Protocol: ChIP

Protocol developed for ChIP-Seq of Ikaros from thymocytes (suspension cells): Based on multiple general ChIP protocols tested and adapted to ChIP-Seq with input from Seth Frietze and the Farnham lab ChIP protocols.

General Considerations before you start:

1. **Cell numbers needed (µg input chromatin):** Will depend on Ab quality and factor to be ChIP'ed (transcription factor, histone modification or polymerase), and planned analysis (single-locus qPCR, locus scanning by qPCR or ChIP-Seq). As a general rule to start: Fix at least 10^7 cells/IP for histones or polII (until optimized), and 10^8 cells/IP for ChIP-Seq of Transcription Factors (TFs).
2. **Ab used for ChIP**: Must be tested prior to performing ChIP. Must work in western and IP. Be aware of cross-reactivity.
3. **Cross-linking:** General protocol: 1% final formaldehyde for 10 min at RT. For some factors, additional cross-linkers can be tried (i.e. if the factor is not binding directly to DNA, but binds by protein-protein interactions to DNA).
4. **Sonication**: Must be optimized for each cell type and each individual sonicator. Information given can be used as a starting point. Note that cell density will affect sonication efficiency, so keep cell number in mind when testing and optimizing.
5. **Washing**: Must be optimized for each Ab. To few washes, or too low stringency will give too high background, while too many washes or too high stringency might lead to loss of signal.
6. **Include appropriate Positive and Negative controls.** Important: If binding sites for your factor are know in the cell type you are using, use those for Positive ctrl.

Testing before you start:

1. Test Ab against your target by Western (and IP if you have multiple Abs available). Also test on lysates from fixed, sonicated cells.

2. Optimize sonication for your cells and your available sonicator.

3. Literature search for putative positive ctrl primers (and corresponding negative ctrls).

4. Consider other Ab's as positive and negative ctrls. For instance, RNA polymerase II is a general good positive control that can be ChIP'ed down at active gene promoters. This will be control that your ChIP experiment can work in general (buffers and experimental protocol ok).

Day 1: Date: .

Step 1. Harvest and count cells:

Adherent cells: Grow in 10 cm dishes. Find approx cell count/dish. Fix directly in dish.

Suspension cells: Grow cells (if cell line) or harvest primary cells fresh.

Count cells and make note: Sample ID: cell#

_____: _____ _____: _____

_____: _____ _____: _____

_____: _____ _____: _____

Step 2. Fixation of cells:

Preparation: Make 2.5 M Glycine: 3.753 g Glycine in 20 ml water (mix to resuspend).

Adherent cells: Fix cells directly in dish containing 10 ml media.

Suspension cells: Resuspend cells in 10 (or increase to 20-40 ml if fixing >10^8 cells) medium in 50 ml conical tube: Fix in high quality formaldehyde at 1 % final conc.

Best to use 16% UltraPure formaldehyde (freeze in aliquots once opened).

Alternative: 37% (use relative new bottle, and store tightly capped).

Fix in 1% formaldehyde: Assuming 10 ml media:

Add 278 µl of 37% Formaldehyde (final 1%).

or 664 µl of 16% Formaldehyde (final 1%).

➔ mix gently at RT for 10 min (use timer for exactly 10 min: if multiple samples, allow 30 sec staggering between samples)

Stop reaction (after 10 min) by adding 525 µl 2.5 M Glycine (final 0.125 M)

Mix at RT for 5 min and spin down cells at 4C. (1200-1400 rpm)

Note: Discard media containing 1% formaldehyde as hazardous waste.

* **Wash** 3 times in ice-cold DPBS (3x 40 ml) @ 4C, then transfer to epp tube for last 1ml wash. For adherent cells: wash on plates w/ 10 ml, then scrape cells off before last wash.

Spin down cells after last wash in epp tube, remove supernatant (DPBS wash) and snap-freeze cell pellet in epp tube in liquid nitrogen (or in ethanol w/ dry ice).

Store at -70 or -80 C.

Note cells#/tube. Date, genotype/cell-type/condition/sample ID.

Alternative: Double cross-linking with DSG or other cross-linkers. (protocol elsewhere).

NB: Spin at 1800 rcf=3000 rpm after DSG cross-linking!

Make list of frozen fix'ed cell pellets, stored at -70 or -80 C:

Tube#	Date	Cell #	Cell type	Condition	Sample ID

Day 2: Date: .

Step 3. Sonication of chromatin:

NB: Keep all chromatin on ice @ 4 C at all times, unless otherwise noted.
NB: This protocol use whole cell extract. An alternative (especially if Ab might cross-react with cytoplasmic proteins) is to first lyse cells, make nuclear extracts and sonicate.

Resuspend frozen cell pellet in 0.5 ml ChIP-Lysis buffer (CLB) w/ PIC + PMSF
(if one pellet/IP) ➔ 10e8 cells/ 0.5 ml CLB
Sonicate in Bioruptur waterbath: 3 x 10 min (3x10 cycles)
a 30 sec ON (setting: HIGH), 30 sec OFF
Sonicate in aliquots a 250 µl in TPX epp tubes (Max volume: 300 µl).
(hard plastic: TPX, epp cat # M-50050, Diagenode))
Alternative: Sonicate 1 ml samples in 14 ml tubes (from Diagenode) w/ probe.
Spin down at max speed to remove cell debris, and transfer to new epp tube.

NB: THIS MUST BE OPTIMIZED for each cell type and individual sonicator machine. Note that sonication efficiency is dependent on cell concentration (read manual), and keep cell concentrations constant after optimization.
First time: Take out aliquots (i.e. 10 µl, see below, step 4) at different times to test size of genomic DNA fragments after different numbers of sonication cycles. Also test samples with different cell concentrations.
Samples Tested: _____

Step 4. Test sonication on gel:

Take out a sample: 10 µl of sonicated sample (if 0.5 ml total ➔ 0.5 % input)
+ 88 µl ChIP Elution buffer (CEB)
+ 12 µl 5 M NaCl
➔ 90 C for 20 minutes
+ 1 µl DNase free RNase: 37 C, 10 min
Purify DNA from Protein with Qiagen PCR purification columns.
Measure DNA conc (nanodrop): _____, _____, _____, _____
Run 3 µg DNA on 1 % gel w ladder to test sonication (fragments 300-1000 bp)
Keep some (_____) as input DNA control (20 µl default)

Step 5. Start IP over night (ON):

Dilute sonicated chromatin: ➔ 5 ml/IP (Use 5 ml FACS tubes or similar)
200 µg chromatin/IP is good for ChIP-Seq of TF (100 µg also works).
Can use less for histones, polII or single locus qPCR.
➔ 0.3 ml chromatin + 4.7 ml ChIP dill buffer = 5 ml/IP + Ab for ChIP
Add Ab: 10-40 µl pr IP (approx 10e8 cells pr IP): _____
This also needs to be tested out and optimized for each Ab.
Seal cap w/ parafilm after adding Ab. Rotate at 4 C ON

Tube #	Sample ID	Cell type	Ab added	µl Ab	Time added

Day 3: Date: .

Step 6. IP Ab-Chromatin with protein A magnetic beads:

Pre-wash beads (use magnet): 3x wash in 1 ml ChIP Dill buffer to equilibrate.
Add beads: 10-50 µl pr IP for histones, polII or single locus qPCR ChIP.
100 µl pr IP for TF for ChIP-Seq
Re-seal 5 ml tube with frash parafilm.
Collect for 1-2 h @ 4C: ____ ➔ ____ = ____

Step 7. Wash & Elute: Optimize wash stringency for each Ab.

Collect ChIP by adding 1.5 ml of IP to epp tube on magnet. Combine (4 times)
Wash: Add wash buffer, Vortex gently/briefly to resuspend pellet, invert 20x to mix, pulse spin ➔ magnet.
2 x 1 ml ChIP Dilution Buffer (0.15 M NaCl)
2 x 1 ml High Salt ChIP Wash buffer (0.5 M NaCl) [High stringency]
2 x 1 ml ChIP Wash Buffer (0.5 M LiCl, w/ or wo/ NaDOC) [High stringency]
2 x 1 ml TE (or ChIP Dialysis Buffer)
Elute: 2 x 100 µl Elution buffer: rotate @ RT for 15 min
Make fresh EB: 42 mg NaHCO3 + 9 ml water + 1 ml 10% SDS = 10 ml EB.

Step 8. Reverse crosslink:

200 µl ChIP elute: + 24 µl 5 M NaCl
Remember input: + EB up to 200 µl + 24 µl 5 M NaCl
@ 65 C over night (ON): @ _____ ➔ _____

Day 4: Date: .

Step 9. RNAse treat and purify DNA:

+ RNase: 1 µl a 10 µg/µl = 1µg): @ 37C for 30 min@ _____ ➔ _____
➔ Purify DNA over Qiagen PCR purification columns or with Ampure beads: Elute in 37 µl EB ➔ collect 34 µl

Step 10. Test ChIP DNA with RT-PCR on known/expected targets:

Take 4 µl of ChIP DNA. Dilute 6 x ➔ Enough for 6 targets w 2 µl duplicates.
Set up RT-PCR rxn w/ primers against expected targets and negative ctrl regions

Step 11. Start preparing DNA-Seq Library: Step 1:

30 µl of ChIP DNA.

Buffers:	**Final Conc.**	**Recipe to make 50 ml:**
ChIP Lysis Buffer (CLB)*:	1 % SDS	5.0 ml a 10% SDS
	50 mM Tris 8	2.5 ml 1 M Tris 8
	5 mM EDTA	0.5 ml 0.5 M EDTA
		42 ml ddH2O
		To total 50 ml CLB with ddH2O

*** NB:** Add fresh Protease inhibitors and PMSF before use.

ChIP Dilution Buffer (CDB)*:	1 % Triton X-100	2.5 ml a 20% Triton-X-100
	20 mM Tris 8	1.0 ml 1 M Tris 8
(=Low Salt Wash Buffer)	2 mM EDTA	200 µl 0.5 M EDTA
	150 mM NaCl	1.5 ml 5 M NaCl
		44.8 ml ddH2O
		To total 50 ml CDB with ddH2O

High Salt Wash Buffer:	1 % Triton X-100	2.5 ml a 20% Triton-X-100
	20 mM Tris 8	1.0 ml 1 M Tris 8
(500 mM NaCl)	2 mM EDTA	200 µl 0.5 M EDTA
	500 mM NaCl	5.0 ml 5 M NaCl
		41.3 ml ddH2O
		To total 50 ml w/ddH_2O

LiCl Wash Buffer:	100 mM Tris-Cl pH 8.0	5.0 ml 1M Tris 8
	500 mM LiCl	10.0 ml 2.5M LiCl
	1% Igepal (aka NP-40)	5.0 ml 10% NP-40
	(1% Deoxycholic Acid	optional - try with and without)
		To total 50 ml w/ddH_2O

ChIP Elution Buffer (CEB):	1 % SDS	5.0 ml a 10%
	50 mM NaHCO3	xxx g (weight fresh)
NaHCO3 MW: 84.01 g/mol		
==> 1M : 84 mg/ml		To total 50 ml CEB with ddH2O

Dialysis Buffer:	2 mM EDTA	200 µl 0.5 M EDTA
	50 mM Tris-Cl pH 8.0	2.5 ml 1 M
	0.2% Sarkosyl	
		To total 50 ml w/ddH_2O

TE:	10 mM Tris 8	0.5 ml 1M Tris 8
	1 mM EDTA	100 µl 0.5 M EDTA
		49.4 ml ddH2O
		To total 50 ml CDB with ddH2O

5 M NaCl:	(MW = 58.44 g/mol)	146.1 g in final 500 ml with ddH2O
2.5 M LiCl:	(MW = 42.3 g/mol)	52.9 g in final 500 ml with ddH2O

ChIP-Ab list:

mouse polII as + ctrl:
RNA Pol2 (CTD4H8, Millipore)
polII (N-20, Santa Cruz: sc-899 X, 200 µg/0.1 ml)
http://www.scbt.com/datasheet-899-pol-ii-n-20-antibody.html

Alternative "better" epp tubes with less adsorption:
phenix research.com mh-815s