

# ChIP-seq protocol

(Updated from Ramesh's protocol [https://link.springer.com/protocol/10.1007/978-1-4939-7003-2\\_7](https://link.springer.com/protocol/10.1007/978-1-4939-7003-2_7))

## TISSUE COLLECTION:

---

- Collect tissue in liquid N<sub>2</sub>, store at -80°C.
- 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 glass 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 H<sub>2</sub>O, 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<sub>2</sub>O) - 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 4°C, 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 4°C (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 4°C (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 too tight for this tube, toxic supernatant!)
- 17) Spin for 5min, 2,000g at 4°C (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 4°C, remove the supernatant completely and incubate on ice for ~1h (**over Lunch!**)
- 20) Centrifuge 5min, 2,000g at 4°C 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 4°C.
- 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 4°C
- 26) **SONICATION (Covaris machine)**
  - a) Check water level
  - b) Remove the metal cross thing from the bottom (if there)
  - c) Choose your preset program (**per sample: 15min .....** ).
  - 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 4°C
- 29) Transfer supernatant to a new 1.5ml tube, centrifuge 5min, 13000g at 4°C
- 30) Transfer supernatant to a new 1.5ml tube, centrifuge 5min, 13000g at 4°C
- 31) **!don't forget!** Take out 20ul for **Post-Sonication control** tube, put to 4°C
- 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 4°C 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 **Nx100ul** beads (for pre-clearing) in **Nx1ml ChIP dilution buffer**, repeat wash
- 35) Resuspend beads in **Nx200ul 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 4°C**, 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 -20°C)
  - ❖ **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 4°C** 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 **4°C**, rotating.

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

- 47) Pellet beads, remove supernatant into a new tube (keep at -20°C 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 4°C, rotating
  - 1x with **High salt wash buffer**, 5 min at 4°C, rotating
  - 1x with **LiCl wash buffer**, 5 min at 4°C, rotating
  - 2x with **TE buffer**, 5 min at 4°C, 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 **65°C**
- 51) Prepare **Elution buffer** (prepared at the last minute for NaHCO<sub>3</sub>)

**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 65°C** (thermomixer).

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

- 53) Spin down at Vmax at 4°C, 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 65°C** (thermomixer). Spin down at Vmax at 4°C. 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 45°C** and **overnight at 65°C** (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

---