-

Meyer 3310 Department of Animal Science, UC Davis	Doc. No	HZ-SP-07
Standard Protocol	PI	Dr. Zhou
Title: ChIP-seq Protocol for animal tissues	Date	June 5 2018

Preparation:

Diagenode iDeal ChIP-seq kit for Histones for 100 reactions (C01010059)

Diagenode iDeal ChIP-seq kit for Transcription Factors for 100 reactions (C01010170)

Buffers from Diagenode iDeal kit:

- **Lysis Buffer:**
  Buffer iL1 and iL2 from
- **Shearing Buffer:**
  Buffer iS1 from the kit
- **TE Buffer**
- **PBS (Add protease inhibitor tablets)**

Pierce™ Methanol-free Formaldehyde Ampules, Thermo Scientific - 16% Formaldehyde (w/v), Methanol-Free 28906)

Tubes used with covaris machine: microTUBE AFA Fiber Snap-Cap 6x16mmm (130ul/tube) (catalog#520077)

Protocol:

Tissue Harvest and Cross-Linking

Preparation:

- Turn on microcentrifuge and set at 4°C.
- Move Dounce homogenizers and homogenizer probes to cold room. Place motor pestle into -80*C.
- Prepare an ice bucket.
- Move the glycine to benchtop and allow to reach room temperature.
- Make 1% formaldehyde in PBS.
- Label 2 mL tubes
- Prepare foil sleeves for the tissues in case you mangle the existing foil sleeves.

- Fill a freezer box with liquid nitrogen for the tissue sleeves.

First pre-make the 1% formaldehyde from 16% formaldehyde: 62.5ul 16% formaldehyde + 937.5ul PBS = 1ml 1% formaldehyde/sample.

1. Transfer tissues into liquid N2 tank. Take 30mg of fresh or frozen tissue to a pre-chilled motor pestle. Grind tissue into powder in liquid N2. Transfer the tissue powder into a clean 2.0-mL tube.
2. Quickly add the **1ml of 1% formaldehyde** to each tube for crosslinking the tissue and immediately invert the tube several times.
3. Incubate the tube on the rotator at **room temperature** for **8 min (This time may vary depending on tissue type, optimization may be needed)**
    a. To test for optimal crosslinking time, test times between 5 min to 10 min if necessary. Generally it is best to avoid over-crosslinking.
4. Quench the cross-linking by adding **100 µL of glycine** (**from the kit**) and incubate the tube on the rotator at **room temperature** for an additional **10 min**.
5. Harvest the tissue by centrifugation at **2000g** for **10 min at 4°C**.
6. Remove the supernatant and add **1 mL** of ice-cold **PBS**. Flick the tube with your finger several times to dislodge the pellet. Pellet might be difficult to dislodge, thus turn tube horizontally then flick.
7. Harvest the tissue by centrifugation at **2000 x *g*** for **10 min at 4°C** and discard the supernatant. Flash freeze the tissue pellet in liquid nitrogen and stored at −80°C or proceed to Step 10 without freezing.

<u>Chromatin Extraction and Shearing</u>

In the cold room:

8. Resuspend by flicking the frozen cross-linked tissue pellet in **1500 µL of iL1 buffer**.
    a. *If the tissue remains in large chunks, homogenize it in a prechilled Dounce homogenizer with a loose pestle (pestle A) five to 10 times.) Amount of lysis buffer is proportionate to amount of cells extracted approximately 1:5 ratio of cell pellet to buffer.*
9. Incubate the tube **for 20 min on ice with occasional mixing**. (*This incubation period allows for swelling of cells to allow for mechanical lysis while keeping nuclei intact.)*
10. Transfer the tissue suspension to a prechilled Dounce homogenizer and homogenize with 30–40 strokes with the tight pestle. **Keep samples on ice.**

11. Transfer the homogenate to a fresh 2.0-mL tube, rinse the Dounce homogenizer with **500 µL of iL1 buffer**, and combine.

12. Centrifuge for **5 min** for **2000g**. Remove supernatant and add **2ml of iL2 buffer.** Incubate for **10 min on ice** with occasional mixing.

In lab:

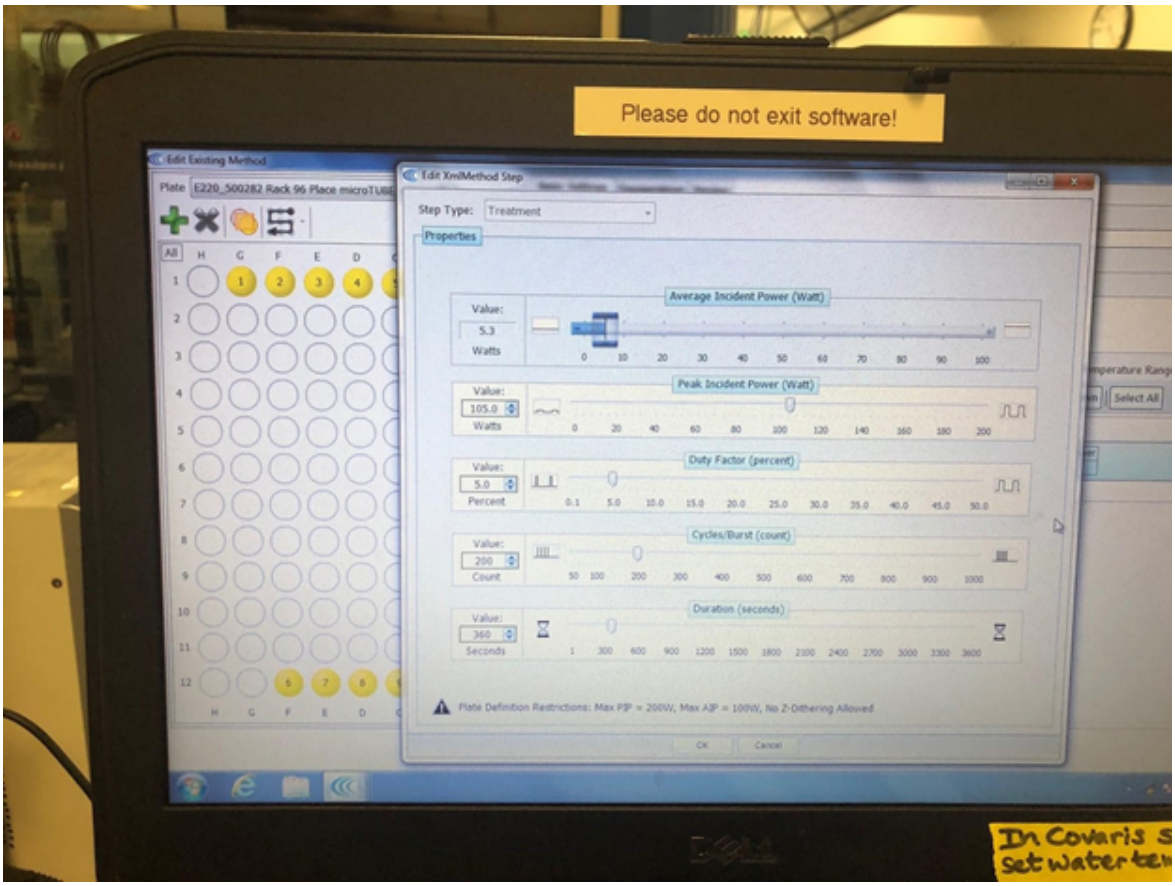
13. Harvest the nuclei by centrifugation at **2000g for 5 min at 4°C**.
    a. *The pellet will be not be as tight against the tube as the original cell pellet and will be mostly white.*
14. Add protease inhibitor cocktail (200X- from kit) to iS1 buffer.
15. Remove the supernatant and resuspend the nuclei via gentle pipetting to break up the pellet in **660 µl of iS1 buffer.**
    a. *Generally 30mg of tissue can be diluted with 660ul of iS1 buffer. If doing more or less tissue, adjust this amount appropriately.*
16. Incubate the tube on **ice for 20 min. with occasional mixing**.
17. Aliquot **130 uL** into three Covaris snap-cap tubes for chromatin shearing. Remove the caps from the tubes using the cap tool and dispense aliquot into tube, then cap tubes.

**When testing for optimal shearing time for a tissue, perform a time increment for shearing that increases 1 min between 5 min and 10 min.

At the DNA Sequencing Core at the Genome Center: Sonication

You must be trained with the sonicator before being allowed to use the machine.

18. Press "Start Position" and wait for machine to adjust. Observe water level for correct height (make sure they cover the tubes) and adjust the water level if needed.
19. Adjust settings to match below:
    a. **Maximum power: 105 W**
    b. **Duty factor: 5.0%**
    c. **Shear time: 6 min (or what was determined from optimal shearing time)**



20. Once completed, remove the samples from the sonicator and store on ice.

QC for shearing:

21. Create a thermocycling program for steps 24 and 25. (65°C for 30 min., 95°C for 5 min., 25 forever) Start the program and pause it to allow the lid to reach the correct temperature.
22. Take a **5-µL aliquot** and dilute it with **15 µL of TE** for an analytical gel (1-1.5% agarose).
23. Treat all reserved aliquots with 1ul of RNase A (10mg/ml). Incubate at **37*C for 30 min**.
24. Next, treat all reserved aliquots with **20 µg or 2ul** of **proteinase K** for **at least 4 hours at 65°C**.
25. Add 2 ul loading dye to each 10ul of the sample, and separate samples on a 1% agarose gel in 1× TAE running buffer.
26. Visualize DNA samples on the agarose gel. ***(Sheared chromatin should be a light smear ranging in size from 200 to 700 bp.)***
27. Additionally, you can purify the the dna using Qiagen PCR purification kit (28104) and

elute with 10-25ul EB buffer, and run on High Sensitivity DNA Bioanalyzer chip.

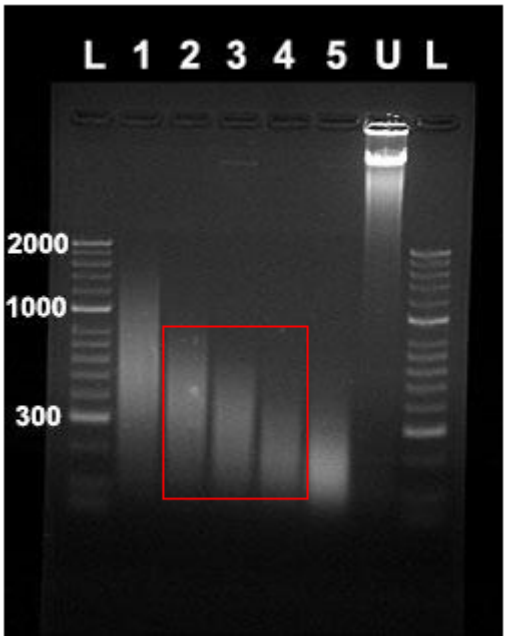
a. Running on bioanalyzer is recommended for troubleshooting tissue (if **undershearing** or **overshearing** is a concern). Once desired bioanalyzer results is achieved, a gel can then be run for comparison to further quality check gels.

This is an ideal time to freeze and store samples for further application at -80C. Samples can be stored for an indefinite amount of time at -80C.

Expected gel picture shown below:

Example figure of quality assessment of sheared chromatin after various number of cycles. As you can see, increasing the number of cycles yields a smaller fragment. Lane U is used as a standard taken from the non-sheared sample demonstrating the quality of the DNA prior to sonication.

Quality Check: If samples appear under sheared (lane 1), then re-shear for additional time. Generally going by increments of 1 min. Lanes 2-3 is optimal. Lane 4 is usable although may be possible over shearing. Lane 5 is also a bit over sheared. It is best to avoid over shearing versus under shearing.



<u>Immunoprecipitation:</u>

<u>Antibodies:</u>

H3K4me3 (comes with Diagenode iDeal Histone kit)

H3K27ac (Diagenode, catalog# C15410196)

H3K4me1 (Diagenode, catalog# C15410194)

H3K27me3 (Diagenode, catalog# C15410195)

CTCF (comes with Diagenode iDeal Transcription Factor kit)

Recommended chromatin input into IP: 1-1.5ug (measured from purified DNA) and dilute to 100 ul if necessary

Recommended antibody input: 0.5ug, increase to 1ug if necessary (i.e. H3K27ac or H3K4me1)

**Immunoprecipitation performed according to kit protocol

QC for immunoprecipitation: Perform qPCR with a positive and negative target for each mark on each sample. If you have enough IP DNA, then qPCR is recommended for 2 positive and 2 negative targets.

Calculate percent input: 2^(Ct(input)- Ct(sample))

Expected results shown below:

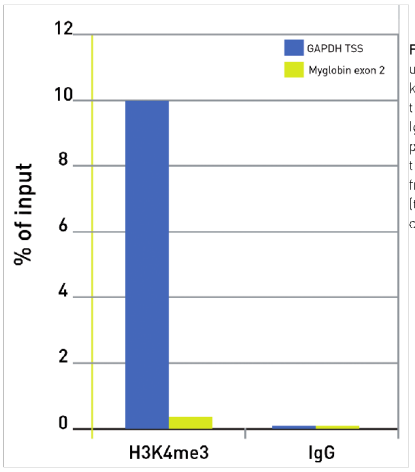


Figure 2: ChIP was performed on human HeLa cells using the control antibodies from the iDeal ChIP-seq kit. Sheared chromatin from 1 million cells, 1 µl of the positive control antibody and 2 µl of the negative IgG control were used per IP. Quantitative PCR was performed with the positive control GAPDH-TSS and the negative control Myoglobin exon 2 primer sets from the kit. The recovery, expressed as a % of input (the relative amount of immunoprecipitated DNA compared to input DNA after qPCR analysis).

Narrow marks: Expected % of input for positive targets above 5% and negative targets below 2%.

Broad marks: Expected % of input varies. Observe that positive target is greater than negative target by ratio of ~ 5.

<u>Current available primers:</u>

Chicken Primers:

primers	sequence	H3K4me3	H3K27me3
GAPDH_F	GTCACGTCCCAGGAGCAG	+	-
GAPDH_R	AGGACCGTGCTAATGAGGAA	+	-
MYOD_F	TTGGTGGAGATCATGCCATA	-	+
MYOD_R	GTTGTGGGCCAGAAACAAGT	-	+

Pig primers:

Porcine genes	porcine build10.2				Expected PCR				
Gene/region	Chr	position forward	position reverse		Product length	**H3K27me3**	**H3K4me3/H3K27ac**	**CTCF**	**Input**
ChIP_Hoxc4	5	20167574-20167593	20167677-20167696	Exon1	122	YES	NO		YES
ChIP_HOXB5_TSS	12	24875377-24875396	24875428-24875447	TSS	70	YES	NO		YES
ChIP_NDUFA4_1	9	88562719-88562738	88562823-88562842	TSS	124	NO	YES		YES
ChIP_TRX1_1	1	282076966-282076985	282077044-282077063	TSS	98	NO	YES		YES
ChIP_NegC2_2	2	44495204-44495223	44495289-44495308		105	NO (or weak)	NO		YES
Chicken genes	galGal5.0								
ChIP_NegCTCF_Chicken	1	76654645-76654666	76654921-76654941		297	NO	NO	NO	YES

Recommend using

Library prep kit:

NEBNext® Ultra™ II DNA Library Prep Kit for Illumina, 96 reactions (E7645L)

Multiplex oligos:

NEBNext Multiplex Oligos for Illumina (Index Primers Set 1) (E7335L)

NEBNext Multiplex Oligos for Illumina (Index Primers Set 2) (E7500L)