ChIP Protocol for immune cells **(modified from H. Zhou, UC-Davis) protocol and Diagenode)**	PI	Dr. Chris Tuggle
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Before starting:
Estimate amount of time to be completed and reserve sonicator.
Order the Diagenode's reagents necessaries for the immunoprecipitation before starting the process.
Use low retention microcentrifuge tubes (250) for this protocol. Fisherbrand, Cat Nº02-681-320.

Cell cross-linking

1. Obtain 1x10^6 cells per population (sorting or counting).
2. Bring 1x10^6 cells up to 1ml of cold PBS.
3. Add 54 µL of 16% fresh formaldehyde (final volume 1% formaldehyde).
4. Incubate on a rotator for 8-10 mins at room temperature.
5. Add 60 µL of 2.5M glycine to tube (150 mM final) to quench cross-linking.
6. Incubate at room temperature for 10 mins.
7. Pellet cells at 2000g for 10 mins at 4ºC.
8. Remove supernatant, resuspend pellet in 1ml cold PBS, transfer to microfuge tube.
9. Pellet cells at 2000g for 10mins at 4ºC.
10. Remove supernatant and flash freeze pellet.
11. Store at -80ºC.

Chromatin Extraction and Shearing

12. Resuspend the cell pellet in 1000 µL of cell lysis buffer.
13. Incubate the tube on ice for 20 mins.
14. Counting the nuclei using the hemocytometer to verify the amount and viability of the nuclei.
    Add 2 µL of the lysate cells (nucleus) to 18 µL of trypan blue to get a dilution factor 1:10 and mix well.
15. Add 10 µL in the space between the coverslip and the chamber.
16. Use the 20x magnification to focus on the grid.
17. Count cells that don't touch the top or left lines in the four squares (Figure 1).

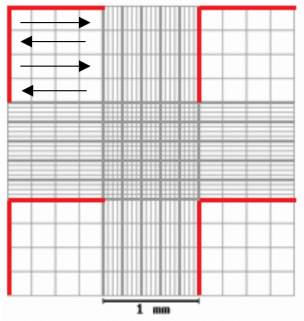

Figure 1. Hemocytometer. Count cells in each square following the lines.

Calculate the number of cells (cells/mL):

$$\text{Cell density} = \frac{\text{(Total cells / 4 squares) x dilution factor (1:10)}}{\text{Square volume (0.0001 mL)}}$$

18. Harvest the nuclei by centrifugation at 2000g for 10 mins at 4°C.
19. Remove the supernatant and resuspend the nuclei in 140 µL of nuclear lysis buffer *(The ChIP-Seq nuclear lysis buffer contains 0.5% SDS to aid in the lysis of nuclei. Some tissues such as brain samples require SDS concentrations of up to 0.5%; this will need to be empirically determined. High concentrations of SDS can inhibit antibody-binding downstream and will require further dilution or dialysis detailed below before using in IPs.).*
20. Incubate the tube on ice for 20 mins.
21. Take a 10 µL aliquot for an analytical agarose gel (1.5%). Store the aliquots on ice until required. This sample is to confirm that the chromatin was intact before isolation.
22. Aliquot 130 µL into the Covaris microtube (130 AFA Fiber, Screw-Cap 25) for chromatin shearing, avoid the bubbles because could interfere on the sonication process. Proceed to sonication in the Covaris sonicator (ME220) using the program 350 bp microtube screw cap (180 seconds for macrophages/tube, peak power 70, duty factor 20%, cycles/burst 1000, Ag power 14).
23. Harvest the sheared chromatin by centrifugation at 2000g for 10 mins at 4°C. Transfer the supernatant (sheared chromatin) to a new Eppendorf tube.
24. Once completed, remove the samples from the sonicator and store on ice at 4°C if you are going to work with them the same day or flash freeze the sample and store at -80ºC.
25. Treat the sonicated samples adding 20 µg or 2 µL of proteinase K (600mAU/mL,cat:71049-4) for 3 hours at 65°C (Reverse the cross-links) and treat with 2 µL RNAse A (10mg/mL, #EN053).
26. Confirm the size of the DNA using the High Sensitivity DNA chip in the Agilent Bioanalyzer *(Sheared chromatin should range in size from 150 to 600 bp.) Examples of sheared chromatin and non-sheared chromatin can be seen in Figure 2. Ideally you would prefer to see non-sheared*

chromatin to maintain a high molecular weight similar to purified DNA. Sheared chromatin should fall within your ideal size range. Increase the time of shearing will need to be optimized based on cell type.

27. Flash freeze the sample and store at -80ºC.

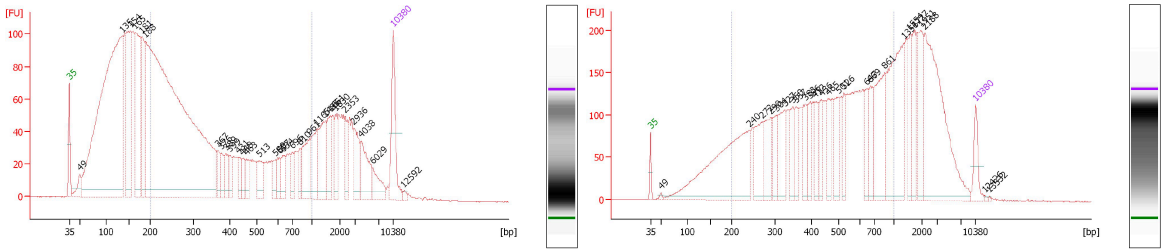

Figure 2. Example of quality assessment of sheared macrophages chromatin. On the left is shown acceptable DNA size distribution (between 150-600 bp) and on the right is shown un-sheared DNA.

<u>After Shearing:</u> Diagenode. *iDeal ChIP-seq kit for Histones.*

Day 1: (From this step, all the reagents are from the Diagenode *iDeal ChIP-seq kit*)
Some steps below should be performed in a cold room to minimize temperature increases.

1. Collect the supernatant which contains the sheared chromatin.
2. Determine the total number of IP's in the experiment (approx. 200.000 cells/nucleus for immune cells).
3. Take the required amount of DiaMag Protein A-coated magnetic beads (20 µL/IP). Dilute the 5x ChIP buffer iC1 with ChIP-seq grade water to obtain 1x ChIP buffer iC1. The total amount of 1x ChIP buffer iC1 needed is 9x (additional aliquot added for pipette error) the volume of beads required for each sample:
   (# of rxns) (20ul beads/rxn) = (vol. for experiment) (x10) = Total Vol. iC1 buffer (Prepared with ratio of Total Vol. C1 buffer needed: water = 4:1)
   Place the diluted ChIP buffer iC1 on ice.
4. Wash the beads 4 times with 2x the volume of ice-cold 1x ChIP buffer iC1 for experiment [i. e. (# of rxns) (20ul beads/rxn) = (vol. for experiment)]: To wash the beads, add 1x ChIP buffer iC1 and resuspend the beads by pipetting up and down several times and place the tubes in the 1.5 ml magnetic rack. Wait for one min to allow the beads to be captured by the magnet and remove the supernatant. Repeat 3 times.
5. After the last wash, resuspend the beads in the original volume 1x ChIP buffer iC1 for experiment [i. e. (# of rxns) (20ul beads/rxn) = (vol. for experiment)].
6. Prepare the ChIP reaction mix for total number of reactions plus one according to Table 1:

Table 1. ChIP reaction mix							
Number. of IP's	5% BSA (µL)	200x Protease inhibitor cocktail (µL)	5x buffer iC1 (µL)	Sheared chromatin (2^5 cells) (µL)	Magnetic beads (µL)	ChIP-seq grade water (µL)	Antibody (µL)
1	6	1.5	56	x µL of sheared chromatin	20	116.5-(x+y)	y

7. Aliquot the ChIP reaction mix to 1.5ml tubes.
8. Volume of sheared chromatin, water and antibody depend on amount/type of cells and concentration of the antibody.
9. Volume and concentration of each antibody necessary for immune cells:

   H3K4me3: 1.5 µg (1.07 µL).

   H3K4me1:1 µg (0.66 µL).

   H3K27ac: 2 µg (0.70 µL).

   H3K27me3: 1 µg (0.69 µL).
10. When preparing the reaction mix, place 1 µL of (more depending on the concentration of sample) of the sheared chromatin aside to be used as an input the next day.
11. Incubate the tubes overnight at 4°C under constant rotation at 40 rpm on a rotating wheel.

Day 2: (Performed in cold room)

12. Incubate the iE1 buffer in room temperature and thaw iE2 buffer (stored in -20C) (solution should be clear once reached room temp). After the overnight incubation, briefly spin the tubes and place them in the magnetic rack. Wait for one min and remove the supernatant. To wash the beads, add 350 µL of Wash buffer iW1, gently invert to resuspend the beads and incubate for 5 mins on a rotating wheel at 4°C.
13. Repeat the wash as described above once with Wash buffer iW2, iW3 and iW4 using the same buffer volume, respectively.

Elution, decross-linking:

14. After removing the last wash buffer, add 400 µL of Elution buffer iE1 to the beads and incubate for 30 mins on a rotating wheel at room temperature.
15. Briefly spin the tubes and place them in the magnetic rack. Transfer the supernatant to a new tube and add 16 µL of iE2 buffer. If running with input (to be performed only once), add 399 µL

buffer iE1 and 16 µL buffer iE2 to 1 µL of the input sample kept aside the day before. Fasten the lids with parafilm or microtube cap locks. Incubate for overnight in a thermomixer at 65°C.

Day 2: DNA isolation

16. Add 2 µL of carrier to each IP and input sample. Vortex briefly and perform a short spin.
17. Add 400 µL of 100% isopropanol to each IP and input sample. Vortex briefly and perform a short spin. Following the addition of isopropanol, the solution may become cloudy. This is not detrimental to your experiment and will not influence the quality or quantity of your purified DNA.
18. Resuspend the provided Magnetic beads and transfer 15 µL to each IP and input sample.
    • Keep Magnetic beads in liquid suspension during storage at 4°C and at all handling steps, as drying will result in reduced performance.
    • The final volume is now 817 µL per IPure reaction.
19. Incubate IP and input samples for 1 hour at room temperature on a rotating wheel (40 rpm).
    Prepare the Wash buffer 1 containing 50% isopropanol:
    Wash buffer 1 (w/o isopropanol): 50ul/sample
    100% isopropanol: 50ul/sample
    = 100ul wash buffer 1/sample
    Total vol.= (# of samples x 100ul wash buffer) + 10% more
    Never leave the bottle open to avoid evaporation.
20. Briefly spin the tubes, place in the DiaMag1.5, wait 1 min and discard the buffer. Keep the captured beads and add 100 µL Wash buffer per tube. Pipette mix to resuspend the beads, transfer to 200ul PCR tubes, and incubate for 5 mins at room temperature on a rotating wheel (40 rpm). Careful not to disturb the captured beads attached to the tube wall. Briefly spin down after incubation.
21. Prepare the Wash buffer 2 containing 50% isopropanol as follows:
    Wash buffer 2 (w/o isopropanol): 50ul/sample
    100% isopropanol: 50ul/sample
    = 100ul wash buffer 2/sample
    Total vol. = (# of samples x 100ul wash buffer) + 10% more
22. Wash the IP and input samples with the Wash buffer 2 as follows:
    Briefly spin the tubes, place into the DiaMag1.5, wait 1 min and discard the buffer. Keep the captured beads and add 100 µL Wash buffer 2 per tube. Pipette mix and incubate for 15 mins at room temperature on a rotating wheel (40 rpm). Careful not disturb the captured beads attached to the tube wall. Briefly spin down after incubation.

Note: This Elution buffer (buffer C) is suitable for direct qPCR analysis, whole genome amplification, chip hybridization and Next-Generation sequencing.

23. One elution in 25 µL (standard procedure).
    Briefly spin the tubes and place them into the DiaMag1.5, wait 1 min and discard the buffer. Keep the captured beads and add 25 µL Buffer C per tube. Invert the 8-tube strip to resuspend the beads and incubate for 15 mins at room temperature on a rotating wheel (40 rpm). Resuspend the pelleted beads using the pipet and make sure that you drop them on the bottom of the tube.
    Spin the tubes and place them into the magnetic rack, wait 1 min and transfer the supernatants into a new labelled 1.5 ml tube. Keep the bead pellets on ice.
24. Place the DNA on ice and proceed to any desired downstream applications, or store it at -20°C.
25. Take 5 µL of IP'd DNA and determine the concentration with 'Quant-IT dsDNA HS assay kit' using the Qubit system.
26. Store the DNA at -20°C until you are ready to analyze it with qPCR or by high throughput sequencing an example can be seen in Figure 3.

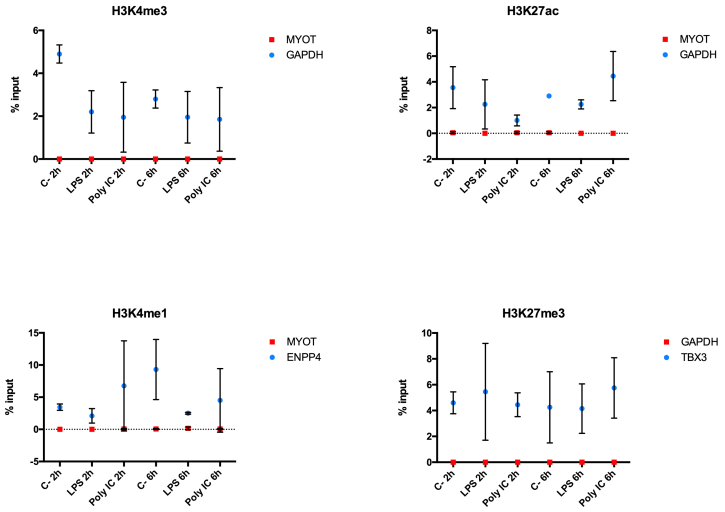

Figure 3. ChIP-qPCR shows enrichment of histone marks at target gene promoter regions for all histone modifications tested. Percent input method was calculated to evaluate the DNA recovery after ChIP using primers for regions associated to each histone mark (blue spots). Non-associated regions (red spots) were used to measure the background.

Reagents list

Preparation:

- **Cell Lysis Buffer:**

50 mM Tris (pH 8.0)
140 mM NaCl
1 mM EDTA
10% glycerol
0.5% NP-40
0.25% Triton X-100

Filter-sterilize using 0.2 um filter system. Store for up to 1 yr at 4°C.
***In addition, if immunoprecipitating with an antibody against the histone modification H3K27ac, include the histone deacetylase inhibitor sodium butyrate at a final concentration of 20 mM (Cat Nº C12020010. Diagenode). Other inhibitors are generally not necessary when immunoprecipitating transcription factors but should be considered when using antibodies against posttranslational covalent modifications.

- **Nuclear Lysis Buffer:**

10 mM Tris (pH 8.0)
1 mM EDTA
0.5 mM EGTA
0.2% SDS concentration can be increased to (0.5% SDS as required)

Filter-sterilize. Store for up to 1 yr at 4°C
TE Buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0)
PBS (8g of NaCl ; 0.2g of KCl ; 1.44g of Na_2HPO_4 ; 0.24g of KH_2PO_4; dissolve to 1L ddH2O; pH=7.4)
*** Add one protease inhibitor according to product description at day of use to all buffers.

Use low retention microcentrifuge tubes (250) for this protocol. Fisherbrand, Cat Nº02-681-320.
proteinase K: 600mAU/mL, Cat Nº. 71049-4. Millipore.
RNAse A: 10mg/mL, DNase-free. Cat Nº. EN0531. ThermoFisher Scientific.
16% Formaldehyde solution (w/v), methanol free. Cat Nº. 28908. Thermo Scientific.
2.5M Glycine solution, ACS grade. Cat Nº. C-4375. Boston BioProducts.
Diagenode ----iDeal ChIP-seq kit for Histones Cat. Nº. C01010051
H3K4me3 polyclonal antibody: Diagenode Cat. Nº. C15410003.
H3K27me3 polyclonal antibody: Diagenode Cat. Nº. C15410195.
H3K27ac polyclonal antibody: Diagenode Cat. Nº. C15410196.
H3K4me1 polyclonal antibody: Diagenode Cat. Nº. C15410194.