Native Chromatin Immunoprecipitation with high throughput Sequencing (N-ChIP-seq) of Sheep Alveolar Macrophages

Protocol adapted from N-ChIP methods described previously (David et al., 2017; Naval-Sanchez et al., 2018; Wagschal et al., 2007).

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Isolate native chromatin

Preparation steps: Cool centrifuges to 4 °C. Prepare and filter-sterilize solutions with 0.2 micron sterile filters, chill solutions to 4 °C. Chill Dounce homogenizer. Label tubes in advance and prepare an ice bucket. Always maintain cell samples on ice or work in a cold room (4 °C).

1) Briefly thaw frozen alveolar macrophages (5×10^7 live counted prior to freezing, estimated 150 mg) on ice after adding 1 mL of ice-cold Dulbecco's phosphate buffered saline (DPBS, Mg^{2+} Ca^{2+} free). Once thawed, obtain cell pellet by centrifugation for 5 minutes at 4 °C, 350 x *g*. Discard supernatant containing cryopreservative (CryoStor CS10, BioLife Solutions, Bothell, WA, USA) rinse with 1 mL ice cold DPBS, gently resuspend cells by flicking the tube, and collected by brief centrifugation at 4 C, 350 x *g*. Repeat wash and spin again for two total washes. Discard all supernatant.
2) Resuspend cell pellet in 2 mL (at least 6X volume of cell pellet) of Nuclei Buffer (0.3 M sucrose, 60 mM KCl, 15 mM NaCl, 5 mM MgCl2, 0.1 mM EGTA, 15 mM Tris-HCl pH 7.5) with addition of 20 µL 100x HALT protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA) (add protease inhibitor fresh just before using to all buffers, caution do not put protease inhibitor on ice it will freeze solid very quickly). Sodium butyrate is added to all buffers at a final concentration of 5 mM to inhibit histone deacetylase when processing chromatin for H3K27ac immunoprecipitation (IP). Pipette up and down briefly until pellet is well suspended (take care to avoid entrapping cells in the pipette tip).
3) Incubate cells in Nuclei Buffer for 5 minutes on ice.
4) Add equal volume of Nuclei Buffer 2 (Nuclei Buffer + 0.4% IGEPAL CA-630 detergent Sigma-Aldrich, St. Louis, MO, USA)) to the cell suspension for a final detergent concentration of 0.2% in the tube. Start a timer for ten minutes and gently mix. After 5 minutes incubation on ice, transfer the cell suspension to a Dounce homogenizer and mix with 30-40 strokes (about 3 minutes) of the tight pestle. Transfer to fresh tubes and rinse the Dounce homogenizer with approximately 1 mL of Nuclei buffer B and transfer that liquid back to the tube to maintain as much of the cells as possible. Split the cell suspension by gently layering each half onto an 8 mL cushion of Nuclei Buffer 3 (Nuclei buffer + 1.2

M sucrose) in 15 mL conical bottom tubes. Two tubes will allow you to balance the centrifuge and provide adequate separation in the sucrose gradient. Carefully avoid mixing the layers when adding the cell suspension to the 15 mL tubes. Make sure to complete this step within 10 minutes of adding Nuclei Buffer 2.

5) Centrifuge at 4000 x *g* for 20 minutes at 4 °C.
6) Very carefully remove tubes from the centrifuge and remove the supernatant layers, discarding, with multiple tip changes so that the detergent layer on top does not contact the nuclei pellet. Nuclei pellet should be white and flocculent compared to cell pellet. Remove as much supernatant as possible without disturbing the nuclei pellet.
7) Add 1 mL micrococcal nuclease buffer (Micrococcal nuclease (Mnase), M0247S, New England Biolabs, Ipswich, MA, USA) to resuspend and combine nuclei pellets into a single tube. Briefly vortex to homogenize the nuclei suspension. Prepared micrococcal nuclease buffer contains 100 microliters 10X Mnase buffer, 10 microliters protease inhibitor cocktail, 10 microliters BSA (provided with Mnase), and 880 microliters of sterile nuclease-free water. Dilute the Mnase with prepared buffer and add 60 Kunitz units of the enzyme to the nuclei suspension.
8) Incubate the nuclei suspension with the Mnase enzyme for 12 minutes at 37 °C on a thermomixer with gentle rotation (300 rpm).
9) Stop the digestion reaction by adding 40 microliters of 0.5 M EGTA for a total concentration of 20 mM EGTA and mix well by flicking the tube. Incubate the digested chromatin on ice for 10 minutes. Sonicate the nuclei suspension/digested chromatin for two, 30 second intervals (30 seconds of rest between) in a probe free, cup horn sonicator at 100% power (260 W) chilled with ice water.
10) Spin the digested chromatin at 20,000 x *g* (maximum) for 10 minutes at 4 °C to pellet the nuclear membranes. Keep the supernatant and transfer it to a clean non-stick tube.
11) Aliquot 50 microliters of the chromatin from the supernatant to check the digestion after phenol:chloroform:isoamyl clean-up, on a 1.5% agarose gel. Use orange loading dye and run at 55 volts with a 100 bp DNA ladder. Chromatin should be mostly mononucleosomes with few dinucleosomes. Fragment analysis can also be completed on a Bioanalyzer (Agilent, Santa Clara, CA, USA) with the high sensitivity DNA chip to ensure fragment lengths between 100-450 bp, with an average size of 150 bp ideal.
12) Aliquot and freeze (-20 °C) 50 microliters (5%) of the chromatin to be used as the input control for sequencing (no immunoprecipitation with antibody).

Prepare antibody-bead complexes (prepare the night before to allow for incubation prior to chromatin preparation)

Preparation steps: Allow 30 minutes plus overnight incubation time to prepare the antibody-beads before preparing your chromatin. Make the bead binding buffer (PBS + 0.2% Tween 20, filter-sterilize, and chill). Prepare rotator in cold room for overnight incubation.

13) Resuspend protein G coupled magnetic beads (Dynabeads, Invitrogen, Waltham, MA, USA) on a rotator for 5 minutes at room temperature. Briefly vortex and spin the tube prior to opening. For each IP aliquot 50 microliters of beads into a microcentrifuge tube and a single 50 microliter aliquot for pre-clearing the chromatin to be used for IP. You can combine up to 8 reactions of beads into a single tube for washing.

14) Carefully resuspend the beads by slowly pipetting up and down before each time you aliquot them from the main stock. Wash beads with 1 mL of bead binding buffer, briefly vortex, briefly centrifuge, place on magnetic rack for 1 minute and remove the liquid. Take beads off magnetic rack quickly without allowing to dry and repeat for a total of 5 washes.
15) For each IP antibody resuspend beads for two replicates in 400 microliters of bead binding buffer into a separate tube. Add antibodies to beads. Rotate beads for pre-clearing without any antibody.
    a. For each IP reaction (multiple by 2X for two animal replicates) use

10 µL (~ 0.95 µg)	CTCF	Cell signaling D31H2XP 3418S	Lot 3
5 µg	H3K27me3	EMD Millipore 07-449	Lot 3018864
5 µg	H3K4me1	Millipore 07-436	Lot 2975223
5 µg	H3K4me3	Abcam 8580	Lot GR3221448-1
5 µg	H3K27ac	Abcam 4729	GR3211959-1

16) Incubate overnight on rotator in cold room 4 °C.
17) Spin briefly, place tubes on magnetic rack for 1 minute, discard liquid, wash beads with 1 mL of bead binding buffer, repeat. Then wash three times with 1 mL of ChIP Incubation buffer (50 mM NaCl, 50mM Tis-HCl 7.5, 5mM EDTA). Resuspend the beads in 50 microliters of ChIP Incubation buffer per IP and add protease inhibitor cocktail. Keep beads on ice until added to chromatin.

Immunoprecipitation

Preparation steps: Prepare three low salt wash buffers, filter sterilize, and chill.

18) After the input chromatin has been aliquoted and frozen, add 50 microliters of washed magnetic beads (no antibody) to the digested chromatin. Mix well by rotating the tube and incubate on ice for 5 minutes. Briefly spin, place on magnetic rack for 1 minute and remove supernatant containing pre-cleared chromatin to a new tube.
19) Aliquot 190 microliters of chromatin into a new tube for each IP reaction, add 50 microliters of magnetic beads bound to antibody, protease inhibitor cocktail and sodium butyrate (for 5 mM total concentration), and 740 microliters of ChIP Incubation buffer to QS a 1 mL total reaction volume.
20) Incubate overnight on a rotating rack in a cold room, 4 °C. Approximately 10 hours.
21) Harvest immunoprecipitated chromatin after a brief spin on the magnetic rack for 1 minute. Remove liquid to a new tube and label as unbound fraction. Wash the IP chromatin bound the antibody-beads on the magnetic rack, with two washes each of increasing salt concentrations (Wash buffers 10 mM EDTA, 50 mM Tris/Cl pH 7.5, + 75 mM, 125 mM, and 175 mM NaCl), discarding the liquid each time. Complete a final wash of the chromatin-antibody-beads with 1X TE, re-spin, place on magnetic rack, and discard all liquid twice.

Elution and DNA clean-up

Preparation steps: Prepare iPure kit buffers according to manufacturer's instructions. Ensure that magnetic clean-up beads are always well mixed with the pipette immediately prior to aliquoting beads and keep cap closed and liquids present as quickly as possible to prevent drying of beads. Chill magnetic rack ahead of time and keep on ice.

22) Following the manufacturer's instructions, the DNA for each reaction sample was eluted in 200 microliter elution buffer and purified as directed, excluding the cross-linking reversal step, with the iPure kit (Diagenode, Liege, Belgium). Input chromatin (non-immunoprecipitated control) was purified with the same kit.
23) Total amount of immunoprecipitated DNA obtained for each sample was determined by Qubit dsDNA HS analysis (Thermo Fisher Scientific).
24) Samples are frozen to -80 °C for overnight storage and shipped overnight on ice to facility for library preparation.

Library preparation

25) Confirm fragmentation and DNA quantity with fluorescence Qubit (Thermo Fisher Scientific) and fragment analysis (Agilent).
26) Use 7.5 ng of DNA to prepare each library with the Truseq ChIP Sample Prep kit (Illumina, San Diego, CA, USA) according to the manufacturer's directions. Reduce PCR to 15 cycles minimize duplication bias. Size select the band for 250-600 bp fragments (which isolates the bulk of DNA fragments of average size 150 bp, accounting for gel shift, and ligated adapters).
27) Multiplex libraries with the indexing adapters included in the kit.
28) Repeat check for library size and concentration with fragment analysis with the High Sensitivity NGS Fragment Analysis Kit (Agilent). Concentration is verified with the StepOnePlus Real-Time PCR System (Thermo Fisher Scientific) and KAPA Library Quantification Kit (Kapabiosystems, Wilmington, MA, USA) according to kit directions.
29) Libraries are diluted to 4 nM with RSB (10 mM Tris-HCl, pH 8.5) then denatured with 0.1 M NaOH, and finally 20 pM clustered in a high-output flow cell with HiSeq Cluster Kit v4 on a cBot (Illumina).
30) After cluster generation, load flow cell onto HiSeq2500 for 50 bp, single end sequencing. Samples are multiplexed between 2-4 libraries per lane to generate greater than 40-80 million, filter-passing reads per sample.

References:

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