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TITLE:

Chromatin Immunoprecipitation Assay Using Micrococcal Nucleases in Mammalian Cells.

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SUMMARY:

Chromatin immunoprecipitation (ChIP) is a powerful tool for understanding the molecular mechanisms of gene regulation. However, the method involves difficulties in obtaining reproducible chromatin fragmentation by mechanical shearing. Here, we provide an improved protocol for a ChIP assay using enzymatic digestion.

ABSTRACT:

To express cellular phenotypes in organisms, living cells execute gene expression accordingly, and transcriptional programs play a central role in gene expression. The cellular transcriptional machinery and its chromatin modification proteins coordinate to regulate transcription. To analyze transcriptional regulation at the molecular level, several experimental methods such as electrophoretic mobility shift, transient reporter and chromatin immunoprecipitation (ChIP) assays are available. We describe a modified ChIP assay in detail in this article because of its advantages in directly showing histone modifications and the interactions between proteins and DNA in cells. One of the key steps in a successful ChIP assay is chromatin shearing. Although sonication is commonly used for shearing chromatin, it is difficult to identify reproducible conditions. Instead of shearing chromatin by sonication, we utilized enzymatic digestion with micrococcal nuclease (MNase) to obtain more reproducible results. In this article, we provide a straightforward ChIP assay protocol using MNase.

INTRODUCTION:

Gene expression in mammalian cells is tightly and dynamically regulated, and transcription is one of the key steps. Gene transcription is mainly regulated by transcription factors and histones. A transcription factor is a protein that binds to specific DNA sequences and controls gene transcription. These factors either promote or inhibit the recruitment of RNA polymerase

II (PolII), which initiates mRNA synthesis from genomic DNA as a template[1]. Histone modifications such as acetylation and methylation of histone tail residues positively and negatively affect gene transcription by changing the chromatin structure[2]. Since alterations in gene expression affect the cellular context, it is essential to examine the molecular mechanisms by which transcription is regulated.

To date, several methods for investigating the regulation of gene transcription are available. Electrophoretic mobility shift assay (EMSA), also called a gel shift assay, is used for analyzing a protein-DNA interaction[3]. A nuclear extract from cells of interest is incubated with a radioactive isotope (for example, ^{32}P)-labeled DNA probe and electrophoresed on a polyacrylamide gel. Its autoradiogram shows that the DNA-protein complex migrates slower than the probe in a gel. In the presence of an antibody against the protein, the DNA-protein-antibody complex migrates in a gel more slowly than the DNA-protein complex. This supershifted band reveals specific binding between the DNA and protein. However, EMSA only determines a specific DNA-protein interaction in a cell-free system, and therefore it remains unknown whether the interaction controls transcription in living cells. The transient reporter assay, commonly called luciferase reporter assay, was developed to address gene expression regulation in cells. Typically, an upstream genomic region of a gene of interest is inserted into a reporter plasmid, transiently transfected into cells, and the reporter activity is measured. A variety of deletion mutants allows the identification of regions that are responsible for gene regulation. Even though a reporter assay is a useful tool for identifying transcription factors and binding DNA sequences controlling transcription, this method has a major disadvantage in that a reporter plasmid is free of chromatin structure and does not reflect “real” transcription machinery. In addition, changes in histone modifications cannot be determined by the system.

The development of the chromatin immunoprecipitation (ChIP) method was based on Jackson and Chalkley’s reports that “whole cell” fixation with formaldehyde preserved chromatin structure[4,5]. Since then, many related techniques have been developed and improved[6]. In ChIP assays, cells are fixed with formaldehyde to cross-link DNA and proteins. The chromatin is fragmented and then immunoprecipitated with antibodies of interest. The immune complex is washed, and DNA is purified. PCR amplification with primers targeted to a particular region of the genome reveals the occupancy of proteins of interest in the genome.

Although ChIP is a powerful tool to identify the interactions of proteins such as transcription factors and modified histones with DNA, the method involves some difficulties, such as a chromatin fragmentation step, in practice. Sonication has been widely used for shearing chromatin; however, it is cumbersome to identify reproducible conditions. Micrococcal nuclease (MNase) treatment is an alternative method for chromatin shearing. MNase is an endo-exonuclease that digests double-stranded, single-stranded, circular and linear DNA and RNA. It is relatively easy to determine the conditions, including the amounts of chromatin and enzyme, temperature, and incubation time, for optimum chromatin fragmentation. We modified and simplified the existing protocols, and we established a straightforward and reproducible method. This paper provides the protocol for a ChIP assay using MNase in mammalian cells.

PROTOCOL:

1. Preparation of reagents

1.1 Make 18.5% paraformaldehyde (PFA) solution. Add 0.925 g of PFA, 4.8 mL of water (use ultrapurified water throughout the protocol) and 35 µL of 1 M KOH in a 50-mL conical plastic tube. Close the cap tightly and heat the tube in a 400-600 mL glass beaker containing approximately 200 mL of water using a microwave. Remove the tube before the water starts boiling and vortex the tube to dissolve PFA. Allow PFA to cool to room temperature and store on ice.

NOTE: Repeat heating and mixing until PFA dissolves completely. Prepare PFA solution immediately before crosslinking (step 2.1.3).

1.2 Make 1.25 M glycine solution. Dissolve 14.07 g of glycine in 150 mL of water and filter through a 0.22 µm pore-size filter. Store at 4 °C.

1.3 Make ChIP cell lysis buffer. Dissolve 378 mg of PIPES, 10.6 mL of 2 M KCl, and 1.25 g of branched octylphenoxy poly(ethyleneoxy)ethanol in water. Adjust pH to 8.0 with 1 M KOH at 4 °C and make up to 250 mL. Filter through a 0.22 µm pore-size filter and store at 4 °C.

1.4 Make micrococcal nuclease (MNase) digestion buffer. To make 1 mL, mix 0.1 mL of 10x MNase digestion buffer, 10 µL of 100x BSA solution, 10 µL of 0.1 M dithiothreitol and 0.88 mL of water.

1.5 Make 1 M $NaHCO_3$. Dissolve 4.2 g of $NaHCO_3$ in 50 mL of water and filter through a 0.22 µm pore-size filter. Store at room temperature.

1.6 Make 10% sodium dodecyl sulfate (SDS). Dissolve 5 g of SDS in 50 mL of water. Store at room temperature.

1.7 Make elution buffer. Mix 15 µL of 1 M $NaHCO_3$, 15 µL of 10% SDS, and 120 µL of water to obtain 150 µL of elution buffer for one sample.

NOTE: Increase the volumes proportionally depending on the number of samples.

1.8 Make 3 M sodium acetate (pH 5.2) solution. Dissolve 24.6 g of sodium acetate anhydrous in water. Adjust pH to 5.2 with acetic acid and make up 100 mL. Filter through a 0.22 µm pore-size filter and store at room temperature.

2. Determination of MNase digestion conditions

NOTE: In the step 2 of protocol, an example using VCaP, human prostate cancer cells is

presented. Any mammalian cell lines can be used; see Note at the steps.

2.1 Preparation of crosslinked chromatin

2.1.1 Maintain VCaP cells in DME-high glucose supplemented with 10% fetal bovine serum (FBS) at 37 °C in a carbon dioxide incubator. Seed VCaP cells at 4 x 10^6 cells in six 6 cm dishes in 4 mL of culture media and culture for 3 days.

NOTE: Use appropriate culture media for each cell line. Up to 10 x 10^6 cells can be seeded in a 6 cm or 10 cm dish. For suspended cells, seed cells in T25 flasks. The number of dishes or flasks depends how many doses are tested for MNase digestion. If 4 doses are tested, prepare 6 dishes or flasks. See also step 2.2.

2.1.2 Detach VCaP cells from one dish using trypsin and count the cell. Calculate cell number in one dish. For suspended cells, remove a small amount of cell suspension from one flask and count cell number.

2.1.3 Add 0.229 mL of 18.5% PFA to a 6 cm dish in 4 mL of culture media at a final concentration of 1%. Gently but thoroughly swirl a dish or flask to distribute PFA evenly. Incubate at room temperature for exactly 10 min.

NOTE: In this step, chromatin and proteins are crosslinked. As PFA is toxic, carry out this step in a chemical fume hood and use proper personal protective equipment.

2.1.4 After 10 min, add 0.47 mL of 1.25 M glycine solution at a final concentration of 125 mM. Incubate at room temperature for 5 min.

NOTE: Glycine neutralizes excess PFA to stop further crosslinking.

2.1.5 Aspirate formaldehyde/glycine/culture media. Wash the cells by adding 4 mL of phosphate-buffered saline (PBS) twice. For suspended cells, transfer the cell suspension to a 15-mL conical tube and centrifuge at 300 x *g* for 5 min at room temperature. Aspirate the supernatant and add one medium volume of PBS and fully suspend. Repeat this step. Dispose liquid wastes properly since they contain formaldehyde.

2.1.6 Prepare PBS containing protease and phosphatase inhibitor cocktail (PIC) by adding 1/100 volume of 100x PIC to PBS. Aspirate PBS from a dish and add 1 mL per dish of PBS containing PIC. Scrape the cells and transfer the cell suspension to a 1.5-mL microcentrifuge tube. For suspended cells, simply transfer the suspension to a 1.5 mL-tube.

2.1.7 Centrifuge tubes at 3,000 x *g* for 5 min at room temperature to recover the cell pellet. Remove the PBS completely using a pipette. Record the cell number per tube and store the cell pellet at -80 °C.

2.2 Cell lysis and MNase digestion

NOTE: The reagent volume in the following steps is based on one tube containing 6 x 10^6 cells. Adjust reagent volume proportionally depending on cell number; when one tube contains 2 x 10^6 cells, use 100 µL of ChIP cell lysis buffer (step 2.2.1), MNase digestion buffer (step 2.2.3), and ChIP dilution buffer (step 2.2.5), and 10 µL of 0.5 M EDTA (pH 8.0) (step 2.2.4).

2.2.1 Thaw the stored cell pellet (VCaP cells, containing 6 x 10^6 cells per tube) prepared in step 2.1.7 on ice. Prepare ChIP cell lysis buffer supplemented with PIC by adding 1/100 volume of 100x PIC to the buffer. Add 300 µL of ChIP cell lysis buffer supplemented with PIC and resuspend the pellet thoroughly. Vortex the tube for 15 s and incubate the suspension on ice for 10 min.

2.2.2 Centrifuge at 9,000 x *g* for 3 min at 4 °C and remove the supernatant completely. Resuspend the pellet in 300 µL of MNase digestion buffer.

2.2.3 Dilute MNase (2,000 gel unit/µL) with MNase digestion buffer to give 50 gel units/µL. Add 0, 0.5, 1, 2, 4 µL of 50 gel units/µL of MNase to the suspension and incubate at 37 °C for exactly 10 min, mixing by inversion every 2.5 min.

NOTE: **Table 1** shows optimal amounts of MNase in several cell lines.

2.2.4 Add 30 µL of 0.5 M EDTA (pH 8.0) to terminate MNase digestion and vortex briefly. Incubate for 5 min on ice. Centrifuge at 9,000 x *g* for 5 min at 4 °C and remove the supernatant completely.

2.2.5 Prepare ChIP dilution buffer supplemented with PIC by adding 1/100 volume of 100x PIC to the buffer. Resuspend the pellet in 300 µL of ChIP dilution buffer supplemented with PIC.

2.2.6 Sonicate the suspension on ice using a sonicator equipped with a microtip probe. Use the following sonication conditions: amplitude 2, processed time 15 s, pulse ON 5 s, pulse OFF 30 s. Take 1 µL of the suspension and spot onto a slide glass and observe them using a microscope. Ensure that the cell structure is almost broken.

NOTE: **Figure 1A** shows representative microphotographs of the VCaP cell suspension before and after sonication. This step is for releasing digested chromatin into the supernatant by breaking nuclear membranes. A power setting should be adjusted to less than 5% of maximum power and try sonication for 5 s three times with more than a 30 s interval. If wattage control is available, adjust power setting to 5 W and process the samples for total 15 s as mentioned above to give total power of 70-75 J. Check whether the cell structure is broken as mentioned above. If not enough, repeat one more time. The condition described above is practically applied to all cell lines tested.

2.2.7 Centrifuge at 9,000 x *g* for 10 min at 4 °C, transfer the supernatant to a new 1.5-mL

microcentrifuge tube, and save 20 µL of the digested chromatin for step 2.3. Store the remainder at -80 °C.

2.3 Reverse crosslinking, purification of DNA, and analysis of digested chromatin

2.3.1 Add 75 µL of water, 4 µL of 5 M NaCl, and 1 µL of proteinase K to a 1.5-mL screw tube. Add 20 µL of digested chromatin from various MNase digestion conditions prepared in step 2.2.7 to tubes containing water, NaCl, and proteinase K. Close the cap tightly, mix completely, and incubate the tube at 65 °C overnight.

NOTE: This step is for the removal of crosslinking between protein and DNA.

2.3.2 Prepare two 1.5-mL microcentrifuge tubes per condition. Add 100 µL of phenol:chloroform:isoamylalcohol (25:24:1) (PCI) to one 1.5-mL tube. Add 10 µL of 3 M sodium acetate (pH 5.2) and 2 µL of glycogen to another tube.

NOTE: The use of chloroform:isoamylalcohol is not necessary[7]. Increasing volume may result in low DNA recovery after ethanol precipitation[8].

2.3.3 Centrifuge the tube containing digested chromatin from step 2.3.1 briefly and add 100 µL of PCI. Close the cap tightly, and vortex vigorously to form emulsion. Centrifuge the tube at maximum speed (e.g., 20,000 x *g*) for 30 s at room temperature.

2.3.4 Carefully take the upper phase containing DNA and add to the tube containing PCI prepared in step 2.3.2. Vortex vigorously and centrifuge the tube as step 2.3.3.

NOTE: If lower phase is contaminated, centrifuge the tube again, or remove most of the lower phase first, centrifuge the tube, and take the upper phase.

2.3.5 Carefully take the upper phase as described in step 2.3.4 and add to the tube containing sodium acetate and glycogen prepared in step 2.3.2. Add 250 µL of ethanol. Mix by inversion and incubate for 10 min at room temperature. Centrifuge the tube at maximum speed (e.g., 20,000 x *g*) for 30 min at 4 °C.

NOTE: Incubation of the solution at room temperature does not affect DNA recovery[8,9].

2.3.6 Confirm the pellets on the bottom of the tube. Carefully remove the supernatant so as not to disturb the pellet and add 500 µL of 70% ethanol. Centrifuge the tube at maximum speed (e.g., 20,000 x *g*) for 5 min at 4 °C.

2.3.7 Remove the supernatant completely and dry the pellet for approximately 5 min at room temperature. Dissolve the pellet in 20 µL of 10 mM Tris·HCl/1 mM EDTA (pH 8.0) (TE). Measure DNA concentration using a UV spectrophotometer.

2.3.8 Mix 0.5 µg of DNA with a gel loading dye and apply to 2% agarose gel. Electrophorese the samples at 100 V in tris-acetate-EDTA buffer until purple dye migrated to the two-third of the gel and stain a gel with 0.5 µg/mL ethidium bromide for 10 min. Take a picture of the gel.

NOTE: See **Figure 2** for detail.

3. Chromatin immunoprecipitation

3.1 Preparation of digested chromatin

3.1.1 Prepare thecells. For adherent cells, seed 2-10 x 10^6 cells per dish in 2 dishes (6 cm or 10 cm) per treatment group and allow the cells to attach to the bottom of dishes for more than 1 day. For suspended cells, seed in 2 T25 flasks per treatment group. Treat the cells as desired.

3.1.2 Take one dish or flask from each group and count cell number as described in step 2.1.2. Prepare crosslinked chromatin as mentioned in steps 2.1.3-2.1.7.

3.1.3 Lyse the cell pellet and MNase digestion as described in steps 2.2.1-2.2.7. Use the optimal amount of MNase to digest chromatin as determined in step 2. Store digested chromatin at -80 °C.

3.1.4 Reverse crosslink 20 µL of digested chromatin and purify DNA as described in step 2.3.8. Measure DNA concentration as described in step 2.3.7. Electrophorese the samples in 2% agarose gel to check the size of digested chromatin as mentioned in step 2.3.

NOTE: The DNA concentration is identical to that of stored digested chromatin prepared in step 3.1.3.

3.1.5 Dilute a reverse cross-linked digested chromatin sample from step 3.1.4 to give 10 ng/µL with water and serially dilute to make concentrations of 1, 0.1, and 0.01 ng/µL. Store at -20 °C.

NOTE: These samples are used for real-time PCR standards.

3.2 Immunoprecipitation

NOTE: In the steps 3.2-3.5 of protocol, anti-trimethylated histone H3 lysine 4 (H3K4me3) occupancy in VCaP cells is determined. See also **Figure 3**.

3.2.1 Thaw digested chromatin from step 3.1.3. Keep all samples on ice.

3.2.2 Prepare ChIP dilution buffer supplemented with PIC by adding 1/100 volume of 100x PIC to the buffer. Dilute digested chromatin to 5 µg/500 µL with ChIP dilution buffer supplemented with PIC. Prepare 1000 µL plus extra for (1) IP with nonimmune IgG (Control IgG), (2) IP with H3K4me3.

3.2.3 Add 5 µL of 5 µg/500 µL digested chromatin to a 1.5-mL screw tube as an input sample (1% of one IP). Store the sample at -80 °C.

3.2.4 Add 500 µL each of 5 µg/500 µL digested chromatin to a 1.5-mL screw tube as an IP sample. Add 2 µg of antibody to the tube. Close the cap and incubate the tube at 4 °C overnight with gentle mixing using a rocking platform.

NOTE: Although optimal antibody amount should be determined empirically, 2 µg per IP is recommended at first.

3.2.5 Add 30 µL of ChIP-grade protein G magnetic beads per IP. Incubate the tube at 4 °C for 2 h with gentle mixing using a rocking platform.

NOTE: Prewash of magnetic beads is not necessary.

3.3 Washing immune complex and reversing crosslinking

3.3.1 Spin down the tube from step 3.2.5 briefly. Place the tube in a polyethylene rack containing neodinium magnets for 1 min. Carefully remove the supernatant by aspiration.

3.3.2 Add 0.5 mL per tube of low salt immune complex wash buffer. Disperse the beads by gently tapping or briefly vortexing the tube. Incubate the tube at 4 °C for 5 min with gentle mixing using a rocking platform. After 5 min, repeat step 3.3.1.

3.3.3 Add 0.5 mL of high salt immune complex wash buffer per tube. Disperse and wash the beads as step 3.3.2. After washing, repeat step 3.3.1.

3.3.4 Add 0.5 mL of LiCl immune complex wash buffer per tube. Disperse and wash the beads as step 3.3.2. After washing, repeat step 3.3.1.

3.3.5 Place the tube in a magnetic rack for 1 min. Remove the remaining supernatant completely.

3.3.6 Add 150 µL of elution buffer to the tube. Vortex the tube to disperse the beads completely. Close the cap and incubate the tube at 65 °C for 30 min, mixing by inversion or vortexing every 5 min to disperse the beads thoroughly.

3.3.7 During the incubation, prepare a 1.5-mL screw tube and add 6 µL of 5 M NaCl and 2 µL of proteinase K. Thaw 1% input sample prepared in step 3.2.3. Add 150 µL of elution buffer, 6 µL of 5 M NaCl and 2 µL of proteinase K to 1% input sample.

3.3.8 After the incubation, spin down the tube. Place the tube in a magnetic rack for 1 min and transfer the supernatant to the screw tube containing NaCl and proteinase K prepared in step

3.3.7.

3.3.9 Close the cap and vortex all IP and input samples to mix completely. Incubate the tube at 65 °C overnight.

3.4 DNA purification

3.4.1 Prepare the tube as described in 2.3.2 and add 150 µL of PCI instead of 100 µL. In another tube, add 12 µL of 3 M sodium acetate (pH 5.2) and 2 µL of glycogen.

3.4.2 Remove the tube from the incubator. Spin down the tube and add 150 µL of PCI.

3.4.3 Vortex vigorously the tube to form emulsion. Centrifuge the tube at maximum speed (e.g. 20,000 x *g*) for 30 s at room temperature.

3.4.4 Carefully transfer 140 µL of the upper phase to the tube containing PCI prepared in step 3.4.1. Repeat step 3.4.3.

NOTE: See also Note in step 2.3.4.

3.4.5 Carefully transfer 120 µL of upper phase to the tube containing sodium acetate and glycogen prepared in step 3.4.1.

NOTE: See also Note in step 2.3.4.

3.4.6 Add 300 µL of ethanol. Mix by inversion and incubate for 10 min at room temperature.

3.4.7 Centrifuge the tube at maximum speed (e.g., 20,000 x *g*) for 30 min at 4 °C. Wash the pellet as described in step 2.3.6.

3.4.8 After drying the pellet as described in step 2.3.7, dissolve the pellet in 50 µL of TE. Store at -20 °C.

3.5 Detection of DNA fragments using real-time PCR

3.5.1 Thaw the following samples: (1) IP with Control IgG, (2) IP with anti-H3K4me3, (3) 1% input sample. Thaw also 4 doses of standards prepared in step 3.1.5 (total of 7 samples).

NOTE: Use standards from the same group for the quantification of DNA amounts in the input and IP samples.

3.5.2 Make PCR working solution for 8 samples. Mix 40 µL of 2x real-time PCR supermix, 8 µL each of 4 µM forward and reverse primers, and 8 µL of water.

NOTE: One PCR reaction mixture contains 5 µL of 2x real-time PCR supermix, 1 µL each of 4 µM forward and reverse primers, and 1 µL of water. Primer sequences are listed in **Table 2**.

3.5.3 Aliquot 8 µL of PCR working solution into one well of a PCR plate. Add 2 µL of samples from step 3.4.8 or standards from step 3.1.5.

3.5.4 Seal the PCR plate and run PCR using the following conditions: 1) 95 °C for 3 min for initial denaturation, 2) 95 °C for 10 s for denaturation, 3) 56 °C for 30 s for annealing, 4) 72 °C for 30 s for extension and data collection, repeat steps 2) to 4) for 55 cycles. After cycling, measure a melting curve of the PCR product. Analyze the data.

NOTE: Raw data for **Figure 3** are shown in **Table 3**. Calculate starting quantity (SQ) of the samples using a standard curve. Multiply SQ in 1% input by 100 to adjust a SQ of 1% input sample to one IP to give adjusted SQ in Input (Eq. 1). Divide SQ in IP sample by adjusted SQ in Input to give % input value (percent input method, Eq. 2). To calculate fold enrichment, divide SQ in IP with anti-H3K4me3 by SQ in IP with control IgG (fold enrichment method, Eq. 3).

Adjusted SQ in Input = (SQ in 1% input) x 100 (1)

Percent input of H3K4me3 = 100 x (SQ in IP with anti-H3K4me3) / (Adjusted SQ in Input) (2)

Fold enrichment of H3K4me3 = (SQ in IP with anti-H3K4me3) / (SQ in IP with control IgG) (3)

REPRESENTATIVE RESULTS:

Digesting chromatin is one of the important steps for a ChIP assay. We used MNase to digest chromatin to obtain a mixture of nucleosome oligomers. In the MNase digestion step, MNase can go through the nuclear membrane and digest chromatin. However, the digested chromatin cannot go through the membrane and remains in the nuclei. To release the digested chromatin from the nuclei, brief sonication is needed. **Figure 1A** shows microphotographs before and after sonication of VCaP cell suspension. Without sonication, the cell structure remains intact, indicating that the chromatin is present in the nuclei. A brief sonication breaks the cell structure, and checking the cells in microphotographs helps to determine the brief sonication conditions. We also represented other examples for brief sonication in 293T cells (**Figure 1B**) the human B-cell acute lymphoblastic leukemia cell line, REH cells (**Figure 1C**) and the human prostate cancer cell line, LNCaP (**Figure 1D**).

Figure 2A shows chromatin fragmentation after treatment with different amounts of MNase in VCaP cells. We treated 6 x 10^6 crosslinked VCaP cell pellets with 0, 50, 100, 200 gel units of MNase in 300 µL of digestion buffer for 10 min at 37 °C. After purification of the digested chromatin, 500 ng of DNA was analyzed on 2% agarose gel and stained with ethidium bromide. Without adding MNase, a smear pattern with a very high molecular weight appeared (lane 1). The addition of MNase gave a ladder pattern (N; a mononucleosome unit), showing that MNase digests internucleosome (lanes 2-5). **Figure 2B** shows an inappropriate digestion pattern.

Overdigestion mainly resulted in mononucleosome production (**Figure 2B**, lane 7). We should find the proper conditions that produce chromatin fragments up to 900 bp (one to five nucleosomes; e.g., lane 5).

To check whether the ChIP assay is performed properly, it is essential to have appropriate controls in the assay. For immunoprecipitation, nonimmune IgGs from the same species as the antibodies of interest are used as a control that shows nonspecific binding to the same region (see discussion). In addition, it is recommended to measure the binding of the proteins (occupancy) in both positive and negative regions. It has been widely accepted that H3K4me3 occupancy is distributed between approximately one kilobase (kb) upstream and downstream of transcription start sites[10,11]. We measured H3K4me3 occupancy in the AR genome spanning approximately 20 kb upstream through 12 kb downstream of the AR transcription start site (AR-TSS) in AR-positive VCaP cells. Digestion pattern of chromatin in VCaP cells used in this experiment was shown in **Figure 3A**, indicating the proper digestion of chromatin. The highest occupancy of H3K4me3 was observed around the AR-TSS and 0.5 kb and 1 kb upstream of the AR-TSS (**Figure 3B**). As long as genes are transcriptionally active, TSSs can be "positive regions". Regions located at 19 kb and 8 kb upstream and 12 kb downstream of AR-TSS, however, had little occupancy of H3K4me3 (**Figure 3B**), indicating that these can be used as "negative regions".

It has been shown that an androgen increases RNA polymerase II occupancy in the PSA promoter and enhancer in LNCaP cells using sheared chromatin by sonication[12,13]. We therefore tested the validity of our protocol by measuring active RNA polymerase II occupancy (phosphorylated RNA polymerase II at serine 5; PolII(pS5)) in the cells. We performed the same experiment to check the reproducibility of our method. LNCaP cells were cultured in steroid-starved medium for 3 days and stimulated with a vehicle or 10 nM dihydrotestosterone (DHT) for 4 h. Active RNA polymerase II occupancy was measured by immunoprecipitation with anti-PolII(pS5), followed by real-time PCR. **Figure 4A** shows a reproducible digestion pattern of chromatin from LNCaP cells in three independent experiments. As shown in **Figure 4B**, DHT significantly increased PolII(pS5) occupancy in the PSA promoter and enhancer when using percent input method. We also calculated the occupancy using fold enrichment method (**Figure 4C**) and found that no significant difference in PolII(pS5) in the PSA promoter was observed with or without DHT treatment. DHT did not affect occupancy in the GAPDH promoter as previously published[14]. Importantly, our data were similar to that obtained from sonication-sheared chromatin samples[12,13].

FIGURE AND TABLE LEGENDS:

Figure 1: Representative microphotographs of crosslinked cell pellets before and after sonication. Crosslinked VCaP (**A**), 293T (**B**), REH (**C**) and LNCaP (**D**) cell pellets were treated with MNase, and pellets were resuspended in ChIP dilution buffer. Before and after sonication, pictures of the suspensions were taken. Scale bar = 200 μm.

Figure 2: Representative agarose gel analysis of digested chromatin. (**A**) Crosslinked chromatin

was prepared from VCaP cells and digested with various amounts of MNase as described in step 2.2. Digested chromatin was reverse crosslinked, purified, and analyzed in a 2% agarose gel. N; a mononucleosome unit. (**B**) Chromatin of VCaP cells was digested with 250 gel units of MNase per 2 x 10^6 cells at 37 °C for 20 min and analyzed as described in **A**. Larger amounts of MNase and longer incubation times caused almost complete digestion of chromatin to form mononucleosomes (150 bp).

Figure 3: H3K4me3 occupancy in the AR genome. Digested chromatin was prepared from VCaP cells. (**A**) Digestion pattern was analyzed using an agarose gel. (**B**) 5 µg of digested chromatin was immunoprecipitated with 2 µg of either normal rabbit IgG or anti-H3K4me3 antibody as mentioned in step 3.1 and step 3.2. Immune complexes were washed and eluted from beads, and reverse crosslinked. Purified DNA fragments were analyzed using real-time PCR with the primer sets listed in **Table 2**.

Figure 4: Androgen increased active RNA polymerase II occupancy in the PSA promoter and enhancer. Steroid-starved LNCaP cells were treated with or without 10 nM DHT for 4 h, and digested chromatin was prepared. (**A**) Digestion pattern of chromatin from LNCaP cells in three independent experiments. (**B**,**C**) Digested chromatin was immunoprecipitated with an anti-PolII(pS5) antibody, and DNA fragments were purified as described for **Figure 3**. The occupancy of active RNA polymerase II in the PSA promoter, enhancer, and GAPDH promoter as a percent input (**B**) and fold enrichment (**C**) was determined using real-time PCR with the primer sets listed in **Table 2**. The results shown are mean ± SE of three independent experiments. (*); $p<0.05$, (**); $p<0.01$ versus 0 nM DHT treatment. NS; not significant versus 0 nM DHT.

Table 1: Optimum amount of micrococcal nuclease in various cell lines. The value represents the amounts of MNase per 2 x 10^6 cells in 100 µL of buffer, 37 °C for 10 min.

Table 2: Paired Primer sequences used for ChIP assay.

Table 3: Raw data from quantitative PCR analysis for Figure 3. Cq: Threshold cycle number, SQ: starting quantity calculated using a standard curve, Adjusted to one IP: multiply SQ in 1% input by 100 as 1% sample volume of one IP is used for PCR, % Input: divide SQ in IP sample by adjusted SQ in Input, Fold enrichment: divide SQ in IP with anti-H3K4me3 by SQ in IP with control IgG.

DISCUSSION:

Although sonication is commonly used to obtain fragmented chromatin, it is time-consuming and cumbersome to identify reproducible conditions. In this protocol, we used MNase digestion because enzyme digestion should be easier to identify reproducible conditions. A brief sonication step after MNase digestion (see step 2.2) was necessary to break the cell membrane and to release the digested chromatin. Therefore, the sonication power in our protocol should be as low as possible. We use the same sonication conditions for all cells we employed (see **Figure 1** and **Table 1**) to obtain complete breakdown of the membrane.

Digestion of chromatin by MNase is a critical step in our protocol, and thus we have exerted great effort to optimize the conditions for the digestion of chromatin inside cells. Digestion activity is determined by the amounts of enzyme and substrate (chromatin) and incubation time. In addition, since ploidy varies among different cells, the optimum conditions for MNase digestion must be identified for each cell type. Once established, the same conditions can be applied irrespective of the cell treatments. We always check the chromatin digestion patterns in each experiment to ensure that the digestion patterns are suitable for ChIP assays, as shown in **Figure 2A**, lane 5.

Some factors affect the results of ChIP assays. It is important to use the right amount of digested chromatin in our protocol. In many protocols, the cell number but not the amount of chromatin for one IP is shown[15,16]. These experimental conditions produce high variability in the amount of chromatin among cells due to ploidy differences. We use 5 µg of chromatin and 2 µg of antibody per one IP in the assay, thus our protocol is more straightforward and clearer than other protocols, although optimum amounts of antibody for IP may be needed. The selection of antibodies is also important; use ChIP assay-validated antibodies.

There are two methods to analyze ChIP PCR data: the percent input method and the fold enrichment method. In the percent input method, signals from IP samples are divided by signals from total chromatin in the IP sample. In the fold enrichment method, signals from IP with a specific antibody such as H3K4me3 are divided by signals from IP with the control IgG. The later method is only applicable when signals from IP with the control IgG are similar and reproducible at multiple targets or identical targets under the different physiological conditions. In practice, the signal levels vary so the fold enrichment value has high variability as shown in **Figure 4C**. Therefore, we do not recommend using the fold enrichment method to represent ChIP data in our method.

MNase favors an euchromatin 'open' environment and is not accessible to a heterochromatin structure, suggesting that MNase digestion may produce some bias. It has been reported that enrichment of lamin A-interacting chromatin domains is different in between sonication-sheared and MNase-digested chromatin preparations[17]. Thus, MNase digestion in ChIP assay may not be suitable to analyze nuclear structure-associated molecules such as lamin A/C and Special AT-rich Sequence Binding Protein 1 (SATB1).

In ChIP assay protocols designed for downstream microarray (ChIP-on-chip) or sequencing (ChIP-seq) analyses, RNase is used during the DNA purification step, although not in protocols designed for PCR analyses[18]. We have not tested whether our protocol is compatible with ChIP-on-chip and ChIP-seq analyses, but we assume that our protocol is applicable when samples are treated with RNase. If RNase treatment is needed, use 2 µL of 10 mg/mL DNase-free RNase A instead of proteinase K in step 3.3 and incubate at 65 °C overnight. Before purifying DNA (step 3.4), add proteinase K and incubate for an additional 1 h at 60 °C.

We routinely carry out ChIP assays with modified histone and transcription factors using the method described here and have shown that the chromatin remodeling factor, AT-rich

interaction domain 5B, regulates AR gene expression by changing the occupancy of PolII(pS5) and H3K4me3 in the AR promoter[19]. We believe that our protocol is technically easier than other ChIP assays and is widely acceptable in molecular biology research.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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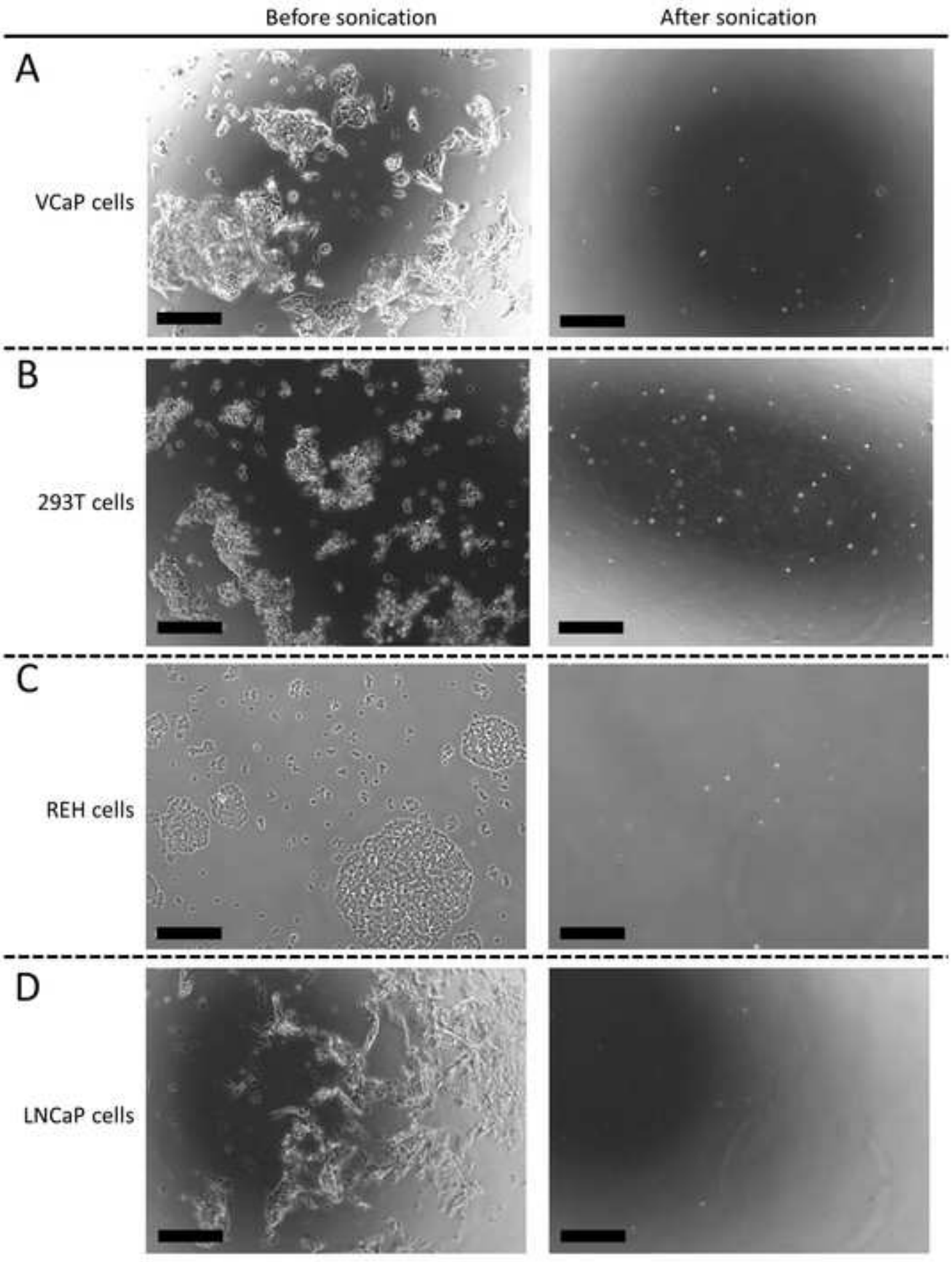

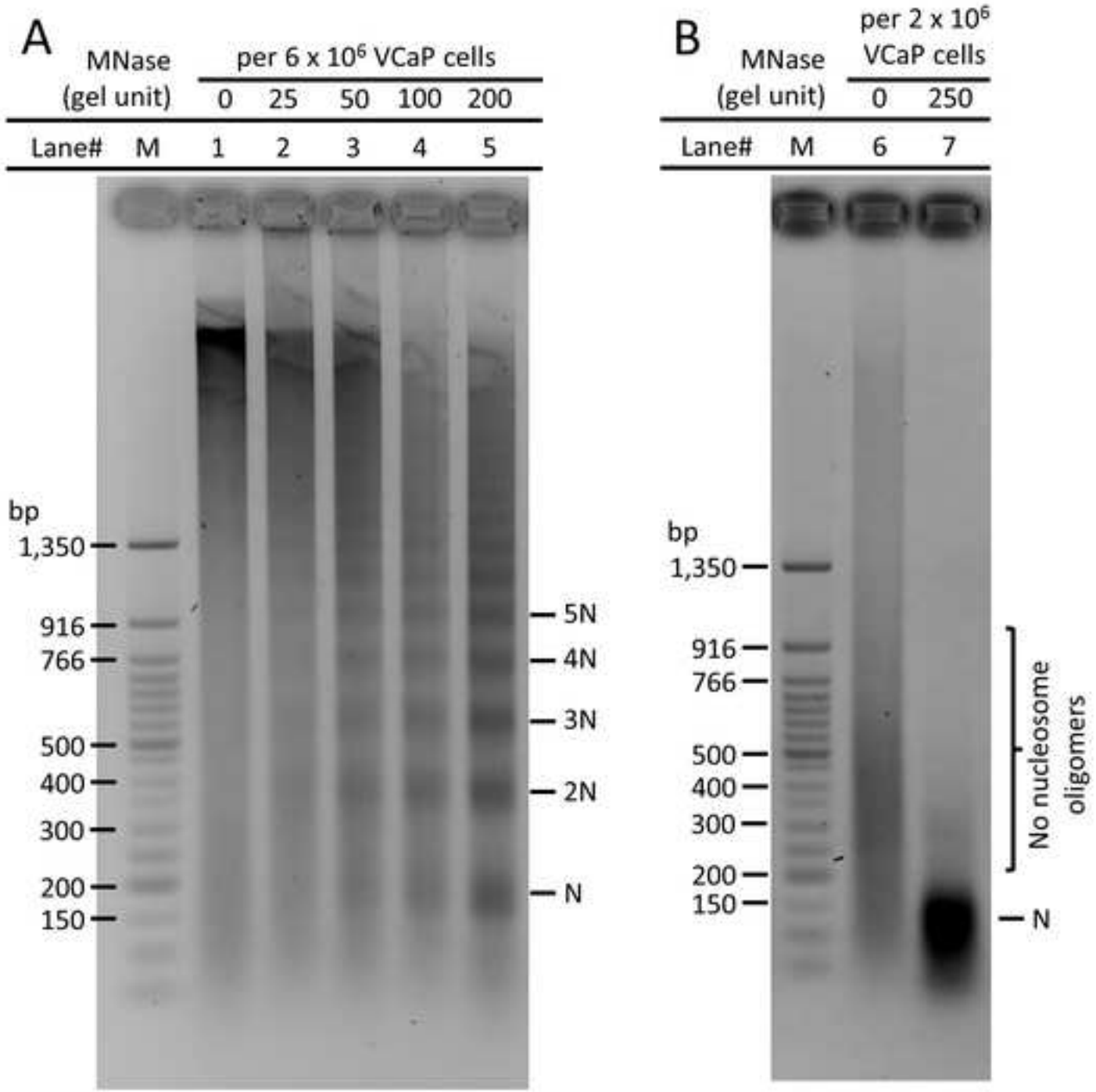

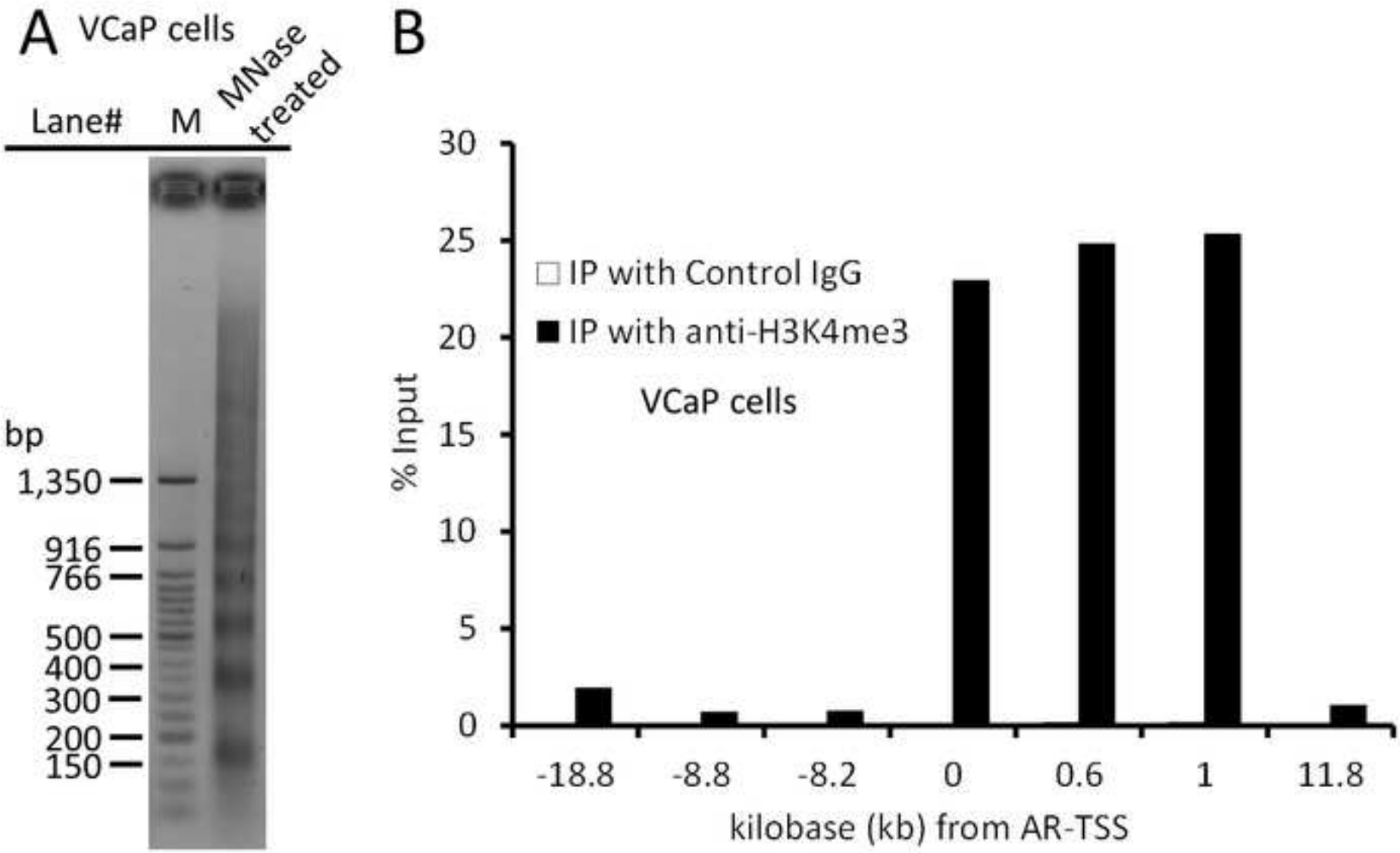

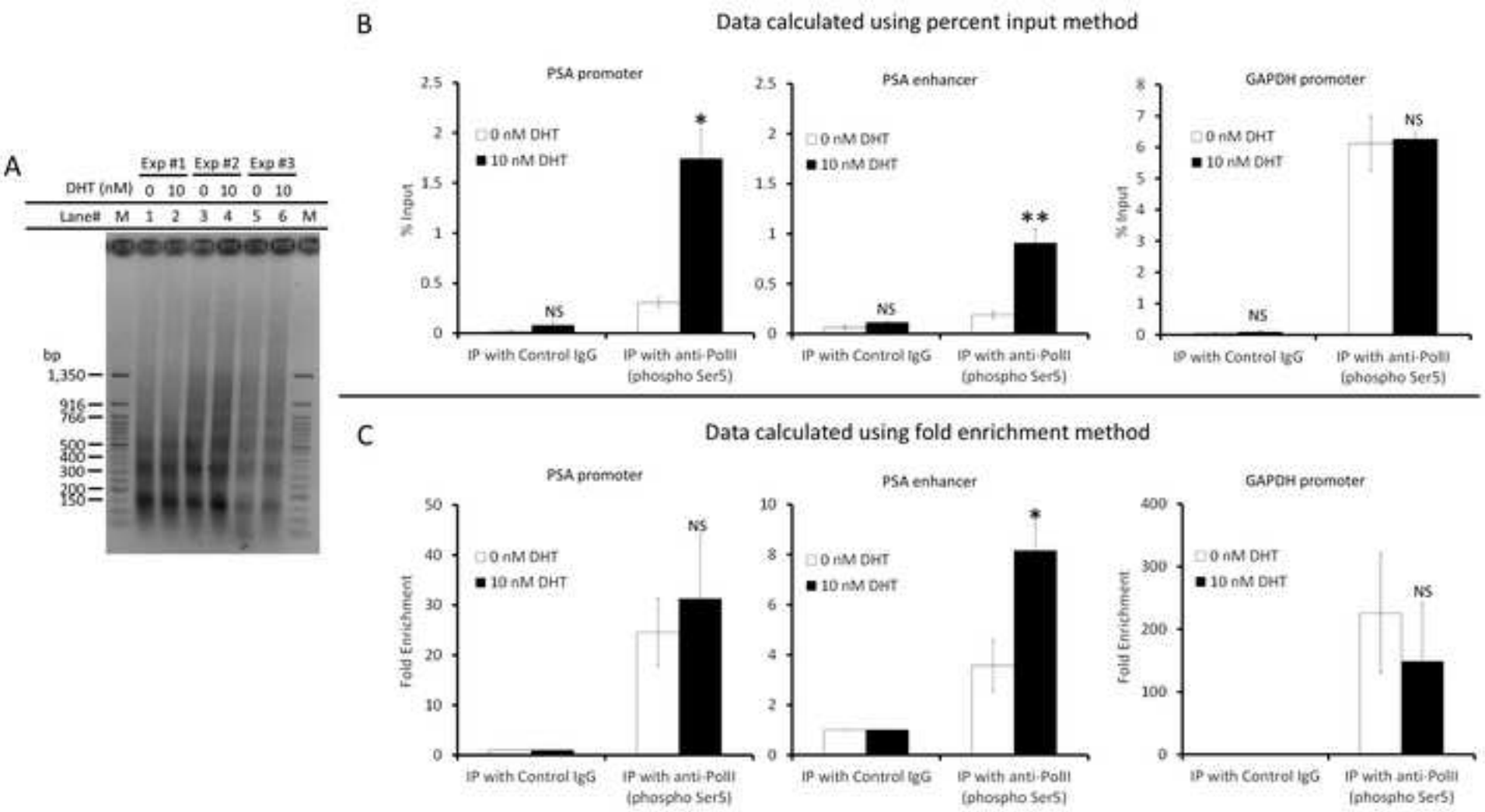

Cell line	gel units per two million cells in 100 µL of buffer, 37 °C for 10 min
LNCaP	267
VCaP	66.7
293T	450
REH	134
22Rv1	400

Table 2

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Primer name		Sequence
AR (-18.8kb)	FWD	ATTTGGAACTGGGAACATCT
	REV	CACCTTCTCTCCTCCACTCT
AR (-8.8kb)	FWD	TAACAGCTTTGCATCCAAGT
	REV	TGAAATCTGGGACTAAAGCA
AR (-8.2kb)	FWD	CAGTGCTATTCCCTTGTGAC
	REV	TTGGACTGGCTCTATCTTGA
AR-TSS (0 kb)	FWD	GCAAACTGTTGCATTTGCTC
	REV	GGCCCTTTTTCCCTCTGTC
AR (0.6 kb)	FWD	CACGACCCGCCTGGTTAG
	REV	TGAAGACCTGACTGCCTTTTC
AR (+1.0kb)	FWD	CCGCAAGTTTCCTTCTCTGG
	REV	CTTCCCAGCCCTAACTGCAC
AR (+11.8kb)	FWD	CCTTGCTTGTGGAACTGTAG
	REV	TTTATTGTCTGGTGCTAGGC
PSA promoter	FWD	CCTAGATGAAGTCTCCATGAGCTACA
	REV	GGGAGGGAGAGCTAGCACTTG
PSA enhancer	FWD	GCCTGGATCTGAGAGAGATATCATC
	REV	ACACCTTTTTTTTTCTGGATTGTTG
GAPDH	FWD	TACTAGCGGTTTTACGGGCG
	REV	TCGAACAGGAGGAGCAGAGAGCGA

Table 3

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AR (-18.8kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	23.49	0.815	81.5	100	
IP with Control IgG	27.68	0.051	0.051	0.062	1
IP with anti-H3K4me3	22.48	1.590	1.590	1.951	31.4

AR (-8.8kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	23.22	0.586	58.6	100	
IP with Control IgG	26.81	0.052	0.052	0.088	1
IP with anti-H3K4me3	23.74	0.414	0.414	0.706	8.0

AR (-8.2kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	23.19	0.643	64.3	100	
IP with Control IgG	26.99	0.048	0.048	0.075	1
IP with anti-H3K4me3	23.63	0.477	0.477	0.742	9.9

AR-TSS (0 kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	25.06	0.657	65.7	100	
IP with Control IgG	28.63	0.050	0.050	0.077	1
IP with anti-H3K4me3	20.70	15.064	15.064	22.944	299.8

AR (0.6 kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	23.86	0.716	71.6	100	
IP with Control IgG	26.67	0.106	0.106	0.147	1
IP with anti-H3K4me3	19.15	17.787	17.787	24.840	168.6

AR (+1.0kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	23.51	0.730	73.0	100	
IP with Control IgG	25.94	0.125	0.125	0.171	1
IP with anti-H3K4me3	19.06	18.486	18.486	25.335	147.8

AR (+11.8kb)	Cq	SQ	Adjusted to one IP	% Input	Fold enrichment
1% Input	24.54	0.876	87.6	100	
IP with Control IgG	29.14	0.033	0.033	0.037	1
IP with anti-H3K4me3	24.47	0.918	0.918	1.048	27.97

Revised Table of Materials

Click here to access/download;Table of Materials;Rev JoVE_Materials (TYamakawa).xlsx

Name of Reagent/ Equipment	Company	Catalog Number	Comments/Description
0.5 M EDTA (pH 8.0)	Thermo Scientific	AM9010	
2 M KCl	Thermo Scientific	AM9010	
2X iQ SYBR Green supermix	Bio-Rad	1706862	
5 M NaCl	Thermo Scientific	AM9010	
50 bp DNA ladder	New England Biolabs	N3236S	
Agarose	Research Product International	A20090	
Branched octylphenoxy poly(ethyleneoxy)ethanol	Millipore Sigma	I8896	IGEPAL CA-630
ChIP-grade protein G magnetic beads	Cell signaling technology	9006S	
Chromatin Immunoprecipitation (ChIP) Dilution Buffer	Millipore Sigma	20-153	Buffer composition: 0.01% SDS, 1.1% Triton X- 100, 1.2mM EDTA, 16.7mM Tris-HCl, pH 8.1, 167mM NaCl.
Gel Loading Dye Purple (6X)	New England Biolabs	B7024S	
Glycine	Bio-Rad	161-0724	Electropheresis grade
Glycogen	Millipore Sigma	G1767	19-22 mg/mL
Halt Protease and Phosphatase Inhibitor Cocktail, EDTA-free (100x)	Thermo Scientific	78445	
High Salt Immune Complex Wash Buffer	Millipore Sigma	20-155	Buffer composition: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 500mM NaCl.
Histone H3K4me3 antibody (pAb)	Active Motif	39915	
LiCl Immune Complex Wash Buffer	Millipore Sigma	20-156	Buffer composition: 0.25M LiCl, 1% IGEPAL CA630, 1% deoxycholic acid (sodium salt), 1mM EDTA, 10mM Tris, pH 8.1.
Low Salt Immune Complex Wash Buffer	Millipore Sigma	20-154	Buffer composition: 0.1% SDS, 1% Triton X-100, 2mM EDTA, 20mM Tris-HCl, pH 8.1, 150mM NaCl.
Magna GrIP Rack (8 well)	Millipore Sigma	20-400	Any kind of magnetic separation stands that are compatible with a 1.5 mL tube is fine.
Micrococcal nuclease	New England Biolabs	M0247S	comes with 10 x buffer (500 mM Tris-HCl, 50 mM CaCl2, pH 7.9 @ 25 °C) and 100 x BSA (10 mg/ml)
NaHCO3	JT Baker	3506-01	
Normal rabbit IgG	Millipore Sigma	12-370	
PIPES	Millipore Sigma	P6757	
Proteinase K	Millipore Sigma	3115887001	
Real-time PCR system	Bio-Rad	CFX96, C1000	
RNA pol II CTD phospho Ser5 antibody	Active Motif	39749	
SDS	Boehringer Mannheim	100155	Electropheresis grade
sodium acetate	Millipore Sigma	S5636	
Sonicator equipped with a microtip probe	QSONICA	Q700	Any kind of sonicators that are compatible with a 1.5 mL tube is fine.
UltraPure Phenol:Chloroform:Isoamyl Alcohol (25:24:1, v/v)	Thermo Scientific	15593031	pH 8.05

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Thank you for reviewing our manuscript (JoVE59375) entitled "Chromatin immunoprecipitation assay using micrococcal nucleases in mammalian cells". We revised our manuscript according to the following editorial comments. We hope that our edits are satisfactory. The following is a point-by-point response to the editorial comments delivered in the rebuttal letter.

Editorial comments:
Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
Answer 1: We checked our manuscript carefully and corrected some grammar issues. We tracked the changes in our revised manuscript.

2. The highlighted protocol steps are over the 2.75 page limit (including headings and spacing). Please highlight fewer steps for filming.
Answer 2: We deleted some steps for filming by erasing highlights. We tracked the changes in our revised manuscript.

3. Please remove all headers from Representative results.
Answer 3: We removed all headers from representative results.

4. Figure 3A: Please add a unit.
5. Figure 4A: Please add a unit.
Answer 4 and 5: We added "bp" (base pair) in the marker lanes.

In addition to these revisions, we revised a sentence in the Discussion part (line 563 in 59375_R1.docx) for clarification.

Again, thank you for careful review of our manuscript. We hope that these revisions persuade you to accept our submission.

Sincerely,

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