ChIP assay - Swaroop Lab, 2013

ChIP protocol (Sooyoung) - For retina tissues (Check: one retina amount of gDNA:)

Generally, use DNA, 5, 10, or 20 ug, but some peoples are using 200 ug also.
H3, H4, 2ug, 2hr, 5 ul PCR - result good
Generally, other antibody - DNA 20 ug, 2 ug antibody, would be OK, but need optimization

Prior to Day1- option

- **Coating protein A beads**

Take enough dynabeads slurry (30ul-50ul each sample) (Invitrogen #100-01D)

Separate beads and buffer with Magnetic Separation Stand (promega # Z5332)

Remove the sup. with loading tip

Wash three times with 1 mL IP dilution buffer

Resuspend beads in 1mL pre-blocking buffer

Incubate at 4°C, greater than 2hr or O/N

Separate beads and buffer with Magnetic Separation Stand and remove the sup.

Wash twice with 1 mL IP dilution buffer

Add IP dilution buffer back to original volume

(Comments: *This protocol is modified specifically for ChIP-Seq. Many protocols suggested that dynabeads don't need blocking. For separation of beads and sup, I first briefly centrifuge it and then put the tube to the magnetic stand. Remove the sup. by loading tips)*

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DAY1

Cell lysis and chromatin DNA extraction

1) Dissect retinas from eye balls, and wash with Cold PBS (X1)
2) Put retinas into 900 ul RT PBS
3) Add 100 ul formaldehyde (from 10% stock) and mix immediately, Incubate and rotate at RT for 15 min
   For histone antibody, 10 min would be proper, but for other antibody the time could be increased upto 30 min. (Check, 10 min, 15 min and 30 min)
4) Add 143 ul **glycine** (from 1M Stock, final 125 mM) and mix immediately and spin down, remove sup
5) Add **0.125 M glycine** to soak retina, rotate 5 min at RT

   Keep 4 'C (on ice) from here
6) Rinse retinas with 1ml Cold 1xPBS twice, centri at 4'C
7) Add **600 ul Cell lysis buffer** and keep on ice for 10 -20 min and Pipet up and down every 5-10 min until no clear tissue can be visible (or use homogenizer)
8) Centri 2500g (3500 rpm), 5 min in Cold centrifuge and remove the sup.
9) Resus pellet in at least 10 vol (600 ul) with cell lysis buffer and Rinse once and centri 2000 g (3500 rpm) again, remove the sup.
10) Re-suspend the nuclear pellet (white) in 200 ul **nuclei lysis buffer** and pipet up and down to disrupt clumps
11) On ice 10-20 min (lay the tube on ice to avoid precipitation of SDS in the nuclei lysis buffer)
12) Add 400 ul **IP dilution buffer**+protein inhibitors

    Possible to freeze at -80'C

Sonication

13) Sonicate –Covaris setting (check: and gel loading)
    Sonication setting (___ pulses of ___ sec, X____, interval _____,)
    Take 10 ul for agarose gel electrophoresis to check the size of fragmented DAN after decross-linking
    Average fragment size should be between 100 bp-600 bp.
14) Centri at 14000 rpm 15 min (5 min, or 10 min, OK)
15) Take the supernatant and centri again at 14000 rpm for 15 min (5 min or 10 min, OK even once centri would be OK). Save 10ul for input DNA (1/10 of working)
    Possible to freeze at -80'C

Antibody binding

16) Preclear the lysate by incubating by constant rotation with dynabeads (Invitrogen, 25 ul) for >2 hours in the cold room.

17) Separate beads and buffer with Magnetic separation stand.
18) Take the sup.
19) Add antibody 5 ug to the sample (Keep the no-antibody control sample at 4'C without adding any antibody), rotate O/N at 4'C.

DAY 2

Dynabeads binding, washing, elution, and decross-linking

20) Add 30 ul bloked dynabeads to each sample (also to no-antibody control tube)
21) Incubate on a rotating wheel at RT 60-90min (or cold room, 6 hrs)
22) Separate beads and buffer with Magnetic separation stand.
23) Remove sup (Beads from no-antibody tube could serve as" no Ab" negative control after washing and elution)
24) Wash the beads twice with 1 ml 1X dialysis buffer
    Add 1 ml buffer, rotate 15 min at RT, separate beads and buffer with Magnetic separation stand, remove the sup. As much as possible with loading tip

25) Wash the beads twice with 1ml IP wash buffer (same as above)
    (comment: Washing step could be held at 4'C with longer time, if non-specific binding still occur, wash 3 times for each buffer followed by 1-3 times TE wash)

Elution and decross-linking

26) Add 150 ul elution buffer to the beads
27) Incubate in 65'C water bath for 10-15 min (vortex every 2-3 min)
28) Separate beads and buffer with Magnetic separation stand
29) Transfer the sup. To a new tube.
30) Repeat elution steps again and combine both elution (300ul)
31) Add 30 ul 3M NaCl (final 250mM) and 1 u 10mg/ml RNaseA (for input sample, add right amount of Nacl and Rnase A)
32) Incubate 65'C, 4-5 hr or O/N (input control samples also need to be decross-linking at the time)
33) Purify ChIP DNA and input DNA with Qiagen PCR purification kit
34) Do qPCR with ChIP sample, no-Ab control and total input

Recipes for solution and material information

1 M glycine (FW = 75.07)

3.75 g / 50 mL dH_2O

filtrate and store at RT

1% formaldehyde in 1x PBS (freshly prepared)

Total	25ml
37% formaldehyde	676 uL
1x PBS	24.324ul

0.125 M glycine in 1x PBS (freshly prepared)

	10 mL	30 mL
1 M glycine	1.25 mL	3.75 mL
1x PBS	8.75 mL	26.25 mL

Proteinase inhibitors

(final conc.)	(per 1 mL solution/buffer)
100 uM PMSF	1 uL of 100 mM
100 ug/mL benzamideine	10 uL of 10 mg/mL
5 ug/mL leupeptin/pepstatin	1 uL of 5 mg/mL

Cell lysis buffer (at 4°C)	(40 mL)
10 mM Tris-Cl (pH 8,1)	400 uL of 1 M (Using Tris Base)
10 mM NaCl	133 uL of 3 M
0.5% NP-40	2 mL of 10%
proteinase inhibitors (freshly add)	
dH_2O	37.47 mL

Nuclei lysis buffer (20 mL)

50 mM Tris-Cl (pH 8.1)	1 mL of 1 M
10 mM EDTA	400 uL of 0.5 M
1% SDS (Sodium Dodecyl Sulfate)	2 mL of 10%
proteinase inhibitors (freshly add)	
dH_2O	16.6 mL

IP dilution buffer (at 4°C) (40 mL)

16.7 mM Tris-Cl (pH 8.1)	668 uL of 1 M
167 mM NaCl	2227 uL of 3 M
1.2 mM EDTA	96 uL of 0.5 M
1.1% Triton X-100	2.2 mL of 20%
0.01% SDS	40 uL of 10%
proteinase inhibitors (freshly add)	
dH_2O	34.77 mL

Pre-Blocking buffer for dynabeads: 1mL

glycogen	10uL (20mg/mL),
BSA	40uL (5mg/mL)
Yeast-tRNA	10uL (20mg/mL)

in 1mL IP dilution buffer

500 mM $NaHCO_3$ (filtrate, store at RT)

$NaHCO_3$ (FW = 84.01)	4.2 g
H_2O	up to 100 mL

1x dialysis buffer (store at 4°C) (100 mL)

50 mM Tris-Cl (pH 8.1)	5 mL of 1 M
2 mM EDTA	400 uL of 0.5 M

0.2% sarkosyl (omit for monoclonal Ab) 1 mL of 20%

The other name is N-lauroylsarcosin solution (SIGMA L7414-10ML)

dH_2O 92.6 mL

IP wash buffer (stored at 4°C) (100 mL)

100 mM Tris-Cl (pH 9.0) (pH 8.0 for monoclonal Ab) 10 mL of 1 M

500 mM LiCl 10 mL of 5M

1% NP-40 10 mL of 10%

1% deoxycholic acid (Sodium deoxycholate Sigma D6750-25G) 5 mL of 20%

dH_2O 65 mL

Elution buffer (40 mL)

50 mM $NaHCO_3$ 4 mL of 500 mM

1% SDS 4 mL of 10%

dH_2O 32 mL of dH_2O

5x PK buffer

50 mM Tris-Cl (pH 7.5) 5 mL of 1 M

25 mM EDTA 5 mL of 0.5 M

1.25% SDS 12.5 mL of 10%

dH_2O 77.5 mL

Glass beads, acid-washed, Sigma G1277-100G

10 mg/mL proteinase K

10 mg/mL glycogen

3 M NaCl

Phenol (saturated with TE)

$CHCl_3$ (+1/25 vol. Iso-amyl alcohol)

EtOH (-20°C)