ChIP protocol

Day 0 – Buffers and materials

- ☐ Make sure you have enough solution for the experiment
- ☐ Place the Metal Block for sonication (see ref materials) in the freezer

Day 1 – Tissue disruption, crosslinking and sonication

Preparations

- ☐ One 15-ml falcon centrifuge set-up at 4˚C / one 1.5/2ml centrifuge set-up at 4˚C
- ☐ Prepare PBS with PIC (see **ChIP buffers**)
- ☐ Complement the lysis buffer with PIC, PMSF and sodium butyrate (see **ChIP buffers**)
- ☐ Ice bucket
- ☐ Dounce homogeniser in ice with 5 ml of PBS with PIC – pestle B

Tissue disruption

1. Transfer one piece of liver (70 mg recommended for liver) into the Dounce homogenizer containing 5 ml of PBS with PIC, immersed in ice
2. Use the pestle B for the homogenization, 10 strokes (stay in the ice as much as possible and try to be as quick as possible [5-10 mins])
3. Transfer the homogenate into a 5-ml tube [1]
4. Centrifuge 3 mins, 2500 g, 4˚C
5. Remove and discard supernatant

   You can control the dissociation of the tissue with an aliquot of 10 µl, under the microscope

Crosslinking

6. Add to the pellet 2.5 ml of formaldehyde solution 1% and resuspend the pellet by pipetting
7. Incubate under constant rotation [2] at room temperature, 5 mins
8. Quench the reaction with 360 µl of glycine 1M (0.125M final). Incubate 10 mins, room temperature, under constant rotation

[1] or a 15-ml canonical Falcon tube

[2] Place the tube in the HulaMixer, 360˚ rotation, 40 rpm. Other options: in horizontal position in a box on the top of an orbital shaker/rocker (60 rpm) or directly between two tubes of a tube roller mixer (60 rpm)

9 Centrifuge 5 mins, 800 g, 4˚C

10 Remove and eliminate in appropriate trash the supernatant

11 Resuspend with 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

12 Centrifuge 5 mins, 800 g, 4˚C

13 Remove the supernatant

14 Resuspend with 5 ml of PBS with PIC by pipetting (quick vortex if necessary)

15 Take an aliquot of 10 µl to count the number of cells in a cell counter without staining the cells, or in a counter chamber under a microscope (we expect 6-8 million of cells total for liver)

16 Centrifuge 5 mins, 800 g, 4˚C

17 Remove the supernatant

18 Resuspend with 1 ml of PBS with PIC and transfer in a 1.5-ml tube

19 Centrifuge 10 mins, 3000 g, 4˚C

20 Remove the supernatant

Cell lysis

21 Resuspend the cell pellet in a ratio of 200 µl of lysis/2 million of cells of lysis buffer (completed with PIC, PMSF and sodium butyrate). Pipet until the pellet is completely dissolved

22 Put on ice for 20 mins. Vortex 5 seconds, medium speed, every 5 mins

23 Take out from the fridge the Qubit kit and Bioanalyser DNA 1000 kit and set-up incubators (37˚C and 68˚C)

Sonication

24 When the lysis is finished, split the suspension in tubes of 200 µl for the sonication

You can control the quality of your nuclei with an aliquot of 5 µl with 5 µl of Trypan blue, under the microscope

25 Place the metal block from the freezer in ice. Keep the tubes in it all along the process

26 Sonicate the chromatin

Time	10 cycles
Duty cycle	5%
Intensity	50%
On/Off	15 sec/45 sec [3]

27 Remove an aliquot of 20 µl and label as “sonication control”.

28 Centrifuge the remaining volume of sonicated chromatin for 10 mins, @ 8 000 g, 4˚C.

29 Transfer at least 150 µl of the supernatant into a clean LoBind 1.5-ml tube

[3] The resting time is 15 seconds multiplied by the number of tubes you have obtained. We advise you to not run more than 6 tubes at the same time. No less than 45 sec.

30 Dilute the chromatin by adding 3 volumes of complete IP buffer (completed with PIC, PMSF and sodium butyrate).

31 Place the tubes of diluted sonicated chromatin under constant rotation at 4˚C.

Test of the sonication

32 To the "sonication control" from step 27, add 180 µl of elution buffer and 2 µl of RNase A (100 mg/ml)

33 Incubate for 10 mins @37˚C.

34 Add 5 µl of proteinase K (20 mg/ml) to digest proteins.

35 Incubate with shaking for 1hr @68˚C in a Thermomixer set at 300 rpm.

36 Purify the DNA using a Qiagen DNA Minelute kit. Elute with 10 µl of elution buffer (EB from kit).

37 Quantify DNA using a Qubit, and run maximum 50 ng (in 1 µl) on a Bioanalyser DNA 1000 Chip. We expect to recover over 60% of the chromatin with a size distribution between 200 and 700 bp

Pool together the tubes with a correct distribution size of fragmented chromatin

You can leave the prepared sonicated chromatin at 4˚C and pursue with the ChIP capture protocol the same day or the next day or you can freeze the sonicated chromatin @ -80 ˚C and keep it for later

Day 2 – Beads preparation and immunoprecipitation

Preparing beads for immunoprecipitation and antibody complexes and for pre-clearing of the samples

Enter the chromatin concentration from Step 37 in excel calculator table "***calcul_nb_IP"***. Follow the instructions below to prepare the beads, noting that the **"bold text"** refers to specific formula containing cells in the excel sheet. Use only LoBind tubes from this step.

PREPARATION OF THE BEADS FOR PRE-CLEARING AND FOR COUPLING;

38 From the excel file, prepare the **"Total mix of beads"**

39 Mix and pulse centrifuge.

40 Place the mixture on a magnet for 2 mins and discard the supernatant.

41 Remove the tube from the magnet and add the appropriate volume of complete IP buffer (from Excel file).

42 Repeat steps 39-41 two additional times.

PREPARATION OF THE BEADS WITH ANTIBODIES OR PRE-CLEARING OF THE SONICATED CHROMATIN;

I. COUPLING ANTIBODIES TO BEADS

43 For each antibody to be used, in a separate tube combine the indicated **"Volume of beads**" with **"Volume of antibodies to add"**.

44 Rotate tubes at 4˚C for 4h or overnight

II. PRE-CLEARING CHROMATIN WITH NAKED BEADS

45 For each capture, combine the **"Volume of beads**" with the corresponding **"Volume of diluted chromatin"**

46 Rotate tubes at 4˚C for 4h or overnight.

47 Stored unused chromatin at -80˚C.

Immunoprecipitation of samples

Pulse spin all the tubes to collect mixtures.

FROM I (coupling AB to beads): These tubes contain the complexed beads + antibodies. Mix well and prepare 110 µl aliquots for each antibody.

FROM II (preclearing): This tube contains the pre-cleared sonicated chromatin. Mix well and prepare aliquots from these tubes according to excel table.

48 Place the tubes containing the complexed beads **(I)** on the magnetic rack and (after 2 min) discard the supernatant. Remove the tubes from the magnet.

49 Place the tubes with the pre-cleared sonicated chromatin **(II)** on magnetic rack and after 2 min transfer the supernatant to the complexed beads (ie tubes from Step 48). Keep one volume of pre-cleared sonicated chromatin correspond, label this **"input control"** and store at 4C.

50 Rotate all other tube mixtures overnight at 4˚C

51 Place a 50 ml tube of absolute ethanol in the freezer overnight. Keep the rest of the complete IP buffer in the fridge for the next day.

Day 3 – Wash beads, decrosslinking and DNA recovery

Preparations

- ☐ Prepare fresh ChIP elution buffer (see ChIP buffers)
- ☐ Prepare 70% ethanol and place it in the freezer
- ☐ Set up a 1.5/2 ml centrifuge to 4˚C
- ☐ Supplement the low salt wash buffer and the high salt wash buffer with sodium butyrate

Wash beads, decrosslinking and purification

Wash beads and elute immunoprecipitated chromatin

52 Pulse spin the tubes from 51, collect the beads using a magnet and (without disturbing the pellet) discard the unbound chromatin containing supernatant.

53 Remove the tubes from the magnet and resuspend beads in 200 µl of complete IP buffer

54 Return the tubes to the magnetic rack, capture the beads and remove the supernatant.

55 Remove the tubes from the magnet and resuspend the beads in 200 µl of low salt wash buffer, put on the magnet, capture the beads and remove the supernatant. Repeat once.

56 Remove the tubes from the magnet and resuspend the beads in 200 µl of high salt wash buffer, put in the magnet, discard supernatant. Repeat once.

57 Pulse spin the tubes, place on a magnet, and very carefully remove the last droplets of salt wash buffer.

58 Remove the tubes from the magnet and resuspend beads in 30 µl of ChIP elution buffer.

59 Elute the DNA from the beads by incubating 65˚C, for 1 ½ hours in a Eppendorf Thermomixer set at 1000rpm.

60 Pulse spin the sample tubes and collect beads by placing tubes on a magnet.

61 Transfer the eluted chromatin to a clean 1.5-ml LoBind tube

62 Wash beads with an additional 70 µl of ChIP elution buffer, and combine with the eluted chromatin from previous step to give 100 µl final volume.

63 Take out the "**input control**" tube from the fridge.

NOTE! The volume of the input is not 100 µl, adjust the following volumes appropriately.

64 Per 100 µl of **"input control"**, add 5 µl of 20% SDS (final conc 1% SDS)

The input tube and the eluted chromatin from IP tubes are ready for DNA purification

Decrosslinking of the input and of the eluted chromatin from IP

65 For 100 µl of solution, add 2 µl of RNase A (100 mg/ml)

66 Incubate 30 mins, 37˚C

67 For 100 µl of solution, add 5 µl of proteinase K (20 mg/ml)

68 Incubate 1h, 68˚C, under agitation (500 rpm)

Purification of the input and of the eluted chromatin from IP

69 Centrifuge one phase-lock gel tube per sample/input at max speed (16000g) for 30 sec to collect gel.

70 In a fume hood, add 1 volume of phenol:chloroform:isoamyl alcohol (25:24:1) to each sample/input. Place on hula shaker at 300rpm for 5 minutes.

71 Pulse spin to collect and transfer the solution to a phase lock gel tube.

72 Centrifuge at 13000 g for 5 mins at room temperature.

73 Transfer the aqueous upper phase into clean 1.5-ml LoBind tubes

74 For 100 µl of solution, add 10 µl of 3M sodium acetate, 275 µl of cold absolute ethanol. Add 1 µl of glycogen in the cap of each tube. Mix well by inverting 20 times

75 Precipitate DNA 1h to overnight at – 80˚C

DNA recovery

76 Warm the Qubit kit to room temperature.

77 Centrifuge the tubes containing precipitated DNA at max speed (13000 g) for 20 mins @4˚C.

78 Carefully remove supernatant, and was the pellet twice with 200 µl ice cold 70% ethanol (centrifuging at max speed for 5 minutes between each wash).

79 Remove supernatant, quick spin the tubes to remove the maximum of the last drops of ethanol and air dry the pellet for maximum 5 min.

80 Resuspend in 30 µl of elution buffer and quantify 1ul using Qubit.

The DNA from the input and chromatin from IP can be stored at – 80˚C, each partner will need to develop qPCR protocols for assessing enrichment of capture vs input.

Stock Solutions:

PBS 1X (1L)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
Tablets of PBS	tablet	5 tablets	1X	
dH_2O		1000 ml		
sodium chloride 5M (250 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
sodium chloride (NaCl : MW = 58.44 g/mol)	powder	73.05 g	5M	
dH_2O		250 ml		
Sodium bicarbonate 0.25M (40 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 3 months**
sodium bicarbonate ($NaHCO_3$: MW = 84 g/mol)	powder	3.36 g	0.25M	
dH_2O		40 ml		
sodium acetate 3M (250 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
sodium acetate ($C_2H_3NaO_2$: MW = 82g/mol)	powder	61.5 g	3M	
dH_2O		250 ml		
sodium butyrate 2.5M (18 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
sodium butyrate ($CH_3CH_2CH_2COONa$: MW = 110 g/mol)	powder	5 g	3M	*dilution of the entire powd*
dH_2O		18,18 ml		
Formaldehyde 1% (250 ml)	**stock**	**mix**	**final concentration**	**conservation 4˚C 1 month**
ssolve 2.5g of paraformaldehyde in 200ml of water, with 5 drops of NaOH to help dissolution, at 60				need of 2.5 ml
move from heat, add 25ml of PBS 10X. Verify the pH (~7.2), complement well until 250ml with PBS				
Filter. Conserve at 4°C. You can also store in aliquots at -20°C				
Glycine 1M (40 ml)	**stock**	**mix**	**final concentration**	**conservation RT 1 month**
Glycine ($C_2H_5NO_2$: MW = 75.07 g/mol)	powder	3 g	1M	need of 400 µl
PBS	1X	40 ml		
Dithiothreitol 1M (32 ml)	**stock**	**mix**	**final concentration**	**conservation -20˚C indefin**
dithiothreitol, DTT ($C_4H_{10}O_2S_2$: MW = 154.25 g/mol)	powder	5 g	3M	dilution of the entire powd
dH_2O		32,415 ml		
7X Proteinase Inhibitor Cocktail	**stock**	**mix**	**final concentration**	**conservation -20˚C indefin**
PIC tablet	tablet	1	7X	
dH_2O		1.5 ml		

Buffers

quantity for one tissue preparation / one ChIP

PBS with PIC	**stock**	**mix**	**final concentration**	**prepare for the day - keep in the ice**
PBS 1X	1X	30 ml	1X	30 ml
PIC tablets	tablets	3 tablets		

Lysis buffer (100 ml)	**stock**	**mix**	**final concentration**	**conservation RT 3 months**
Tris-HCl pH 8.0	1M	5 ml	50mM	1 ml
EDTA	0.5M	2 ml	10mM	*If precipitation of the SDS, warm and agitate a*
SDS	20%	5 ml	1.0%	
dH_2O		88 ml		

To complement with PIC, PMSF and sodium butyrate the day of use

- mix 845 µl of lysis buffer with 140 µl of PIC solution 7X, 10 µl of 0.1M PMSF and 5 µl of 2.5M sodium butyrate => lysis buff

Complete IP buffer (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4°C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	10 ml
EDTA	0.5M	400 µl	2mM	
Triton X-100	100%	100 µl	0.1%	
NaCl	5M	3 ml	150mM	
dH_2O		94.5 ml		

To complement with PIC, PMSF and sodium butyrate the day of use

- mix 9.896 ml of complete IP buffer with 1 tablet of PIC, 100 µl of 0.1M PMSF and 4 µl of 2.5M sodium butyrate => complet

ChIP elution buffer (2 ml)	**stock**	**mix**	**final concentration**	**prepare for the day**
sodium bicarbonate	0.25M	800 µl	100mM	1 preparation when needed
SDS	20%	100 µl	1%	
dH_2O		1100 µl		

Low salt wash solution (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4°C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	5 ml
EDTA	0.5M	400 µl	2mM	
NaCl	5M	3 ml	150mM	
Triton X-100	100%	1 ml	1%	
SDS	20%	500 µl	0.1%	
dH_2O		93.1 ml		

To complement with sodium butyrate the day of use

- mix 4.998 ml of low salt wash solution with 2 µl of 2.5M sodium butyrate => low salt wash solution

High salt wash solution (100 ml)	**stock**	**mix**	**final concentration**	**conservation 4°C 3 months**
Tris-HCl pH 8.0	1M	2 ml	20mM	5 ml
EDTA	0.5M	400 µl	2mM	
NaCl	5M	10 ml	500mM	
Triton X-100	100%	1 ml	1%	
SDS	20%	500 µl	0.1%	
dH_2O		86.1 ml		

To complement with sodium butyrate the day of use

- mix 4.998 ml of high salt wash solution with 2 µl of 2.5M sodium butyrate => high salt wash solution

Elution buffer (40 ml)	**stock**	**mix**	**final concentration**	**conservation RT indefinite**
Tris-HCl pH 8.0	1M	400 µl	10mM	1 ml
dH_2O		39.6 ml		

Ethanol 70% (2 ml)	**stock**	**mix**	**final concentration**	**prepare one day before DNA precipitation**
Absolute ethanol	100%	1.4 ml	70%	2 ml
dH_2O		0.6 ml		