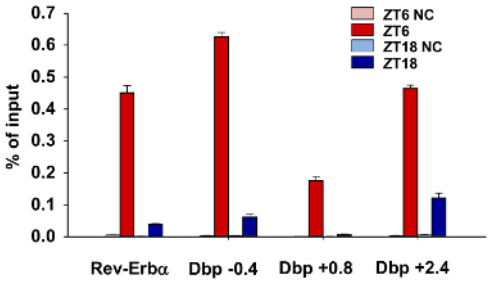

Figure 3: Representative qPCR Results for BMAL1 ChIP with Mouse Skeletal Muscle Samples Collected at ZT6 and ZT18. Data are presented as mean ± SEM. Dbp -0.4, +0.8 and +2.4 indicate locations of the E-Box elements on the *Dbp* gene. NC: negative control with IgY. The temporal pattern of BMAL1 binding is consistent with previous results showing BMAL1 binding peak at around ZT6[18]. The forward and reverse primers are as follows. Rev-erba: 5'-GTAGACTACAAATCCCAACAATCCTG, and 5'-TGGAGCAGGTACCATGTGATTC; Dbp -0.4: 5'-ACACCCGCATCCGATAGC, and 5'-CCACTTCGGGCCAATGAG; Dbp +0.8: 5'- ATGCTCACACGGTGCAGACA, and 5'-CTGCTCAGGCACATTCCTCAT; Dbp +2.4: 5'- TGGGACGCCTGGGTACAC, and 5'- GGGAATGTGCAGCACTGGTT. Please click here to view a larger version of this figure.

Discussion

Here we describe a robust method where cross-linked skeletal muscle tissues were used to isolate high-quality nuclei. Sequential filtration was carried out to effectively separate nuclei from debris, and ultrasonic acoustic energy from dish-shaped transducer sheared the chromatin for ChIP analysis. The results showed circadian time-specific binding of BMAL1 to target promoters.

ChIP can be employed to capture real-time protein occupancy on genomic DNA when cross-linking takes place. To take advantage of this potential, we aimed to develop a method to allow cross-linking of skeletal muscle at the time of tissue dissection and to streamline the nuclei isolation without gradient ultracentrifugation. Due to the difficulty of homogenizing fiber-rich skeletal muscle compared with soft tissues such as liver, we minced muscle tissue in ice-cold PBS and then homogenized the sample in a formaldehyde buffer. After quenching, tissue suspension was centrifuged and rinsed with ice-cold base buffer to rinse out any remaining formaldehyde. Nuclei were released by Dounce homogenization, and the homogenates were sequentially filtered to gradually remove cell debris and myofibrils. We devised the series of filtration to minimize filter clogging which could adversely impact yield. Only very short myofibrils remained when the sequential filtration was completed.

The sonication and ChIP procedures were adapted from a previous report[12] with modifications including sonication timing and SDS buffer amount. The dish-shaped sonicator allows exposure to centralized ultrasonic wave for samples in glass vials in a cold water bath. Compared with probe sonicators, this sonicator controls the sample temperature to avoid overheating, and also prevents sample cross-contamination. If probe sonicators are used, optimal sonication conditions need to be determined empirically. We also reduced the amount of SDS buffer since the yield of muscle chromatin is lower than that in liver[12]. Several protocols[28,31,32] include incubation on ice or at room temperature prior to sonication. However, in our experience, pre-incubation on ice did not improve the sonication efficiency. In fact, in some instances the sonication was compromised. It is possible that residual myofibrils entangled the chromatin DNA during incubation and attenuated sonication efficacy. With immediate sonication after nuclei suspension in SDS lysis buffer, we succeeded in obtaining progressive chromatin fragmentation with increasing sonication cycles (**Figure 2**).

We validated the quality of chromatin with ChIP qPCR. As shown in **Figure 3**, the BMAL1 promoter occupancy was robust at ZT6 and minimal at ZT18, consistent with previously shown BMAL1 circadian promoter binding[18]. This functional assay confirmed the quality of nuclei and chromatin. In recent years, rapid development in NGS opened up a new horizon for ChIP application where ChIP-seq can quantitatively interrogate genomic binding with high sensitivity[17]. Particularly for ChIP-seq, high-quality nuclei and chromatin are required to consistently capture protein-DNA

interaction. The procedure described herein may constitute a valuable resource for ChIP-seq studies using skeletal muscle. Of note, ChIP-seq library preparation requires additional measures to improve signal resolution, such as PAGE-based size selection[18].

In conclusion, we developed a filtration-based protocol to prepare high-quality nuclei from cross-linked skeletal muscle. We remove the need for ultracentrifugation, making it easily applicable. In addition to ChIP for genes of interest, the nuclei and chromatin prepared as described can be broadly applicable to ChIP-seq studies.

Disclosures

The authors have nothing to disclose.

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