ChIP Protocol for General Use

Part I: Sonication Optimization and Cell Number Normalization

Day 1 – Nuclei extraction and sonication

*all steps should be done on ice

1) Prepare Solutions
   a) Add Complete Protease Inhibitors (PI) to Cell Lysis Buffer (1mL per $3x10^6$ cells)
   b) Add Complete Protease Inhibitors (PI) to Nuclei Lysis Buffer.
   c) Add Complete Protease Inhibitors (PI) to ChIP Dilution Buffer.

   *to speed things up, add the PI tablets to ~1mL of each buffer and let it dissolve at room temp or with vortexing or mix on a rotator at RT
2) Cross-linked Cell Preparation
   a) Fresh cell pellets can be used directly, while frozen cell pellets are taken from -80°C freezer, placed in an ice bucket on the diagonal so that the pellet is up. Thaw this way for at least 30'.
3) Resuspend thawed cells with 1mL per $3x10^6$ cells of prepared Swelling Buffer + PI. If there are several aliquots of cells combine them all in a 15mL tube.
4) Incubate on ice for at least 10' – flick occasionally to resuspend.
5) After 10' ensure the cells are completely re-suspended by pipetting with 200µL tip. Aliquot cells equally into 1.5mL tubes with $3x10^6$ cells per aliquot.

   Note: if you have enough cells to do the optimization, you can aliquot up to $2x10^7$ cells in each tube. Branson works well for $2x10^7$ in 1ml volume!
6) Spin tubes at 2,500 x g for 5' at 4°C to pellet nuclei. Remove supernatant with pipette.
7) Resuspend one aliquot of nuclei in 300µL per $3x10^6$ cells with Nuclei Lysis Buffer + PI. Remaining cells should be put at -80°C, indicate on tube that Swelling Buffer has been used.
8) Incubate on ice for 10' at least.
9) If using the **Bioruptor** for sonication test, put cells into bioruptor and at each time point remove a 20µL aliquot, replacing this with Nuclei Lysis Buffer + PI. Freeze remaining chromatin at -80°C.
   a) Pre-cool the re-circulating baths to ensure the water is at 4°C when you begin sonication.
   b) All positions must be filled with 300µL aliquots to ensure even sonication.
   c) Set power to 'high' and run with 30s on / 30s off.
   d) Typical time course is 20-60' with a sample being removed every 10'.
10) If using **Branson,** increase volume of lysed cells to 1mL with Nuclei Lysis Buffer + PI. At each time point, take a 20µL aliquot, replacing with Nuclei Lysis Buffer + PI. Freeze remaining chromatin at -80°C.
    a) Pre-cool the re-circulating baths to ensure the water is at -6°C when you begin sonication
    b) Load preset #20 on the Branson – 40% amplitude 0.7s on 1.3s off.
    c) Typical time course is 2-12' with a sample being removed every 2'.
11) Let sonicated samples sit on ice for 10'. Spin down at 4°C, maximum speed for 10'.

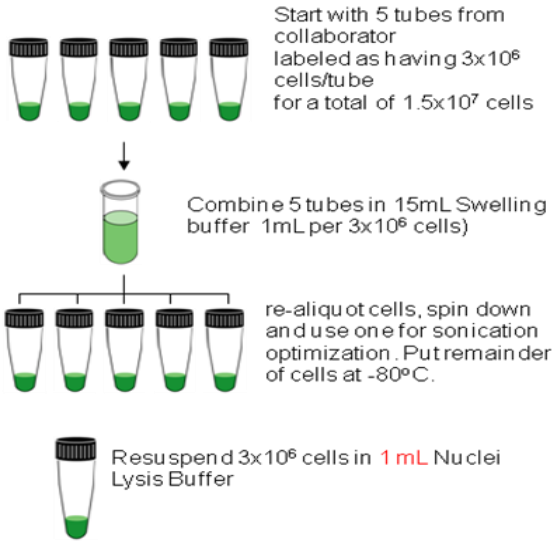
12) Remove supernatant into a new eppendorf tube and increase volume to 500µL with H_2O.

13) Add 80µL of Reverse Crosslink Salt Mix, cover top of tube with parafilm, and incubate in 65°C water bath for at least 5 hours or overnight.

Day 2 – DNA extraction and cell number normalization

1) Add 580µL of 4°C phenol:chloroform:isoamyl-OH, vortex 30s, spin HS, 5'. Remove top aqueous layer, transfer to new tube.
2) Add about 500µL of 4°C chloroform: isoamyl alcohol 25:1, vortex 30s, spins HS, 5'. Transfer aqueous layer to new tube.
3) Precipitate DNA by adding 1/10 volume of 3M sodium acetate (pH 5.0), 1µL glycogen and 2 volumes 100% ethanol. Mix well; incubate 2 hours at -80°C or overnight at -20°C.
4) Spin samples at maximum speed 45' 4°C, dump supernatant, add 500µL 70% etOH, spin 15' at 4°C, remove supernatant and additional etOH with a pipette and let air dry.
5) Resuspend samples in 32µL TE and quantify with Qubit.
6) Run 20ng on FlashGel and 1ng on Bioanalyzer HS DNA chip.
7) Include the step 6 info in file "Bioanalyzer Global List of Bioanalyzer WCEs" that locates in our shared folder**.**
8) If cells are from an outside collaborator you will need to determine if the given cell number is accurate. Use the following example for **<u>Branson Sonication</u>** to determine how many cells you truly have present in your sample. This cell number will help to ensure you do not over-saturate your sample with antibody.

Cell Counting



•<u>Principle: 6 pg DNA/each cell</u>

•In theory, 20 ul aliquot from 1 mL sonicated solution (containing 3e6 cells) contains 6e4 cell, 360 ng DNA. If DNA is resuspended in 32 ul, this leads to a concentration of 11.25ng/µL.

•In actual experiment, you just reverse the above process to estimate the start cell number from the DNA concentration. It should account for a 30% loss of DNA due to sample processing (phenol extraction/ethanol prep).

•The following is one example:

1	DNA concentration (ng/ul)	3.5
2	Total DNA volume (ul)	32
3	Total amount of DNA (ng)	112
4	[Chromatin] in 20 ul sonication aliquot (ng/ul)	5.6
5	Sonication volume (ul)	1000
6	Total chromatin in 1000 ul sonication aliquot (ug)	5.6
7	Total chromatin after 30% process loss adjustment (ug)	8
8	The estimated start cell# (total DNA /6 pg)	1.33E+106

•Based on this result you can set up your ChIP's with the appropriate cell # and antibody concentration.

Part II: Sonication and Chromatin Immunoprecipitation

DAY 1 – Sonication and primary antibody incubation

1) Prepare Solutions
   a) Add Complete Protease Inhibitors (PI) to Swelling Buffer (300µL per $3x10^6$ cells)
   b) Add Complete Protease Inhibitors (PI) to Nuclei Lysis Buffer (300µL per $3x10^6$ cells)
   c) Add Complete Protease Inhibitors (PI) to ChIP Dilution Buffer.

   *to speed things up, add the PI tablets to ~1mL of each buffer and let it dissolve at room temp or with vortexing

2) Sonicate cells for predetermined amount of time from **Part I**:
   i) Fresh cell pellets can be used directly, while frozen cell pellets are taken from -80°C freezer, placed in an ice bucket on the diagonal so that the pellet is up. Thaw this way for at least 30'.
   ii) **If you optimized your cells using this protocol skip steps iii – vi.**
   iii) Lyse cells as before with 300µL per $3x10^6$ cells of prepared Swelling Buffer + PI. If there are several aliquots there is no need to combine them since you have already done this during optimization.
   iv) Incubate on ice for 10' – flick occasionally to resuspend
   v) After 10' ensure the cells are completely resuspended by pipetting with 200uL tip.
   vi) Spin tubes at 2,500 x g for 5' at 4°C to pellet nuclei, remove supernatant with pipette.
   vii) Resuspend each thawed aliquot of nuclei in 300µL per $3x10^6$ cells with Nuclei Lysis Buffer + PI.
   viii) Incubate on ice for at least 10'.

If using the **Bioruptor** for sonication:
   a) Pre-cool the re-circulating baths to ensure the water is at 4°C when you begin sonication.
   b) All positions must be filled with 300µL aliquots to ensure even sonication.
   c) Set power to 'high' and run with 30s on / 30s off. Sonicate for predetermined time point. Put samples on ice for 10'.

If using **Branson** for sonication**:**
   a) Increase volume of lysed cells to 1mL with Nuclei Lysis Buffer + PI.
   b) Pre-cool the re-circulating baths to ensure the glycerol water is at -6°C when you begin sonication.
   c) Load preset #20 on the Branson – 40% amplitude 0.7s on 1.3s off. Sonicate for predetermined time point. Put samples on ice for 10'.

3) After sonication, spin samples at max speed for 10' at 4ºC. A pellet of insoluble material and SDS should form.

4) After centrifugation, collect supernatant from all sonicated samples (per cell line) and combine in a 15mL tube. Remove 50µL for WCE and store at 4ºC until ready for reverse cross-linking.

   Note: be careful; don't disturb the pellet when you collect the supernatant. Otherwise, it will increase your background!

2) Reference Table 1 for IP reaction and antibody volumes. Dilute each sample to appropriate volume with CDB + PI and add appropriate antibody volume. Incubate IP's at 4ºC on rotator at setting of ~10rpm.

Table 1

	Mark	Minimum cell # test in Broad	Supernatant (ml)	CDB (ml)	Total IP reaction volume (ml)	Antibody (ul/ChIP)	Dry bead volume (ul)
1	H3K4me1	3×10^6	x	x	3	1.5	10
2	H3K9ace	3×10^6	x	x	3	1.5	10
3	H3K27me3	1.5×10^6	x	x	3	1.5	10
4	H3K4me2	3×10^6	x	x	3	1.5	10
5	H3K9me3	5×10^5	x	x	1	1	10
6	H3K27ace	3×10^6	x	x	3	1.5	10
7	H3K4me3	5×10^5	x	x	1	1	10
9	CTCF	3×10^6	x	x	3	1.5	10
10	H3K36me3	5×10^5	x	x	1	1	10
11	H4K20me1	3×10^6	x	x	3	1.5	10

Note: if the sonication volume is too big, you can add NaCl to final concentration 167 mm instead of diluting with CDB.

DAY 2 – Chromatin extraction and reverse cross-linking

*Use 1.5mL tubes for all steps

1) Determine what bed volume of Protein-A Sepharose beads you need based on Table 1. Remove twice the required bed volume from the stock tube and put in an eppendorf tube.
   a) Spin 3,000 x g 30s and check 'bed volume'. Remove supernatant and put back into stock Protein-A vial. The bed volume needs to be enough for all of your ChIPs based on Table 1.
   b) Wash beads 2x in 2 volumes of cold CDB + PI.
   c) Resuspend beads to make 50/50 slurry.
   d) Add the appropriate volume of beads to each IP based on Table 1.
   e) Incubate for 1 hour on rotator at ~10rpm in cold room (4°C).

2) After 1 hour incubation, spin samples at ~3,000 x g for 1' at 4º C. Aspirate supernatant, being sure not to disturb the bead pellet. You can leave a small amount of buffer and remove it with pipette tip.
   i) Beads can stay on ice for 1-2 hours.

3) Re-suspend the beads in 500µl of Low Salt Immune Complex Wash Buffer. Pipette into new eppendorf tube and save this tip in the original tube. Incubate on ice for 5' and flick tube ~2x during incubation.

   Note: Always transfer the bead to the new tube to decrease the possibility of increasing the background!

   a) After incubation, spin samples 3000 x g for 30s at room temp. Aspirate supernatant, taking care not to disturb the bead pellet.

b) With clean tip, add another 500µl Low Salt Buffer to original tube with tip inside. Transfer this to the new tube. This will ensure that remaining beads are successfully removed.

c) After incubation, spin samples 3000 x g for 30s at room temp. Aspirate supernatant, taking care not to disturb the bead pellet.

4) Re-suspend the beads in 1mL of High Salt Wash Buffer. Incubate 5' on ice, spin, and aspirate supernatant.

5) Repeat previous step.

6) Re-suspend the beads in 1mL of LiCl Immune Complex Wash Buffer to wash. Incubate 5' on ice, aspirate supernatant.

7) Repeat previous step **but** during this incubation, remove tubes to bench top to equilibrate to RT.

8) Re-suspend beads in 1mL of RT TE pH 8.0 (10mM Tris-HCl, 1mM EDTA). Incubate 5' RT and aspirate supernatant.

9) Repeat previous step. Use pipette to remove any residual TE going into the next step.

10) Re-suspend bead pellet in 250µL of freshly prepared ChIP Elution Buffer, lay down the tube on horizontal shaker and shake at RT for at least 10'. Spin 8,000 x g for 1'. Remove supernatant and pipette into a clean eppendorf tube. Add another 250µL of elution buffer to the beads, repeat incubation and spin. Remove supernatant and add to previous 250µL of elution buffer (total volume = 500µL).

11) To the WCE <u>only</u>**,** add water to final volume 500µL.

12) Add 80µL Reverse Crosslinking Salt Mixture to ChIP and WCE samples. Mix well, seal with parafilm and incubate at 65º C water bath for at least 5 hours or overnight.

DAY 3/4 – Phenol extraction / ChIP quantification

1) After incubation at 65º C, remove the samples and bring to room temperature. Perform the following phenol/chloroform extractions:
   a) Add 580µl of 4°C phenol: chloroform: isoamyl-OH vortex 30s, spin max speed 5'. Remove top aqueous layer, transfer to new tube.
   b) Add about 500µl of 4°C chloroform: isoamyl alcohols 25:1, vortex 30s, spin max speed 5'. Remove top aqueous layer, transfer to new tube.
   c) Precipitate DNA by adding 1/10 volume of 3M sodium acetate (pH 5.0), 1µL glycogen and 2 volumes 100% etOH. Mix well; incubate overnight at -20°C or 2 hours at -80°C.

2) After overnight or 2 hour incubation, spin samples at max speed for 45' at 4°C.

3) Aspirate ethanol taking care with pellet. Add 1 ml 70% ethanol and spin at max speed for 15'. Aspirate ethanol and remove remaining ethanol by pipet. Allow pellets to air dry for ~10-15'. Re-suspend in 36µL 1x TE pH, 8.0.
   a) If you think your yield will be very low you can resuspend in a lower volume.

4) Use 2µl of each sample to quantify the yields with Invitrogen Qubit.

Buffers and Solutions for ChIP

Cell Lysis Buffer
20 mM Tris-HCl ph8.0
85 mM KCl
0.5% NP 40

Nuclei Lysis Buffer
10 mM Tris–HCl, pH 7.5
1% NP-40
0.5% sodium deoxycholate
0.1% SDS

Chip Dilution Buffer
0.01% SDS
1.1% Triton X-100
1.2mM EDTA
16.7mM Tris-HCl pH 8.1
167mM NaCl

Low Salt Immune Complex Wash Buffer
0.1% SDS
1% Triton X-100
2mM EDTA
20mM Tris-HCl pH 8.1
150mM NaCl

High Salt Wash Buffer
0.1% SDS
1% Triton X-100
2 mM EDTA
20mM Tris, pH 8.1
500 mM NaCl

LiCl Immune Complex Wash Buffer
0.25M LiCl
1% NP40
1% deoxycholate
1mM EDTA
10mM Tris-HCl pH 8.1

Reverse Crosslinking Salt Mixture
250 mM Tris-HCl, pH 6.5
62.5 mM EDTA pH 8.0
1.25 M NaCl
5mg/ml Proteinase K
62.5 ug RNase A

TE Buffer pH 8.0
10mM Tris-HCl, pH 8.0
1mM EDTA pH 8.0

Elution buffer with (10ml)
0.5 to 1% SDS
0.1 M $NaHCO_3$
Prepare buffer fresh each time.

4 X loading buffer
50mM Tris pH8.0
40mM EDTA
40% (w/v) sucrose
0.25% orange G