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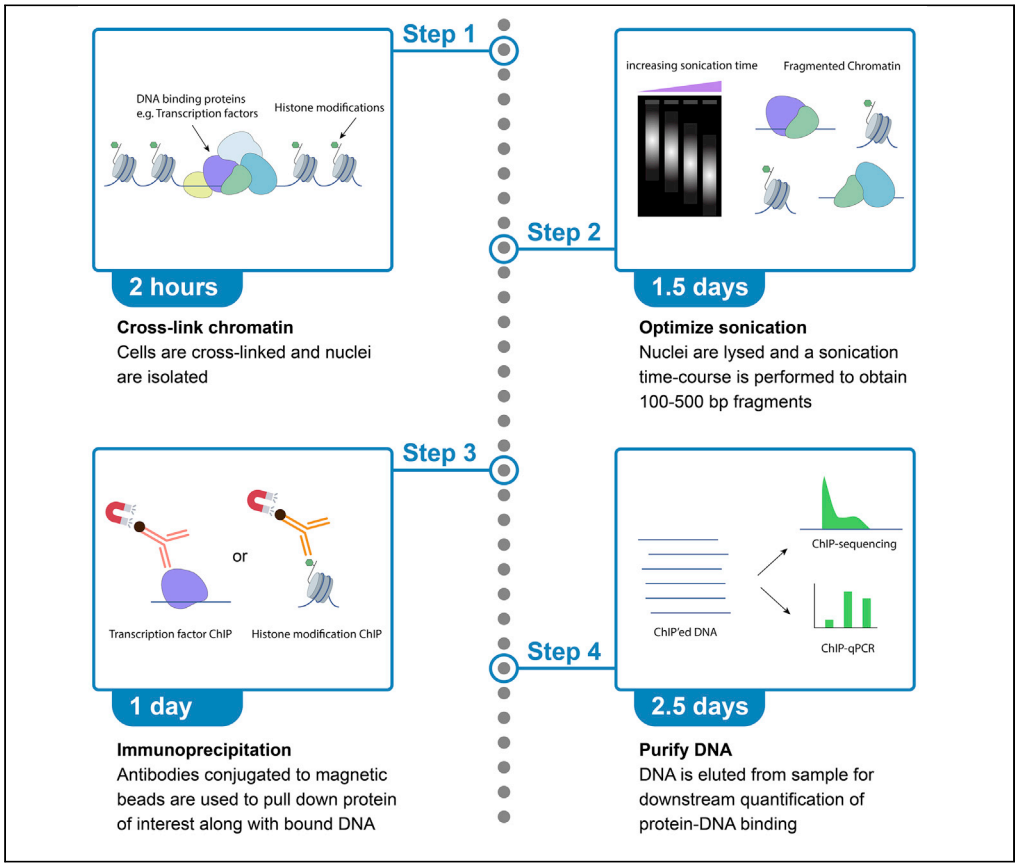
Protocol

Chromatin immunoprecipitation of transcription factors and histone modifications in Comma-Dβ mammary epithelial cells



Chromatin immunoprecipitation (ChIP) is used to study interactions between proteins and DNA. Nuclear lysates are prepared, and chromatin is fragmented by sonication. Antibodies are used to purify a protein of interest (e.g., a transcription factor or histone mark) along with any bound DNA. The genomic binding sites can then be mapped by sequencing the bound DNA (ChIP-seq) or by qPCR if binding sites are already known. ChIP requires optimization for each cell type, and success is highly antibody dependent. This protocol can be adapted to other cell lines with careful optimization.

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Highlights

Chromatin immunoprecipitation (ChIP) is used to study protein-DNA interactions

Careful optimization for each cell line is required

This protocol describes how to optimize and perform ChIP in mammalian cells

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Chromatin immunoprecipitation of transcription factors and histone modifications in Comma-Dβ mammary epithelial cells

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SUMMARY

Chromatin immunoprecipitation (ChIP) is used to study interactions between proteins and DNA. Nuclear lysates are prepared, and chromatin is fragmented by sonication. Antibodies are used to purify a protein of interest (e.g., a transcription factor or histone mark) along with any bound DNA. The genomic binding sites can then be mapped by sequencing the bound DNA (ChIP-seq) or by qPCR if binding sites are already known. ChIP requires optimization for each cell type, and success is highly antibody dependent. This protocol can be adapted to other cell lines with careful optimization.

For complete details on the use and execution of this protocol, please refer to Holliday et al. (2021).

BEFORE YOU BEGIN

The protocol below describes the specific steps for using Comma-Dβ normal murine mammary epithelial cells. However, we have also used this protocol in human breast cancer cell lines HCC70 and MDA-MB-468.

Cell culture

Timing: 3 days

1. Seed 1.5×10^6 Comma-Dβ cells into 15 cm tissue culture dishes in 25 mL cell culture media (DMEM/F12 media supplemented with 2% FBS, 10 mM HEPES, 0.125 IU/mL Insulin and 5 ng/mL mEGF)
   a. Drip cells around the dish and use figure-8 motions for even distribution
   b. Set up 1 dish per ChIP
   c. Set up an extra plate for estimating cell number
   d. Incubate at 37°C
2. After 72 h cells should be 70%–80% confluent and ready for harvesting
3. Trypsinize cells from the extra dish for 2–3 min at 37°C and quench with cell culture media. Count the cells for an estimate of cell number per dish.

Note: A sub-confluent dish of Comma-Dβ cells is approximately 15×10^6 cells

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
HEB antibody	Santa Cruz Biotechnology	sc-357 X
H3K27me3 antibody	Merck Millipore	07-449
H3K4me3 antibody	Active Motif	39159
H3K27ac antibody	Active Motif	39034
Chemicals, peptides, and recombinant proteins		
cOmplete™ ULTRA Tablets, EDTA-free	Merck	5892791001 /5892953001
TE buffer	Thermo Fisher Scientific	12090015
16% Paraformaldehyde (formaldehyde) aqueous solution	ProSciTech Pty	C004
Glycine	Astral Scientific Pty Ltd.	BIOGB0235
Trypan Blue Solution, 0.4%	Thermo Fisher Scientific	15250061
SDS Solution, 10% Sodium Dodecyl Sulfate Solution	Thermo Fisher Scientific	BP2436200
Tris base	Sigma-Aldrich	77-86-1
EDTA	Sigma-Aldrich	E9884
IGEPAL	Sigma-Aldrich	I8896
$MgCl_2$	Sigma-Aldrich	M8266
NaCl	Sigma-Aldrich	S9888
LiCl	Sigma-Aldrich	213233
Sodium deoxycholate	Sigma-Aldrich	D6750
Sodium bicarbonate	Sigma-Aldrich	S5761
Nuclease-Free Water	Thermo Fisher Scientific	AM9932
Triton-X-100	Sigma-Aldrich	T8787
Proteinase K	New England Biolabs	P8107S
RNase A	QIAGEN	158922
Phenol-chloroform-isoamyl alcohol mixture	Sigma-Aldrich	77617
Sodium acetate	Sigma-Aldrich	S2889
Ethanol	Sigma-Aldrich	E7023
GlycoBlue™	Thermo Fisher Scientific	AM9516
ChIP-grade Protein A/G Magnetic Beads	Thermo Fisher Scientific	26162
Bovine serum albumin	Sigma-Aldrich	A7906
PBS	Thermo Fisher Scientific	14190144
NuPAGE™ LDS Sample Buffer (4×)	Thermo Fisher Scientific	NP0007
NuPAGE™ Sample Reducing Agent (10×)	Thermo Fisher Scientific	NP0009
100 bp DNA Ladder	Promega	G2101
Critical commercial assays		
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32851
TruSeq ChIP Library Preparation Kit	Illumina	IP-202-1012
Experimental models: cell lines		
Comma-Dβ cells	Joseph Jeffery (University of Massachusetts, Amherst, MA, USA)	N/A
Other		
Bioruptor® Plus sonication device with 1.5 mL tube holder	Diagenode	B01020001
2 mL Dounce with tight pestle	Merck	D8938-1SET
DynaMag™-2 Magnet	Thermo Fisher Scientific	12321D
Phase Lock Gel Tubes (light)	Quantabio 5Prime	10847-800
Qubit Fluorometer	Thermo Fisher Scientific	Q33238
NanoDrop 2000/2000c Spectrophotometer	Thermo Fisher Scientific	ND-2000
Cell scraper	Corning (or equivalent)	CLS2010 (or equivalent)

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Microscope and hemocytometer	Various	N/A
Pipette boy and serological pipettes	Various	N/A
Pipettes and tips	Various	N/A
50 mL Falcon conical tubes	Corning (or equivalent)	CLS430921 (or equivalent)
Eppendorf tubes (1.5 mL and 2 mL)	Eppendorf	T9661 and EP0030123620
Eppendorf DNA LoBind tubes (1.5 mL)	Eppendorf	EP0030108051
Rotating platform	labForce (or equivalent)	1165U03 (or equivalent)
Heat block	Eppendorf (or equivalent)	T3317 (or equivalent)
Centrifuge	Various	N/A

MATERIALS AND EQUIPMENT

Nuclei extraction buffer

Prepare on the day of experiment, keep on ice.

Reagent	Final Concentration	Amount
Tris-HCl pH 7.5 (1 M)	10 mM	100 µL
NaCl (5 M)	10 mM	20 µL
$MgCl_2$ (1 M)	3 mM	30 µL
EDTA pH 8 (0.5 M)	0.1 mM	2 µL
10% IGEPAL	0.5%	500 µL
Nuclease-free water	n/a	9.35 mL
Mini protease inhibitor cocktail tablet	1×	1 tablet
Total	**n/a**	**10 mL**

Sonication buffer

Prepare on day of experiment. Keep at 20-25°C to prevent precipitation of SDS.

Reagent	Final Concentration	Amount
Tris-HCl pH 8 (1 M)	50 mM	500 µL
SDS (10%)	1%	1 mL
EDTA pH 8 (0.5 M)	10 mM	200 µL
Nuclease-free water	n/a	8.3 mL
Mini protease inhibitor cocktail tablet	1×	1 tablet
Total	**n/a**	**10 mL**

IP dilution buffer

prepare on the day of experiment, keep on ice.

Reagent	Final Concentration	Amount
Tris-HCl pH 8 (1 M)	16.7 mM	167 µL
SDS (10%)	0.01%	10 µL
Triton-X-100 (10%)	1%	1 mL
NaCl (5 M)	167 mM	334 µL
EDTA pH 8 (0.5 M)	1.2 mM	24 µL
Nuclease-free water	n/a	8.47 mL
Mini protease inhibitor cocktail tablet	1×	1 tablet
Total	**n/a**	**10 mL**

Low salt wash buffer

Store at 4°C. Keep on ice on day of protocol.

Reagent	Final Concentration	Amount
EDTA pH 8 (0.5 M)	2 mM	200 µL
SDS (10%)	0.1%	500 µL
Triton-X-100 (10%)	1%	5 mL
Tris-HCl pH 8 (1 M)	20 mM	1 mL
NaCl (5 M)	150 mM	1.5 mL
Nuclease-free water	n/a	41.8 mL
Total	**n/a**	**50 mL**

High salt wash buffer

Store at 4°C. Keep on ice on day of protocol.

Reagent	Final Concentration	Amount
EDTA pH 8 (0.5 M)	2 mM	200 µL
SDS (10%)	0.1%	500 µL
Triton-X-100 (10%)	1%	5 mL
Tris-HCl pH 8 (1 M)	20 mM	1 mL
NaCl (5 M)	500 mM	5 mL
Nuclease-free water	n/a	38.3 mL
Total	**n/a**	**50 mL**

LiCl wash buffer

Store at 4°C. Keep on ice on day of protocol.

Reagent	Final Concentration	Amount
EDTA pH 8 (0.5 M)	1 mM	100 µL
Tris-HCl pH 8 (1 M)	10 mM	500 µL
LiCl (5 M)	250 mM	2.5 mL
IGEPAL (10%)	1%	5 mL
Sodium Deoxycholate (10%)	1%	5 mL
Nuclease-free water	n/a	36.9 mL
Total	**n/a**	**50 mL**

ChIP elution buffer

Prepare fresh. Make 1 M sodium bicarbonate (0.42 g dissolved in 5 mL nuclease-free water) immediately before addition.

Reagent	Final Concentration	Amount
SDS (10%)	1%	600 µL
Sodium bicarbonate (1 M)	100 mM	600 µL
Nuclease-free water	n/a	4.8 mL
Total	**n/a**	**6 mL**

RIPA buffer

Store at 4°C.

Reagent	Final Concentration	Amount
Tris-HCl pH 7.5 (1 M)	50 mM	5 mL
IGEPAL or Triton-X-100 (10%)	1%	10 mL
Sodium Deoxycholate (10%)	0.5%	5 mL
SDS (10%)	0.1%	1 mL
NaCl (5 M)	140 mM	2.8 mL
dH_20	n/a	76.2 mL
Total	**n/a**	**100 mL**

STEP-BY-STEP METHOD DETAILS

Harvesting and cross-linking nuclei

Timing: 2 h

In this step cells are harvested by scraping, crosslinked in solution and nuclei are extracted. Nuclei can be frozen down for later use. The protocol below is for 1 ChIP assay.

1. Working on ice, aspirate media from 150 mm dish of sub-confluent cells and add 3 mL ice-cold PBS (pH 7.4) containing protease inhibitors (PBS + PI)

   Note: Perform all steps on ice unless otherwise stated

   Note: The amount of input material can be increased when performing ChIP for lower abundance proteins such as transcription factors, and needs to be optimized on a case-by-case basis. If ChIP signal is low then increasing the amount of input material may help improve signal over background (see Troubleshooting section).

2. Scrape cells off the dish and transfer the suspension into a 50 mL falcon tube
   a. Perform long strokes in perpendicular planes then scrape around the outside, being sure to scrape the entire surface of the dish for maximum recovery.
3. Wash dish with 10 mL PBS and transfer the wash solution into the same tube
4. Pellet cells by centrifugation at 500 ×*g* for 5 min at 4°C
5. Aspirate supernatant and loosen the cell pellet by flicking three times
6. Resuspend cells in 2 mL of PBS + PI by pipetting with a P1000 3 times
7. Pass cell suspension gently through a 19-gauge needle 10 times to break up large clumps of cells. Avoid bubbles.
8. Add 23 mL PBS + PI and invert 3 times to mix.
9. Working in a fume hood, add 1.67 mL of 16% paraformaldehyde (final concentration 1%). Invert tube 4 times.
10. Incubate for 15 min at 20°C–25°C to crosslink the chromatin.
11. Quench crosslinking reaction by adding 1.77 mL 2 M glycine (final concentration 125 mM). Invert 4 times to mix.
12. Incubate for 5 min at 20°C–25°C
13. Centrifuge at 500 ×*g* for 5 min at 4°C, aspirate supernatant and resuspend in 10 mL PBS + PI. Pipette up and down 3 times.
14. Repeat above wash step two more times for a total of 3 washes.
15. Centrifuge at 500 ×*g* for 5 min at 4°C and aspirate supernatant.
16. Resuspend in 0.5 mL nuclei extraction buffer. Pipette to mix.
17. Incubate on ice for 10 min.
18. Meanwhile chill Dounce homogenizer tube on ice
19. Transfer cell suspension into Dounce homogenizer tube and perform 20 strokes with a tight pestle

a. For 1 stroke plunge the pestle to the bottom of the tube and rotate 180°

20. Remove a 10 μL aliquot and mix with 10 μL trypan blue in an eppendorf tube and use a hemocytometer to check nuclei extraction (Figure 1).
    a. Repeat Dounce step if necessary until most nuclei are extracted

Note: Clean Dounce homogenizer and pestle thoroughly with dH_20 and 70% ethanol between uses if extracting nuclei from different conditions. Soak in 1 M NaOH for 16–20 hr after use to clean.

21. Transfer suspension to a new 1.5 mL eppendorf tube
22. Wash nuclei by centrifuging at 1000 ×*g* for 5 min at 4°C, aspirate supernatant and re-suspend nuclei pellet in 300 μL PBS + PI
23. Repeat centrifugation as in step 22 and aspirate supernatant. Nuclei can be stored at −80°C or continue with either sonication optimization or chromatin immunoprecipitation below.

Pause point: Crosslinked nuclei pellet can be stored at −80°C for several months.

Optimizing sonication

Timing: 1.5 days

In this step the optimal sonication time to generate 100–500 bp DNA fragments is determined. The sample is sonicated in increments and aliquots are taken at various time points, DNA is reverse cross-linked and size determined by agarose gel electrophoresis. This step only needs to be performed once per cell line and sonicator and not for every ChIP experiment.

24. Turn on sonicator cooling system or add ice to water bath
25. Thaw nuclei pellet on ice
26. Add 300 μL of sonication buffer to nuclei pellet
27. Incubate for 5–10 min on ice
28. Pipette to mix

Note: It is normal for suspension to be clumpy

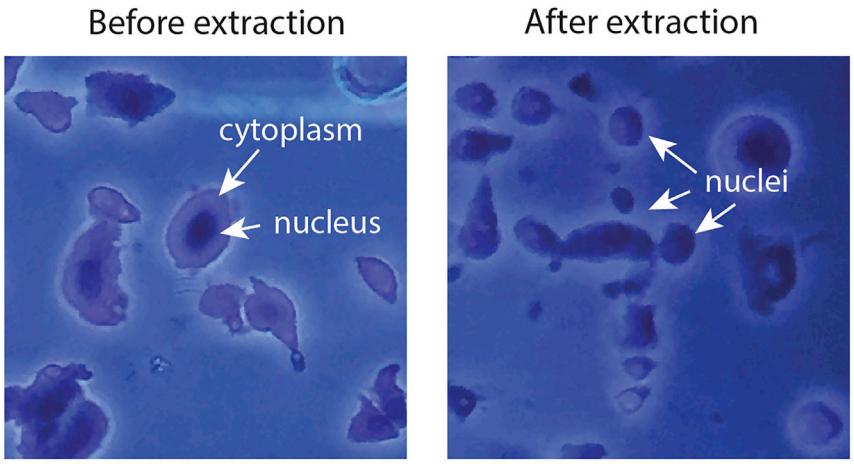

Figure 1. Cells stained with trypan blue before and after homogenization in nuclei extraction buffer with 40 strokes
Note that trypan blue is expected to stain all nuclei under these conditions.

29. Vortex sample and briefly centrifuge to collect the liquid at the bottom of the tube.

    Optional: Take a 10 μL aliquot before sonication for unsonicated control.

30. Sonicate on high for 5 cycles
    a. 1 cycle = 30 sec on and 30 sec off
    b. Add balance tubes containing 300 μL water to the remaining positions
31. Remove sample tube, vortex, and briefly centrifuge. Remove a 10 μL aliquot into an eppendorf tube and place on ice
32. Return sample volume to 300 μL by adding 10 μL sonication buffer. Vortex and briefly centrifuge.
33. Sonicate for a further 5 cycles.
34. Repeat steps 31–33 every 5 cycles up to 30 cycles, transferring the aliquot into a new eppendorf tube each time.
    a. At the end of the time course there will be 6 tubes: 5, 10, 15, 20, 25, and 30 cycles.

    Note: If using an ice bath sonicator, replenish ice when it is low to ensure samples do not overheat, as this will denature the proteins.

35. To each 10 μL aliquot add 90 μL nuclease free water, 4 μL 5 M NaCl and 1.25 μL Proteinase K (20 mg/mL)
36. Incubate at 65°C for 16–20 hr to reverse crosslinks and remove protein
37. The following day add 2.5 μL RNase (4 mg/mL) and incubate at 37°C for 1 h.
38. Extract DNA using 2 mL Phase Lock Gel (PLG) tubes
    a. Spin PLG tubes for 20–30 sec at 12,000–16,000 ×*g* to pellet the gel
    b. Transfer sample to PLG tubes
    c. Working in fume hood, add equal volume of phenol-chloroform-isoamyl alcohol mixture to each sample and shake vigorously for 15 sec or until the solution is a uniform, milky white.
    d. Spin at 12,000–16,000 ×*g* for 5 min at 20–25°C
    e. Transfer the aqueous phase to a LoBind eppendorf tube

39. Perform ethanol precipitation
    a. To each sample add 1 μL GlycoBlue and 1:10 volume of 3 M sodium acetate and mix well

    Note: The GlycoBlue will aid in visualization of the DNA pellet which will be very small.
    b. Add 2.5× volumes of 100% ethanol
    c. Incubate at −80°C for 4–20 hr
    d. Spin for 15 min at top speed (13,000 – 22, 000 ×*g*) at 4°C
    i. Orientate hinge of tubes on the outside so pellet can be located.
    e. Remove supernatant
    f. Wash pellet with 1 mL 80% ethanol.
    i. Do not disturb pellet.
    g. Spin at top speed (13,000 – 22, 000 ×*g*) for 5 min at 4°C
    h. Remove supernatant. Use a P10 to remove all traces of ethanol without disturbing the pellet
    i. Air dry pellet on bench for 5 min or until no ethanol is visible
    j. Resuspend DNA in 10 μL nuclease free water

40. Quantify DNA concentration using a NanoDrop spectrophotometer
41. Load 0.5−1 μg of DNA on a 2% agarose gel with a 100 bp ladder
42. Choose the number of cycles that shears DNA to 100–500 bp in size (Figure 2).

Immunoprecipitation

Timing: 1 day

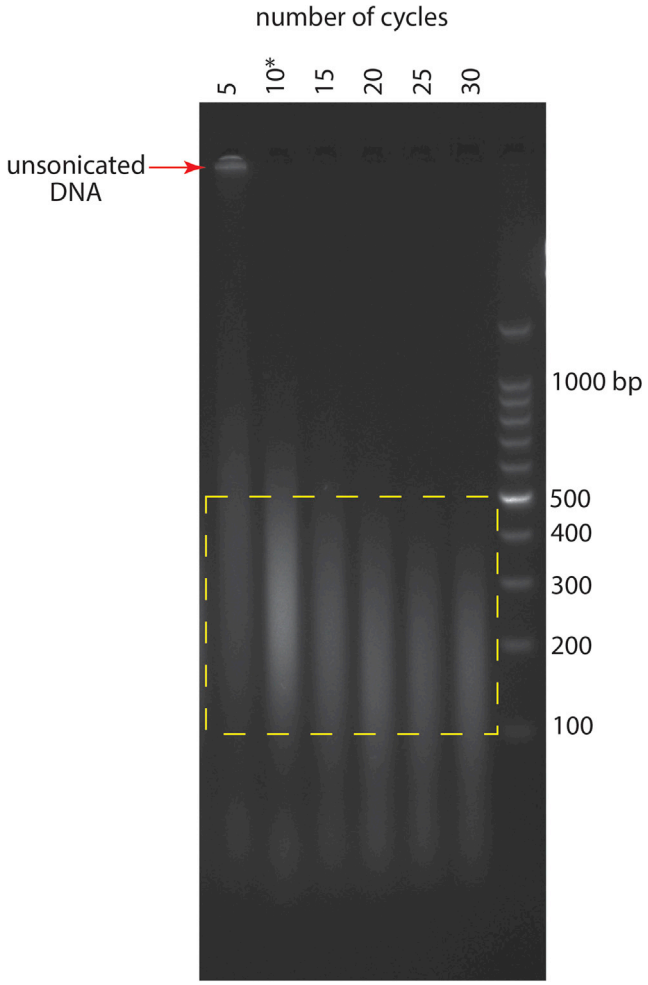

Figure 2. Sonication optimization time-course samples run on a 2% agarose gel with 100 bp ladder
Fragments between 100–500 bp are required for ChIP. 10 cycles is the optimal number of cycles in this case as the majority of the DNA lies within the desired range of 100–500 bp.

This step details the immunoprecipitation of protein/DNA complexes from sonicated nuclear lysates. We recommend using ChIP-grade antibodies if they are available. In this study we used antibodies raised against H3K4me3 (active promoter mark), H3K27me3 (repressive chromatin mark), H3K27ac (active enhancer mark) and the bHLH transcription factor HEB. If ChIP-grade antibodies are not available we recommend checking the ability of the antibody to pull down its target protein under the crosslinking conditions described in this protocol. At the very minimum antibodies should be validated by western blotting or immunofluorescence.

43. Add 1 mL 0.5% BSA in PBS to a 2 mL eppendorf tube

Note: 2 mL tubes are preferred to 1.5 mL tubes for better mixing during immunoprecipitation.

44. Aliquot 50 μL Pierce ChIP-grade protein A/G magnetic beads per ChIP into the 2 mL tube and briefly vortex.

⚠ CRITICAL: Mix beads by vortexing just before aliquoting as the beads settle rapidly

45. Place tube on magnetic rack and allow beads to separate for approximately 30 sec. Remove supernatant with a pipette.
46. Repeat wash with another 1 mL 0.5% BSA in PBS, vortex tube, place on magnetic rack and wait for beads to separate, then remove supernatant.
47. Add 350 μL 0.5% BSA in PBS to washed beads and remove from magnetic rack.
48. Add antibody to bead solution

Note: Amount is antibody dependent, generally 10 μg per ChIP is a good starting point

49. Vortex tubes and place on rotating platform at 4°C for a minimum of 4 h
    a. During the 4 h incubation, proceed with nuclei sonication and clearing steps below
50. Thaw nuclei pellet on ice
51. Add 300 μL of sonication buffer and incubate for 5–10 min on ice.
52. Pipette to mix
53. Vortex and briefly centrifuge sample.
54. Sonicate on high for pre-optimized number of cycles as previously determined in step 42
55. Centrifuge sample for 5 min at top speed (13,000 – 22, 000 ×*g*) at 4°C
56. Transfer supernatant into a 2 mL eppendorf tube and dispose of the pellet
57. Remove a 10 μL aliquot and store at −80°C

⚠ CRITICAL: Do not forget to set aside the sonication check sample as it is important to check that the fragments are in the appropriate size range

58. Add 200 μL dilution buffer to the sonicated lysate to bring volume to 500 μL
59. Wash 15 μL of magnetic beads two times with 1 mL 0.5% BSA in PBS, then remove supernatant
60. Add the diluted lysate to the beads and place on rotating platform for at 1.5 h at 4°C to clear

Note: pre-clearing helps to remove proteins that bind non-specifically to the beads

61. Place bead/lysate mix on magnetic rack and transfer the cleared lysate into a new 2 mL tube.
62. Remove 10 μL aliquot of the sample and store at −80°C.

Note: This is the "total input" and represents what your sample looks like without enrichment of your protein of interest

⚠ CRITICAL: Do not forgot to set aside the total input sample as this is the control for the immunoprecipitation

63. Retrieve the antibody/bead solutions and wash three times in 0.5% BSA in PBS. Remove supernatant.
64. Transfer the cleared lysate into the tube containing the washed antibody-bound beads.
65. Place on the rotating platform and incubate for 16–20 hr at 4°C.
66. The following day either validate successful IP by western blotting, or proceed to elute DNA.

Validating IP antibodies by western blotting

Timing: 1–2 days

For the first time performing ChIP experiments, we recommend checking the ability of the antibody to pull down its target protein under the crosslinked conditions used in the ChIP protocol by western blotting.

67. Place bead/lysate mix on magnetic rack. Remove supernatant.

Optional: keep the supernatant to check depletion of protein of interest

68. Wash beads three times with RIPA buffer
    a. Ensure you remove as much of the last wash as possible by briefly spinning tubes down and use a P10 to remove all supernatant
69. Resuspend beads in 80 μL 2× NuPage sample buffer for 1 dish of cells

Note: For the purpose of western blotting, the amount of input material, beads, and antibody can be quartered. In this case add 20 μL of sample buffer to beads, which is enough for 1 western blot lane.

Note: Using 2× loading buffer rather than 1× loading buffer is thought to help remove protein from the beads. The input sample is also made up with 2× loading buffer so that the western blot runs evenly.

70. Thaw input sample and make up in 2× NuPage sample buffer to 20 μL
71. Heat samples at 85°C for 10 min
72. Place IP tubes on magnet rack to separate beads from the supernatant which contains the protein
    a. This step is not necessary for the input
73. Load 20 μL input and 20 μL IP samples onto an SDS-PAGE gel
    a. Do not load the beads onto the gel
74. Perform western blot as described on the antibody datasheet.

Eluting DNA

Timing: 2.5 days

In this section, chromatin is eluted from the beads and DNA-protein crosslinks are reversed overnight. The next day, DNA is cleaned up by ethanol precipitation. We advise performing ethanol precipitation overnight to increase yield. This protocol is based on the Millipore ChIP Assay Kit (#17-295)

75. Place bead mixture on magnetic rack and remove the supernatant
76. Wash beads for 5 min on a rotating platform at 20°C–25°C with 1 mL of each of the following buffers. Following washing place tube on magnetic rack, remove supernatant, and add the next wash buffer in the following order:
    a. Low salt wash buffer – one wash
    b. High salt wash buffer – one wash
    c. LiCl wash buffer – one wash
    d. TE buffer – two washes
77. Add 100 μL ChIP elution buffer.

⚠ CRITICAL: Make the sodium bicarbonate stock immediately before use

78. Rotate on rotating platform at 20°C–25°C for 15 min
79. Place sample on magnetic rack and transfer supernatant into a new eppendorf tube

⚠ CRITICAL: Do not discard the supernatant as this contains the eluted DNA

80. Add another 100 μL of ChIP elution buffer to the beads and rotate for a further 15 min. Place on magnetic rack and combine supernatant with the first eluate for a total of 200 μL.
81. Thaw the total input and sonication check aliquots and add 190 μL nuclease free water.
82. Reverse crosslinking of all samples (ChIP, input and sonication check) by adding 8 μL of 5 M NaCl and 2.5 μL of Proteinase K (20 mg/mL). Pipette to mix. Incubate at 65°C for 16–20 hr.

83. After incubation, centrifuge briefly.
84. Add 5 μL of RNase A (4 mg/mL) to each sample and incubate at 37°C for 1 h
85. Extract DNA using 2 mL PLG tubes
    a. Spin PLG tubes for 20–30 sec at 12,000–16,000 ×*g* to pellet the gel
    b. Transfer sample to PLG tubes
    c. Working in fume hood, add equal volume of Phenol-chloroform-isoamyl alcohol mixture to each sample and shake vigorously for 15 sec
    d. Spin at 12,000–16,000 ×*g* for 5 min at 20°C–25°C
    e. Transfer the aqueous phase to a LoBind eppendorf tube
86. Perform ethanol precipitation
    a. To each sample add 1 μL GlycoBlue and 1:10 volume of 3 M sodium acetate and mix well
    b. Add 2.5× volumes of 100% ethanol
    c. Incubate at −80°C for 16–20 hr.
    d. Spin for 15 min at top speed (13,000 – 22, 000 ×*g*) at 4°C.
        i. Orientate hinge of tubes on the outside so pellet can be located.
    e. Remove supernatant
    f. Wash pellet with 1 mL 80% ethanol
        i. Do not disturb pellet
    g. Spin at top speed (13,000 – 22, 000 ×*g*) for 5 min at 4°C
    h. Remove supernatant. Use a P10 to remove all traces of ethanol without disturbing the pellet
    i. Air dry pellet on bench for 5 min or until no ethanol is visible

⚠ CRITICAL: Remove as much ethanol as possible but do not over dry the pellet

87. Resuspend in 20 μL TE buffer

Pause point: ChIP'ed DNA can be stored at −20°C for several months.

88. Determine DNA concentration using the Qubit Fluorometer with the dsDNA HS Assay Kit according to the manufacturer's protocol.

Note: Input concentrations should be in the range of 300–800 ng/μL. As this is above the detection limit of the HS kit (10 pg/μL to 100 ng/μL), first dilute the input samples 1:10 (1 μL input + 9 μL nuclease-free water) before performing quantification.

Note: ChIP'ed DNA concentrations will be low (0.1–2.5 ng/μL) and sometimes undetectable (see Troubleshooting section)

89. Check fragment size of the sonication check sample from step 57 by DNA gel electrophoresis

EXPECTED OUTCOMES

Following ChIP, DNA can be sequenced to map the genomic binding sites of your protein of interest. Before performing sequencing, qPCR may be performed as a quality control to see if the ChIP worked. However, this is not always practical, for example, if the DNA binding sites are not known.

For ChIP-seq we used the TruSeq ChIP library prep kit and NextSeq system (Illumina) with 75 bp paired-end reads, aiming for a sequencing depth of 30 M reads for TFs and 50–60 M reads for histone marks. Note that the minimum requirement is 5 ng ChIP DNA but we have had success with as little as 2 ng. We recommend using the entire ChIP volume and 50 ng DNA for the input control. The recommended number of PCR cycles (18 cycles) was used for amplification during library prep.

Bound regions are visualized as a pile up of reads, which form peaks when viewed on a genome browser. See Figure 3 for an example of ChIP-seq peaks on the Integrative Genomic Viewer (IGV) (Robinson et al., 2011). The HEB transcription factor occupies regions that overlap with marks of active promoters (H3K4me3) and enhancers (H3K27ac), suggesting that HEB regulates expression of these genes.

LIMITATIONS

The success of this technique is very dependent on the antibodies used for immunoprecipitation and validation of the antibodies can be laborious and time consuming.

High amount of input material is required for lowly expressed proteins, which can be a limitation if using rare cell/tissue types.

Sonication conditions need to be determined for every cell line.

TROUBLESHOOTING

Problem 1

DNA over or under sonicated (step 89). Examples of under-sonicated DNA and over-sonicated DNA are shown in Figure 2, lane 1 and 6 respectively.

Figure 3. Snapshot of chromosome 10 (mm10 genome) from the Integrative Genomics Viewer (IGV) displaying HEB (pink), H3K4me3 (green), H3K27ac (blue), and H3K27me3 (orange) ChIP-seq peaks

Input (gray) was used as a negative control. RefSeq genes shown in blue.

Potential solution

Make sure optimization is performed on the same cell density as the experimental sample, as cell density will affect the efficiency of sonication.

Problem 2

No DNA detected after ChIP (step 88).

Potential solution

Sometimes the amount of DNA is below the detection limit of the Qubit HS kit. We still recommend using the DNA for preparing libraries, using the entire amount. If there is no enrichment in the sequenced libraries see the potential solutions to Problem 3.

Problem 3

No enrichment is seen in sequenced libraries prepared using ChIP DNA (step 87)

Potential solution

- This could be due to several reasons relating to the ChIP:
- Not enough starting material. Try increasing the number of cells used in step 1.
- Failure of immunoprecipitation. Try increasing the amount of antibody used in step 48. Check by western blotting to make sure the antibody is able to pull down its target (step 67).
- Over-sonication of DNA, which can disrupt chromatin integrity and denature antibody epitopes. Sonicate for less time at step 54.
- Take care not to lose the DNA pellet during ethanol precipitation (step 86).

Problem 4

Enrichment is seen in unexpected regions in sequenced libraries prepared using ChIP DNA (step 87)

Potential solution

- Background signal may be a result of non-specific binding during immunoprecipitation. Try reducing amount of cells and/or antibody used (step 1 and 48 respectively).
- Over fixation can also result in artifactual DNA binding. Try reducing the crosslinking time (step 10).
- Under-sonication resulting in large DNA fragments can result in low peak resolution, increase sonication time to achieve fragments between 100–500 bp (step 54).

Problem 5

Enrichment is seen in the libraries prepared from input DNA (step 62)

Potential solution

Certain problematic regions of the genome, such as repetitive elements, appear to have enrichment of signal due to the amplification of noise. These so called 'blacklist' artifact regions should be filtered out bioinformatically.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact Alexander Swarbrick (a.swarbrick@garvan.org.au)

Materials availability

This study did not generate new unique reagents.

Data and code availability

The ChIP-seq data generated using this protocol in the study (Holliday et al., 2021) are available on the GEO repository with the accession number GSE149969.

Text for Footnote 0

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AUTHOR CONTRIBUTIONS

H.H. wrote this protocol with extensive advice from A.K. All authors proofread the manuscript. A.S. supervised the work.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Holliday, H., Roden, D., Junankar, S., Wu, S.Z., Baker, L.A., Krisp, C., Chan, C.-L., Mcfarland, A., Skhinas, J.N., Cox, T.R., et al. (2021). Inhibitor of Differentiation 4 (ID4) represses mammary myoepithelial differentiation via inhibition of HEB. iScience *24*, 102072.

Robinson, J.T., Thorvaldsdóttir, H., Winckler, W., Guttman, M., Lander, E.S., Getz, G., and Mesirov, J.P. (2011). Integrative genomics viewer. Nat. Biotechnol. *29*, 24–26.