Protocol

Chromatin Immunoprecipitation with Fixed Animal Tissues and Preparation for High-Throughput Sequencing

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Chromatin immunoprecipitation coupled with high-throughput sequencing (ChIP-Seq) is a powerful method used to identify genome-wide binding patterns of transcription factors and distribution of various histone modifications associated with different chromatin states. In most published studies, ChIP-Seq has been performed on cultured cells grown under controlled conditions, allowing generation of large amounts of material in a homogeneous biological state. Although such studies have provided great insight into the dynamic landscapes of animal genomes, they do not allow the examination of transcription factor binding and chromatin states in adult tissues, developing embryonic structures, or tumors. Such knowledge is critical to understanding the information required to create and maintain a complex biological tissue and to identify noncoding regions of the genome directly involved in tissues affected by complex diseases such as autism. Studying these tissue types with ChIP-Seq can be challenging due to the limited availability of tissues and the lack of complex biological states able to be achieved in culture. These inherent differences require alterations of standard cross-linking and chromatin extraction typically used in cell culture. Here we describe a general approach for using small amounts of animal tissue to perform ChIP-Seq directed at histone modifications and transcription factors. Tissue is homogenized before treatment with formaldehyde to ensure proper cross-linking, and a two-step nuclear isolation is performed to increase extraction of soluble chromatin. Small amounts of soluble chromatin are then used for immunoprecipitation (IP) and prepared for multiplexed high-throughput sequencing.

MATERIALS

It is essential that you consult the appropriate Material Safety Data Sheets and your institution's Environmental Health and Safety Office for proper handling of equipment and hazardous materials used in this protocol.

RECIPES: Please see the end of this protocol for recipes indicated by <R>. Additional recipes can be found online at <http://cshprotocols.cshlp.org/site/recipes>.

Reagents

When applicable, buffers can be prepared at a 2× concentration to allow for addition of various inhibitors and different concentrations of sodium dodecyl sulfate (SDS).

Agarose gel (1% agarose)

Prepare in 1× TAE with 0.2 µg/mL ethidium bromide.

Antibodies to protein of interest

Many suppliers offer "ChIP-grade" versions of their antibodies that have high concentrations (~1 mg/mL). The use of more dilute antibodies or those not affinity-purified is not recommended.

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Bead binding buffer <R>

Cell lysis buffer for ChIP-Seq <R>

ChIP-Seq dilution buffer <R>

ChIP-Seq elution buffer <R>

ChIP-Seq nuclear lysis buffer (see Step 24) <R>

ChIP-Seq wash buffer <R>

DNA loading dye (e.g., Orange Loading Dye [NEB B7022S])

Standard DNA loading dye recipes call for bromophenol blue, which migrates at the same rate as the desired chromatin fragments. We recommend using a loading dye that migrates more quickly through the gel (e.g., Orange G).

Ethidium bromide (10 mg/mL)

Formaldehyde (37%)

Glycine (2.5 M)

Liquid nitrogen (optional; see Steps 18 and 38)

Magnetic Protein G or Protein A beads (Dynabeads from Invitrogen/Life Technologies)

Phosphate buffered saline (PBS), prepared from powder (Sigma-Aldrich P5368) and prechilled to 4°C before use

Proteinase K (20 mg/mL)

QIAquick PCR purification kit (Qiagen)

qPCR primers

Properly designed qPCRs are critical for assessing the quality of each ChIP. Primers should flank a known binding site for a transcription factor or region known to contain high levels of a particular histone modification. We typically design qPCR primers with Primer3Plus (<http://www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi/>). Amplicon length should be set between 85 and 150 bp. Use an optimal primer size of 20 ± 2 nt, an optimal melting temperature of $60^\circ\text{C} \pm 2^\circ\text{C}$ with no more than 3°C difference in melting temps between primers, and an optimal GC content of $50\% \pm 10\%$. All primers should be tested with a dilution series of genomic DNA to confirm a single product and good efficiency over a range of template concentrations.

Quant-iT PicoGreen dsDNA Assay Kit (Invitrogen/Life Technologies)

RNase A (10 mg/mL, DNase-free)

Sodium acetate (3 M, pH 5.2)

Sodium butyrate (1 M) (optional; see Step 19)

Sodium dodecyl sulfate (SDS) (20%)

SYBR Green qPCR Master Mix (Invitrogen/Life Technologies)

TAE <R>

TE buffer for ChIP-Seq <R>

Tissue to be examined

This protocol is optimized for small amounts of fresh or frozen tissue. One IP is generally performed with 10^4 to 10^5 cells. This amount of material can be obtained from one litter of E10.5 mouse limb buds (i.e., 8–10 forelimb buds), five to ten 20- μm thick sections of frozen tumor block, or 1–5 mg of fresh tissue. However, the protocol has successfully been used for tissue amounts ranging from 25 E11.5 mouse limb buds (~ 1 million cells) to two E33 human limb buds ($\sim 50,000$ cells). If substantially more tissue is used, further dissection of the tissue may be required or empirical optimization of the protocol for increased cell number may be needed. Performing a ChIP on previously studied material in tandem may be necessary to confirm successful IP.

Equipment

Disposable plastic pestles (Kontes) or Polytron homogenizer with 3.5-mm tip (Kinematica)

Dounce homogenizer with tight and loose pestles (2 mL; Kontes)

Prechill before starting protocol.

Fluorometer (NanoDrop 3300)

Other sensitive double-strand DNA detection methods and detectors are available such as the Qubit system from Invitrogen.

Gel electrophoresis apparatus

Heat block (95°C)
Incubator (4°C) or cold room
Incubator or heat block at 37°C and 65°C
Magnetic stand for precipitation of beads (Invitrogen)
Microcentrifuge (refrigerated or prechilled to 4°C)
Microcentrifuge tubes (1.5 and 2.0 mL)
Nonstick 1.5- and 2.0-mL tubes (Ambion)
qPCR apparatus
Rotator
Sonicator

We use a Misonix S4000 sonicator with attached 431A cup horn. A chilled Fisher Isotemp 3006D recirculator pump continuously cycles cold water through the cup horn to dissipate any heat generated during sonication. Alternatively, ice water can be kept in the cup horn and changed frequently.

Thermomixer (Eppendorf)

METHOD

Preparation of Antibody Beads

This portion of the protocol requires ~30 min followed by an overnight incubation. It can be performed a day in advance of chromatin extraction (or at any point if frozen chromatin aliquots are to be used).

1. Resuspend magnetic Protein G (or Protein A) beads on a rotator for 5 min at room temperature.
2. For each IP to be performed, aliquot 50 μ L of beads into a microcentrifuge tube.
If several IPs will be performed using the same antibody, combine up to eight reactions worth of beads in a single 2-mL tube.
3. Wash the beads with 1 mL of bead binding buffer by resuspending the beads, placing the tubes on the magnetic stand for 1 min, and removing the bead binding buffer. Repeat.
If combining multiple aliquots of beads in a single tube, increase number of washes to five.
4. Resuspend the beads in 200 μ L of bead binding buffer per IP.
If performing eight IPs, resuspend the beads in 1.6 mL of bead binding buffer in a 2-mL tube.
5. Add up to 10 μ g of the desired antibody per IP.
The amount of antibody will vary considerably and will need to be empirically tested. Scale accordingly as above for multiple aliquots of beads.
6. Incubate the beads and antibody on the rotator overnight at 4°C.
7. Wash the beads twice with 1 mL of bead-binding buffer and then twice with 1 mL of ChIP-Seq dilution buffer. Perform each wash by placing the tubes on the magnetic stand for 1 min and then removing the supernatant.
Increase number of washes to five bead-binding buffer washes and five ChIP-Seq dilution buffer washes if multiple reactions were combined above.
8. Resuspend the beads in 50 μ L of ChIP-Seq dilution buffer per IP to be performed.
9. Store the beads at 4°C until the chromatin is prepared.

Tissue Harvest and Cross-Linking

This procedure takes ~1 h, not including any time necessary to harvest or dissect tissue.

10. Transfer fresh or frozen tissue to a clean 1.5-mL tube containing 250 μ L of ice-cold PBS.
11. Briefly homogenize the tissue by hand to yield chunks 0.5 mm³ or smaller. Use a disposable plastic pestle or a handheld Polytron homogenizer fitted with 3.5-mm tip.

12. Add ice-cold PBS to a final volume of 1 mL.
13. Cross-link the tissue by adding 27 μ L of 37% formaldehyde (1% final) to each tube and immediately invert the tube several times.
14. Incubate the tube on the rotator at room temperature for 15 min.
15. Quench the cross-linking by adding 67 μ L of 2.5 M glycine (150 mM final) and incubate the tube on the rotator at room temperature for an additional 10 min.
16. Harvest the tissue by centrifugation at 2000g for 10 min at 4°C.
17. Remove the supernatant and add 1 mL of ice-cold PBS. Flick the tube with your finger several times to dislodge the pellet.
18. Harvest the tissue by centrifugation at 2000g for 10 min at 4°C and discard the supernatant. Flash freeze the tissue pellet in liquid nitrogen and store at -80°C.

Alternatively, proceed to Step 19 without freezing.

Chromatin Extraction and Shearing

This procedure requires ~5 h.

19. Resuspend the frozen cross-linked tissue pellet in six volumes of ice-cold cell lysis buffer for ChIP-Seq. Use the markings on the 1.5-mL tube to provide an estimate of the pellet volume. If the pellet is very small, resuspend it in 300 μ L of cell lysis buffer.

Cell pellets at this stage will frequently adhere to the insides of pipette tips. It may be necessary to resuspend the pellet by flicking the tube several times instead of by pipetting. If the tissue remains in large chunks, homogenize it in a prechilled Dounce homogenizer with a loose pestle (pestle A) five to 10 times.

20. Incubate the tube on ice for 20 min.

This incubation period allows for swelling of cells to allow for mechanical lysis while keeping nuclei intact.

21. Transfer the tissue suspension to a prechilled Dounce homogenizer and homogenize with 30–40 strokes with the tight pestle (pestle B).

22. Transfer the homogenate to a fresh nonstick 1.5-mL tube, rinse the Dounce homogenizer with 500 μ L of cell lysis buffer, and combine.

23. Harvest the nuclei by centrifugation at 2000g for 5 min at 4°C.

The pellet will not be as tight against the tube as the original cell pellet and will be mostly white.

24. Remove the supernatant and resuspend the nuclei in five volumes of ice-cold ChIP-Seq nuclear lysis buffer (based on the volume of the original cell pellet in Step 19).

The ChIP-Seq nuclear lysis buffer contains 0.2% 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.

25. Incubate the tube on ice for 20 min.

26. Take a 5- μ L aliquot from each sample and dilute it with 15 μ L of TE for an analytical gel.

Store the aliquots on ice until required. This sample is to confirm that the chromatin was intact before isolation.

27. Place the tube in the sonicator bath set at 2°C.

In our hands, sonicating a volume of 300 μ L per tube with the Misonix 431A cup horn has performed very consistently, so we advise dividing larger samples into multiple tubes for sonication. Place at least two and up to eight tubes in the tube holder in the sonicator.

28. Adjust the water level so that the tubes just touch the surface of the cup horn.

29. Sonicate the samples at amplitude 20 with 10-sec pulses and 10-sec rest for a total sonication time of 30 min.

30. Remove the samples from the sonicator and store on ice.

31. Take a 5- μ L aliquot and dilute it with 15 μ L of TE for an analytical gel.
32. Treat all reserved aliquots with 10 μ g of RNase A for 15 min at 37°C.
33. Treat all reserved aliquots with 20 μ g of proteinase K for 30 min at 65°C.
34. Reverse the cross-links for 5 min at 95°C and allow samples to cool slowly to room temperature.
These steps are required to ensure proper migration of the DNA on the agarose gel; otherwise, smearing or migration at much higher molecular masses than expected will occur.
35. Add loading dye to each sample, and separate samples on a 1% agarose gel in 1 $\times$ TAE running buffer.
36. Visualize DNA samples on the agarose gel.
Sheared chromatin should range in size from 200 to 500 bp. See Troubleshooting.
37. Centrifuge the samples from Step 30 at 16,000g for 10 min at 4°C to remove insoluble material.
38. Transfer the soluble sheared chromatin to fresh nonstick 1.5-mL tube. Take a final 5- μ L aliquot and dilute it with 15 μ L of TE for estimation of DNA concentration.
Chromatin can be frozen in liquid nitrogen and stored at -80°C until needed.
39. Treat the aliquot with RNase A, proteinase K, and cross-link reversal as above (Steps 32–34).
40. Purify the chromatin aliquot with the QIAquick PCR Purification Kit, and measure the DNA concentration with PicoGreen and a NanoDrop 3300 Fluorospectrometer. Prepare a 1 $\times$ PicoGreen stock and follow the manufacturer's instructions for setting up a standard curve on the NanoDrop instrument.

Binding of Antibody to Target Antigen

This procedure requires ~10 min followed by an overnight incubation.

41. Dilute 30–50 μ g of chromatin from Step 38 with ChIP-Seq dilution buffer in a fresh nonstick tube to reduce the SDS concentration <0.1% and achieve a final volume of 450 μ L.
The amount of chromatin to be used will be highly antibody- and target-dependent and must be determined empirically, with 1–50 μ g as a general range. Histones are highly abundant and widespread across the genome, whereas transcription factors are much less abundant and have specific binding sites.
42. Reserve 5%–10% of diluted chromatin as an input sample and store at 4°C.
Input must be reserved to assess enrichment via qPCR and to provide a necessary control for comparing sequence data to identify true binding sites or regions of enrichment.
43. Add 50 μ L of the prepared antibody beads from Step 9 to each tube of diluted chromatin.
44. Incubate tube on the rotator overnight at 4°C.

IP of Chromatin

This procedure requires ~2 h followed by an overnight incubation. Perform all washes and incubations in a cold room.

45. Remove the tube containing chromatin and the antibody beads from the rotator, place it on the magnet, and let it stand for 1 min.
46. Remove the supernatant and transfer it to a fresh tube if required for additional IPs or troubleshooting.
47. Wash the beads from each IP with 1 mL of ChIP-Seq wash buffer by pipetting the beads up and down or inverting the tube several times to mix.
48. Place the tube on the rotator and incubate for 3 min.
49. Place the tube on the magnet to precipitate the beads and remove supernatant.
50. Repeat Steps 47 through 49 for a total of eight washes.
51. Perform one wash with 1 mL of TE. Remove the supernatant.

52. Resuspend the beads in 85 μL of ChIP-Seq elution buffer.
53. Elute the chromatin at 65°C with agitation on the thermomixer for 10 min.
The beads will settle if not agitated frequently. If a thermomixer is not available, mix the beads by flicking the tubes with your finger every 2 min.
54. Place the tube on the magnet and transfer the eluate to a fresh nonstick tube.
55. Repeat elution with a second volume of 85 μL of ChIP-Seq elution buffer and combine eluates.
56. Incubate the combined eluates at 65°C overnight to reverse the cross-links.
57. For each input control (see Step 42), add ChIP-Seq elution buffer to a final volume of 170 μL and incubate overnight at 65°C.

Purification of Chromatin

This procedure requires ~4 h.

58. Add 10 μg of RNase A to each tube of input and IP chromatin and incubate for 1 h at 37°C.
59. Add 200 μg proteinase K diluted in 120 μL TE to each tube and incubate for 2 h at 65°C.
60. Transfer each reaction to a 2-mL nonstick tube and add five volumes of PBI from the QIAquick PCR Purification Kit. Add 10 μL of 3 M sodium acetate to adjust the pH and generate a bright yellow color.
Phenol:chloroform extraction and ethanol precipitation of DNA can be substituted here if preferred. However, depending on the amount of chromatin obtained, a significant volume of sample may be required for subsequent high-throughput sequencing library generation. Phenol carryover may inhibit initial end repair steps and result in low-quality libraries.
61. Follow the QIAquick PCR Purification Kit instructions and elute the DNA with 50 μL of EB into a fresh nonstick 1.5-mL tube.
EB can be prewarmed to 50°C before elution to maximize recovery.
62. Quantify the DNA recovered by IP and in the input sample with PicoGreen and a fluorospectrometer.
63. Dilute a portion of the ChIP and input samples with dH_2O to the same concentration in nonstick tubes (50–500 pg/ μL).

Analysis by qPCR

64. Analyze target regions by qPCR using the following reaction:

Template DNA from either input or IP	1 μL
Primer mix (forward and reverse primers premixed at 10 μM each)	2.5 μL
2 $\times$ SYBR Green qPCR Master Mix	10 μL
dH_2O	to 20 μL

65. Run the samples on the qPCR machine with standard conditions for SYBR Green. Adjust conditions as your instrument and reagents require.
 - i. Melt the template for 10 min at 95°C.
 - ii. Amplify the target by performing 40 cycles of 15 sec at 95°C and 1 min at 60°C, measuring the fluorescence after each cycle.
 - iii. Examine the melting curve to ensure that a single product is made in each reaction.
A good reference for performing qPCR is Bustin et al. (2009).
66. Measure enrichment by comparing Ct values between input and ChIP reactions.

Depending on the type of factor being immunoprecipitated, fold enrichments versus input can vary considerably. For histone modifications, four- to 16-fold enrichments are acceptable. For transcription factors and other sequence specific DNA-binding proteins, enrichments would be expected to be quite higher, up to 60-fold.

Preparation of Samples for High-Throughput Sequencing

67. Dilute 10 ng of ChIP or input DNA with dH₂O to a final volume of 30 µL in a fresh nonstick tube for high-throughput sequencing library preparation.

Both ChIP and input samples must be sequenced to identify real binding peaks or enriched regions from more abundant open chromatin in the input sample.

68. Follow the manufacturer's instructions for preparing libraries with a kit.

We routinely use kits from NEB (e.g., E6240) for sequencing on Illumina instruments. Many library prep kits for different sequencing instruments are now commercially available.

TROUBLESHOOTING

Problem (Step 36): Chromatin is not successfully sheared to fragments between 200 and 500 bp.

Solution: Sonication (Step 29) is the most critical step to a successful ChIP. Fragments longer than 500 bp may result in very wide peaks and a high degree of genomic background; in contrast, oversonication can destroy protein complexes and result in no IP. For each new tissue type, sonication must be optimized to achieve consistent levels of chromatin shearing. Amplitude 20 is a good general starting point, but can be increased as needed based on the shearing level observed in the agarose gel. Depending on the number of tubes tested in the 431A cup horn, the amplitude should be adjusted to obtain ~12 to 15 W output per tube during operation. This level is sufficient to properly shear most samples, but more difficult samples such as fibrous tumors will require higher amplitudes and a longer processing time. During optimization stages, take 5-µL aliquots (dilute with 15 µL of TE) at regular intervals to determine the proper length of sonication time.

RELATED INFORMATION

ChIP-Seq has been used extensively to study genome-wide histone modification profiles and transcription factor occupancy in cultured human and murine cells (Neph et al. 2012; Thurman et al. 2012; Shen et al. 2012). Few studies have used tissue for ChIP-Seq and those that have reported the use of large amounts of tissue and milligram quantities of soluble chromatin for a single experiment (Visel et al. 2009; Blow et al. 2010). Use of this protocol to identify active enhancers marked with histone H3 lysine 27 acetylation (H3K27ac) in early murine and primate limb development is described in Cotney et al. (2013).

RECIPES

Bead-Binding Buffer

Phosphate-buffered saline (PBS)
Tween 20 (0.2%)

Dissolve one pouch of powdered PBS (Sigma-Aldrich P5368) in 1 L of dH₂O, and sterilize by autoclaving. Add Tween 20 to a final concentration of 0.2%, and filter-sterilize. Store for up to 1 yr at 4°C.

Cell Lysis Buffer for ChIP-Seq

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. Store for up to 1 yr at 4°C. Add one protease inhibitor tablet or 2 mL of 25× inhibitor stock solution as directed by the manufacturer (cOmplete EDTA-Free; Roche) per 50 mL of buffer immediately before use. In addition, if immunoprecipitating with an antibody against the histone modification H3K27ac, include the histone deacetylase inhibitor sodium butyrate at a final concentration of 5 mM. Other inhibitors are generally not necessary when immunoprecipitating transcription factors but should be considered when using antibodies against posttranslational covalent modifications.

ChIP-Seq Dilution Buffer

0.01% SDS
1.1% Triton X-100
1.2 mM EDTA
16.7 mM Tris (pH 8.1)
167 mM NaCl

Filter-sterilize. Store for up to 1 yr at 4°C. Add one protease inhibitor tablet or 2 mL of 25× inhibitor stock solution as directed by the manufacturer (cOmplete EDTA-Free; Roche) per 50 mL of buffer immediately before use.

ChIP-Seq Elution Buffer

50 mM Tris (pH 8.0)
10 mM EDTA
1% SDS

Filter-sterilize. Store for up to 1 yr at 4°C.

ChIP-Seq 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. Add one protease inhibitor tablet or 2 mL of 25× inhibitor stock solution as directed by the manufacturer (cOmplete EDTA-Free; Roche) per 50 mL of buffer immediately before use.

ChIP-Seq Wash Buffer

100 mM Tris (pH 8.0)
500 mM LiCl (or NaCl, for a lower-stringency wash)
1% NP-40
1% deoxycholic acid

Filter-sterilize. Store for up to 1 yr at 4°C. Add one protease inhibitor tablet or 2 mL of 25× inhibitor stock solution as directed by the manufacturer (cOmplete EDTA-Free; Roche) per 50 mL of buffer immediately before use.

TAE

Prepare a 50× stock solution in 1 L of H₂O:
242 g of Tris base

57.1 mL of acetic acid (glacial)
100 mL of 0.5 M EDTA (pH 8.0)

The 1× working solution is 40 mM Tris-acetate/1 mM EDTA.

TE Buffer for ChIP-Seq

50 mM Tris (pH 8.0)
10 mM EDTA

Filter-sterilize or autoclave. Store for up to 1 yr at 4°C.

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