Chromatin Immunoprecipitation (ChIP) Protocol

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Introduction

This protocol is used to verify the enrichment of protein(s) on chromatin. It is good for the detection of transcription factors and proteins from complexes that function in transcriptional regulation.

Materials

ChIP fixation buffer
ChIP lysis buffer 1, 2 and 3
2X IP buffer (prepare fresh right before use)
RIPA buffer for ChIP
TEN buffer
Elution buffer
Protease inhibitors (PMSF, leupeptin, pepstatin, aprotinin)
2.5 M glycine
1X PBS pH 7.2-7.4
5M NaCl
Sonication tubes (15 mL TPX (polymethylpentene) (Diagenode, cat #: M-UN-15) **NOTE:** alternatively, the cheaper polystyrene tubes can also be used (BD Falcon, cat #: 352099)
Diagenode Bioruptor (Diagenode, cat #: UCD-200 TO)
Protein G Dynabeads
TE Buffer
BSA
Qiagen Qiaquick columns

Protocol

Adherent cells:

1. Remove media from plates and add 15 mL of Fixation buffer. (for 15 cm dishes)
2. Allow crosslinking for 10 min @ RT. Put plates on top of the rocker so that Fixation buffer is well absorbed.
3. Quench the formaldehyde by adding 750uL of 2.5M Glycine (0.125M final). Media will turn yellow immediately due to change in pH.
4. Remove the media and rinse cells with 15 mL of cold PBS.
5. Remove the PBS and add 5mL more.
6. Scrape the cells and collect on a tube.
7. Centrifuge 5 min @ 2000 x g.
8. Remove the supernatant.
9. If desire snap freeze the cell pellet and store at -80ºC.

Cell suspension:

1. Use Accutase (or mild trypsin) to detach adherent cells.
2. Centrifuge 3 min @ 2000x g.
3. Resuspend cell pellet with culture media and count cell number.
4. Dilute into 30 million cells per 10 mL.

Crosslinking:

5. Add Formaldehyde to cell suspension to 1% concentration.
6. Allow crosslinking for 10 min @ RT.
7. Quench the formaldehyde by adding 500 μL of 2.5 M Glycine (0.125 M final). Media will turn yellow immediately due to the pH change.
8. Centrifuge 5 min @ 2500 x g, remove the media and rinse cells with 10 mL of cold PBS.
9. Centrifuge 5 min @ 2500 x g, remove the supernatant. **NOTE:** at this point the cells are really sticky, aspirate carefully snap freeze the cell pellet and store at -80ºC.

Chromatin Preparation:

10. Re-suspend cells in 15 mL of ChIP Lysis buffer 1 per 300 mg of cells.
11. Place on an end over end rotator for 10 min @ 4ºC.
12. Centrifuge @ 500 x g for 5 min @ 4°C.
13. Remove the supernatant and add the same amount of ChIP Lysis buffer 2 that was added in step 10.
14. Place on an end over end rotator for 10 min @ 4ºC.
15. Centrifuge @ 500 x g for 5 min @ 4°C.
16. Remove the supernatant and add 1.5 mL of ChIP Lysis buffer 3 (transfer to 15mL-polystyrene tubes (glass like tube) before sonication). Do not use regular plastic 15 ml falcon tubes.
17. Sonicate using a Bioruptor in 10 cycle sets (15-17 cycles works well for ESCs) at max setting using 30 sec ON, 30 sec OFF cycles. Total 10 mins. **NOTE:** This needs to be determined for each cell type!
18. Check size of DNA fragments loading 5-10 μL of chromatin on a 1.5% agarose gel. For regular ChIP-qPCR experiments try to get sizes of ~1.5Kb (1-2kb) of cross-linked material. For ChIP-seq experiments try to get sizes of ~1 kb (0.5kb-1.5kb) of cross-linked material. As samples were crosslinked, the fragment size will not reduce to 150-300bp (app one nucleosome size). It is also important that all samples show similar size of fragmented DNA. If accurate measurement of size is needed, reverse the crosslink by incubating material at 65ºC O/N in Elution Buffer. Add 190 μL of elution buffer + 20 μg of proteinase K to 10 μL of chromatin sample and incubate O/N in the incubator. For ChIP-qPCR ~500bp sizes are OK, for ChIP Seq ~150-300bp (this part accounting for >80% is ok) is needed.
19. Centrifuge @ Max speed for 10 min @ 4°C. Take the supernatant into fresh e-tube (low-binding tubes).
20. The chromatin can now be either stored at -80ºC or the protocol can be continued.
21. To quantify the chromatin measure absorbance at 260 and calculate an approximate [DNA] (the sample will contain proteins and RNA, but this is a good approximation). OD260/280 should be about 1.7 - For 3x10^7 cell normally get 1-1.5 ug/ul. For one IP (next step) you should use between 25-200 μg of chromatin (Usually, ~5 million cells for high abundance protein per IP, for low abundance proteins one could scale up to 10 million cells per IP).

Immuno-Precipitation:

22. Prepare 5 μL of Dynabeads Protein G (not bed volume) per μg of antibody for each sample. Calculate how much you will use the Dynabeads protein G and prepare for the experiment.
23. Wash the beads with TE Buffer twice.
24. Block the beads with 1 mg/mL BSA in TE (make it fresh). 1hr @ 4ºC with rotation.
25. Wash the beads with TE Buffer 5 times.
26. Resuspend the beads to a 50% slurry after the last wash. These blocked beads can be stored at 4ºC, but they should be used within 1-2 weeks.
27. To pre-clear the chromatin, use 20 μL of the previously prepared slurry per IP. Incubate at 4ºC for 1 hr.
28. Spin and transfer the supernatant. This is the chromatin sample you will use for IP. Save some of material for using as an input later on.

	Final			
* 2x IP buffer	1x			
** Chromatin sample	50-200ug			
Antibody (your target) or ***control IgG	****2-5ug			
***** Homemade Drosophila chromatin				
***** H2Av antibody (specific for drosophila)				

* IP conditions will be: 1% NP-40, 0.25% Sarkosyl, 100 mM NaCl. Usually the final concentration of chromatin will be 0.1-0.5mg/mL.

** You need to determine the amount of chromatin sample. You may start from 100ug. All IP samples should start with same amount of chromatin sample.

*** Remember to setup your IgG or pre-immune serum control. Or Knockout control.

**** This amount needs to be optimized, usually titrate so that the antibody is about 1%-10% of the total chromatin amount
***** Drosophila chromatin and H2Av antibody is for spike-in control.

29. Incubate O/N (or at least 4 hrs) at 4ºC.
30. After incubation O/N some of the sample could have precipitated is remove this before proceeding with the addition of beads. Spin your sample at max speed (15-20K x g) for 10 min and transfer the supernatant to another tube.
31. Add 5 μL of the previously prepared Dynabead slurry (not bed volume) per μg of antibody and incubate at 4ºC for 1-2 hrs with end over end rotation.
32. Wash the beads 5 times with 750-1000 μL of cold RIPA buffer for ChIP. Mix for 2 min between each wash. **NOTE:** It is very important to **NOT** over wash the beads for fear of losing material.
33. Wash once with TEN buffer (for ChIP-WB testing, use [NaCl] = 200 mM) without incubation.
34. At this point process in parallel a 10% input sample to analyze your IPs afterwards.
35. **(Optional)** To test antibody by WB, add 15 μL Elution buffer (4% SDS), and 15 μL 2 x Loading buffer, boil (98 °C) for 30 min in PCR machine, then load all.
36. Add 200 μL of Elution buffer + 5 μg of RNAse A per ChIP and 20 μg of Proteinase K. Incubate at 37°C for 30 min and then place at 65ºC overnight. This step is necessary for the reversal of the crosslinking. If possible use a thermomixer to agitate while incubating. NOTE: proteinase K is not active at 37°C.
37. Spin and collect the supernatant.
38. Use the Qiaquick PCR purification columns to collect the immunoprecipitated chromatin. Follow the manufacturer's instructions. Now go to Chip-seq library preparation protocol
39. Elute the chromatin from the columns using 30 μL of TE buffer.
40. Analyze your IPs by qPCR using 1-2 μL of this material or proceed to library preparation. To quantify and normalize also use dilutions of the input material (I usually use 10%, 1% and 0.1% of Input)

RECIPES

ChIP Fixation Buffer

	Initial	Final	200 mL	500 mL	1L
HEPES pH 7.6	1 M	10 mM	2 mL	5 mL	10 mL
NaCl	5 M	15 mM	0.60 mL	1.50 mL	3 mL
Formaldehyde	37%	1%	5.41 mL	13.51 mL	27.03 mL
EDTA	0.5 M	0.15 mM	60 μL	150 μL	300 μL
EGTA	0.5 M	0.075 mM	30 μL	80 μL	150 μL
DMEM			191.91 mL	479.76 mL	959.52 mL

ChIP Lysis Buffer 1

	Initial	Final	50 mL	100 mL	200 mL
HEPES pH 7.6	1 M	50 mM	2.5 mL	5 mL	10 mL
NaCl	5 M	140 mM	1.4 mL	2.8 mL	5.6 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
Glycerol	100%	10%	5 mL	10 mL	20 mL
NP-40	10%	0.5%	2.5 mL	5 mL	10 mL
Triton-X	10%	0.25%	1.25 mL	2.5 mL	5 mL
ddH_2O			37.25 mL	74.5 mL	149 mL

Right before use add: 0.2 μM PMSF, 1 μg/mL Pepstatin A, 1 μg/mL, Leupeptin, 1 μg/mL Aprotinin

ChIP Lysis Buffer 2

	Initial	Final	50 mL	100 mL	200 mL
Tris-HCl pH 8.0	1 M	10 mM	0.5 mL	1 mL	2 mL
NaCl	5 M	200 mM	2 mL	4 mL	8 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
EGTA	0.5 M	0.5 mM	0.05 mL	0.1 mL	0.2 mL
ddH_2O			47.35 mL	94.7 mL	189.4 mL

Right before use add: 0.2 μM PMSF, 1 μg/mL Pepstatin A, 1 μg/mL, Leupeptin, 1 μg/mL Aprotinin

ChIP Lysis Buffer 3

	Initial	Final	50 mL	100 mL	200 mL
Tris-HCl pH 8.0	1 M	10 mM	0.5 mL	1 mL	2 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
EGTA	0.5 M	0.5 mM	0.05 mL	0.1 mL	0.2 mL
N-Lauryl Sarcosine	10%	0.5%	2.5 mL	5 mL	10 mL
ddH_2O			46.85 mL	93.7 mL	187.4 mL

Right before use add: 0.2 μM PMSF, 1 μg/mL Pepstatin A, 1 μg/mL, Leupeptin, 1 μg/mL Aprotinin

RIPA Buffer for ChIP

	Initial	Final	50 mL	100 mL	200 mL
HEPES pH 7.6	1 M	25 mM	1.25 mL	2.5 mL	5 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
LiCl	4 M	0.5 M	6.25 mL	12.5 mL	25 mL
NP-40	10%	1%	5 mL	10 mL	20 mL
N-Lauryl Sarcosine	10%	0.1%	0.5 mL	1 mL	2 mL
ddH_2O			36.9 mL	73.8 mL	147.6 mL

Right before use add: 0.2 μM PMSF, 1 μg/mL Pepstatin A, 1 μg/mL, Leupeptin, 1 μg/mL Aprotinin

2X IP Buffer (Prepare fresh)

	Initial	Final	2 mL	5 mL	10 mL
NP-40	10%	2%	400 μL	1 mL	2 mL
NaCl	5 M	200 mM	80 μL	200 μL	400 μL
PMSF	0.1 mM	1 μM	20 μL	50 μL	100 μL
Leupeptin	1 mg/mL	2 μg/mL	4 μL	10 μL	20 μL
Aprotinin	1 mg/mL	2 μg/mL	4 μL	10 μL	20 μL
Pepstatin	1 mg/mL	2 μg/mL	μL	10 μL	20 μL
TE (pH 8.0)			1.488 mL	3.72 mL	7.44 mL

TE Buffer

	Initial	Final	50 mL	100 mL	200 mL
Tris pH 8.0	1 M	10 mM	0.5 mL	1 mL	2 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
ddH_2O			49.4 mL	98.8 mL	197.6 mL

TEN Buffer

	Initial	Final	50 mL	100 mL	200 mL
Tris pH 8.0	1 M	10 mM	0.5 mL	1 mL	2 mL
EDTA	0.5 M	1 mM	0.1 mL	0.2 mL	0.4 mL
NaCl	5 M	50 mM	0.5 mL	1 mL	2 mL
ddH_2O			48.9 mL	97.8 mL	195.6 mL

Elution Buffer

	Initial	Final	5 mL	10 mL	20 mL
$NaHCO_3$	1 M	100 mM	0.5 mL	1 mL	2 mL
SDS	20%	1%	0.250 mL	0.5 mL	1 mL
NaCl	5 M	200 mM	0.2 mL	0.4 mL	0.8 mL
ddH_2O			4.05 mL	8.1 mL	16.2 mL

Add 20 μg of Proteinase K per sample