**Micro ChIP assay – Dahl & Collas, Nat Protocols, 2008.**

[**http://www.nature.com/nprot/journal/v3/n6/full/nprot.2008.68.html**](http://www.nature.com/nprot/journal/v3/n6/full/nprot.2008.68.html)

**1. Cell Collection/Fixation**

*Collection*

-Remove media from cell plates

-Wash twice with sterile PBS

-Remove PBS and add trypsin to each plate (4ml for 100mm, 6ml for 150mm dishes…)

-Place cell plates in incubator for 5 mins and then check to see if all of the cells are floating

 -if not, return to incubator and keep checking at 2 minute intervals

-Once all cells are floating, add an equal amount of media to each plate

-Collect all like cells/media/trypsin into labeled tubes.

 *Cell Counting* (*Recommended Method)*

-Place 10 ul of sample onto the counting slide (hemocytometer) with cover slip on.

-Count cells in each of the 5 quadrants (see schematics).



$Counting in 5 quadrans X 2.10^{3}=C × 10^{6}cells/ml$

$Total nb of cells=C.10^{6 }X x ml (vol in which cells are collected) $

-Spin cells 2000g for 5 min 4C. Resuspend in PBS and calculate volume to add to get 10^6 cells/ml based on calculations. Aliquot in 1.5ml eppendorf tube, 1ml (1 million cells) per tube.

-Repeat wash with PBS. Resuspend in adequate volume of PBS. Spin 2000g, 5 min, 4C.

*RNA isolation*

**It is highly recommended to keep some of the cells harvested as detailed above for gene expression quantification by real-time PCR. Take one aliquot (one million cells) for each condition/plate harvested previously to allow side comparison between ChIP and gene expression results. This can also allow you to validate the efficiency of a treatment (e.g. PDGF-BB) prior to perform ChIP or ChIP-seq experiments.**

-Take one aliquot after centrifugation and remove PBS.

-Add 500 ul Trizol in the fume hood then place tubes at -80c until isolation.

*Cell fixation*

-Remove PBS, leaving the pellet.

-Prepare **20mM Na-butyrate PBS**: 980ul PBS + 20 ul 1M Na-butyrate per mL (+1mM PMSF and 1X protease inhibitor mix final)

-Put 500 ul of 20mM Na-butyrate PBS into each tube

-Add **31.25 ul PFA** (16% initial concentration. 1% final concentration)

-let sit 10 min on bench

-Add **25 ul glycine 2.5M** (125mM final concentration).

-let sit 10 min on bench

-Spin at 2000g for 5 min at 4c

-Remove supernatant, leaving pellet

-Wash 3 times with 500 ul of 20mM Na-butyrate PBS, vortexing on a low setting briefly to resuspend pellet

-Remove all of the liquid in the tubes (as much as possible without sucking up pellet) and store at -80c until needed.

**2. Chromatin Immunoprecipitation (see annex 1 for solution recipes)**

*Chromatin preparation*

-Add **120ul** of room temperature **complete lysis buffer**. Vortex for 2x 5s, leave on ice for 5 min

-Sonicate on ice for time determined by sonication optimization (**see annex 2**). 12 min for A404 cells with 30s ON/30s OFF on HIGH power.

**Make sure that the sonicator is used properly: 6 tubes at a time with equal volume, waterbath filled with cold DI water… in the sonicator.**

-Add **400 ul complete RIPA ChIP** buffer to the tubes (which contain ~140 ul lysate) and mix by vortexing

-Centrifuge at 12,000g for 10 min at 4c, carefully collect the supernatant (chromatin) and transfer it into a clean 1.5 ml tube chilled on ice for each sample

-leave around 50 ul at the bottom of the tubes so as not to disturb the pellet

-Add **410 ul complete RIPA ChIP** buffer **to the remaining pellet**, vortex and spin at 12,000g for 10 min at 4c

-Remove the supernatant and pool with the first supernatant. Vortex gently.

-Aliquot isolated chromatin: **100u**l/1.5ml tube, and store samples at -80c

*Bead preparation (see Nat Prot paper p1037)*

-Determine what type of Dynabeads to use: Dynabead Protein A *vs* Protein G.

-For 16 ChIPs, place 180ul of well-suspended Dynabeads stock solution into a 1.5ml tube, place in the magnetic rack, allow beads to be captured, remove buffer, remove the tube from the rack and add **500ul of RIPA** buffer (**non-complete).** Put on **r**otator at room temperature for 5min. Spin down, capture beads on rack, remove buffer, and repeat the wash with 500ul RIPA buffer.

-Resuspend beads in **170ul RIPA buffer**.

-Aliquot **90ul RIPA buffer** (non-complete) into 200ul PCR tubes (one tube per IP), add **10ul** **of washed beads**.

-Add adequate primary antibody or IgG control in each tube. Usual amount of antibody = 2ug/IP.

-Place on rotator for 2h at 4C.

 *Immunoprecipitation*

*Day 1*

-Add the chromatin to be used to the magnetic bead/antibody mixture prepared above

-Place the tubes on rotator at 40 r.p.m. at 4c overnight

*Day 2*

-Spin down the tubes to clear the lids of any fluid

-Place tubes in magnetic rack (chilled) and remove supernatant

-Add **100 ul ice-cold non-complete RIPA buffer**. Resuspend by vortexing gently and then place on rotator for 5 min

-Repeat washes 2 additional times

- Remove the supernatant, add **100 ul TE buffer. Transfer the ChIP material in TE buffer to a clean tube. This is critical to enhance specificity and reduce background of the ChIP assay.** Rotate for another 5 min

-Centrifuge for 1s

-Grab **INPUT samples** (samples that haven’t been treated with antibodies/magnetic beads) for each time point used from freezer

-Add **150 ul complete Elution buffer (100ul 10% SDS, 20ul Na-Butyrate and 2.5ul Prot K per mL)** to each tube with beads and **200 ul complete elution** buffer to each input sample tube

-Vortex and incubate for 2h at 68c on the heat block, vortexing every 30min or so.

-Take the input samples. Spin down. Add **200 ul elution buffer** (**total volume now 500 ul**).

-Take IP samples. Spin down. Put on magnetic rack and collect the supernatant and place it into a clean 1.5 ml tube

-Add **150 ul elution buffer** to the tubes with the beads and incubate for 5 min at 68c.

-Put tubes on magnetic rack and collect the supernatant and combine it in first supernatant tube

-Add **200 ul elution buffer** to the supernatant tube (**total volume now 500 ul**)

 *DNA extraction*

-Add an equal amount (for the amounts above this would be 500 ul) of phenol-chloroform to all of the tubes (including input samples). Vortex. Incubate 5min room temperature on rotator.

-Centrifuge at 15,000g for 10 min 4C.

-Transfert ~460 ul of the top phase into a new tube

-Add an equal volume (~460 ul) of chloroform into each tube. Vortex, incubation 5min at RT, and centrifuge again for 10 min at 15,000g

-Transfer the top phase into a new tube

-Add 1 ml 96%, 44 ul 3 M NaAc (pH 7.0) and 12 ul 5 mg/ml linear acrylamide to each tube

-Incubate at -80c overnight

*Day 3*

-Thaw the tubes and centrifuge at 20,000g for 15 min at 4c.

-Remove the supernatant, add 1 ml of 70% ethanol at -20c and vortex briefly to wash the pellet

-Centrifuge at 20,000g for 10 min at 4c

-Remove the supernatant, wait complete ethanol evaporation and resuspend the DNA in 50 ul TE buffer (or Ultrapure water if using all within several weeks).

-Either store (-20c) or use for RT PCR.

**Annex 1. Solution recipes**

**Have to add 1mM PMSF, protease inhibitor mix and 20mM Na- butyrate right before use (calculate amounts to use)**

**Values given in ml are from stock solutions**

|  |  |
| --- | --- |
| **Start Concen.** | **RIPA ChIP Buffer Stock** |
| 1 M1 M0.5 M0.5 MFrom stock10%10% | 1 ml – 10mM Tris-HCL (pH 7.5)14 ml – 140 mM NaCl200 ul – 1 mM EDTA100 ul – 0.5 mM EGTA1 ml – 1% Triton X - 1001 ml – 0.1% SDS1 ml - 0.1% Na-deoxycholate |
|  | 18.3 ml + 81.7 ml of water = 100 ml total |

**Have to add 1mM PMSF, protease inhibitor mix and 20mM Na- butyrate right before use (calculate amounts to use)**

**Values given in ml are from stock solutions**

|  |  |
| --- | --- |
| **Start Concen.** | **Lysis Buffer Stock** |
| 1 M0.5 M10% | 5 ml - 50mM Tris-HCL (pH 8.0)2 ml - 10 mM EDTA10 ml - 1% SDS |
|  | 17 ml + 83 ml of water = 100 ml total |

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 5 ul PMSF

For Lysis buffer and RIPA ChIP buffers 10 ul protease inhibitor **per mL of buffer**

 20 ul Na-butyrate

|  |  |
| --- | --- |
| **Start Concen.** | **Elution Buffer Stock** |
| 1 M0.5 M1 M | 2 ml - 20mM Tris-HCL (pH 7.5)1 ml - 5 mM EDTA5 ml – 50 mM NaCl |
|  | 8 ml + 92 ml of water = 100 ml total |

**Have to add 20mM Na-butyrate, 1% SDS and 50 ug/ml proteinase K right before use (calculate amounts to use)**

**Values given in ml are from stock solutions**

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 100ul 10% SDS

For Elution buffer 2.5 ul Proteinase K **per mL of buffer**

 20 ul Na-butyrate

Protease-inhibitor in common drawer of fridge

-1 tablet in 1 ml of Ultrapure H20 for a 100X stock solution

**Start Concen.**

200mM PMSF

100x Protease inhib

1M Na-butyrate

Take out PMSF early and put into water bath (60C)

**Annex 2. Sonication optimization**

**Use treated and non-treated cells to unsure that treatment does not affect sonication. Do not forget to use one non-sonicated sample as control.**

-Add **120ul** of room temperature **complete lysis buffer**. Vortex for 2x 5s, leave on ice for 5 min

Sonication: 30s ON/30s OFF on HIGH power.

Typical optimization protocol for one million cells aliquots: 0, 10, 12 and 14 min.

-Add **400 ul complete RIPA ChIP** buffer to the tubes (which contain ~140 ul lysate) and vortex.

-Centrifuge at 12,000g for 10 min at 4c, carefully collect the supernatant (chromatin) and transfer it into a clean 1.5 ml tube chilled on ice for each sample.

-Add **600ul complete Elution buffer** (final volume 1000ml). Incubate 68C for 2h.

-**Split the total volume in 2 tubes.**

-Add **500ul of phenol chloroform per tube**. Vortex, incubate on rotator 5min at room temperature, centrifuge 15,000g 5min.

-Transfer the upper phase to a new tube. Add an equal volume of chloroform. Vortex, incubate on rotator 5min at room temperature, centrifuge 15,000g 5min.

-**Repool** upper phases from the duplicate tubes in a new tube.

-Add **900ul of 100% EtOH, 68ul of NaAc 3M and 15ul of linear acrylamide.** Vortex and incubate overnight at -20C (or -80).

*Next Day*

-Cool down 4c centrifuge

-spin tubes 20 minutes at 20,000g (at 4c)

-Discard Supernatant

-Add ~700 ul 70% EtOH to each tube. Use special molecular biology Ethanol>

-Spin tubes 10 minutes at 20,000g (at 4c)

-Dry pellet by leaving open and allowing too dry for ~30 min

-Add 20 ul H2O and resuspend pellet

-Add 4 ul loading buffer, vortex and load on gel (1.5%)

Delphine’s sonication optimization 10/02/2014

Sonication done with one million cell aliquots in 120ul of Lysis Buffer in 1.5b eppendorf tubes.

Time of

sonication

**0**

**12**

**10**

**14**

**0**

**12**

**10**

**14**

**0**

**12**

**10**

**14**

**500**

**400**

**300**

**200**

**100**

**bp**

**A404**

**Rat SMC**

**Mouse SMC**

**500**

**400**

**300**

**200**

**100**

**Suggested conditions of sonication: 14min, 30s ON/30s OFF. One million cells in 120ul Lysis buffer.**