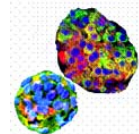


SOP



Title:	Chromatin immunoprecipitation				
Protocol #:	1.4	Submitted:	050510	Approved:	200610
Category:	MB	Author(s): ¹	MPD, MVJ	Checked by:	AAH

Reagents (in order of use):

1. 37% formaldehyde
2. 2M glycine
3. Phosphate buffered saline (PBS)
4. TBE buffer
5. EtBr
6. Protease inhibitor cocktail (PIC)
7. Wash buffer 1
8. Wash buffer 2
9. Lysis buffer
10. RIPA buffer
11. Protein A/G plus bead cocktail
12. 20 mg/ml Salmon sperm DNA
13. Bovine serum albumin (BSA)
14. DEPC treated water
15. Antibodies (H3K4-Me2/ H3K9-Me2/ H3K4-Me3/H3K9-Me3/ H3-Ac, etc)
16. Tris-EDTA buffer (TE)
17. 10% SDS
18. 1M Sodium bicarbonate
19. Dithiothreitol (DTT)
20. 4 N NaCl
21. 0.5 M EDTA
22. 1 M Tris-HCl, pH 6.5
23. 1 mg/ml proteinase K
24. Phenol : chloroform : isoamyl alcohol
25. Chloroform
26. 10 mg/ml Glycogen
27. 3 M Sodium acetate, pH 5.2
28. Absolute ethanol

Equipment

1. Centrifuge
2. Sonicator
3. Eppendorf rocker (at 4°C)
4. Thermomixer
5. Vortex
6. Dry bath
7. Water bath
8. Nanodrop instrument for quantification of DNA
9. Real time PCR instrument
10. Pipettes, tips, eppendorfs, cell scrapers

SOP

Procedure:

(A)CROSSLINKING:

1. Crosslink cells by adding 37% formaldehyde directly to a T75 flask such that the final concentration of formaldehyde in the flask is 1% (Add 0.26 ml of 37% formaldehyde to 10 ml of medium in a T75).
2. Keep flask on a rocker at 37°C for 10 min.
3. Add 0.625 ml of 2M glycine in the same medium in the flask.
4. Keep flask on a rocker at 37°C for 10 min.
5. Scrape the cells off the flask surface using a cell scraper and take this scraped cell suspension in a 15 ml conical tube.
6. Centrifuge the suspension at 2500 rpm for 4 min at 4°C and discard the supernatant.
7. Wash cells with cold PBS thrice by centrifuging at 2500 rpm for 4 min at 4 °C.
8. Remove any traces of PBS by brief spin at 10,000 rpm for ~30 sec.
9. This pellet can be stored at -80°C until further use.

(B)SONICATION:

1. Add 500 µl of Wash buffer 1 (Stock PIC is 10x, so add 50 µl of it to 450 µl of pre-made wash buffer 1, just before use) to the pellet and suspend pellet by pipetting. Tap gently and keep on ice for 5 min.
2. Spin at 3000 rpm, 4°C for 5 min. Discard supernatant.
3. Add 500 µl of Wash buffer 2 (containing PIC) to the pellet and suspend pellet by pipetting. Tap gently and keep on ice for 5 min.
4. Spin at 3000 rpm, 4°C for 5 min. Discard supernatant.
5. Add 1 ml of Lysis buffer (containing PIC) and keep on ice.
6. Sonicate the sample giving pulses of 5 sec alternating with pulse off for 5 sec at an amplification setting of 60 %. Vary the duration of sonication (number of minutes for the above sonication cycle) depending on the sample.
Remember: It is necessary to optimize the sonication time according to the cells in use to get the right size of DNA fragments required for ChIP.
7. After sonication, spin the sample at 13,000 rpm at 4°C for 5min. Keep tubes on ice.
8. Load 5 µl of sample (with 1 µl of 6x loading dye) on a 1% agarose gel (along with a 100 bp DNA ladder as well as a 1 kb ladder in other wells).
9. Electrophorese at 70 V for 1.5 hrs.
10. Size of DNA fragments should be between 600 bp and 1.5 kb for a good ChIP efficiency (If the DNA smear is well above this size range, repeat sonication till you get a smear in that range).

(C)CHROMATIN IMMUNOPRECIPITATION:

1. Quantify DNA in your sample (using a nanodrop instrument).
2. Add 10 µl of pre-made bead cocktail to the 1 ml sonicated suspension.

SOP

3. Keep the tube on an eppendorf rocker for 2 h at 4°C.
4. Spin sample at 5000 rpm at 4°C for 5 min.
5. Divide 1 ml of sample into aliquots ('x' number of tubes for antibodies + 1 isotype control antibody + 1 input control tube; 100 µl is sufficient for input control).
6. Make up volume in each eppendorf to 500 µl with lysis buffer.
7. Add 2 µl of 1 mg/ml antibody to respective eppendorf (H3K4-Me2/ H3K9-Me2/ H3K4-Me3/ H3K9-Me3/ H3-Ac/ H4-Ac, etc).
8. Keep on an eppendorf rocker at 4°C, overnight (and freeze the input control tube at -20°C).
9. Take out tubes from the rocker the next day and add 20 µl of bead cocktail to each tube.
10. Keep on the rocker for 2-4 h at 4°C.
11. Centrifuge eppendorfs at 5000 rpm, 4°C for 5 min.
12. Discard supernatant and wash pellet with RIPA buffer (containing PIC) thrice.
13. Discard supernatant and wash pellet with Tris-EDTA buffer (TE) thrice.
14. Prepare fresh elution buffer and add 100 µl of elution buffer to each eppendorf (Do not keep tubes on ice as SDS precipitates at 4°C).
15. Keep tubes on a thermomixer at 37°C, 1200 rpm for 10 min.
16. Spin tubes at 13,500 rpm for 5 min at 25°C.
17. Take supernatant in new eppendorfs.

(D) REVERSE-CROSSLINKING:

1. Add 5 µl of 4 N NaCl to each tube (in 100 µl, ie 0.05 volume).
2. Thaw out input control tube and add 5 µl of 4 N NaCl to it.
3. Keep tubes on 65°C dry bath for minimum 4 h.
4. Add 0.025 volume of 0.5 M EDTA (ie, 2.5 µl), 0.05 volume of 1 M Tris-HCl, pH 6.5 (ie, 5 µl) and 100 µg/ml proteinase K (10 µl of stock 1 mg/ml).
5. Keep on a water-bath at 45°C for 1 hr.

(E) DNA ISOLATION:

1. Make up volume in each eppendorf to 500 µl using DEPC treated water.
2. Add equal volume (500 µl) of phenol : chloroform : isoamyl alcohol and shake the tubes vigorously.
3. Spin at 12,000 rpm for 5 min at 25°C.
4. Take upper aqueous layer in separate eppendorf and add equal volume (500 µl of chloroform) and shake vigorously.
5. Spin at 12,000 rpm for 5 min at 25°C.
6. Take upper aqueous layer in separate eppendorf.
7. Add 20 µg of glycogen, 0.1 volume of 3 M sodium acetate, pH 5.2 and 2 volumes of cold absolute ethanol (ie, 2 µl of 10 mg/ml glycogen, 17 µl of 3 M sodium acetate and 1 ml of cold absolute ethanol).
8. Mix by pipetting/ inverting tubes.
9. Keep at -20°C overnight or for a couple of hrs at -80°C for precipitation.

SOP

10. Take out tubes from -20°C the next day and centrifuge them at 12,000 rpm for 5 min at 4°C.
11. Discard supernatant and add 1 ml of 75% ethanol. Vortex for 2-3 sec.
12. Centrifuge at 12,000 rpm, 4°C for 5 min.
13. Discard supernatant and keep tube on a dry bath (37°C) for 10 min or less (till pellet is dry).
14. Add 20 µ of DEPC treated water to pellet and mix by pipetting.
15. Carry out real-time PCR using primers for your gene promoter of interest and run the same for a control, say GAPDH gene promoter).

Anticipated results (and Calculations):

1. Ct values are obtained after running the quantitative real time PCR.
2. Subtract these Ct values from 39 (to obtain Ct value over detectable).
3. Calculate 2 to the power values for each of these net Ct values to get fold over detectable (FOD).
4. Normalize these values with respect to the input control by dividing FOD values for all the antibodies individually by the FOD value for input control (for each promoter region that you are testing). This gives you your input-normalized-fold over detectable values (INFOD).
5. Now normalize these INFOD values for gene promoters with respect to those for GAPDH promoter by dividing the INFOD values for the promoter region tested by the INFOD value for GAPDH gene promoter. New values are your Input-&-GAPDH-normalized FOD values (IGNFOD).
6. Similarly, divide these IGNFOD values for the various antibodies used by the IGNFOD for isotype control. The resultant values are your final values.
7. Plot these final values in Graphpad Prizm/Sigmaplot for comparison.

Note:

These calculations are based on my analysis and interpretation method for the ChIP data. Different papers in the literature use different calculations based on their experimental design (Some of the papers that I came across in my reading had (1) used only 1 or 2 of the three controls that have been mentioned above: Input control, Isotype control or GAPDH control, and hence the difference in calculations, or (2) Had not normalized with respect to the isotype control and had instead plotted these values separately on the same graph).

SOP

Supplementary data:	1. Composition of buffers.
----------------------------	----------------------------

Wash Buffer 1:

	Stock	Working	For 100 ml
Triton X 100	10 %	0.25 %	2.5 ml
EDTA	500 mM	10 mM	2 ml
EGTA	100 mM	0.5 mM	0.5 ml
HEPES	2500 mM	10 mM	0.4 ml
Sodium butyrate	500 mM	10 mM	2 ml
PIC	10X	1X	10 ml
Autoclaved Water			82.6 ml

Wash Buffer 2:

	Stock	Working	For 100 ml
NaCl	4 M	0.2 M	5 ml
EDTA	500 mM	1 mM	0.2 ml
EGTA	100 mM	0.5 mM	0.5 ml
HEPES	2500 mM	10 mM	0.4 ml
Sodium butyrate	500 mM	10 mM	2 ml
PIC	10X	1X	10 ml
Water			82.5 ml

Lysis buffer:

	Stock	Working	For 100 ml
NaCl	4m	150%	3.75 ml
Tris-HCl	1 mM	25 mM	2.5 ml
EDTA	500 mM	5 mM	1 ml
Triton X-100	100%	1%	1 ml
SDS	10%	0.1%	1 ml
Sodium deoxycholate	10%	0.5%	5 ml
Sodium butyrate	500mM	10 Mm	2 ml
PIC	10X	1X	10 ml

SOP

Water 73.75 ml

RIPA buffer:

	Stock	Working	For 100 ml
SDS	10 %	0.1 %	1 ml
Sodium deoxycholate	10 %	1 %	10 ml
NaCl	4M	150 mM	3.75 ml
Na-PO4 buffer	1M	10 mM	1 ml
EDTA	500ml	2 mM	0.4 ml
Sodium vanadate	1M	0.2 mM	0.02 ml
IGEPAL	100 %	1 %	1 ml
Water			82.83 ml

Protein A/G plus bead cocktail:

Take 100 μ l of 50 % bead slurry. Let the beads settle down and then discard the upper storage buffer. Add 1 μ l of 20 mg/ml salmon sperm DNA. Add 100 μ g of BSA (10 μ l of 10 mg/ml BSA prepared in DEPC treated water). Make up the volume to 200 μ l with DEPC treated water.

Anticipated results

N/A

Representative image / picture

N/A

References:

Joglekar MV, Joglekar VM, Joglekar SV and Hardikar AA (2009), Human fetal pancreatic insulin-producing cells proliferate in vitro. [*J. Endocrinology*](#) 201(1):27-36, Epub 2009 May 6