



CHIP-reCHIP Protocol for Mapping Bivalent Chromatin

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ABSTRACT

This is a detailed protocol for performing sequential ChIP-reChIP to map H3K4me3-H3K27me3 bivalent chromatin regions. It has been optimised using mouse embryonic stem cells and so may need to be refined based on your cell type of interest. For more details please refer to the accompanying manuscript (Ho *et al.* 2023).

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MANUSCRIPT CITATION:

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A low-input high resolution
sequential chromatin
immunoprecipitation method
captures genome-wide dynamics
of bivalent chromatin. *Epigenetics*
Chromatin. 2024 Feb 10;17(1):3.
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Protocol status: Working

We use this protocol and it's
working. Some steps (noted) may
require optimisation for different
cell lines.

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ChIP

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GUIDELINES

Note: As a standard throughout the protocol, we recommend using low bind tubes and tips, and advise that all buffers be prepared using RNase and DNase free reagents. Furthermore, while most commercially available antibodies will have a recommended volume to use, we recommend validating and titrating antibody volumes and cell numbers prior to starting. Finally, we recommend designing and testing quantitative PCR primers to amplify positive and negative control regions, ensuring they have good efficiencies and melt-curves. PCR products should be between 80-120 base pairs. Validated primer sequences for mouse Embryonic Stem Cells can be found in the accompanying manuscript.

GENERAL WORKFLOW

- A. Chromatin fixation (2 hours)
- B. Chromatin fragmentation and Primary antibody incubation (2 hours hands on time with overnight incubation)
- C. First elution, buffer exchange and secondary antibody incubation (2 hours hands on time with overnight incubation)
- D. Secondary elution, de-crosslinking, and DNA purification (4 hours)

KEY REAGENTS & RESOURCES

Reagent or Resource	Source	Catalogue No.
16% Formaldehyde (w/v), Methanol free	Life Technologies	28908
Glycine	Sigma	G8898
Sodium deoxycholate	Sigma	30970
Lithium Chloride solution 8M	Sigma	L7026
MNase	NEB	M0247S
RNaseA	NEB	T3018-2
ProteinaseK	NEB	P8107S
Protein A DynaBeads	Thermo Fisher	10002D
Protein LoBind 1.5ml tubes	Eppendorf	0030108442
Magnetic Rack	Invitrogen	12321D
Protease Inhibitor cocktail	Roche	05892791001
Amicon Ultra	Millipore	MPUFC5003BK
NEBNEXT Ultra II DNA Library Prep Kit	NEB	E7645L

PROTOCOL MATERIALS

-  ProteinaseK **New England Biolabs Catalog #P8107S** Step 1
-  Sodium deoxycholate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #30970**

Step 1

-  MNase **New England Biolabs Catalog #M0247S** Step 1
-  Protein A DynaBeads **Thermo Fisher Scientific Catalog #10002D** Step 1
-  Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8898** Step 1

-  NEBNext Ultra II DNA Library Prep Kit for Illumina - 96 rxns **New England Biolabs Catalog #E7645L**

Step 1

-  Lithium Chloride solution 8M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L7026**

Step 1

-  RNAse A **New England Biolabs Catalog #T3018-2** Step 1
-  Protease Inhibitor cocktail **Roche Catalog #05892791001** Step 1
-  16% Formaldehyde (w/v), Methanol free **Life Technologies Catalog #28908** Step 1

BEFORE START INSTRUCTIONS

Prepare Buffers as outlined below:

- NP buffer
- Complete Chromatin Immunoprecipitation buffer
- Low salt wash buffer
- High salt wash buffer
- LiCl wash buffer
- Elution buffer

NP BUFFER

Reagent	Final Concentration	Amount in 100ml
1M Tris pH7.4	10mM	1ml
Sorbitol	1M	18.217g
1M NaCl	50mM	5ml
1M MgCl ₂	5mM	0.5ml
1M CaCl ₂	1mM	0.1ml
IGEPAL	0.075%	75ml
ddH ₂ O		Up to 100 ml

CHROMATIN PRECIPITATION BUFFER

Reagent	Final Concentration	Amount in 500ml
1M Tris pH7.4	20mM	10ml
0.5M EDTA	2mM	2ml
1M NaCl	150mM	75ml
Triton X100	0.1%	0.5ml
ddH ₂ O		Up to 500ml

LOW SALT WASH BUFFER

Reagent	Final Concentration	Amount in 500ml
1M Tris pH8	20mM	10ml
0.5M EDTA	2mM	2ml
5M NaCl	150mM	15ml
Triton X100	1%	5ml
10% SDS	0.1%	5ml
ddH ₂ O		Up to 500ml

HIGH SALT WASH BUFFER

Reagent	Final Concentration	Amount in 500ml
1M Tris pH8	20mM	10ml
0.5M EDTA	2mM	2ml
5M NaCl	500mM	50ml
Triton X100	1%	5ml
10% SDS	0.1%	5ml
ddH ₂ O		Up to 500ml

LiCl WASH BUFFER

Reagent	Final Concentration	Amount in 500ml
8M LiCl	250mM	15.625ml
IGEPAL	1%	5ml
10% deoxycholate	1%	50ml
0.5M EDTA	1mM	1ml
1M Tris pH7.4	10mM	5ml
ddH ₂ O		Up to 500ml

ELUTION BUFFER

Reagent	Final Concentration	Amount in 10ml
1M Tris pH7.4	10mM	0.1ml
0.5M EDTA	1mM	0.02ml
10% SDS	1%	1ml
ddH ₂ O		Up to 10ml

Key Resources & reagents

1

- ☒ 16% Formaldehyde (w/v), Methanol free **Life Technologies Catalog #28908**
- ☒ Glycine **Merck MilliporeSigma (Sigma-Aldrich) Catalog #G8898**
- ☒ Sodium deoxycholate **Merck MilliporeSigma (Sigma-Aldrich) Catalog #30970**
- ☒ Lithium Chloride solution 8M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #L7026**

- ✖ MNase New England Biolabs Catalog #M0247S
- ✖ RNase A New England Biolabs Catalog #T3018-2
- ✖ ProteinaseK New England Biolabs Catalog #P8107S
- ✖ Protein A Dynabeads Thermo Fisher Scientific Catalog #10002D
- ✖ Protease Inhibitor cocktail Roche Catalog #05892791001
- ✖ NEBNext Ultra II DNA Library Prep Kit for Illumina - 96 rxns New England Biolabs Catalog #E7645L

Equipment

Amicon Ultra-0.5 Centrifugal Filter Unit	NAME
Centrifugal Filter Unit	TYPE
Millipore	BRAND
UFC5003BK	SKU
https://www.merckmillipore.com/GB/en/product/Amicon-Ultra-0.5-Centrifugal-Filter-Unit,MM_NF-K_UFC5003BK	LIN K

Equipment

Protein Lobind 1.5mL tubes	NAME
Tubes	TYPE
Eppendorf	BRAND
0030108442	SKU
https://www.eppendorf.com/au-en/eShop-Products/Laboratory-Consumables/Tubes/Protein-LoBind-Tubes-p-0030108116	LINK
1.5mL, snap-cap, Protein LoBind, PCR clean, colourless	SPECIFICATIONS

Equipment

DynaMag-2	NAME
Magnet	TYPE
Invitrogen	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D#/12321D	LINK

Chromatin Fixation

2h

2

Note

Before you start: pre-warm DMEM; pre-cool centrifuge to 4 degrees; add Roche cOmplete EDTA-free protease inhibitor tablets to PBS/EDTA and keep on ice until required.

Note

Note: all steps must be performed on ice or at 4 degrees unless otherwise specified.

Note

Note: chromatin fixation can also be performed on cells in suspension by resuspended a pellet of a known number of washed live cells directly into the 1% formaldehyde solution and incubating for 8 minutes on a rocker, quenching with glycine as below and then mild centrifugation to get a fixed cell pellet (rather than scraping cells).

- 3 Grow cells until they are 70-80% confluent across several plates. One will be used for counting and the remaining for fixation (collection plates).
- 4 Collect and count cells on the counting plate: Remove media from the counting plate and wash once with DPBS.
- 5 Add  1 mL of Trypsin or appropriate dissociation reagent per 10cm plate and incubate until cells lift off plate as single cells.
- 6 Quench trypsin with  5 mL of media.

7 Take aliquot and count cells to determine total cell number per plate and therefore the total number of cells overall.

8 Prepare [M] 1 %(w/v) formaldehyde solution by adding \ddagger 0.625 mL of [M] 16 %(w/v) formaldehyde solution to \ddagger 9.375 mL of prewarmed DMEM per 10cm collection plate.

Note

Scale up volumes if you have more than one 10cm collection plate.

9 Remove media and wash cells on collection plates with DPBS.

10 Cross link cells by adding \ddagger 10 mL of [M] 1 %(w/v) formaldehyde solution per plate of adherent cells for 8 min at room temperature.

11 Quench with 1ml [M] 1 Molarity (M) Glycine per plate of adherent cells to a final concentration [M] 125 millimolar (mM), for 5 min at room temperature.

12 Pour off medium and wash cells with \ddagger 10 mL of ice cold DPBS.

13 Pour off DPBS, scrape cells in residual PBS and transfer to low-bind 1.5ml tube. Pool cells from all plates into one tube.

14 Centrifuge for at 500xg at 4 degrees for 10 minutes  500 x g, 4°C, 00:10:00 . 10m

15 Remove supernatant and resuspend cells to 2×10^7 cells per 1ml in PBS/5mM EDTA containing protease inhibitors. Aliquot by adding  0.1 mL cell slurry (corresponding to 2×10^6 cells) per low-bind tube.

16 Pellet aliquots by centrifugation at 500xg at 4 degrees for 5 minutes  500 x g, 4°C, 00:05:00 . 5m

17 Remove supernatant and snap freeze on dry ice or in liquid nitrogen. Store pellets at  -80 °C for up to 6 months .

Chromatin fragmentation and primary antibody incubation - Day 1 1d 2h

18

Note

Before you start: pre-cool centrifuge; **add Roche cOmplete EDTA-free protease inhibitor tablets to ChIP buffer and NP buffer** and keep on ice until needed.

Note

Note: Unless otherwise specified all procedures should be carried out between 2-8 degrees

Note

Note: NP buffer composition and cell lysis conditions may need to be optimised depending on your cell type.

Note

Note: volumes below are to process one sample corresponding to one vial of 2×10^7 cells and will result in input, in-line H3K4me3, in-line H3K27me3, IgG-IgG reChIP and bivalent K4-K27 and K27-K4 reChIP samples that can be further processed by qPCR and/or library preparation for sequencing. If you have more than one sample (e.g. biological replicate or other condition), scale volumes accordingly. We typically do not process more than 4 samples at any time.

Note: sonication can be used instead of MNase digestion. We routinely use both fragmentation methods in performing reChIP experiments with similar results.

19**Bead preparation – 30 minutes**

Prepare a sufficient amount of Protein A dynabeads for chromatin preclear and antibody binding. For each sample in the reChIP experiment you will need $\text{150 } \mu\text{L}$. This corresponds to:

- $\text{20 } \mu\text{L}$ of beads to preclear 2×10^6 cells.
- $\text{120 } \mu\text{L}$ of beads for antibody-complex formation ($\text{20 } \mu\text{L}$ per antibody-complex of which there are 6 reactions in total: 2xIgG, 2x H3K27me3, 2xH3K4me3)
- $\text{10 } \mu\text{L}$ for pipetting errors

19.1

Take 150 mL Protein A dynabeads, place on magnetic rack and remove supernatant.

19.2

Remove tube from rack and resuspend in $\text{500 } \mu\text{L}$ of cold ChIP buffer containing freshly added protease inhibitor cocktail, discard buffer.

Repeat steps 2-3 for a total of 3 washes.

After 3 washes resuspend in $\text{150 } \mu\text{L}$ of cold ChIP buffer.

Keep chilled on ice until needed later.

20 Binding Antibody to beads – 15 minutes preparation time plus at least 3 hours incubation

Label 6 low-bind tubes (2X IgG, 2X H3K4me3 and 2X H3K27me3)

20.1 Add 500 µL of cold ChIP buffer to each tube.**20.2** Add 20 µL of pre-washed Protein A dynabeads to each tube.**20.3** Add appropriate antibody to each tube - 1 µg IgG (Invitrogen), 10 µL H3K27me3 (CST 9733), 2 µL H3K4me3 (Millipore 07-473).

Note: If using other antibodies the volumes will need to be titrated to maximise signal:noise.
Adding too little antibody will not capture all chromatin containing the modification of interest.
Adding too much antibody increases the background non-specific binding.

20.4 Incubate bead/antibody mix at 4 °C on a rotator for at least 3 hours 03:00:00 . 3h**21 Chromatin preparation – 1 hour preparation time plus at least 3 hours incubation** 4h**21.1** Thaw 1 vial of 2×10^6 crosslinked cells on ice

21.2 Resuspend cell pellet (2×10^6 cells) in $\text{97.68 } \mu\text{L}$ NP buffer supplemented with $\text{0.7 } \mu\text{L}$ of $[M] 55$ millimolar (mM) beta-mercaptoethanol, $\text{1.82 } \mu\text{L}$ of $[M] 0.1$ Molarity (M) spermidine and freshly added protease inhibitor cocktail.

21.3 Fragment chromatin with MNase: prepare MNase master mix ($\text{20 } \mu\text{L}$ per sample)

- $\text{12 } \mu\text{L}$ 10X MNase buffer
- $\text{1.76 } \mu\text{L}$ 100mM DTT
- $\text{3.84 } \mu\text{L}$ dH₂O
- $\text{2.4 } \mu\text{L}$ MNase

21.4 Add $\text{20 } \mu\text{L}$ of MNase master mix to each tube of 2×10^6 cells in NP buffer.

21.5 Incubate at 37°C with shaking at 600 rpm for 7.5 - 15 min. 15m

Note

Note: This amount of MNase and digestion time will need to be titrated for each cell line. These conditions have been optimised to yield predominantly mono nucleosomal DNA for 2×10^6 mouse embryonic stem cells. If preferred sonication can be used to fragment chromatin instead.

21.6 During digestion prepare STOP buffer:

- $\text{15 } \mu\text{L}$ $[M] 100$ millimolar (mM) EDTA
- $\text{15 } \mu\text{L}$ $[M] 1\%$ (v/v) Triton / $[M] 1\%$ (v/v) deoxycholate solution per sample

21.7 Add $\text{26.4 } \mu\text{L}$ of STOP buffer to each sample to stop the MNase digestion.

- 21.8 Incubate on ice for 5 minutes.  00:05:00 5m
- 21.9 Vortex each tube for 30 seconds each.
- 21.10 Bring volume up to  600 µL by adding  473.6 µL cold ChIP buffer containing protease inhibitor cocktail.
- 21.11 Add  20 µL of pre-washed protein A Dynabeads from **step 19** to the chromatin and incubate 3h for at least 3 hours at  4 °C on rotator to pre-clear the chromatin. This is critical to reduce non-specific binding and decrease background signal.  03:00:00
- 22 Overnight incubation with primary antibody – 30 min and overnight 1d 0h 30m
- 22.1 Take all bead-antibody tubes from the 4-degree rotator.
- 22.2 Back at the bench place the pre-cleared chromatin sample and 1x IgG, 1 x H3K4me3 and 1 x H3K27me3 bead-antibody complexes per sample on the magnet rack until the solution clears.

Note

We recommend sitting the magnetic rack on ice to keep cool during these steps. Alternatively, these steps can be performed in a cold room.

22.3 The remaining bead-antibody mixes can be stored at 4 degrees until needed on day 2.

22.4 Take $\text{10 } \mu\text{L}$ of pre-cleared chromatin supernatant to a separate tube and label as 5% input control. Keep at 4°C until day 3.

22.5 Remove the supernatant from the antibody-bead complexes on the magnetic rack and discard.

22.6 Add $\text{200 } \mu\text{L}$ of the pre-cleared chromatin supernatant to each antibody/bead mixture.

22.7 Top up each chromatin/antibody/bead mixture with $\text{300 } \mu\text{L}$ of ChIP buffer to final volume of $\text{500 } \mu\text{L}$.

22.8 Incubate Overnight at 4°C on a rotator.

15m

First elution, buffer exchange and secondary antibody incubation – Day 2

1d 2h

23

Note

Before you start: Prepare 30ml of ChIP buffer containing protease inhibitor cocktail. Prepare 5ml of elution buffer containing protease inhibitor cocktail; pre-cool centrifuge to 4 degrees.

Collect chromatin-antibody-bead samples from overnight 4 °C rotation.

24 Wash chromatin-antibody-bead complexes a total of 9 times using the following steps keeping the samples cool by either placing magnet on ice or working in a cold room:

- a. Place tubes on magnets rack and wait for solution to turn clear.
- b. Carefully remove supernatant while on magnet making sure you do not disturb the beads. Make sure you do not let the beads dry out.
- c. Resuspend beads in 500ml of low salt buffer.
- d. Repeat steps a-c for a total of 3x low salt buffer washes, 3x high salt buffer washes, 2x LiCl buffer washes and 2x 1xTE washes.

25 Elute washed complexes in 100 µL elution buffer containing fresh protease inhibitor cocktail for 30 min at 37 °C on a thermomixer. 00:30:00

Note

If you do not have access to a shaking heat block, gently flick the tubes periodically during the incubation to ensure beads remain suspended in solution.

26 During elution step prepare 3x Amicon Ultra buffer exchange columns by adding 500 µL Milli-Q H₂O columns and spinning at 14000 x g for 30 min at 4 °C. 00:30:00

27 After chromatin elution, place samples on magnetic rack and wait for sample to turn clear. Move supernatant containing chromatin to a new low-bind tube.

- 28 Take 10% volume (\ddagger 10 μL) from each chromatin IP as an in-line single ChIP control into a new low-bind tube, label and store at \ddagger 4 °C until day 3.
- 29 Bring each remaining chromatin sample up to \ddagger 300 μL with ChIP buffer containing protease inhibitor cocktail.
- 30 Decant the H₂O from the prepared Amicon Ultra filters and add each chromatin sample to a separate filter.
- 31 Spin at $\ddot{\circ}$ 14000 x g for 30 min at \ddagger 4 °C. 30m
 00:30:00
- 31.1 Carefully decant flowthrough and discard.
- 32 Add \ddagger 500 μL ChIP buffer containing protease inhibitor cocktail and spin at 14000g for 30 minutes at \ddagger 4 °C. 30m
 00:30:00
- 32.1 Carefully decant flowthrough and discard.
- 33 Repeat steps 32 and 32.1 for a total of 2 washes.

- 34 Recover as much chromatin sample as possible from within the Amicon Ultra filter device. (This is usually around  50 µL).
- 35 Bring volume up to  500 µL with ChIP buffer containing protease inhibitors.
- 36 Take antibody bound beads for the second incubation from  4 °C and place on magnetic rack.
- 37 Wait for the solution to turn clear and remove the supernatant.
- 38 Add appropriate chromatin samples to appropriate antibodies:
- Add the IgG chromatin sample to the IgG-bead complexes.
 - Add the H3K4me3 chromatin to the H3K27me3-bead complexes.
 - Add the H3K27me3 chromatin to the H3K4me3-bead complexes.
- 39 Incubate overnight at  4 °C with rotation.

Second elution, de-crosslinking and DNA purification – Day 3

4h

- 40 Wash chromatin-antibody-bead complexes a total of 9 times using the following steps keeping the samples cool by either placing magnet on ice or working in a cold room:
- a. Place tubes on magnets rack and wait for solution to turn clear.
 - b. Carefully remove supernatant while on magnet making sure you do not disturb the beads. Make sure you do not let the beads dry out.

c. Resuspend beads in $\text{500 } \mu\text{L}$ of low salt buffer.

d. Repeat steps a-c for a total of 3x low salt buffer washes, 3x high salt buffer washes, 2x LiCl buffer washes and 2x 1xTE washes.

41 Elute complexes and reverse crosslinks in $\text{100 } \mu\text{L}$ elution buffer for a minimum of 2.5 hours up to **2h 30m** overnight at 65°C on a thermomixer. 02:30:00

Note

Note: no protease inhibitors are required for this elution step.

42 Collect the 5% input sample ($\text{10 } \mu\text{L}$) and three in-line total control samples ($\text{10 } \mu\text{L}$ each) from 4°C storage.

43 Add $\text{90 } \mu\text{L}$ elution buffer to bring the final volume of each control to $\text{100 } \mu\text{L}$.

44 Place at 65°C for 2.5 hours on a thermoshaker alongside the reChIP samples to de-crosslink. **2h 30m**
 02:30:00

45 After de-crosslinking, place all tubes on the magnetic rack.
You should have a total of 7 tubes corresponding to input, 3x in-line total controls and 3x reChIPs.

46 When the solution turns clear move supernatant to new low-bind tubes.

- 47 Add $\text{2 } \mu\text{L}$ RNaseA (NEB) to each tube and incubate at 37°C for 30 min. 00:30:00 30m
- 48 Add $\text{2 } \mu\text{L}$ Proteinase K (NEB) to each sample and incubate at 37°C for 1 hour. 01:00:00 1h
- 49 Purify DNA using Ampure beads. 20m
- Bring Ampure beads to room temperate prior to use.
 - Add beads to sample in a 1:1.8 ratio (e.g. $\text{180 } \mu\text{L}$ beads to $\text{100 } \mu\text{L}$ sample) and pipette up and down to mix.
 - Incubate at room temperate for 5 minutes. 00:05:00
 - Place tubes on magnetic rack for 5 mins and remove supernatant. 00:05:00
 - While tubes are on the rack wash the beads with $\text{400 } \mu\text{L}$ of freshly prepared [M] 80 % (v/v) ethanol in molecular grade water, remove ethanol leaving only bead-bound DNA.
 - Repeat step e for a total of 2 washes.
 - While the tubes are on the magnet, allow beads to air dry for up to 5 minutes. Note – it is important not to over dry here. Proceed to DNA elution before the beads start to crack.
 - To elute DNA, remove tubes from the magnetic rack and resuspend beads in **Xm1** (see step 50 below) of [M] 10 millimolar (mM) Tris-HCL pH8.0.
 - Incubate at room temperature for 5 minutes. 00:05:00
 - Place tubes back on the magnetic rack for 5 minutes and transfer DNA solution to new tube. 00:05:00
- 50 For downstream qPCR analysis elute in $\text{60-80 } \mu\text{L}$ [M] 10 millimolar (mM) Tris-HCL pH8.0 and use $\text{1 } \mu\text{L}$ per qPCR reaction.
- 51 For downstream NGS elute in $\text{20 } \mu\text{L}$ [M] 10 millimolar (mM) Tris-HCL pH8.0.
- Use $\text{1 } \mu\text{L}$ of eluate to determine the DNA concentration using a Qubit fluorometer.

- b. Use \ddagger 2 μL for qPCR analyses of positive and negative control regions (dilute 4x to give final volume of \ddagger 8 μL and use \ddagger 1 μL per reaction).
- c. Use remaining eluate (\ddagger 17 μL) to prepare libraries using NEBNext® Ultra™ II DNA Library Prep Kit or similar following manufacturer's instructions.