Aleksandra E. Kornienko

ChIP-seq protocol

(updated from (Yelagandula et al., 2014) protocol)

TISSUE COLLECTION:

- Collect tissue in liquid N2, store at -80/-70C.
- Grind the samples with mortar and pestle, or with the Retsch machine.
- 2 grams of tissue is sufficient for 6 ChIP reactions (300mg per one IP)
- Start with 1-2g of material for each sample (~2-5 ml of frozen powder) in a 50ml falcon
    - ✓ Calculate how to prepare all the buffers in advance
    - ✓ Check that you have Covaris tubes (for sonication)
    - ✓ prepare buffers/premixes in advance (can be stored in the fridge for 6 months)
    - ✓ Covaris shearing buffer will go white in the fridge - heat up to 55°C for 10 min before starting the experiment

DAY 1

(start at 9am to have the lunch break at 12am and finish at 5pm)

1. Preparation on Day 1:

- ✓ Precool the Falcon tube centrifuge and the 5ml centrifuge to 4°C. Set the program - 5min 2000g 4°C
- ✓ Put the rotating machine to the cold room
- ✓ Prepare Miracloth (squares for funnel) and Buffers
- ✓ Prepare Covaris class cap tubes
- ✓ Prepare a big bucket with ice
- ✓ Prepare Formaldehyde 37%
- ✓ Prepare dilutions (used in buffers):
    1. 2M Glycine (prepare fresh, 0.15g in 1ml H2O, 0.9g in 6ml) - **2ml per sample!**
    2. B-Mercaptoethanol (toxic)
    3. TRITON 10% - 5ml per sample
    4. 25xComplete (Dissolve 1 tab per 2ml H_2O) - 3ml per sample

✓ **Prepare buffers for Day1:**

1. M1 buffer (toxic) – 25 ml per sample
2. M2 buffer (toxic) – 28 ml per sample

3. M3 buffer (toxic) – 2.5 ml per sample
4. Covaris Shearing Buffer – 12 ml per sample
5. ChIP dilution buffer - 8ml per sample

2. CHROMATIN IMMUNOPRECIPITATION:

FIXATION

1) Frozen powder (1-5ml) should be in a 50-ml Falcon tube.
2) Work in the fume hood!
3) Add **10 ml** of **M1 buffer** buffer, mix by inverting
4) Fill tube with **M1 buffer** up to **20 ml** total volume, mix again
5) Add **37% Formaldehyde** to a final concentration of **1%** (27.03ul per 1ml, **540.60ul per 20 ml**), close the tube and seal with parafilm (because toxic evaporations)
6) Put the tubes on ice
7) Quickly bring the tubes on ice to the cold room, insert into the rotator
8) Incubate at 4ºC, 10min (rotating)
9) Quickly bring the tubes on ice back to the fume hood
10) Stop fixation: add fresh **Glycine 2 M** to a final concentration of **0.125 M** (62.5ul Glycine per 1ml, **1.28 ml Glycine per 20 ml**)
11) Bring back to the cold room, incubate another 5 min at 4ºC (rotating)

NUCLEI EXTRACTION

12) Put the squares of Miracloth filters into a 50ml tube. Wet with **2 ml M1 buffer**
13) in the hood: Filter nuclei through funnel with one Miracloth layer into a 50ml tube, wash with the rest of **M1 buffer.** Trash the filters.
14) Spin the 50ml tube for 5min, 2,000g at 4ºC (large centrifuge)
15) Precool the M2 and M3 buffers on ice.
16) in the hood: Pour out the supernatant (Formaldehyde waste!), carefully resuspend the pellet in **4.5ml M2 buffer.** Transfer to a 5ml safe-lock Eppendorf tube. (5ml would be to tight for this tube, toxic supernatant!)
17) Spin for 5min, 2,000g at 4ºC (5ml centrifuge)
18) Repeat washes **5 times** with **4.5ml M2 buffer**. The supernatant must be clear and transparent at the end. The nuclei will be greenish-whitish.

19) Resuspend nuclei in **2ml M3**, spin 5min, 2,000g at 4ºC, remove the supernatant completely and incubate on ice for ~1h (over Lunch!)
20) Centrifuge 5min, 2,000g at 4ºC after lunch to remove supernatant.

during the break:

- ❖ go down to Bell's lab to switch on and precool the Covaris sonicator (must be done 45 min before the sonication!).
- ❖ Precool the 1.5ml centrifuge
- ❖ prepare Pre-sonication and Post-sonication control tubes (1.5ml Eppendorf)

LYSIS AND SONICATION

21) Add protease inhibitors to Covaris Shearing Buffer Premix.
22) Slowly add **5ml CiA Covaris Shearing Buffer** along the sides of the tube (from the top) to wash all salt. Do not disrupt the pellet! Centrifuge 3min, 1,200g at 4ºC.
23) Repeat the washing once
24) Resuspend the pellet in 0.9ml **CiA Covaris Shearing Buffer** (with freshly added protease inhibitors). Transfer to Covaris glass tube.
25) **!don't forget!** Transfer 20ul to **Pre-sonication control** tube, put to 4ºC
26) SONICATION (Covaris machine in the Bell lab)
    a) Check water level
    b) Remove the metal cross thing from the bottom (if there)
    c) Choose your preset program (Covaris E220 Focused-ultrasonicator for 15 min at 4 °C with the following settings: duty factor of 5.0, peak incident power of 140 ; 200 cycles per burst) .
    d) Sonication will take 15min x number of samples
27) Bring the tubes on ice to the fume hood
28) Transfer to 1.5ml tube, centrifuge 5min, 13000g at 4ºC
29) Transfer supernatant to a new 1.5ml tube, centrifuge 5min, 13000g at 4ºC
30) Transfer supernatant to a new 1.5ml tube, centrifuge 5min, 13000g at 4ºC
31) **!don't forget!** Take out 20ul for **Post-Sonication control** tube, put to 4ºC
32) Measure the volume and add **ChIP dilution buffer** to an appropriate final volume
    a) **M** - number of antibodies per sample
    b) Final volume=**M**x540ul+100ul (for Input) +100ul (pipetting reserve)

2 antibodies	1.3ml

3 antibodies	1.9ml
4 antibodies	2.4ml
5 antibodies	2.9ml
6 antibodies	3.5ml

33) Samples can be stored at 4ºC for up to a week

PRE-CLEARING AND IMMUNOPRECIPITATION;

N is number of plant samples

34) Prepare **Dynabeads Protein A magnetic beads**: wash a total of **N**x**100ul** beads (for pre-clearing) in **N**x**1ml ChIP dilution buffer,** repeat wash
35) Resuspend beads in **N**x**200ul ChIP dilution buffer (2x dilution)**
36) **Pre-clearing:** Add **200ul beads** to each chromatin sample (vortex between each pipetting to have equal amounts) - 3.5ml sample + 0.2ml beads=3.7ml in a 5ml tube
37) Bring the samples to the cold room, **incubate 1h at 4ºC**, rotating
    In the meantime: Dissolve **1 tablet of Complete in 2ml MiliQ** water
38) Split each sample (if volume >2ml) into 2x2ml tubes for magnet binding
39) Pellet beads with a magnet and **transfer supernatant** into a new tube
40) Put this new tube onto the magnetic rack again to remove residual beads. **Supernatant is your sample!**

    Antibody binding to the chromatin:

41) Split each of the samples into N parts into **2 ml Eppendorf LoBind tubes**:
    - **100ul** INPUT fraction (put to -20ºC)
    - **NNx** ChiP reactions (**NNx** different antibodies), **540ul** each
42) Add **20ul** of (antibody+ChIP dilution buffer) and 23ul Complete to each ChIP-reaction tube. **Generally 5ug of antibody should be enough**
43) Incubate **overnight at 4ºC** in the cold room, rotating

DAY 2:

(takes about 6 hours. Start at 9 to have a break from 10 to 13 and finish at 16)

> prepare warm clothes to work 1 hour in the cold room

Buffers for Day2:

- Low Salt Buffer
- High Salt Buffer
- LiCl Buffer
- TE Buffer
- Elution Buffer
- 10x Reverse Crosslink Mix

BEADS BINDING

44) You will need 50ul of beads per ChIP reaction (~N samples x 5)

45) Using magnetic rack wash **the beads** 2 times with 1ml ChIP dilution buffer Resuspend the beads in the same volume of **ChIP dilution buffer** as the initial bead volume, add **50ul of beads** per tube of chromatin+Antibody (12 tubes)

46) Incubate **3h (over lunch) at 4ºC**, rotating.

WASHES (takes ~1.5 hours, work in the cold room!)

47) Pellet beads, remove supernatant into a new tube (keep at -20ºC as **UNBOUND** fraction)

48) Proceed with the following washing steps (wash by rotating, not by centrifugation! pellet the beads using magnetic rack) with 1ml buffer per tube:

- 2x with **Low salt wash buffer**, 5 min at 4ºC, rotating
- 1x with **High salt wash buffer**, 5 min at 4ºC, rotating
- 1x with **LiCl wash buffer**, 5 min at 4ºC, rotating
- 2x with **TE buffer**, 5 min at 4ºC, rotating, change tubes for the last wash.

ELUTION AND REVERSE CROSSLINK:

49) Change the thermomixer head to 2ml-tube one

50) Prewarm the thermomixer to **65ºC**

51) Prepare **Elution buffer** (prepared at the last minute for $NaHCO_3$)

52) Elution:

- **ChIP** samples: resuspend in **250ul Elution Buffer**
- **Input**: take 100ul Input sample and add **400ul Elution buffer**
- **Pre-sonication** and **post-sonication** samples: 20ul + **480ul Elution buffer**

Vortex briefly to mix, incubate **15 min at 65ºC** (thermomixer).

in the meantime: prepare **10x Reverse Crosslink Mix**

53) Spin down at Vmax at 4ºC, carefully transfer supernatant to a new 1.5ml LoBind Eppendorf tube.

54) Repeat elution: add **250ul Elution Buffer** to the bead pellet in ChIP samples. Vortex briefly to mix, incubate **15 min at 65ºC** (thermomixer). Spin down at Vmax at 4ºC. Transfer supernatant to the same 1.5ml LoBind Eppendorf tube containing the supernatant from the previous step => total **500ul** of eluates
55) Precipitate the residual beads (there might be some left after centrifugation) on the Magnarack and transfer the supernatant into a new **1.5ml LoBind Eppendorf tube**.
56) Reverse Crosslink:
57) Add 51ul of Reverse Crosslink Mix to 500ul sample, input and pre-/post-sonication samples (24 samples)
58) Incubate **3h at 45ºC and overnight at 65ºC** (14-16h) using thermomixer program.

DAY 3:

RNase TREATMENT AND DNA PURIFICATION

59) Add **10ug RNase A** (Fermentas, 10ug/ul) =**1ul** to each tube, incubate at room temperature for 30min
60) Purify using the Qiagen PCR purification kit Qiaquick (add 1/10 of **3M Sodium Acetate: 54ul**), elute in 36ul EB.
61) PCR - check if everything worked
