OESTREICH LAB CHIP PROTOCOL

<u>Buffers</u> (*all starred (*) buffers must be autoclaved before use*)

10X Cell Lysis Buffer*: 100mL
0.214g Potassium Acetate (KOAc) = 10mM
0.147g Magnesium Acetate (MgAc) = 15mM
10mL 1M Tris, pH 7.6 = 100mM in solution
add ultrapure H_2O to 100mL

5M NaCl*: 500mL
146.1g NaCl
add ultrapure H_2O to 500mL

Nuclei Lysis Buffer* ("NLB"): 100mL
5mL 1M Tris, pH 8.0 = 50mM
2mL 0.5M EDTA = 10mM
5mL 20% SDS = 1% SDS
add ultrapure H_2O to 100mL

IP Dilution Buffer*: 200mL
100uL 20% SDS = 0.01%
2.2mL Triton x100 = 0.909%
480uL 0.5M EDTA = 1.19mM
3.34mL 1M Tris, pH 8.0 = 16.7mM
6.68mL 5M NaCl = 166mM
add ultrapure H_2O to 200mL

Tris-EDTA* (TE): 1L
10mL 1M Tris, pH 7.6 = 10mM in solution
2mL 0.5M EDTA = 1mM in solution
add ultrapure H_2O to 1L

PMSF: 5mL
0.1742g/10mL = 0.1M solution in 100% isopropanol,
aliquot and store at -20C. Bring to room temperature before use.

Aprotinin: 1mL (we purchase 10mg)
10mg/mL in molecular grade water,
aliquot and store at -20C

Leupeptin: 1mL (we purchase 10mg)
10mg/mL in molecular grade water,
alquot and store at 4C

5X Proteinase K (PK) Buffer*: 100mL
5mL 1M Tris, pH 7.5 = 50mM in solution
5mL 0.5M EDTA = 25mM in solution
6.25mL 20% SDS = 1.25% in solution
add ultrapure H_2O to 100mL

1X Dialysis Buffer*: 1L (for polyclonal antibodies)
4mL 0.5M EDTA = 2mM in solution
50mL 1M Tris, pH 8.0 = 50mM in solution
10mL 20% Sarkosyl
add ultrapure H_2O to a final volume of 1L

IP Wash Buffer*: 1L (for polyclonal antibodies)
100mL 1M Tris, pH 9.0 = 100mM in solution
21.2g lithium chloride (LiCl)
10mL Igepal CA-630 (CMC 0.08mM) = 0.8uM in solution
10g deoxychloic acid
add ultrapure H_2O to a final volume of 1L

Elution Buffer*: 100mL
0.42g sodium bicarbonate ($NaHCO_3$)
5mL 20% SDS = 1% in solution
add ultrapure H_2O to a final volume of 100mL

Consumables/equipment needed:
Sterile 1.5mL screw-cap tubes (green cap)
Sterile 2mL screw-cap tubes (blue cap)
Acid-washed glass beads (215-300um size, Sigma-Aldrich cat# G1277)
Sterile Dounce Tissue Grinders, 15mL, "B" plunger (1 per sample, Fisher Scientific cat# K885300-0015)
Refrigerated microcentrifuge
Diagenode Bioruptor Sonication System (in cold room)
Electronic water baths
Dynabeads (Protein A (ThermoFisher cat# 10002D) or Protein G (ThermoFisher cat# 10004D))
200 Proof Ethanol (for use with ChIP only)
High-concentration RNase A (Roche cat# 11579681001)
Proteinase K (Roche cat# 03115828001)
Phenol/Chloroform
Chloroform
Glycogen (Roche cat# 10901393001)
Molecular grade water
Dynabead magnet

DAY 1

0. Before you begin:
    a. Pre-chill the refrigerated centrifuge to 4°C.
    b. Bring PMSF (in -20°C freezer) to room temperature (so all of the crystals dissolve), ~15min.
    c. Place leupeptin (in 4°C fridge) on ice.
    d. Thaw aprotinin (in -20°C freezer) and place on ice.
    e. Thaw pellets for ChIP on ice.
    f. Unwrap dounces and place them on ice (leave the pestle in its autoclave pouch).

    Tube setup for Day 1:
    g. Label a 15mL conical for each sample.
    h. Label 3, 2mL (blue-capped) tubes for each sample.
    i. Label one 2mL (blue-capped) tube for each sample, and include "NLB" for nuclei lysis buffer.
    j. Label 3, 1.5mL microcentrifuge tubes for each sample and weigh out 40mg glass beads per tube
    k. Label one 2mL (blue-capped) tube for your sheared chromatin (1 per sample)
    l. Label one 2mL (blue-capped) tube for each IP you plan to do

NOTE: KEEP SAMPLES ON ICE THROUGHOUT THE ENTIRE PROCEDURE (unless otherwise noted).

1. Make **Cell Lysis Buffer** for the number of pellets you need (per pellet = 5mL 1X Cell Lysis Buffer + 50uL PMSF):
   For a standard (2-sample) experiment:
   9mL molecular grade water (aliquot for ChIP only)
   1mL 10X Cell Lysis Buffer
   100uL PMSF
   10uL aprotinin
   10uL leupeptin
   Invert to mix, avoid forming bubbles.

2. Disrupt the cell pellets by flick mixing gently but thoroughly (this makes it easier to resuspend in Cell Lysis Buffer).

3. Add 5mL of **Cell Lysis Buffer** to each pellet (keep on ice), and invert to mix. Avoid forming bubbles. Incubate on ice for 20 minutes, inverting every 5 minutes.

4. Transfer (pour) cell suspension into the dounce and dounce 10 times with the pestle size "B". (When transferring, be sure to wipe off the outside of the 15mL conical tube to prevent ice/ water from dripping into the dounce.) Use a separate, sterile dounce for each sample.

5. Pour dounced sample into a new, labeled 15mL conical tube. (When transferring, be sure to wipe off the outside of the dounce to prevent ice/ water from dripping into the 15mL conical.)

6. Aliquot roughly equal volumes into the 3 labeled 2mL tubes you prepared for each sample (~1.6mL); setting the p1000 to 800uL makes this easier. Add a mark to the side of each tube as a reference (place those marks on the outside when centrifuging) so you know where the pellet will form. Centrifuge samples for 5min at 5000rpm and 4°C.

7. Carefully remove and discard supernatant using the p1000 (or aspirate). Do not touch or disturb the pellet (nuclei).

8. Supplement **Nuclei Lysis Buffer ("NLB")** with protease inhibitors, directly in the 2mL tubes you labeled, 1 tube per sample:
   (in each tube)
   625mL NLB
   6.25uL PMSF per tube
   0.625uL aprotinin
   0.625uL leupeptin

9. Using 500uL of **NLB**, sequentially resuspend all nuclei pellets for a given sample, and transfer back to the "NLB" tube you labeled (with the remaining, supplemented **NLB**). Repeat for all samples. Incubate on ice for 10 minutes.

10. Portion the lysed nuclei into equal volumes (200uL) in each of the 3, labeled, 1.5mL eppendorf tubes with 40mg , per sample. It is critical that the volumes in each of these tubes be equal. Repeat for all samples.

11. For sonication using the Bioruptor: *(Note that this protocol has been optimized for primary T cells; based on your samples of interest, this will need to be modified.)*
    1. Take a bucket of ice with you to the cold room, along with a clean, 250mL plastic beaker.
    2. Evenly space samples in the metal sample holder of the Bioruptor and screw down firmly (but not too firmly).
    3. Sonicate samples on HIGH (30 seconds on/ 30 seconds off) for 5 minutes.
    4. Remove the sample holder from the Bioruptor and place samples (still in the holder) into the ice.
    5. Using the 250mL beaker, remove as much water as possible from the Bioruptor reservoir and place in the labeled gallon container (for reused distilled water).
    6. Replace the water you removed with COLD (4°C) water using the 250mL beaker. Be sure not to fill past the line indicated in the reservoir.
    7. Place the samples (in the sample holder) back in the Bioruptor.
    8. Repeat steps #3-7 two more times (for a total of 15 minutes of sonication, with fresh, cold water every 5 minutes).

12. When finished, place the samples on ice to transport them back to the lab. Centrifuge the samples at maximum speed (14,800 rpm) for 10 minutes at 4°C, to pellet unwanted debris from lysis and sonication.

    Note: If the samples have become too cold, SDS may precipitate out of the nuclei lysis buffer. If this happens, use your hands to warm the sample up just enough to get the SDS back into solution before centrifugation.

13. Without disturbing the pellets that have formed, transfer the supernatants of all three aliquots of a given sample to a single, labeled, 2mL (blue-capped) tube. This is now your sheared chromatin.

14. Decide on the volume of chromatin you'd like to use for each IP. This will depend on the number of IPs you're planning to do. You will also need to save 20% of the volume of a single IP to use as "total" (this is the sample immediately following sonication, which will be reintroduced later in the procedure).

    Examples: Since the recovered volume after sonication will be ~600uL, the volume we'd use for 3 IPs would be 175uL per IP, with 35uL reserved for "total". For 4 IPs, we would use 125uL per IP, with 25uL reserved for "total".

15. Label tubes appropriately for IP (1 blue-capped, 2mL tube per IP). Label the tube of sonicated sample "PS total" for "post-sonicated total".

16. Place the volume of chromatin you calculated per IP (in step #13) in each labeled IP tube, for each sample. Place the remaining "PS total" samples in the 4°C ChIP box for use later.

17. Supplement 4mL of **IP Dilution Buffer** with protease inhibitors, as follows:
    4mL IP Dilution Buffer
    40uL PMSF
    4uL aprotinin
    4uL leupeptin

18. Add **IP Dilution Buffer** to each IP tube, at 2x the volume of chromatin you used. (For example, for 175uL chromatin per IP, you'd add 350uL of **IP Dilution Buffer** supplemented with protease inhibitors.)

19. Add the **appropriate antibody** to each IP, at the microgram amount optimized for each (must be determined empirically for each antibody). Rotate overnight at 4°C.

DAY 2

0. Before you begin:
    a. Pre-chill the refrigerated centrifuge to 4°C.
    b. Bring **PMSF** (in -20°C freezer) to room temperature (so all of the crystals dissolve), ~15min.
    c. Pre-heat the a waterbath to 65°C.
    d. Place **200 Proof Ethanol** (aliquot for ChIP only, in 50mL conical) at -20°C to chill.
    e. Place a bench soaker pad on your bench, setup the aspirator if not already prepared.
    f. Unbox and set out the Dynabeads magnet.

1. Quick-spin samples (310rpm) and place on ice.

2. Label one 1.5mL eppendorf tube per IP (or number them—just keep track of which sample is which).

3. Swirl the appropriate **Dynabeads** (Protein A or Protein G, depending on host species of antibodies for a given experiment, stored at 4°C) gently to resuspend, and place 70uL (using the p200) of beads in each of your labeled tubes.

4. Open the tubes in the eppendorf tube rack and then place on the magnet for ~1 minute. Slowly aspirate off the supernatant, being careful not to touch the mag beads.

5. Using a single pipet tip (without touching the tubes) eject 400uL of **Wash and Bind Buffer** over the beads in each tube, while still on the magnet. (This ensures that the beads do not dry out.)

6. Remove the tubes from the magnet and use a fresh tip for each tube to resuspend the beads in the buffer.

7. Open the tubes in the eppendorf tube rack and then place on the magnet for ~1 minute. Slowly aspirate off the supernatant, being careful not to touch the mag beads.

8. Add the appropriate IP sample to each of the tubes with the mag beads. Be sure to add all IP samples to the beads first (changing tips each time), *then* go back and resuspend (using a fresh tip each time) to ensure that the beads do not dry out.

9. Rotate the resuspended mag beads + IPs at 4°C for 1 to 1.5 hours. When there are ~15 minutes left in the incubation, supplement both **1X Dialysis Buffer** and **IP Wash Buffer** with **PMSF** by adding 80uL of PMSF to 8mL of each respective buffer in a 15mL conical tube (1:100 dilution of PMSF). Invert to mix. Keep on ice.

10. After the incubation, centrifuge the IP samples at 3000rpm for 1 minute at 4°C. Bring the centrifuge to room temperature afterwards, for the next steps.

11. Open the tubes in the eppendorf tube rack and then place on the magnet for ~1 minute. Slowly aspirate off the supernatant, being careful not to touch the mag beads.

12. Perform 4 washes as follows:
    (Use a single pipet tip to add the buffers listed below to all of the tubes at once, without touching the tubes/ samples. Then, remove the tubes from the magnet and resuspend each sample in the given buffer using a fresh pipet tip for each sample. Place back on the magnet for ~1 minute, then slowly aspirate the supernatant (remember to change tips between samples) before moving on to the next wash.):
    1. 500uL **1X Dialysis Buffer**
    2. 500uL **IP Wash Buffer**
    3. 500uL **IP Wash Buffer**
    4. 250uL **1X Dialysis Buffer**

13. After resuspending in the final wash (250uL **1X Dialysis Buffer**), transfer the resuspended magnetic beads to a fresh, labeled 1.5mL eppendorf tube (1 per sample).

14. Place the tubes on the magnet for ~1 minute, then slowly aspirate off the supernatant.

****At this point, turn off the aspirator.****

15. Using a single pipet tip, add 150uL (using the p1000) of **Elution Buffer** to each sample all at once, without touching the tubes or samples. Then, remove the tubes from the magnet and use a fresh tip each time to resuspend each sample.

16. Incubate samples at 65°C for 15 minutes.

17. Remove the samples from the water bath and centrifuge at 3000rpm for 1 minute at room temperature.

18. Open the tubes in an eppendorf tube rack and then place tubes on the magnet for ~1 minute. Pipet supernatant (eluate) into a fresh, labeled, 1.5mL eppendorf tube using the p200.

19. Repeat steps #15-18, combining eluates from both elution steps. Leave the first eluate at room temperature during the second elution from the beads.

20. Centrifuge samples at 14,800rpm for 4 minutes at room temperature. Without disturbing the pellet, transfer supernatant (~300uL) to a labeled 2mL screw-cap (blue capped) tube for each sample.

21. At this point, the "Total" samples need to be added back into the experiment. Label a 1.5mL screw-cap (green capped) tube for each sample set (one for each "Total" sample you will have). Take the "PS Total" samples from the 4°C fridge, and transfer the 20% volume you calculated on Day 1 (step 13) to the newly labeled tube.

    a. Example: If the IP volume was 175uL, the "Total" volume (what you'd take from the "PS Total" samples you reserved on Day 1) would be 35uL.

22. Bring the "Total" volume up to 300uL with **Elution Buffer**.

23. Make **RNase A Mastermix** as follows (for each sample +1):
    12uL 5M NaCl
    1uL High-concentration RNase A

24. Add 13uL of **RNase A Mastermix** to each sample and tap the wall of each tube gently to mix.

25. Place tubes at 65°C for at least 4 hours.

26. Quick-spin samples to collect condensation, and add 800uL of ice-cold **200 Proof Ethanol** to each tube. Invert 10 times to mix and incubate overnight at -20°C.

DAY 3

0. Before you begin:
    a. Pre-chill the refrigerated centrifuge to 4°C.
    b. Pre-heat 42°C water bath to 45°C.
    c. Place **200 Proof Ethanol** in the -20°C freezer.
    d. Label 1 set of 1.5mL eppendorf tubes per sample.
    e. Label 1 set of 1.5mL screw cap tubes (green caps) per sample.

f. Be sure that you have **Phenol/Chloroform** prepared and at 4°C.

1. Centrifuge precipitated samples at 14,800rpm for 20 minutes at 4°C.

2. Remove 950uL of supernatant and discard—be careful that the pellet does not slip.

3. Centrifuge at 14,800rpm for 2 minutes at 4°C.

4. Remove remaining supernatant, again being careful to avoid the pellet.

5. Allow pellets to air-dry for 30-60 minutes (until residual ethanol is gone but pellet is not over-dried).

6. Resuspend sample in 100uL of **TE**.

7. Make up a **PK Mastermix** as follows:
    a. Per sample +1 = 25uL 5X PK buffer, 1.5uL PK (I made up 75uL 5X PK buffer + 4.5uL PK)

8. Add 26.5uL of **PK Mastermix** to each sample and flick gently to mix. Quick spin to collect at the bottom of the tube, and incubate at 45°C for 1-2 hours.

9. Remove samples from water bath and quick spin to collect any condensation. Add 175uL **TE** to bring each sample volume up to 300uL.

10. Add 300uL **Phenol/Chloroform** (*bottom layer in the stock only*, pipet up and down once in the solution to remove any residual top layer from the pipet tip).

11. Vortex vigorously for 5 seconds to mix.

12. Centrifuge samples at 14,800rpm for 5 minutes at RT to separate layers completely.

13. Using a p200 set to 165uL, remove the top layer in 2 pulls and transfer to the appropriately labeled 1.5mL eppendorf tube.

14. Add 290uL of **Chloroform** (only). Repeat steps #11-12. Repeat step #13 but, this time, transfer the top layer to the appropriately labeled 1.5mL screw cap tube (green cap).

15. Make up **Glycogen Mastermix** as follows:
    a. Per sample +1 = 30uL 5M NaCl + 1uL glycogen (I made up 90uL 5M NaCl + 3uL glycogen)

16. Add 31uL of the **Glycogen Mastermix** to each tube prior to the last extraction spin; add the samples to the glycogen mastermix.

17. Add 750uL of ice-cold **200 Proof Ethanol** to each sample. Invert thoroughly. Incubate at -20°C overnight, OR at -80°C for at least 1 hour.

18. Centrifuge samples at 14,800rpm for 20 minutes at 4°C. Remove 950uL of supernatant and discard.

19. Centrifuge again for 2-3 minutes and remove remaining ethanol with p200 pipet. Do not disturb the pellet.

20. Allow pellets to air dry for 30 minutes to 1 hour (until residual ethanol is gone but the pellet is not over-dried).

21. Resuspend final DNA pellet in 41uL **Molecular Grade Water** (aliquot for ChIP only). Resuspend by repeat pipetting and vortexing. Allow samples to sit for a minimum of 1 hour at RT or overnight at 4°C before use. Store at 4°C for future use.