

# Chromatin Immunoprecipitation (ChIP)

Day 1

## A) DNA shearing

### 1. Samples

Dissect tissue (One Mouse OBs) of interest and transfer to an eppendorf containing 0.5 ml of **dissecting media** (on ice) or PBS but **without serum**. Chop the tissue in small pieces with scissor.

### 2. Crosslink protein DNA complexes *in vivo*

Transfer into 15 ml tubes and add 9.2 ml of dissecting media and 1% of formaldehyde (sigma F1635, less than 1 year old) (270  $\mu$ l of formaldehyde of 37% solution) rotate a RT for **8 min**. Fill tube with tissue pieces. Incubate at room temperature for 8 min with rotation. *(The extent of cross-linking is critical and depends on the protein of interest. Too much cross-linking may mask epitopes and too little cross-linking may lead to incomplete fixation. The concentration of formaldehyde, the length of cross-linking or the temperature of cross-linking can all be adjusted.)*

### 3. Quench cross-links

Add 0.125M (0.5 ml of a 2.5 M) glycine to fixed cell and incubate at room temperature for **5 min** with rotation.

### 4. Harvest cells

Centrifuge cells (2 min at 1000 rpm) and discard supernatant. Wash cells with 10ml ice-cold **1X PBS** with protease inhibitors (**XI**) and rotate 5 min. and spin cells down, again and discard supernatant. Wash cells again with 5ml ice-cold **1X PBS** with protease inhibitors (**XI**) and rotate 5 min. and spin cells down, again and discard supernatant. Place cells on ice. *(Cells can keep on ice for a few hours, if you are collecting many samples for a time course. Alternatively, cells may be frozen in liquid nitrogen and placed at -80°C).*

### 5. Lyse cells

Resuspend cell pellet gently with a pipette in 750 $\mu$ l Lyses buffer (**SDS Lyses buffer**) with protease inhibitors 1x and **divide each sample in two** and start to homogenize 20 sec with electric homogenizer and incubate on ice 4°C for 30min. (3 ml SDS Lysis Buffer + 30  $\mu$ l of protease inhibitor)

### 6. Shear chromatin

Using a Branson 250 Sonifier with a microtip at a power setting of 4 and a 60% duty cycle, sonicate extracts for 30x 10sec pulses. In between 20sec. pulses, let samples sit on ice for at least 2min. This should shear chromatin to a final average size of 500bp. *(Your sonicator will have to*

*be calibrated to yield the desired final average length of DNA). (Use extracts of 350µl for sonication)*

### **7. DNA shearing Checking**

To check the DNA shearing, take an aliquot of DNA add 0.3M of NaCl of a 5M stock solution and incubate a 65°C ON.

**(50µl DNA + 3µl NaCL )**

### **Day 2**

The next day add 150 µl of water and extract DNA with phenol/chloroform and precipitate in 2 vol. ethanol 100% or 1 vol isopropanol, add 2 µl of Pellet paint (NF-Co-precipitant). Centrifuge 5 min at 13000 rpm. Wash pellet with ethanol 70%, vortex briefly and spin 5 min at 13000 rpm. Resuspend in 15 µl and analyse on a 2% agarose gel, use 1µl of SYBR-green I (Nucleic acid gel stain) (1:100 in DMSO) for each sample. A smir must be observed between 200-600 bp.

### **B) Immunoprecipitation (IP)**

#### **8. Clarify samples**

Centrifuge samples at 12000 rpm for 10min at 4°C. Transfer supernatant to a fresh 1.5ml microcentrifuge tube. (Optional) centrifuge samples again for 15min at max speed at 4°C. The samples can be stored at -80°C after freezing them in liquid nitrogen for weeks before proceeding to the IP.

**9.** Save an aliquot of samples for protein input that will be analyzed in western blot and another aliquot for DNA input (same procedure than the note above).

**10.** Dilute samples 10 times in IP dilution buffer (460µl chromatin + 4094µl IP dilution buffer). Adding protease inhibitors 1x (46µl).

#### **11. Preclear extracts**

Add Protein A sepharose beads (Upstate 80µl /2.5 ml chromatin) of to each tube and incubate on a rotation wheel for 1h at 4°C. Centrifuge samples at 2000 rpm for 1 min at 4°C and then transfer supernatant to a fresh tube.

#### **12. Immunoprecipitation**

Divide supernatant in 1.5 ml samples in 2 ml tubes and include “no antibody”, “MeCP2” and “control antibody (beta-galactosidase). Add the primary antibody against the protein of interest to the extract. Anti MeCP2 antibody: 20µl that is equivalent to 20ug Upstate Ab and 10ug Pevsner Ab (pevsner’s works better than Upstate), control antibody: same quantity of unrelated rabbit IgG anti β-galactosidase (100 µl)(10ug /50µl). Incubate ON by rotation at 4°C. (Preliminary immunoprecipitation experiments should be performed to determine the appropriate amount of antibody to be used).

### **Day 3**

**13.** Then add 60µl volume Protein A sepharose beads. Incubate on rotating wheel for 1hr at 4°C. Centrifuge sample for 1 min at 2000 rpm at 4°C. **Keep the no antibody supernatant as total input chromatin.**

**14. Wash immunoprecipitates**

Wash beads **twice** with 1 ml **low salt buffer**.

Wash beads **twice** with 1 ml **high salt buffer**.

Wash beads **twice** with 1 ml **IP wash buffer**.

Wash beads **twice** with 1 ml **TE1x**.

For each wash rotate for 3min and centrifuge at 2000 rpm 1min, discard supernatant.

**15. Elute immunoprecipitates**

After last wash, elute antibody/protein/DNA complexes by add 200µl **Elution buffer** (1%SDS/0.1M NaHCO<sub>3</sub> pH8.0 **new**). Shake on vortex for 15 min, spin at 13000 rpm for 3 min. Transfer supernatant to clean tubes. Repeat 2 times with 150µl **Elution buffer** more, vortex 10 min each and combine eluted in the same tube.

5 mL **elution buffer** = 4 mL H<sub>2</sub>O + 500µl SDS (10%) + 500µl

NaHCO<sub>3</sub>(1M).

**16. Reverse cross-links**

To the three eluted samples (500µl each) and Input (1.5 ml separate into 500µl/tube); Add 5M NaCl to final concentration of 0.3M. Incubate samples ON at 65°C to reverse formaldehyde crosslinking.

(500µL DNA + 30µL NaCl)

**Day 4**

**17. Proteinase K treatment**

Add 5x PK buffer (125µl/500µl) and 40µg/ml (1.25 µl/tube). Proteinase K solution (20mg/ml) to each sample and incubate for 2hrs at 45°C.

**18. Purify DNA**

Add 650µl 25:24:1 phenol/chloroform/isoamyl alcohol. Vortex vigorously for 1min. Separate phases by centrifugation at max speed for 3 min. at room temperature. Transfer aqueous phase to a fresh tube and add 550 µl 100% isopropanol, 55µl of sodium acetate and 2 µl of glycogen. Mix and store at -20°C overnight.

**Day 5**

**19.** Centrifuge at max speed for 15min at room temperature. Discard the supernatant and add 1ml ethanol 70% centrifuge at max speed for 15min and dry pellet. Re-suspend DNA in 100µl water and store at -20°C

**20.** Analyze data by Real-Time PCR assay or methylation assay or microarray analysis.

**Solutions:**

***Dissecting media buffer; MEM-AIR***

1PKG DMEN with high glucose

4.8 g Hepes , sodium salt

1 L dd H<sub>2</sub>O

pH:7.4 filter sterile and store at 4°C

***SDS Lysis Buffer***

50mM Tris-HCL, pH8.0	5 ml (1M)
10mM EDTA	2 ml (0.5 M)
1% SDS	10 ml (10%)
dd H <sub>2</sub> O	83 ml

0.22µm filtered

**Store at room temperature.**

add 1x protease inhibitors fresh during experiment

**Dilution IP buffer:** to dilute 10 times

0.01% SDS	0.1 ml (10%)
1.1% Triton X-100	1.1 ml (100%)
16.7mM Tris-Hcl pH 8.0	1.67 ml (1M)
1.2 mM EDTA	0.24 ml (0.5M)
167mM NaCl	3.34 ml (5M)
dd H <sub>2</sub> O	93.55 ml

0.22µm filtered

Store at 4°C

add 1x protease inhibitors fresh during experiment.

**Protease inhibitors stock solution (100X)**

Aprotinin 1 µg/ml

Pepstatin A 1 µg/ml

PSMF 1mM

**Protein-A sepharose:** Upstate PAS

It could be prepared

1X Dialysis Buffer

50 mM Tris-Hcl pH 8.0

2 mM EDTA

0.2% N-lauril sarcosine

**Low Salt Wash Buffer**

0.1% SDS	1 ml (10%)
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0.1% Triton X-100	0.1 ml
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150 mM NaCl	3 ml (5M)
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2mM EDTA	0.4 ml
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20mM Tris-HCl pH 8.0	2 ml (1M)
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dd H <sub>2</sub> O	93.5 ml
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0.22µm filtered

Store at 4°C

**High Salt Wash Buffer**

0.1% SDS	1 ml (10%)
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0.1% Triton X-100	0.1 ml
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500 mM NaCl	10 ml (5M)
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2mM EDTA	0.4 ml
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20mM Tris-HCl pH 8.0	2 ml (1M)
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dd H <sub>2</sub> O	86.5 ml
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0.22µm filtered

Store at 4°C

***IP Wash Buffer***

0.5 M LiCl	2.1195g
1% NP-40	1 ml
1% deoxycholic acid	1g
100mM Tris-Hcl <b>pH 9.0</b>	10ml (1M <b>pH 9.0</b> )
dd H <sub>2</sub> O	≈84 ml
0.22µm filtered	
Store at 4°C	

***Elution Buffer***

1% SDS	1ml (10%)
0.1M NaHCO <sub>3</sub> pH8.0	1 ml (1M)
dd H <sub>2</sub> O	8 ml
<b>Fresh</b> Elution buffer	

***5X PK Buffer***

50 mM Tris-Hcl <b>pH 7.5</b>	5 ml (1M)
25 mM EDTA	5 ml (0.5M)
1.25% SDS	12.5 ml (10%)
dd H <sub>2</sub> O	77.5 ml
Store at 4°C	

***TE Buffer***

10 mM Tris_HCl p.H 8.0	1ml (1M)
1mM EDTA	0.2 ml (0.5M)
dd H <sub>2</sub> O	98.8 ml
0.22µm filtered	
Store at 4°C	

***3M Sodium Acetate***

24.6 g in 80 ml of H<sub>2</sub>O.  
Adjust the pH to 5.2 with glacial acetic acid.  
Adjust the volume to 100 ml  
Dispense into aliquots and sterilize by autoclaving.  
Maniatis B.13

***Other Reagents***

37% Formaldehyde  
Proteinase K  
Glycogen  
Phenol: Chloroform: Isoamyl Alcohol  
Isopronol  
70% ethanol  
PBS (1X)