Chromatin Immunoprecipitation (ChIP) Protocol for Soybean Seeds

Reference: Pelletier, J.M et. Al. (2017) LEC1 sequentially regulates the transcription of genes involved in diverse developmental processes during seed development. Proc. Natl. Acad. Sci. USA, 114:32, E6710–E6719.

MATERIALS

- milliTUBE 1 ml AFA Fiber (Covaris part # 520135)
- Covaris focused-ultrasonicator model S2
- Refrigerated Floor top Centrifuge
- Water bath 37 ℃
- Water bath 65 ℃
- Dynal Biotech Rotary Shaker
- Nalgene® 190-2520 Syringe Filter with SFCA Cellulose Acetate Membrane (Sterile, Diameter: 25mm, Pore Size: 0.2µm, Thermo Scientific)
- Dynabeads Protein A (ThermoFisher, cat#10002D or 10001D, store at 4C)
- Agencourt AMPure XP beads (Beckman, cat#A63880, store at 4C)

NOTE:

- This protocol works well with soybean early maturation embryos.
- Bioruptor or Covaris conditions should be optimized beforehand in order to achieve desired fragment size distribution.
- Chromatin yields vary widely between tissue types. Chromatin amounts isolated with this procedure should be determined empirically and the amount of tissues adjusted in consequence for the proper chromatin: antibody ratio. Different chromatin concentrations will have different sonication efficiencies!!!
- You need to decide how many ChIP reactions to do first. Design the ChIP reaction number based on (i) the antibody number you are going to test, and, (ii) the number of different Ab amount to test.
- For new Ab, we normally test with 2 ug and 8 ug of Ab.
- For Covaris, the maximum number of ChIP reaction that one 1 ml Covaris milliTUBE can contain using early maturation stage embryo is three ChIP reactions, 0.6 g powder (0.2 g EM stage embryo powder for one ChIP reaction).
- “Dynabeads Protein A, Invitrogen (now Thermo Scientific)” is good to bind heavy chain of Ab boosted in rabbit, but not good for Ab boosted in mouse.
- For Ab is boosted in mouse, there are three ways to process

- Use "Dynabead Protein G, Invitrogen (now Thermo Scientific)" to bind heavy chain of Ab boosted in mouse.
- Use "Recombinant Protein A/G, Pierce (now Thermo Scientific)" which is able to bind Ab boosted in rabbit or mouse.
- Use anti-mouse Ab boosted in rabbit (rabbit anti-mouse Ab) first to bind to heavy chain of mouse Ab, then use Protein A to bind to rabbit Ab which binds to mouse Ab.

STOCK SOLUTIONS

2 M sucrose	
1 M Tris pH 8	store at RT
0.5 M EDTA	store at RT
1 M MgCl2	store at RT
20% Triton X-100	store at RT
10% SDS	store at RT
5 M NaCl	store at RT
4 M LiCl	store at RT
NP-40 (IGEPAL CA-630, sigma, Cat# I3021-5ML)	store at RT
Sodium Deoxycholate (DOC)	store at RT
BME (β-Mercaptoethanol)	store at RT
0.1M PMSF (phenylmethylsulfonyl fluoride)	store at -20C (1mL per aliquot, dissolved in isoperpanol)
Proteinase Inhibitor Cocktail	store at -20C (aliquot)
Proteinase K (ThermoFisher, cat#25530-015)	store at 4C
RNaseA (Sigma, Cat# R4642-10MG)	store at -20C
Dynabeads™ Protein A (ThermoFisher, Cat# 10001D)	store at 4C
AmPure XP Beads (Beckman, Cat# A63880)	store at 4C
Isoamyl alcohol (VWR, Cat# TCI0289-25ML)	store at RT
GlycoBlue (Invitrogen, Cat# AM9515)	store at -20C (aliquot)
Buffer saturated Phenol for DNA extraction (cat # 15513-039, Invitrogen)	store at 4C
RNase A (Sigma; cat# R-5500)	store at -20C
Protease Inhibitor Cocktail (Sigma, cat# P9599-1ML)	store at -20C (200 µl per aliquot)

NOTE: The half-life of a 20 mM aqueous solution of PMSF is ~35 min at pH 8.0. Thus, (i) due to a short half-life, fresh solution must be added at every purification step; (ii) this short half-life means that aqueous solutions of PMSF can be safely discarded after they have been rendered alkaline (pH >8.6) and stored for several hours at room temperature. An alternative to PMSF is Pefabloc SC, which is an irreversible serine protease inhibitor, and is used at the same concentration as PMSF, but is nontoxic and stable in aqueous solutions.

PROCEDURES

DAY ONE: Tissue Collection & Crosslinking

This section covers the tissue collection, cross-linking and preparation of the tissues prior to the ChIP. The crosslinking time recommended below is for soybean early-maturation stage embryo. Please test with the tissue of interest for the appropriate crosslinking time.

Solutions for Crosslinking

Buffer A	Amount	Stock
	For 1 L	
0.4 M sucrose	136.9 g	
10 mM Tris pH 8	10 ml	1 M
1 mM EDTA	2 ml	0.5 M
Add ddH2O to 1L		

Stop solution	Amount
	For 100 ml
1.25 M Glycine	9.38 g
Add ddH2O to100 ml	

1. Collect Soybean seed pods containing the target seed stage for ChIP. Stage the seeds, dissect out the embryo and collect it in Buffer A (without formaldehyde). Make a note of volume of Buffer A. Keep Buffer A on ice while collecting. Collect tissues for 20-30 minutes and proceed to the crosslinking.

2. Add formaldehyde (not older than 6 months) to Buffer A to a final concentration of 1% and invert to mix.

   Example:

37% formaldehyde	Buffer A
27.5 uL	1 mL
55 uL	2 mL
110 uL	4 mL

NOTE: 37% formaldehyde with methanol must not be older than 6 months once it is opened.

3. Vacuum-infiltrate the tissues for 10 min in the vacuum oven (no heat) at max vacuum (~23-25 mg Hg).

4. Quench the crosslinking by adding 1/10 volume of 1.25 M Glycine (stop reagent). Invert to mix. Then vacuum-infiltrate for an additional 5 minutes.

5. Rinse the tissues 2 times with Milli-Q (deionized) water, then blot on Whatman paper to remove as much water as possible. Flash freeze in liquid Nitrogen and proceed to step 6 or store at -80°C until ready to proceed. The batches can later be combined to ensure sufficient amount of tissues.

6. Once enough materials are obtained, grind tissues in liquid Nitrogen to a fine powder using a mortar and pestle, grind a bit beyond when you think it is fine enough. Store the powder in 50 ml Falcon tube at -80°C until ready to start.

DAY TWO: Chromatin Isolation, Nuclei Lysis, DNA Shearing (~9hr for 2 samples, if more than 2 samples, add 15 min for each extra sample because the shearing time is 15 min per sample)

Recommended schedule:
Chromatin Isolation, Nuclei Lysis and DNA Sharing (at least 3hr)
Examination of Shearing Result (~6hr)

NOTE:

- The amount of buffer used below is enough for TWO ChIP samples and 0.2 g of tissue is used for each sample. Each sample is used for one ChIP reaction. Read the note below if you use more than 0.2g of tissue.
- A day before making solution, put ddH2O in 4°C fridge for making solution in next day.
- All the following buffers should be FRESH made and chilled on ice on the day of use.
- Keep all solutions and suspensions as cold as possible at all times.
- Both BME (β-Mercaptoethanol) and PMSF (phenylmethylsulfonyl fluoride) are toxic.
- Do NOT add BME and PMSF when making solution. Add them right before using the solution.

Solutions for DAY TWO (Chromatin Isolation, Nuclei Lysis & DNA Shearing)

(1) Extraction Buffer 1 (EB1)	Amount	Stock
	For 50 ml	
0.4 M sucrose	10 ml	2 M
10 mM Tris-HCl pH 8	0.5 ml	1 M
Add ddH_2O to 50, 100 or 250 ml and keep on ice before use.		
5 mM BME	17.5 µl	14.3 M
1 mM PMSF	0.5 ml	0.1 M

NOTE: (i) Fresh made and put on ice (ii) add BME and PMSF right before using the solution.

(2) Extraction Buffer 2 (EB2)	Volume	Stock
	For 10 ml	
0.25 M sucrose	1.25 ml	2 M
10 mM Tris-HCl pH 8	100 µl	1 M
10 mM MgCl2	100 µl	1 M
1% Triton X-100	500 µl	20%
Fill ddH2O to 10 ml and keep on ice before use.		
5 mM BME	3.5 µl	14.3 M
1 mM PMSF	100 µl	0.1 M

NOTE: (i) Fresh made and put on ice (ii) add BME and PMSF right before using the solution.

(3) Extraction Buffer 3 (EB3)	Volume	Stock
	For 10 ml	
1.7 M sucrose	8.5 ml	2 M
10 mM Tris-HCl pH 8	100 µl	1 M
2 mM MgCl2	20 µl	1 M
0.15% Triton X-100	75 µl	20%
Fill 1.2 ml ddH2O to 10 ml and keep on ice before use.		
5 mM BME	3.5 µl	14.3 M
1 mM PMSF	100 µl	0.1 M

NOTE: (i) Fresh made and put on ice (ii) add BME and PMSF right before using the solution.

(4) Shearing Buffer:	Volume	Stock
	For 10 ml	
10 mM Tris-HCl pH 8	100 µl	1 M
1 mM EDTA	20 µl	0.5 M
0.1% SDS	100 µl	10%
Fill 9.78 ml ddH2O to 10 ml and keep on ice before use.		
1 mM PMSF	100 µl	0.1 M

NOTE: (i) Fresh made and put on ice (ii) add PMSF right before using the solution.

1. Turn on the centrifuge and set the temp to 4℃ and place the correct rotor in, then close the door to let the centrifuge to cool down (step 2~6 will take at least 30 min).

2. Prepare the solutions shown above and keep them on ice. Label BME and/or PMSF on the cap of tube or bottle to be checked off when added.

3. Take the powder out from -80℃ and leave the 50ml tube with powder in liquid nitrogen or on dry ice when you measure 0.2 g of ground power in 50 ml Falcon tube.

4. In the hood, add 0.5 ml PMSF and 17.5 ul BME into 50 mL EB1 before using EB1.

5. Add 20 ml of ice-cold EB1 to 0.2 g of ground powder in 50 mL Falcon tube. Vortex (or shake) till homogenous, place on ice.

   NOTE:
   - Regardless of the sonication method, the bottom line is to get a nice diluted slurry, so adjust the buffer volume based on different tissues.
   - If you have more material, use 40ml for 0.6g, 50ml for 1g, or 100ml for 2g.
   - For 1 ml Covaris milliTUBE (part # 520135), it can hold materials up to three ChIP reactions when using early maturation embryos.
   - For embryo proper, 0.2 g early maturation embryo can get 25 ug chromatin for one ChIP reaction.

6. In the hood, filter solution through 4 layers of Miracloth with funnel, into a new 50ml Falcon tube.

7. Bring the filtered solution on ice and centrifuge solution for 20 min @ 4,000 rpm at 4℃.

8. In 4℃ cold room:
    a) Remove supernatant.
    b) In the hood, add _100_ ul PMSF and _3.5_ ul BME into_10_ mL EB2 before using EB2.
    c) Add ice-cold _1_ ml EB2 (with PMSF and BME) for _1_ ChIP reactions in 50 mL Falcon tube.
    d) Resuspend pellet by gentle pipetting.
    e) Transfer and aliquot to 1.5 ml Eppendorf tubes, 1ml per tube.

    NOTE:
    - We use one eppendorf tube for nuclei isolation/process for one ChIP reaction.
    - The volume of EB2 buffer (containing PMSF and BME) depends on the number of ChIP reaction: 1 ml of EB2 for one ChIP reaction, 3ml for three ChIP reactions ~0.6g of tissue).
    - Triton X-100 in EB2 can destroy chloroplast.

9. In 4℃ cold room: Centrifuge at 12,000 x g for 10 min at 4℃.
    Optional: If the pellet after step 5 is still green, repeat the EB2 wash:
    a) Discard supernatant
    b) Add 1 ml ice-cold EB2 (with PMSF and BME) into 1.5 mL eppendorf tube to resuspend pellet by gentle pipetting.
    c) Centrifuge at 12,000 x g for 10 min at 4℃

    NOTE: There are two layers in the pellet, (i) Upper layer is green and transparent, which is chloroplast and nuclei. (ii) Bottom layer is white pellet which is protein. Repeat EB2 wash can clean chloroplast thoroughly and repeat EB wash does not hurt. (iii) Depending on the sample, you may need to adjust the speed. For example, 14,000 x g for EM seed coat, and 12,000 x g for EM embryo. If the pellet is too tight, it is hard to resuspend it. If the pellet is too loose, you may lose some when removing supernatant.

10. In 4℃ cold room:
    a) Remove supernatant.
    b) Add _100_ ul PMSF and _3.5_ ul BME into _10_ mL EB3.
    c) Add 400 µl ice-cold EB3 (with PMSF and BME) to one 1.5 mL eppendorf tube.
    d) Resuspend pellets by gentle pipetting.
    e) If you would like to count nuclei, keep 5 µl to count nuclei number.

11. In 4℃ cold room: In a clean Eppendorf tube, add ice-cold 400µl EB3 (with PMSF and BME) as the bottom layer. Then add all "resuspended pellet from previous step 6" on top of this bottom layer.

    NOTE: Carefully adding "resuspended pellet from previous step 6" without disturbing the bottom layer of new 400µl EB3.

12. In 4 ℃ cold room: Spin solution for 45min to 1 hr @ top speed in bench-top microfuge at 4℃.

13. In 4 ℃ cold room:
    a) Remove supernatant.
    b) Add 100 ul PMSF into 10 mL Shearing Buffer.
    c) add 800 µl chilled Shearing Buffer to resuspend the nuclei pellet and transfer to Covaris milliTUBE. Adjust the final volume to 1 ml for one shearing sample by adding more chilled Shearing Buffer.

    NOTE: If there is more than one ChIP reaction from the same tissue, you can combine at more three ChIP reactions into one shearing reaction. Thus, using the same solution to resuspend pellets to combine pellets in three tubes:
    a) Add 800 µl Shearing Buffer into the 1st tube to resuspend pellet carefully to avoid bubbles.
    b) Then, transfer the resuspend nuclei from 1st tube into the 2nd tube to resuspend the pellet
    c) Finally transfer the resuspend nuclei from 2nd tube into the 3rd tube to resuspend the pellet.

14. Transfer nuclei into one 1 ml Covaris milliTUBE. If it is not full, fill the Covaris milliTUBE with Shearing Buffer to the top of the Covaris milliTUBE and avoid bubbles. Following the Covaris instruction below to process samples on Covaris "E220" with these settings: 140 Peak Incident Power, 5% Duty factor, 200 Cycles per Burst for the appropriate time to achieve good fragmentation. The total process time is ~15 min per sample.

    NOTE: Good fragmentation is the fragment sizes are below 500bp and ideally centered around 200-300bp.

15. After shearing, transfer chromatin to 1.5ml eppendorf tube. Then add 32.6 µl 5 M NaCl and 54.3 µl 20% Triton X-100 into 1 ml shearing reaction.

    CRITICAL:
    - The difference between shearing buffer and ChIP buffer is in that ChIP buffer has additional 150 mM NaCl and 1% Triton X-100. Thus, add stock 5 M NaCl and 20% Triton X-100 accordingly for the shearing product.
    - If "more than one ChIP reactions" was prepared in "1 ml sheared reaction in one Covaris milliTUBE", after adding NaCl and Triton-X-100, add appropriate volume of ChIP Buffer to reach "1 ChIP reaction amount of sheared chromatin in one ml" is obtained (1 ChIP/ml). Mix well and aliquot to get 1 ml in one eppendorf tube. Take a look the example below to learn how to adjust.

    Example: (the "sheared chromatin amount" for "three" ChIP reactions is in one 1 ml Covaris milliTUBE in the shearing process)
    a) Transfer 1 ml sheared chromatin from Covaris milliTUBE to one 15 ml Falcon tube.
    b) Add 32.6 µl 5 M NaCl and 54.3 µl 20% Triton X-100 into 1 ml sheared chromatin.
    c) Add 2 ml ChIP Buffer (with 150 mM NaCl and 1% Triton X-100) into sheared chromatin to get total volume is ~3 ml. Mix by pipetting.
    d) Aliquot 3 ml into three eppendorf tubes, 1 ml per tube.

16. 1st centrifugation to clean the sheared chromatin.
    a) Centrifuge chromatin samples in a microfuge at 4℃ 10 min max speed to take away protein debris.
    b) Transfer supernatant to fresh tube.
    c) Reserve 100 µl supernatant as shearing control to check shearing result. For the same tissue within the same shearing reaction, one shearing control (100 µl) is enough.

    NOTE:
    - You can flash freeze the sheared chromatin used for ChIP reaction and store it at -80C for a later use (ChIP) once you have verified the shearing.
    - 100 µl shearing control is 10% of 1 ml of one ChIP reaction. You may calculate the recovery rate accordingly.

17. Examine shearing result (for 100ul of sheared chromatin, make adjustment if more than 100 ul)
    a) Make fresh 20 mg/ml Proteinase K:
        1) Add _1_ mg Proteinase K into eppendorf tube.
        2) Add _50_ µl 1xTE into the tube.
        3) Spin down to bring Proteinase K powder on the tube wall to the bottom.
        4) Mix by gentle pipetting.
        5) Put on ice.

    NOTE: it is hard to measure little amount of Proteinase K. Just try to measure 1~3 mg Proteinase K powder.

    b) Perform de-crosslink procedure. The sample without de-crosslink will be stored on ice for phenol:chloroform extraction.
        1) Add _4_ µl 5M NaCl and _1_ µl RNaseA into the sample, mix samples by gentle pipetting and then incubate at 37 ℃ for 30 min. Self-digest Proteinase K at 37 ℃ for 30 min at the same time.
        2) After 30 min, add 1µl of Proteinase K, mix samples, and then incubate at 65℃ for 1h. Keep the other Proteinase K on ice.
        3) Repeat Proteinase K treatment: Add 1µl Proteinase K, mix, and incubate at 65℃ for 1h. Keep the other Proteinase K on ice.

    c) Extract DNA with phenol:chloroform for all samples.
        1) In the hood, prepare _2_ ml chloroform:isoamyl alcohol (24:1) in glass scintillation vail.
        2) In the hood, Prepare _2_ ml phenol:chloroform:isoamyl alcohol (25:24:1) in glass scintillation vail.
        3) In the hood, Add equal volume 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1), vortex 30 sec and centrifuge 2 min at max speed.
        4) Transfer supernatant to a fresh tube. In the hood, add equal volume 100 µl chloroform:isoamyl alcohol (24:1), vortex 30 sec, spin 1 min at max speed.
        5) Transfer supernatant to a fresh tube for Ethanol Precipitation Clean-Up. Take a record of the volume of supernatant.

d) Clean-Up with Ethanol precipitation for De-crosslink and No de-crosslink samples.
   1) Add 1/10 volume _____ µl of 3M sodium acetate (NaOAc, pH5.2)
   2) Optional: add __2__µl GlycBlue (15mg/ml), and mix by flicking.
   3) Add 3 volume of ice-cold 100% EtOH.
   4) Invert to mix, spin down. Precipitate -80°C 1 hr or at -20°C overnight.
   5) Centrifuge samples 30 min, max speed, 4°C
   6) Remove supernatant, wash pellets with 1 ml ice-cold 70% EtOH, centrifuge at 4°C for 5 min with max speed (5-15 min is fine).
   7) Remove as much EtOH as possible without disturbing pellet, air dry pellets for minimum of 15-20 min or until all EtOH has evaporated
   8) Resuspend DNA pellet with 30 µl 10mM Tris pH 8.5 (EB buffer in Qiagen kit), vortex, place on ice.
   9) Make 1:100 dilution and read concentrations with PicoGreen on FluoroDrop.
   10) Run the sheared control on 1.5% agarose gel to check if there is any un-sheared chromatin.

DAY THREE: Chromatin Immunoprecipitation (~2hr)

Solutions for DAY THREE (Chromatin Isolation and Immunoprecipitation)

(1) ChIP Buffer:	Volume For 10 ml	Stock
10mM Tris-HCl pH 8	100 µl	1 M
1mM EDTA	20 µl	0.5 M
0.1% SDS	100 µl	10%
150 mM NaCl	300 µl	5 M
1% Triton X-100	500 µl	20%
Fill ddH2O to 10 ml and keep on ice before use.		
1 mM PMSF	100 µl	0.1 M

NOTE: (i) Fresh made and put on ice. (ii) Do NOT add PMSF till chromatin shearing is done. (iii) the difference between Shearing vs ChIP Buffer is ChIP Buffer has additional NaCl and Triton X-100.

1. 2nd centrifugation:
   a) Take sheared chromatin out from -80C and store on ice.
   b) Repeat centrifugation as 1st centrifugation in a microfuge at 4℃ 10 min max speed
   c) During the 2nd centrifugation, take "Protease Inhibitor Cocktail" out of -20 ℃ freezer and keep at RT to warm back to RT.
   d) Transfer supernatant of the 2nd centrifugation result to 1.5 ml non-stick microfuge tubes (low retention tube).

2. Add appropriate volume of Proteinase Inhibitor Cocktail to get final 1:100 dilution: Adding 10 µl Proteinase Inhibitor Cocktail per 1 ml supernatant. Mix well and keep it on ice.

   NOTE: Using non-stick tubes significantly reduces carryover of non-antibody bound chromatin.

3. Equilibrate Dynabead-Protein A beads (Protein A beads) with ChIP Buffer:
   a) Add _____ µl of Protein A beads (30 ul Protein A beads * ChIP reaction number) into 1 ml ChIP Buffer
   b) Mix by pipetting.
   c) Put tube on magnetic stand till clean.
   d) Take away supernatant carefully to avoid disturbing the bead pellet.
   e) Repeat step (a) to (d) for additional two times, total three times.
   f) Add _____ µl of ChIP buffer (30 ul ChIP buffer * ChIP reaction number) to resuspend bead pellet.

   NOTE: For one ChIP reaction, take 30 µl Dynabead-Protein A beads from original stock bottle. After three washes, resuspend bead pellet with 30 µl (the original volume) ChIP buffer for one ChIP reaction.

4. Pre-clear chromatin sample:
   a) Add 30 µl of equilibrated Dynabead-Protein A to each sample
   b) Mix on a rotating mixer at 4℃ for 1 hour at 20rpm.

5. In 4 ℃ cold room, clear samples by placing tubes on magnet stand, 2 min at 4℃. Pull out cleared chromatin and combine samples of the same genotype/treatment/bioreps.

6. Reserve Pre-clear chromatin sample as ChIP INPUT CONTROL and add Ab into ChIP reaction:
   a) Measure volume and adjust volume with ChIP Buffer, if needed, to get 1 ChIP reaction/1 ml.
   b) Reserve Pre-clear chromatin by taking 10% input (1/10 volume of ChIP reaction volume) to a tube to freeze at -20℃ till next and continue in Day FOUR. In this case, take 100 µl (10% of 1 ml)

   NOTE: In the case of testing more than one antibody with the same sheared chromatin form the same process, only one input control is needed.

   d) Add desired amount of antibodies to each tube. Typical antibodies amounts are 1-10µg.

   NOTE: We usually test 2 ug and 8 ug for the same Ab.

7. Incubate chromatin plus antibodies on a rotating mixer wheel (~20 rpm), 4 hours to overnight at 4℃.

DAY FOUR: Washing and Elution After Immunoprecipitation (~9hr)

In this section, we will capture the immune complexes using Protein-A beads, remove non-specific interactions and reverse the cross-links.
If using anti TF Ab boosted in mouse and if using Protein A to capture mouse Ab, add 10 µg Rabbit Anti-Mouse antibodies to IPs and rotate at 4℃ for 4hrs.

NOTE: Fresh made ChIP Buffer on the same day!!!

Solutions for DAY FOUR (Wash and Elution After Immunoprecipitation)

(5) ChIP Buffer:	Volume	Stock
	For total 10 ml	
10mM Tris-HCl pH 8	100 µl	1 M
1mM EDTA	20 µl	0.5 M
0.1% SDS	100 µl	10%
150 mM NaCl	300 µl	5 M
1% Triton X-100	500 µl	20%

Add 8.98 mL ddH2O to 10 ml and keep on ice before use.

1 mM PMSF	100 µl	0.1 M

NOTE: (i) Fresh made and put on ice. (ii) Do NOT add PMSF till you use this solution. (iii) You can make this one first and make other buffers during the 2hr incubation.

(6) Low Salt Wash Buffer:	Volume	Stock
	For total 10 ml	
0.2% SDS	200 µl	10%
0.5% Triton X-100	250 µl	20%
2 mM EDTA	40 µl	0.5 M
20 mM Tris-HCl pH 8	200 µl	1 M
150 mM NaCl	300 µl	5 M

Add 9.01 mL ddH2O to 10 mL
NOTE: Fresh made and put on ice.

(7) High Salt Wash Buffer:	Volume	Stock
	For total 10 ml	
0.2% SDS	200 µl	10%
0.5% Triton X-100	250 µl	20%
2 mM EDTA	40 µl	0.5M
20 mM Tris-HCl pH 8	200 µl	1M
500 mM NaCl	1 ml	5M

Add 8.31 mL ddH2O to 10 mL
NOTE: Fresh made and put on ice.

(8) LiCl Wash Buffer:	Volume	Stock
	For total 10 ml	
0.25 M LiCl	625 µl	4 M
0.5% Sodium Deoxycholate (DOC)	50 mg	powder
1 mM EDTA	20 µl	0.5 M
10 mM Tris-HCl pH 8	100 µl	1 M
0.5% NP-40 (IGEPAL CA-630)	50 µl	100%

Add 9.2 mL ddH2O to 10 mL

NOTE:

- Fresh made and put on ice.
- DOC and NP-40 are stored at RT on chemical shelf.
- DOC is easy to precipitate, so add powder directly into the LiCl Wash Buffer instead of making stock.
- Add NP-40 last. NP-40 is sticky, so cut the tip head. After adding NP-40, put on rotator for ~ 15 min and then put on ice

(9) TE Buffer:	Volume	Stock
	For total 10 ml	
10 mM Tris pH8.0	100 µl	1 M
1 mM EDTA	20 µl	0.5 M

Add 9.88 mL ddH2O to 10 mL

NOTE: Fresh made and put on ice.

(9) Elution Buffer:	Volume	Stock
	For total 1 ml	
50 mM Tris pH8.0	50 µl	1 M
10 mM EDTA	20 µl	0.5 M
1% SDS	100 µl	10%

Add 830 uL ddH2O to 1 mL

NOTE: Do not put on ice, otherwise SDS will precipitate.

1. Add __100__ ul PMSF in __10__ mL ChIP buffer.

2. Capture immune complexes by using Dynabead-Protein A beads:
   a) Equilibrate Dynabead-Protein A beads by ChIP Buffer:
      1) Add ____ uL of Dynabead-Protein A beads into 1 ml ChIP Buffer. Total ChIP buffer needed = (40 µl Protein A beads) * (Number of ChIP reaction)
      2) Mix by pipetting.
      3) Put tube on magnetic stand ~ 1 min till clean.
      4) Take away supernatant carefully to avoid disturbing the bead pellet.
      5) Repeat step (a) to (d) for additional two times, total three times.
      6) Add ____ uL of ChIP buffer to resuspend bead pellet to get 40 µl for one ChIP reaction. Total ChIP buffer needed = (40 µl Protein A beads) * (Number of ChIP reaction)
   b) Capture immune complexes by adding 40 µl of Dynabead-Protein A beads (equilibrated in ChIP Buffer *freshly made this day!) and rotating at 4 ℃ for 2h.

3. Prepare Proteinase K: During the capture, prepare 20 mg/ml Proteinase K and self-digest at 37℃ for 30 minutes. Keep on ice.

   NOTE: it is hard to measure little amount of Proteinase K. Just try to measure 1~3 mg Proteinase K powder. Usually add 50 ul for 1 mg Proteinase K.

4. For INPUT CONTROL(100 uL in ChIP buffer):
   a) Take out INPUT from -20℃ freezer reserved in Day2.
   b) Add 4 µl 5M NaCl and 1 µl RNaseA into INPUT CONTROL. Mix well.
   c) Incubate at 37 ℃ for 30 min then keep on ice until the ChIP samples are ready for 1st Proteinase K treatment because ChIP samples and INPUT CONTROL will do 1st Proteinase K treatment together.

5. For ChIP sample, in 4℃ cold room:
   a) After capture is done at step 2(b), recover beads with magnetic stand for 2 min at 4℃.
   b) Washing steps:
      1) Add 1 ml "Low Salt Wash Buffer", and then put on rotator for 5 min. Put on the magnet stand ~1 min till beads supernatant is clear. Then pipet out supernatant.
      2) Add 1 ml "High Salt Wash Buffer", and then put on rotator for 5 min. Put on the magnet stand ~1 min till beads supernatant is clear. Then pipet out supernatant.
      3) Add 1 ml "LiCl Wash Buffer", and then put on rotator for 5 min. Put on the magnet stand ~1 min till beads supernatant is clear. Then pipet out supernatant.
      4) First TE wash: Add 1 ml "TE buffer", and then put on rotator for 5 min. Put on the magnet stand ~1 min till beads supernatant is clear. Then pipet out supernatant.
   c) 2nd TE wash:
      1) Adding 1 ml TE buffer to resuspend bead pellet
      2) Put on rotator for 5 min.
      3) Transfer beads to a new tube to reduce background that might have stuck to the tubes.
      4) Put on magnetic stand
      5) Remove all residual TE. Now, ready for elution.

6. 1st Proteinase K treatment.
   a) Add Proteinase K
      - ChIP sample: Elute complexes from beads with 100 µl Elution Buffer, add 4 µl 5M NaCl and 1 µl Proteinase K. Resuspend beads by gentle pipetting to avoid bubbles form SDS in elution buffer.
        NOTE: ChIP buffer contains 150 mM NaCl already, but more NaCl still needs to be added for crosslink reversal.
      - INPUT CONTROL: Add 1µl of Proteinase K to INPUT CONTROL from step 4(c).
   b) Incubate at 65℃ for 1h.

7. 2nd Proteinase K treatment:
   a) Add 1µl Proteinase K to the ChIP sample and INPUT CONTROL,
   b) Mix and incubate at 65℃ for 1h.

8. In the hood:
   a) prepare _2_ ml chloroform:isoamyl alcohol (24:1) in glass scintillation vail.
   b) Prepare _2_ ml phenol:chloroform:isoamyl alcohol (25:24:1) in glass scintillation vail.

9. For ChIP samples, there may be some beads left in the solution. To get rid of beads, centrifuge ChIP samples 1 min at max speed, transfer supernatants to new tubes. Repeat one more time to make sure all Protein A beads are removed.

   NOTE:
   - When transferring ChIP supernatant, taking out supernatant carefully with small volume for multiple times to avoid disturbing beads pellet.
   - For the last amount of supernatant, let the part of tube with beads touch the magnetic stand and take the last part of supernatant.

10. For ChIP samples, INPUT and Shearing controls:
    a) In the hood, Add equal volume 100 µl of phenol:chloroform:isoamyl alcohol (25:24:1)
    b) vortex 30 sec and centrifuge 2 min at max speed.

    NOTE:
    - Take phenol from the bottom layer of the buffer saturated phenol, if there are two layers.
    - If the sample has different volume, use the equal amount of phenol:chloroform:isoamyl alcohol.

11. Transfer supernatant to a fresh tube. In the hood, add 100 µl chloroform:isoamyl alcohol (24:1), vortex 30 sec, centrifuge 1 min at max speed. Transfer supernatant to a fresh tube.

12. Clean samples using Agencourt AMPure XP beads (protocol adapted from NuGen Ultralow Library Systems)

    NOTE: For library construction, use beads purification, as it is cleaner than ethanol precipitation.

    a) Take the AMPure XP beads beads out of fridge. Vortex it. Take_____ul of beads per reaction into 1.5 microcentrifuge tube (200 ul per reaction, take 10 ul extra beads). Let stand at RT for 5 min.
    b) Add 200 uL of beads to 100 uL of reaction. Mix thoroughly (pipetting more than 10 times) and let stand at RT for 15 min.
    c) Place tubes on a Magnet separator for 15 minutes to separate beads from solution.
    d) While waiting, make _____ml 80% ethanol (600 ul per reaction)
    e) Aspirate 290 ul cleared solution from the reaction tubes and discard (leave ~10 ul in the tube). This step is performed while the reaction tubes are situated on the Magnet separator.

f) Dispense 300 µL of 80% **FRESH MADE** ethanol to each tube, and incubate for 30 seconds at room temperature. This step is performed while the reaction tubes are situated on the Magnet separator. Aspirate out the ethanol and discard. Repeat for a total of two washes.
g) Place the reaction tubes on bench top to air-dry for 15 min or in laminar flow for 7 min. Be sure to allow beads to dry completely (if it is dry, you may some cracks on the bead pellet).
h) Add 31 uL elution buffer (EB or 10 mM Tris pH 8) to each tube. Pipette to mix beads 10 times and EB buffer thoroughly and let stand RT for 2 mins.
i) For ChIP DNA, keep 3.5 ul for checking DNA concentration with PicoGreen and running on BioAnalyzer using high sensitivity D1000 ScreenTape. Store the rest of DNA at -20 for library construction.
j) For input Control DNA, make 1:100 dilution which will be used for checking DNA concentration with and running on BioAnalyzer using high sensitivity D1000 ScreenTape.

NOTE:

- If you have known targets, quantitative PCR can be used to verify the enrichment of ChIP DNA.
- We use Nugen Ovation Ultralow DR System to construct sequencing library. The detailed protocol is same as the protocol from the manufactory.

