ChIP in pancreatic islets

Protocol optimised by Nikolina Nakic

Based on protocols published in Pasquali et al 2013 and van Aresbergen et al 2013.

This is a basic protocol for preparing chromatin from pancreatic islets or cell lines and preforming chromatin immunoprecipitation using antobodies agains histone modifications or transcription factors. The amount of antibody used for the immunoprecitpitation should be optimised for each antibody. The solution compositions and reagent origin are described at the end of the protocol.

I. FIXATION

Prepare:

- for islets - protein low binding Eppendorfs (note – fixated islets in PBS tend to stick to the walls of the tube, to prevent losing material use the protein low bind tubes if available)
- if no protein low binding Eppendorfs available, prepare 10% BSA
- for cells – 15ml Falcons, cell scrapers
- centrifuge at 4 ºC (table-top for islets, both table-top and big centrifuge for cells)
- formaldehyde 37% (**note** – formaldehyde has a short shelf life; mind the expiration date and don't use a bottle opened more than 4 months ago)
- glycine, 2M
- PBS room temperature
- PBS aliquot on ice
- PI (protease inhibitor cocktail), 50x
- dry ice/EtOH bath or liquid nitrogen

Islets:

- collect the islets in a low protein binding or normal Eppendorf (400-1200 IEQ per tube) kept on ice

- wash 3x in 1ml of cold PBS, 1 min 100g centrifugations

- remove the tube from ice and proceed at RT

- resuspend gently in 1ml RT PBS, add 27ul of formaldehyde 37%, invert gently and incubate with gentle shaking for (precisely!) 10min

- quench the reaction with 64ul of 2M glycine, incubate with gentle shaking for 5min (note – glycine doesn't completely quench the reaction so proceed immediately after the 5min run out)

- centrifuge at 4 ºC, 2min at 1000g, remove supernatant (**note** – check for islets leftover in the supernatant, if necessary re-centrifuge at 3rpm)

- wash 2x with 1ml of cold PBS (**note** – if you don't have protein low-binding Epps and the islets keep sticking, add 100ul of 10% BSA to the washes)

- wash 1x with 1 ml of cold PBS + PI, spin 2min at 2000g

- remove all the supernatant and snap freeze the pellet in liquid nitrogen or dry-ice/ethanol bath

Cells on a plate:

- remove the medium from the plate and wash cells 3x with RT PBS

- cover cells with 10ml of RT PBS (10cm plates) / 20ml of RT PBS (15cm plates)

- add 270ul (10cm plate)/ 540ul (15cm plate) of formaldehyde 37% and incubate at RT with gentle shaking for (precisely!) 10min

- quench the reaction with 640ul of 2M glycine (10cm plate)/ 1280ul of 2M glycine (15cm

plate), incubate with gentle shaking for 5min

- wash remove PBS mix, put plate on ice and wash 2x with 10ml of cold PBS

- cover cells with 5ml of cold PBS, scrape the cells and transfer them to a fresh 15ml Falcon

- rinse the plate with fresh 5ml of cold PBS and transfer to the Falcon

- if you want to aliquot the cells, add 1ml of 5% BSA, resuspend and aliquot

- wash 1x with 10ml of cold PBS + PI

- remove all the supernatant and snap freeze the pellet in liquid nitrogen or dry-ice/ethanol bath

II. SONICATION

Prepare
- Lysis Buffer on ice
- TE Buffer on ice
- PI (protease inhibitor cocktail) 50x, thaw on ice
- 1ml syringe with 30G needle (one per sample)
- waterbath sonicator with ice cold water (**note** – do not have ice in the bath)
- centrifuge at 4ºC

- prepare Lysis Buffer with fresh PI, 500ul per sample to sonicate

- add 500ul of Lysis buffer with PI to a tube containing a frozen pellet of 400-1200 fixed islets, and allow the pellet to thaw on ice with occasional shaking (minimum 15min, to allow cells to lyse)

- resuspend the islets with a 30G needle syringe until there is no more visible islets left (**note** – complete lysis and homogenization are crucial for efficient sonication!)

- sonicate the samples in the waterbath for 10min total

2 x 5min (change ice water after 5min), 40sec ON, 20sec OFF, MEDIUM strength (**note** – fixation of the islets is not always identical and the sonication may need to be optimized for your sample)

- check the sonication on an aliquot that corresponds to minimum 100IEQ, as described below

- spin the rest of the sonicated chromatin at full speed at 4 ºC for 5 min to clear the extract of debris and unlysed cells and store the supernatant in -80’C

III. CHECKING DNA FRAGMENT SIZE

Prepare pre-de-cross linking

- centrifuge at 4ºC
- heating block / water bath at 65ºC
- TE buffer
- RNAse A, 10 mg/ml
- Proteinase K, 20 mg/ml

Prepare post-de-cross linking

- centrifuge at RT
- Phenol Cholorofom (Phenol:Chlorophorm:Isoamyl Alchocol)
- Chloroform
- Na Acetate, 3M, pH5.2
- ice-cold 100% EtOH
- glycogen, 5 mg/ml

Prepare post-precipitation

- centrifuge at 4 ºC
- speed-vac at RT
- ice-cold 75% EtOH

- add TE to make 300 μl and spin at full speed in the microfuge at 4 ºC for 5 min to clear the extract of debris and unlysed cells. Transfer the supernatant to a new tube.

- add 1.5 µl RNAse A to each tube, flick the tube to mix and incubate at 65 ºC for >30 min

- add 4.5 µl Proteinase K + 12 ul of NaCl, flick the tube to mix, and incubate at 65 ºC for 5 hr or ON

- add 60 µl TE to make 360 µl.

- add 450 µl Phenol Cholorofom, vortex to mix; spin at room temperature at maximum speed for 5 min, transfer the top phase to the new tube

- add 450 µl Cholorofom, vortex to mix; spin at room temperature at maximum speed for 5 min, transfer the top phase to the new tube

- add 1/10 volume of Na Acetate, 2.5 volume of ice-cold 100 % EtOH and 1 µl glycogen; invert several times and incubate at -20ºC for > 2h (**note** – for ChIP DNA ON recommended)

- spin at 4ºC at maximum speed for 20 min and remove the supernatant

- wash with 1ml of ice-cold 70% EtOH; spin at 4ºC at maximum speed for 5 min and remove the supernatant

- speed vac 10 min at RT (**note** – don't overdry the pellet!)

- resuspend in appropriate volume of H_20 (usually 10-15ul for ChIPed DNA, 20ul for checking fragmentation) (**note** – don't dilute the ChIP DNA too much if you plan to check fragment size distribution on Bioanalyser)

- incubate at 37ºC for 30 minutes with vigorous shaking

- use 1-2 µL of the resuspended DNA to measure DNA concentration using Qubit

- run the resuspended DNA on a 1% agarose gel in 0.5X TBE buffer (100 V, 45min hour) to check sonication
(**note** – don't over run the gel as you'll lose visibility of the small fragments on a prestained gel)

- check the DNA size on the UV illuminator

(**note** – you're aiming for a smear with fragments from 150-500bp; it is very important that the size range is similar across samples that are going to be compared to each other at some point!)

ChIP

Prepare pre 1st-Ab

- centrifuge at 4ºC
- rotor at 4ºC
- sepharose protein A and protein G beads (keep on ice)
- 10% BSA
- tRNA (10 ml/ml)
- ChIP Working Buffer on ice
- ChIP Dilution Buffer on ice
- PI (protease inhibitor cocktail) 50x, thaw on ice
- primary antibody

Prepare post1st-Ab

- centrifuge at 4ºC
- secondary Ab (if necessary)
- ChIP Buffers on ice - Lysis, Dilution, Washing (Hi Salt, Low Salt, LiCl)
- TE buffer on ice

Prepare post washing

- centrifuge at RT
- rotor at RT
- freshly prepared Elution Buffer at RT

Beads preparation

- prepare beads a 50:50 mixture of sepharose A and sepharose G beads, enough for preclearing and the immunoprecipitation (3 x 10ul/sample)

(**note** – the volume refers to the total volume of the beads slurry which is made of 50% beads and 50% buffer the beads are in)

- wash beads 3x in 1ml of cold Working Buffer, centrifugations of 2min at 400g at 4ºC

- after the last wash leave the appropriate volume of the buffer above the beads (50% of the original beds slurry volume)

Chromatin preclearing

- thaw frozen chromatin sample on ice, or use fresh chromatin

- dilute your chromatin with ChIP Dilution buffer + fresh added PI in the following ratio - chromatin : dilution buff = 1:4

- add the protein A + G-agarose slurry (20ul per tube, 200-500 IEQ of chromatin per tube) to your diluted chromatin and top up with Working buffer to 1ml

- incubate with rotation for 2h at 4ºC

- centrifuge at 400g for 2 min at 4ºC, transfer the supernatant to a new Eppendorf tube

- separate an appropriate amount of chromatin for your INPUT - 5 or 10% of your ChIP and store in -80'C
(**note** – if you're performing several ChIPs with different starting amounts of chromatin, you can remove 5 or 10% for the ChIP with the largest starting amount and use it diluted as input for smaller ChIPs)

Primary Ab

- aliquot the precleared chromatin for your ChIP and top up with Working buffer + PI to 1ml

- add 50ul of BSA (and if dealing with < 200IEQ, add 5ul of tRNA)

- add the primary antibody to each ChIP and incubate while rotating at 4 ºC overnight (**note** – the amount of antibody should be optimized for every ChIP, the most common amount is 1–1,5 ug per 500-1000IEQ)

- in parallel, block the rest of your beads by topping them up to 1ml with the Working buffer and adding 50ul of BSA (**note** – blocking can be done 1h - O/N)

Secondary Ab / Beads

- prepare blocked beads – spin down the beads for 2min at 400g, remove the supernatant but leave 50% of the original slurry volume

- add secondary Ab to your chromatin if necessary and incubate for 2 h at 4ºC
(for example, goat antibodies require a mouse-anti-goat Ab as the proteins A and G don't have strong affinity for goat Ab-s)

- Add 10 μL of blocked Protein A + G slurry to each tube and incubate for 2 h at 4ºC

Washing

- all centrifugations are done for 2 min at 400g at 4ºC

- perform 3 washes, in sequence, each one with 1ml of cold buffer for **5min** and with gentle shaking, discard the supernatant after each wash:
    - **Low salt** Immune Complex Wash Buffer
    - **High salt** Immune Complex Wash Buffer
    - **LiCl** Immune Complex Wash Buffer

- wash the beads 3x with 1 ml ice-cold **TE** buffer (short) and discard the supernatant after each wash

- prepare fresh Elution Buffer at room temperature

- place the sample at room temperature, elute by adding 150 μL of Elution buffer to the beads,

vortex briefly

- rotate for 15 min at RT. Centrifuge at 500g for 3 minutes at RT, transfer supernatant to a fresh 1.5 mL Eppendorf

- re-elute by adding 150 μL of Elution buffer, vortex briefly.

- centrifuge at 2000g for 2 minutes at RT, combine both eluates in the same tube.

DNA isolation

- remember to get your **INPUT** aliquot for parallel DNA isolation!!! - thaw and top up to 300ul with TE buffer

- proceed with RNAseA treatment, reverse crosslinking/ProteinaseK and DNA isolation as described above in the DNA fragment check section

Reagents and Solutions:

Lysis buffer: (50 ml)
2% Triton X-100 (10 mL 10% Triton X-100)
1% SDS (5 mL 10% SDS)
100 mM NaCl (1 mL 5M SDS)
10 mM Tris-HCl pH 8.0 (500 μL 1M Tris-HCl)
1 mM EDTA (100 μL 0.5M EDTA pH 8.0)
1x protease inhibitor cocktail (add just before adding the buffer to the pellet)

Dilution Buffer: (50 mL)
50 mM Hepes pH8.0 (2.5 mL 1M Hepes-KOH pH 8.0)
140 mM NaCl (1.4 mL 5M NaCl)
1 mM EDTA (100 μL 0.5 M EDTA pH 8.0)
0.75% Triton X-100 (3.75mL 10% Triton X-100)
0.1% Na-deoxycholate (50mg Na-deoxycholate)
1x protease inhibitor cocktail (add just before adding the buffer to the pellet)

Working Buffer (prepare before use)
1 : 4 ~ Sonication Buff. : Dilution Buff. (for example, 2ml Lysis B. + 8ml Dilution B.)
1x protease inhibitor cocktail (add just before adding the buffer to the pellet)

Low Salt Immune Complex Wash Buffer: (50 mL)
1% Triton X-100 (5 mL 10% Triton X-100)
150 mM NaCl (1.5 mL 5M NaCl)
20 mM Tris-HCl, pH 8.0 (1mL 1M Tris-HCl pH 8.0)
0.1 % SDS (500 μL 10% SDS)
2mM EDTA (200 μL 0.5M EDTA pH 8.0)

High Salt Immune Complex Wash Buffer: (50 mL)
500 mM NaCl (5mL 5M NaCl)
1% Triton X-100 (5mL 10% Triton X-100)
20 mM Tris-HCl, pH 8.0 (1mL 1M Tris-HCl pH 8.0)
0.1 % SDS (500 μL 10% SDS)
2 mM EDTA (200 μL 0.5 M EDTA pH 8.0)

LiCl Immune Complex Wash Buffer: (50 mL)
0.25 M LiCl (2.5 mL 5M LiCl)
1% deoxycholate sodium (0.5g deoxycholate sodium)
10 mM Tris-HCl, pH 8.0 (500 µL 1M Tris-HCl pH 8.0)
1% NP40 (5mL 10% NP40)
1 mM EDTA (100 µL 0.5 M EDTA pH 8.0)

TE 1x (50 mL)
10 mM Tris-HCl, pH 8.0 (500µL 1M Tris-HCl pH 8.0)
1 mM EDTA (100 µL 0.5 M EDTA pH 8.0)

Elution buffer: (5mL)
Add water before (4mL)
1% SDS (500 µL 10% SDS)
0.1M $NaHCO_3$ (500 µL 1M $NaHCO_3$)
Prepare at room temperature

Stock solutions:
- Sodium Acetate 3M, pH 5.2
- Ethanol 70% (Store at -20˚C)
- Ethanol 95% (Store at -20˚C)
- Ethanol 100% (Store at -20˚C)
- Protease inhibitor cocktail (Roche, cOmplete EDTA-free Protease Inhibitor Cocktail Tablets, 50x)
- Glycogen (Invitrogen, #10814-010; prepare 5 mg/ml aliquots from the original 20 mg/ml stock; store at -20ºC)
- Proteinase K 20 mg/ml (Roche, #03 115 852 001)
- RNAse A 10 mg/ml (Qiagen, #19101)
- BSA 10% (Sigma-Aldrich, #A3059; for 10ml of 10% stock solubilise 1g of powder BSA in 10ml of dH_2O; store 1ml aliquots at -20ºC)
- Tris-HCl 10 mM pH 8.0 (dissolve 6.055g tris (hydroxymethyl) aminomethane in 40ml dH_2O. Add 6N HCl to adjust pH. Add dH_2O to 50 mL)
- 1M Hepes-KOH pH 8.0 (Sigma-Aldrich, #H7523-50G; dissolve 11.92g HEPES (N-2-hydroxyethylpiperazine-N'-ethanesulphonic acid) in 40ml dH_2O. Add 5N KOH to adjust pH. Add dH_2O to 50 mL)

- 5M LiCl (dissolve 10.60g LiCl little by little in 40 mL dH_2O. Add dH_2O to 50 mL)
- NP40 (Igepal CA-630, Sigma-Aldrich, # I3021)
- Tris-saturated Phenol (usb, #75831)
- Chloroform (Sigma-Aldrich, #288306)
- Formaldehyde 37% (Merck, #344198)
- 2M Glycin (MP, #808831; prepare 50mL of 2M stock by dissolving 7.507g of glycine powder in 50ml of dH_2O)
- Protease inhibitor cocktail (cOmplete, EDTA-free Protease Inhibitor Cocktail Tablets), 50x