Chromatin Immunoprecipitatipn of histone marks

This protocol has been used successfully for immunoprecipitation of sheared pig liver chromatin with antibodies to histone marks H3K4Me3 and H3K27ac

Protocol for preparation of sheared chromatin is at link below;
ftp://ftp.faang.ebi.ac.uk/ftp/protocols/assays/ROSLIN_SOP_Tissue_Fixation_and_Sonication_of_Chromatin_for_ChIP_20161115.pdf

Equipment and reagents

PBS 1x, Ca2+ and Mg2+ free, containing 5mg/ml BSA
Dynabeads IgG Sheep Anti-Rabbit 2ml, Invitrogen Cat# 11203D
Anti-Histone H3(trimethyl K4)-ChIP grade Rabbit polyclonal, AbCam Cat# ab8580
Rabbit polyclonal to Histone H3 (acetyl K27), AbCam Cat# ab4729
Rabbit Control IgG-ChIP grade, AbCam Cat# ab46540
Triton X-100
Sodium deoxycholate 10%
TE pH8.0 1x
cOmplete EDTA-free protease inhibitor (Roche Cat#05056489001)
HEPES pH8.0 (1M), VWR Cat#A6906.0125
Lithium chloride 8M
EDTA 0.5M
SDS 10%
Nuclease-free water
Ethanol
phenol/chloroform/isoamyl alcohol 25:24:1, Sigma Aldrich Cat#7617
Glycogen 20mg/ml molecular biology grade
Proteinase K 20mg/ml, Qiagen Cat# 19133
Rnase A 10mg/ml 1ml Biotech grade, VWR E866-1ML
Dyna Mag-96 side, 0.2ml magnet, Life Technologies Cat# 1233D
0.2ml skirted 96-well PCR plate
8-strip tube lids
1.5ml DNA lobind tubes
Magnetic rack for 1.5ml tubes
Pipettes and filter tips
Rotating mixer
Nanodrop
Qubit fluorimeter
Agilent Tapestation and HS D1000 kit

Procedure

Day 1

Preparation of beads, binding of primary Ab, followed by binding of chromatin

Before starting
Chill PBS/BSA on ice
Chill magnet on ice
Perform all steps with magnet on ice
Vortex Dynabeads for 30secs immediately prior to pipetting

For immunoprecipitation of H3K4Me3, H3K27ac and IgG control for 3 sheared chromatin biological replicates, each in duplicate (see template for samples X.Y and Z, page 3)

1. Add 11ul per sample to 18 wells of a 96-well plate
2. Place plate on magnet for 1 min or until all beads appear to be captured on magnet.
3. Slowly remove supernatant with a pipette without disturbing the beads
4. Wash beads x 3 with 150ul cold PBS/BSA. Perform all washes as follows;
    a. Add 150ul cold PBS/BSA and seal wells with 8-strip PCR caps
    b. Remove plate from magnet and invert several times to re-suspend beads
    c. Pulse spin v. briefly in plate spinner to remove residual liquid from lids
    d. Place plate on magnet for 1 min
    e. Remove supernatant with multi-channel pipette
5. After the final wash, add cold PBS/BSA (150ul minus volume required for 3ug antibody/IgG) to beads
6. With plate on magnet, **add 3ug Ab or 3ug control IgG to appropriate wells, see plate template page 3**
7. Remove plate from magnet and incubate for 3hours with rotation at 4C in cold room
8. Towards the end of the incubation time, prepare 2x Binding Buffer, recipe below, and place on ice

Reagents for 2 xBinding Buffer	**Stock concentration**	**Final concentration 1x**	**Vol per sample 100 µL**
Triton-X 100	10%	1%	20 µL
Sodium deoxycholate	10%	0.10%	2 µl
cOmplete EDTA-free protease inhibitor	50x	1x	4 µL
TE	1x	--	74 µL

- Prepare 130ul sheared chromatin at 200ng/ul in 1 xTE for each sample and place on ice. 100ul (20ug) of sheared chromatin is used for each IP and remainder is retained at 4C as 20ul is required for preparation of input DNA (see step 21)

9. Post incubation, place plate on magnet for 1 min, then remove supernatant
10. Wash the beads x3 with 150ul cold PBS/BSA as in step 4 above
11. After the final wash, add 100ul of binding buffer and 100ul (20ug) of sheared chromatin to appropriate wells containing washed beads. **See plate template page 3**. Incubate overnight with rotation at 4C in cold room

Plate template for Ab/chromatin combinations, sheared chromatin X, Y and Z

	Column 1	Column 2	Column 3
A	X-H3K4Me3	X-H3K27ac	X-IgG
B	X-H3K4Me3	X-H3K27ac	X-IgG
C	Y-H3K4Me3	Y-H3K27ac	Y-IgG
D	Y-H3K4Me3	Y-H3K27ac	Y-IgG
E	Z-H3K4Me3	Z-H3K27ac	Z-IgG
F	Z-H3K4Me3	Z-H3K27ac	Z-IgG

Day 2

Post IP washing of beads, elution of bound chromatin and reversal of crosslinks

12. Prepare RIPA buffer immediately before use. Add the stock solutions in the order listed below and chill on ice

Reagents for RIPA Buffer	**Stock Concentration**	**Final Concentration**	**Volume per 1000 µL**
Hepes, pH 8.0	1 M	50 mM	50 µL
NP-40	10%	1%	100 µL
Sodium Deoxycholate	10%	0.70%	70 µL
LiCl	8 M	0.5 M	62.5 µL
50x cOmplete EDTA-free protease inhibitor	50x	1x	*20* µL
EDTA	0.5 M	1 mM	2 µL
dH2O	--	--	695.5 µL

- Set Thermomixer to 65C

13. Place the plate containing the labelled beads and chromatin on magnet on ice for 2mins
14. Wash the beads x 5 with 150ul ice cold RIPA buffer
15. Wash the beads x 1 with cold 1x TE
16. After removal of TE wash, add 150ul of ChIP elution buffer, recipe below

Reagents	**Stock Concentration**	**Final Concentration**	**Volume for 50 mL ChIP ELUTION BUFFER**
Tris, pH 8.0	1 M	10mM	0.5 mL
EDTA	0.5 M	1mM	0.1 mL
SDS	10%	1%	5 mL
dH2O	------	------	44.4 mL

17. Transfer each beads/elution buffer mixture from the plate wells to 1.5ml tubes
18. Incubate at 65C for 20mins at 1300rpm in a Thermomixer
19. Post incubation, spin tubes briefly to remove liquid from lids
20. Place tubes on magnetic rack for 2mins**. Transfer SUPERNATANT containing immunoprecipitated chromatin** to a new 1.5ml DNA lobind tube
21. Incubate samples plus input chromatin (see below) at 65C overnight to reverse crosslinks

- For input control samples, use non-IP chromatin retained at 4C in step 8. Add 20ul of Input chromatin to 130ul of ChIP elution buffer and incubate O/N at 65C with IP samples

<u>Day 3</u> Preparation of ChIP and Input DNA

Before starting DNA prep
Chill 100% EtOH on ice
Prepare fresh 70% EtOH and chill on ice
Thaw glycogen and place on ice
Chill centrifuge and 1.5ml rotor to 4°C
Equilibrate Phenol:Chloroform:Isoamyl alcohol to RT
Label 6 x phase lock tubes plus 6 x 2ml DNA lo bind tubes
Chill 1 x TE pH8.0

22. Add 250ul chilled 1 x TE to each sample.
23. Add 8ul of 10mg/ml RNAse A (final conc 0.2mg/ml). Incubate at 37c for 1 hour.
24. Add 8ul of 20mg/ml Proteinase K (final conc 0.4mg/ml). Incubate at 55C for 1 hour.
25. Prepare 6 Phase Lock tubes by spinning gel to bottom of tube at max speed for 1min in microfuge
26. IN FUME HOOD, add 400ul Phenol:Chloroform:Isoamyl alcohol to each phase lock tube
27. Add samples to Phase Lock tubes and mix by inversion until content appears white and uniform.
28. Spin 4 mins RT at max speed in microfuge
29. If aqueous phase is cloudy, extract again. Transfer top aqueous layer to 2ml DNA lo bind tube
30. Add 16ul of 5M NaCl (final conc 200mM) and 2ul of 20mg/ml glycogen to each sample. Vortex x 4 short pulses to mix
31. Add 920ul cold 100% Ethanol and vortex briefly
32. Place at -80C for 2h
33. Spin 15 mins, 4C, 20,000G. N.B. ensure tube hinges face outside of rotor for location of pellet.
34. Remove supernatant. Wash pellet with 1ml cold 70% ethanol. Spin 5mins, 4C, 20,000G
35. Remove ethanol with 1ml tip. Pulse spin and remove residual ethanol with 200ul tip.
36. Dry pellet for 5mins RT
37. Resuspend pellets of ChIP DNA in 22ul 10mM tris pH 8.0. Leave to resuspend O/N at 4C

<u>Day 4</u> QC of ChIP, IgG and Input DNA

38. Measure DNA quality on the Nanodrop
39. Check fragment size **of Input only** on 1.3% agarose gel.
40. Check fragment size of ChIP, IgG and Input on Agilent Tapestation with HS D1000 kit following manufacturer's instructions. Average size of fragments should be ~200bp (100-300bp)
41. If QC OK, pool duplicate samples to give ~40ul per sample
42. Where possible prior to ChIP-seq, run qPCR with suitable primers, for your tissue/Ab combination to check for enrichment of ChIP DNA relative to Input and IgG controls
43. Store ChIP DNA, control IgG DNA and Input DNA at -20C.