CNON Cell Culture

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Growing a single CNON cell line from the defrost step to 12x15cm plates will use 500mL of 4506 media. It is recommended that you have at least 750mL of 4506 media and 200mL of Coon's media available prior to starting this protocol. CNON should always be passaged at or before they are 80-90% confluent to ensure that cells are maintained in the exponential growth phase. Growing cells beyond this point can lead to changes in gene expression and epigenetic marks, and can potentially cause cells to differentiate or enter senescence. It is important to note that the CNON are primary human cells derived from biopsy material and have not been tested for potential pathogens; therefore, they should always be handled with caution.

A. Day 1: Defrosting CNON cells

Note: It is extremely important to keep Matrigel on ice at all times and keep all cultureware/media coming in contact with Matrigel pre-chilled/ice-cold. The Matrigel will start to gel above 10°C. Warm media in 37˚C water bath prior to use.

1. Prepare one 10cm plate per vial with an even, thin layer of 375 µl Matrigel (1:10) per plate.
2. Incubate plates with Matrigel at 37˚C for at least 10 min.
3. Place the CNON sample vial in a 37˚C water bath for no more than 5 min.
4. Once thawed, transfer the cells into a new 15mL tube.
5. Rinse CNON sample tube with 4mL of Coon's media into 15mL tube.
6. Centrifuge cells at 2000rpm (470rcf) for 7 min.
7. Discard supernatant by pipetting.
8. Resuspend cells with 10mL of 4506 media.
9. Pipet 10 mL of cells (from a single vial) into the Matrigel-coated 10cm plate prepared in steps 1 and 2.

B. Day 2:

1. Replace media with 10mL of fresh 4506 media.
2. Cells should reach 80-90% confluence by Day 3-4 after thawing and be ready to passage from one 10cm to three 10cm plates.

C. Day 3 or 4: Passage from one 10cm to three 10cm plates

1. Prepare three 10cm plates (per each vial of thawed cells) with an even, thin layer of 375µl Matrigel (1:10) per plate.
2. Incubate the new Matrigel-coated plates at 37˚C for at least 10 min.
3. Discard media from each 10cm plate containing CNON cells (from step A9).
4. Pipet 2mL of 1x Dispase into each CNON-containing 10cm plate to cover the whole surface of the plate.
5. Incubate the Dispase-containing plates at 37˚C for 30-60min.
6. After 30 min, use the microscope to check if the cells have detached. If not, let the sample sit in incubator for up to an hour.
7. Scrape cells to detach from surface using sterile cell scraper.
8. Collect all of the Dispase and cells using a pipet and put the cells from one 10 cm plate in one 15mL tube.

9. Pipet 2mL Coon's media into each scraped plate to detach and collect any remaining cells in the same 15mL tube by pipetting; combine with the original 2 mL of scraped cells. Using a microscope, make sure to check that you have harvested nearly all of the cells on the plate. If not, add more Coon's media and scrape the plate again.
10. Centrifuge the cells for 7 min at 2000rpm (470rcf).
11. Aspirate or pipette off the supernatant being careful not to disturb the pellet.
12. Add 3 mL of 4506 media to the pellet and resuspend cells.
13. Pipet 9 mL of 4506 media into each of the three Matrigel-coated 10cm plates prepared in steps 1 and 2.
14. Pipet 1 mL of resuspended cells into each of the three Matrigel-coated 10cm plates.
15. Cells should reach 80-90% confluence in 2-3 days (Day 5-7 after thawing) and then should be ready to passage from the three 10cm plates to six 15cm plates. Do not allow cells to overgrow.

D. Day 5-7: Passage from three 10cm to six 15cm plates

1. Prepare six 15cm plates (per defrosted vial of cells) with an even thin layer of 750 µL Matrigel (1:10) per plate.
2. Incubate at 37˚C for at least 10 min.
3. Discard media from the three 10cm plates containing CNON cells.
4. Pipet 2 mL of 1x Dispase into each 10cm plate to cover the whole surface of the plates containing CNON cells.
5. Incubate the Dispase-containing plates at 37˚C for 30-60min.
6. After 30min, use the microscope to check if the cells have detached. If not, let the sample sit in incubator for up to an hour.
7. Scrape cells to detach from surface using sterile cell scraper.
8. Collect all of the Dispase and cells using a pipet and combine the cells from the 3 10 cm plates in a 50 mL tube.
9. Pipet 4mL Coon's media into each of the six scraped plates to detach and collect any remaining cells; combine with the original scraped cells in the same 50mL tube. Make sure to check that nearly all cells have detached under the microscope. If not, add more Coon's media and scrape the plate again.
10. Centrifuge the sample for 7 min at 2000rpm (470rcf).
11. Aspirate or pipette off the supernatant being careful not to disturb the pellet.
12. Pipet 12mL of 4506 media into the pellet and resuspend cells.
13. Pipet 18mL of 4506 media into each of the six Matrigel coated 15cm plates prepared in steps 1 and 2.
14. Pipet 2mL of resuspended cells into each of the six Matrigel coated 15cm plates prepared in steps 1 and 2.
15. Cells should reach 80-90% confluence in 2-3 days (Day 8-10 after thawing) and then should be ready to passage from six 15 cm plates to twelve 15 cm plates. Do not allow cells to overgrow.

E. Day 8-10: Passage from six 15cm to twelve 15cm

1. Prepare twelve 15cm plates with an even thin layer of 750µL Matrigel (1:10) per plate.
2. Incubate at 37˚C for at least 10 min.
3. Discard media from each of the six 15cm plates containing CNON cells.
4. Pipet 2mL of 1xDispase into each 15 cm plate containing CNON cells to cover the whole surface of the plate.
5. Incubate the cells at 37˚C for 30-60min.
6. After 30min, use the microscope to check if the cells have detached. If not, let the sample to sit in incubator for up to an hour.
7. Scrape cells to detach from surface using sterile cell scraper.
8. Collect all of the Dispase and cells using a pipet and combine the cells from all six 15 cm plates in a 50 mL tube.

9. Pipet 4mL of Coon's media into each of the six scraped plate to detach and collect any remaining cells; combine with the original scraped cells in the same 50mL tube. Make sure to check that nearly all cells have detached under the microscope. If not, add more Coon's media and scrape the plate again.
10. Centrifuge the sample for 7 min at 2000rpm (470rcf).
11. Aspirate or pipette off the supernatant being careful not to disturb the pellet.
12. Pipet 24mL of 4506 media into the pellet and resuspend cells.
13. Pipet 18mL of 4506 media into each of the twelve Matrigel coated 15cm plates prepared in steps 1 and 2.
14. Pipet 2mL of resuspended cells into each of the twelve Matrigel coated 15cm plates.
15. Cells should be ready to harvest for ChIP-seq and/or NOMe-seq when they are 80-90% confluent within 3-4 days; If cells are more confluent than 80%, they should be repassaged before harvesting for gene expression of epigenomic experiments.

F. Freezing cell stocks

1. Plate one 10cm plate and allow cells to reach 80-90% confluence.
2. Discard media.
3. Pipet 1mL of 1x Dispase into the 10 cm plate to cover the whole surface of the plate.
4. Incubate the cells at 37˚C for 30-60min.
5. After 30 min, use the microscope to check if the cells have detached. If not, let the sample sit in incubator for up to an hour.
6. Scrape cells to detach from surface using sterile cell scraper.
7. Collect all of the Dispase and combine the cells from both plates in one 15mL tube.
8. Pipet 2mL Coon's media into each plate to detach and collect any remaining cells; combine with the original scraped cells in the same 15mL tube. Make sure to check that nearly all cells have detached under the microscope. If not, add more Coon's media and scrape the plate again.
9. Centrifuge the sample for 7 min at 2000rpm (470rcf).
10. Aspirate or pipette off the supernatant being careful not to disturb the pellet.
11. Resuspend cells in 6mL freezing medium.
12. Transfer 1mL to each of 6 cryovials and freeze at -80˚C overnight in a cryo-freezing container.
13. The next day, transfer cryovials to liquid nitrogen storage.

Media Preparation:

(Components marked with * are working stocks)

Matrigel (1:10)*

Coon's media	4500µL
Matrigel	500µL

*Thaw Matrigel on ice at 4˚C overnight
*Use prechilled tips and media to make dilution
*Store at -20˚C

Dispase (10x)

Dispase	0.1g
PBS	fill to 10mL

*Filter sterilize
*Store at -20˚C
*Light sensitive

Dispase (1x)*

Dispase (10x)	1mL
PBS	9mL

*Store at -20˚C
*Light sensitive

Sodium selenite (100µg/mL)

Sodium selenite	1mg bottle
Coon's media	add 10mL

*Store at -20˚C

Sodium selenite (1:10)*

Sodium selenite (100µg/mL)	1mL
Coon's media	9mL

*Store at 20˚C

Thyroxine (100µg/mL)

Thyroxine	1mg bottle
Coon's media	add 10mL

*Store at -20˚C

Thyroxine (1:10)

Thyroxine (100µg/mL)	1mL
Coon's media	9mL

*Store at -20˚C

Thyroxine (1:100)*

Thyroxine (1:10)	1mL
Coon's media	9mL

*Store at -20˚C

Transferrin (50mg/mL)

Transferrin	100mg bottle
Coon's media	add 2mL

*Store at -20˚C

Transferrin (10mg/mL)*

Transferrin (50mg/mL)	1 mL
Coon's media	4mL

*Store at -20˚C

Coon's media (1L)

Millipore H_20	800mL
Sodium bicarbonate	2.68g
Ham's F-12	11.5g (one bottle)

- Use stir bar to mix
- Adjust to pH 7.2 with HCl
- Fill to 1L
- Filter sterilize using 0.22µM low protein binding membrane

4506 Media (500 mL)	**Volume**	**Final Conc.**
Coon's Media	455mL	--
Fetal bovine serum	30mL	6%
Transferrin (Human) (10mg/mL)	250µL	5µg/mL
Insulin (10mg/mL)	50µL	1µg/mL
Hydrocortisone (50µM)	100µL	10nM
Sodium selenite (1:10)	125µL	2.5ng/mL
Thyroxine (1:100)	20µL	40pg/mL
Antibiotic-Antimycotic (100x)	5mL	1%
Bovine pituitary extract (13mg/mL)	400µL	50µg/mL
Endothelial Cell Growth (15mg/bottle)	5 bottle	150 µg/mL

- Note: dissolve ECG powder in each bottle in 2mL of Coon's media
- Combine all the reagents for 4506 in a beaker
- Filter the media using 0.22µm low protein binding membrane
- Store at 4˚C

Freezing media (100mL)	**Volume**	**Final Conc.**
4506 Media	75mL	75%
FBS	20mL	20%
DMSO	5mL	5%

- Combine all the reagents for freezing media in a beaker
- Filter the media using 0.22µm low protein binding membrane
- Store at -20˚C

Reagents Used:

Reagent	Vendor	Catalog number
100X Antibiotic-Antimycotic (100 mL)	Life Technology	15240062
Fetal Bovine Serum, Qualified, USDA Approved Regions, 500 mL	Life Technology	26140079
Nutrient Mixture F-12 HAM	Sigma Aldrich	F6636-10X1L
Transferrin (Human)	Sigma Aldrich	T8158-100MG
Hydrocortisone solution (50 UM)	Sigma Aldrich	H6909-10ML
Sodium selenite G-Irradiated, BIOXTRA	Sigma Aldrich	S9133-1MG
L-Thyroxine sodium salt pentahydrate (gamma-irradiated)	Sigma Aldrich	T0397-1MG
Insulin Human, Recombinant	Sigma Aldrich	I2643-25MG
Pituitary extract, Bovine	Sigma Aldrich	P1476-25ML
Endothelial cell growth, 10 bottles	Millipore	02-102
Matrigel (10mL)	Corning	354234
Dispase	Gibco	17105-041

Farnham Lab ChIP & Library Construction Protocol for CNON cells

(October 4, 2016 version)

We are using CNON cells for this project (see CNON cell growth protocol). Before beginning the experiment, record all cell information (e.g patient number, passage number, unusual growth conditions, etc) for future reference. Note that cells should be cross-linked at 80% confluence with ~10% of the cell population in mitosis, which indicates a healthy cell population. **Overgrowth of cells will result in failed ChIPs.** We are growing twelve 15cm plates per sample for this project to perform several histone ChIPs and NOMe-seq on the same cell population. We anticipate that roughly 100 ug of chromatin can be obtained from the twelve plates. We require 10µg of chromatin for each histone ChIP; also keep in mind that up to 5µg of chromatin will be needed every time you check the fragment size after sonication. **Use filtered tips throughout protocol to avoid DNA contamination in sequencing results**.

A. Crosslinking Cells: Day 0/1

1. In a chemical hood, add 540µL formaldehyde (from the 37% stock bottle) directly to the culture media (20 mLs) of each 15cm plate to a final concentration of 1%. Place the adherent CNON cells on a shaking platform in the chemical hood for no more than 10 minutes at room temperature. **Do not cross-link for longer periods since this WILL cause cells to form aggregates that do not sonicate efficiently.** Note: Media containing formaldehyde should be treated as hazardous waste until glycine inactivates formaldehyde in next step.
2. Stop crosslinking reaction by adding glycine to a final concentration of 0.125M. We use 2.2mL of a 10X (1.25M) stock solution per 15cm plate. Continue to agitate ≥5 min @ RT.
3. Pour off media and rinse plates twice with ice-cold 1XPBS. Use a cell scraper to scrape cells into a 50mL tube on ice, using the residual PBS on plates. Use a few extra mLs of PBS containing protease inhibitors to collect all cells from the plate.
4. Centrifuge the 50mL tube of crosslinked cells at 430 rcf for 5 min at 4ºC. Leave ~1mL of supernatant to transfer cell pellet to a labeled 1.5mL tube. Note: It is important to carefully aspirate supernatant so as to not lose cells.
5. Centrifuge the 1.5mL tube crosslinked cells at 430 rcf for 5 min at 4ºC. Carefully remove all supernatant using a pipette or aspirator. Do not pour off supernatant. Note: It is important not to leave any remaining supernatant as doing so can result in cell lysis and loss of chromatin.
6. Snap freeze cell pellet in liquid nitrogen and store at -80°C.
7. Cells may now be used immediately (preferred method) for a chromatin preparation or snap frozen in liquid nitrogen and stored at -80°C. Freezing cells at this stage can result in a loss of chromatin, as some nuclei will inevitably rupture. Additionally, any excess PBS not removed in previous step will rupture more cells resulting in additional loss of material.

B. Chromatin Sonication: Day 1

1. Prepare in advance CLB* (Cell Lysis Buffer plus additional required reagents). First, add Igepal (10µL NP40 per mL of stock CLB) to water, agitate at 37°C to dissolve, and then cool on ice. Then add PIPES and KCl and protease inhibitors (see recipes for details on protease inhibitors).
2. Resuspend cross-linked cells on ice by pipetting a few times with 500µL of ice-cold CLB*. Then, transfer cells to a 15mL tube and add 20x the volume of the cross-linked cell pellet with cold CLB* by washing the 50ml tube several times with 500µl of ice-cold CLB* and adding to the 15 ml tube.
3. Incubate on ice for 15 minutes to allow cells to swell and lyse. If using frozen cross-linked cells, thaw them on ice; keep all cells and chromatin samples on ice at all times.
4. Homogenize cells using the appropriate size (2mL or 7mL) glass dounce homogenizer (type B) to break open the cells and release nuclei. Homogenize cells on ice with 20 strokes. Place sample back into previous tube.
5. Centrifuge the cross-linked cells at 430 rcf for 5 min at 4°C. Discard the supernatant. Use ~500µL of CLB* to transfer sample into a new 1.5mL and repeat spin down. Discard supernatant. You may snap-freeze the nuclear pellet and store at -80°C for later use. **This is the preferred stopping point for sample prep.**
6. Resuspend pellet in nuclei lysis buffer plus protease inhibitors with 5X the volume of the pellet present. Nuclei concentrations that are too high can lead to inefficient sonication and nuclei that are too dilute will result in higher amounts of SDS which will then require much larger volumes of IP dilution buffer (making sample handling more labor intensive).
7. Incubate on ice for 30 minutes. You may snap-freeze the nuclei in nuclei lysis buffer to aid in the lysis of

cells before continuing with sonication.

8. Sonicate cells in cold environment to achieve an average chromatin length of 200-1000bp. Sonication conditions should be optimized for each cell type. For CNON cells, we commonly sonicate 10 minutes (~10 pulses of 30 seconds at the high setting on a Bioruptor Pico using appropriate weight of sonication beads, 30 second pause in between pulses). Volumes between 0.5mL and 2mL are sonicated in 15mL polystyrene tubes and volumes between 0.1 and 0.3mL are sonicated in specialized 1.5mL polystyrene tubes.
9. Transfer sonicated material into 1.5mL tubes (use multiple 1.5mL tubes if volume is more than 1.4mL) and centrifuge at maximum speed for 10 minutes at 4°C. (If samples were sonicated in polystyrene tubes, be sure to transfer to polypropylene tubes for the hard spin, otherwise the tubes will likely crack.) Carefully transfer the supernatant (sonicated chromatin) to a new tube while avoiding cell debris. Keep sonicated material (chromatin and cell debris) at 4°C while performing quantification and determining chromatin size. You may need to recombine the cell debris pellet and supernatant (containing the chromatin) to continue further sonication of all the material.

C. Determining Chromatin Size & Quantification: Day 1

1. Transfer 40µl of sonicated chromatin to a 500µL tube.
2. Add 60µL ChIP elution buffer and 12µL of 5M NaCl.
3. Boil samples in water bath for 15 minutes to reverse cross-links.
4. Allow sample to cool down, add 1µL DNase-free RNase (Thermo Scientific cat# EN0531), and incubate 10 minutes at 37ºC. This step is important because the presence of RNA results in false estimation of chromatin size.
5. Purify DNA using a PCR purification kit (Qiagen cat# 28106), elute in 30µL EB. Measure DNA concentration by NanoDrop and calculate the chromatin yield. Cells typically yield ~2µg of material per 5x10^5 cells.
6. Run 1.5-2µg of DNA on a 1.5% agarose gel to visualize average size of chromatin. MAKE SURE TO USE 10X ORANGE G LOADING DYE as using a different loading dye leads to a bleaching out in the lane where the dyes are present and makes it impossible to determine sonication efficiency. If the chromatin is larger than ~1000bp, resuspend the cell debris pellet with the chromatin and repeat steps 1-5 until desired range of 200 to 1000bp is obtained. If samples are taking much longer than 20 minutes of sonication to achieve desired size, do not proceed. This is an indication that over-crosslinking has occurred and your cross-linking protocol may need to be modified to decrease the amount of cross-linking. Alternatively, the instrument being used for sonication may have lost motor power, which does happen with normal wear and tear on sonication instruments.

D. Chromatin Immunoprecipitation: Day 1

1. Use 10-20µg of chromatin for each Histone ChIP (20µg of chromatin may be preferable for Histone ChIPs that are more difficult, such as broad marks).
2. Remove 500ng of chromatin for the **Input** sample and bring to a total volume of 150µl with ChIP elution buffer. Store this amount at -20ºC until the next day and then reverse the cross-links in the Input chromatin at the same time the crosslinks in the ChIP samples are reversed.
3. Dilute chromatin with at least 5-fold ice-cold IP dilution buffer containing protease inhibitors. This ensures that the SDS concentration is not too high for antibody binding.
4. Add antibody specific to the Histone Mark of interest to capture the protein/chromatin complexes. **Record amount of chromatin and antibody, the catalogue number and lot number of antibodies for future reference!**
5. Incubate on a rotating platform at 4°C overnight.

Chromatin Amount	Antibody name	Antibody Provider	Antibody Cat#	Antibody Amount
10µg	H3K4me3	Cell Signaling Technology	9751S	10µL
10µg	H3K27ac	Active Motif	39133	4µg
10µg	H3K27me3	Cell Signaling Technology	9733	10µL
20µg	CTCF	Active Motif	61311	5µL

20µg	CTCF	Cell Signaling Technology	3418S	10µL

E. Capture Antibody/Chromatin Complex and Reverse Crosslinks: Day 2
------All the following steps are carried out at room temperature------

1. Wash beads in 1mL IP wash buffer 1 (IPWB1) twice and resuspend with starting volume of beads.
2. Add 15µL magnetic protein A/G beads per Histone ChIP sample and incubate on a rotating platform for 2 hrs at 4°C. We use magnetic protein A/G bead mix from Pierce Protein A/G (#88803). **These magnetic beads are DIFFERENT THAN PCR CLEAN UP AMPURE BEADS!**
3. Allow beads to settle for 1 min in a magnetic separation rack, then carefully remove the supernatant.
4. Wash magnetic beads two times with IP wash buffer 1 (RIPA buffer without protease inhibitors); take tubes out of magnetic rack and mix by inverting the tube. Efficient washing is critical to reduce background. Avoid cross-contamination of samples and loss of magnetic beads.
5. Wash magnetic beads three times with IP wash buffer 2 (IPWB2), take tubes out of magnetic rack and mix by inverting the tube. ***Transfer to a new tube for your final wash. This will help eliminate background since a lot of DNA ends up sticking to the walls of the tube and the elution step will release this "sticky" DNA into your sample. Switch to a new tube on your last wash. Remove all wash solution after final wash by spinning down and using 10µL pipette.**
6. Elute antibody/chromatin complexes by adding 75µL IP elution buffer. Shake on vortexer for 30 minutes, setting 2.
7. Allow beads to settle for 1 min in a magnetic separation rack. Carefully transfer supernatant containing antibody/chromatin complexes to a new tube.
8. Repeat steps 6 and 7, but combine the second 75µL elution with the first to obtain a total of 150µL of material.
9. At this point, thaw the Input sample(s) from the previous day. Add 20µL of 5M NaCl per 150µL elution buffer (to give approx. 0.6M final concentration) for each ChIP and Input.
10. Incubate all samples at 67°C overnight to reverse formaldehyde crosslinks.

F. DNA Purification: Day 3

1. Purify DNA with a Qiagen MinElute PCR clean up kit, one column per sample. Elute each sample with 50µL EB.

ChIP Buffers List

Protease inhibitor stock solutions (stored at -20ºC) Note: Protease inhibitors are added to ice-cold solutions at time of use only. Do not add protease inhibitors to IP wash and elution buffers. A mixture of protease inhibitors (1) or a protease inhibitor cocktail may be used as described below.

1) Aprotinin (10mg/mL), Leupeptin in water (10mg/mL), 100 mM PMSF in isopropanol, 1M DTT in PBS.
   [Volume PI per Buffer/Solution Volume = Aprotinin (1µL/mL, *we use 13µL/mL of our VWR Aprotinin soln (cat# 80603-220) that is stored at 4ºC*), Leupeptin (1µL/mL), PMSF (10µL/mL), DTT (1µL/mL)]
2) 200X Protease Inhibitor Cocktail, VWR catalogue #80053-852

Cell Lysis Buffer
5mM PIPES pH 8.0
85mM KCl
1% Igepal/NP40 (10µL/mL), agitate at 37°C to dissolve
Add protease inhibitors when cold

Nuclei Lysis Buffer
50mM Tris-Cl pH 8.1
10mM EDTA
1% SDS
Add protease inhibitors when cold
SDS will fall out of solution when cold, this does not affect solution as long as adequate mixing occurs before use

IP Dilution Buffer (RIPA)
50mM Tris pH 7.4
150mM NaCl
1% Igepal/NP40 (10µL/mL)
0.25% Deoxycholic acid
some brands of Deoxycholic acid do not dissolve in solution, we use Fisher Scientific cat# BP349
1mM EDTA pH 8.0
Add protease inhibitors when cold

IP Wash Buffer 1
50mM Tris pH 7.4
150mM NaCl
1% (10µL/mL) NP40
0.25% Deoxycholic acid
some brands of Deoxycholic acid do not dissolve in solution, we use Fisher Scientific cat# BP349
1mM EDTA pH 8.0

IP Wash Buffer 2
100mM Tris-Cl pH 9.0
500mM LiCl
1% Igepal/NP40
1% Deoxycholic acid
some brands of Deoxycholic acid do not dissolve in solution, we use Fisher Scientific cat# BP349

ChIP Elution Buffer
50mM $NaHCO_3$
1% SDS

Library Construction Protocol

This protocol describes the preparation of libraries using the **KAPA Hyper Prep Kit from KAPA Biosystems (#KK8505)**. Libraries are prepared from the ChIP sample, as well as from Input DNA from the corresponding cell type. It is not necessary to make a fresh Input library every time. You may save library reagents and use an Input library from the same cell type prepared previously for checking enrichment levels by qPCR in the last step. All clean up steps are with PCR Clean Up Ampure magnetic beads (Agencourt Ampure, Beckman Coulter, cat# A29152), **not to be confused with the magnetic protein A/G beads used in the previous ChIP portion of the protocol.**

Step 1: End-Repair and A-Tailing

This step ensures that all DNA fragments are converted to blunt-ended, 5'-phosphorylated DNA.

A) Combine and mix the following components in a PCR tube:

50µL	ChIP DNA (entire ChIP sample) -or- 100ng Input DNA (10µL) and 40µL EB
7µL	End Repair and A-Tailing Buffer
3µL	End Repair and A-Tailing Enzyme Mix
60µL	Total Volume

B) Mix thoroughly and centrifuge briefly.

C) Incubate in a thermocycler with the following profile:

20˚C → 30 min
65˚C → 30 min
10˚C → ∞

D) **Proceed immediately to the next step.**

Step 2: Illumina Barcode Adapter Ligation (BIOO Adapters)

BIOO supplies their NEXTflex™ DNA Barcodes adapters at 25µM, so make a 1:17 dilution (final concentration 1.5µM).

A) Combine and mix the following components in PCR tube:

60µL	End Repair and A-Tailing Reaction Product
9µL	EB
30µL	Ligation Buffer
1µL	BIOO Adapter (1.5µM)
10µL	DNA Ligase
110µL	Total Volume

B) Mix thoroughly and centrifuge briefly

C) Incubate at 20˚C for 15 min

D) **Proceed immediately to the next step.**

Step 3: Post-Ligation Clean Up

A) Perform a low ratio (0.8x) bead clean up by combining the following:

110µL	Adapter Ligation Reaction Product
88µL	Ampure Magnetic Beads
198µL	Total Volume

B) Thoroughly mix beads and incubate 5-15 min to bind DNA to beads.

C) Place tube on a magnet to capture beads until solution looks clear. Beads may take several minutes to pellet.

D) Carefully remove and discard supernatant. **Do not allow the beads to dry.**

E) Wash 2x with 200µL 80% freshly made ethanol. During all washes, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping.

F) Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.

G) Resuspend beads with 30µL of EB and incubate for 2 min at room temperature to elute DNA off the beads.

H) Place tube on magnet and transfer clear solution to a new tube.
I) Perform a second low ratio (0.8x) bead clean up with 24µL Ampure magnetic beads.
J) Thoroughly mix beads and incubate 5-15 min to bind DNA to beads.
K) Place tube on a magnet to capture beads until solution looks clear. Beads may take several minutes to pellet.
L) Carefully remove and discard supernatant. **Do <u>not</u> allow the beads to dry.**
M) Wash 2x with 200µL 80% freshly made ethanol. During all washes, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping.
N) Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.
O) Resuspend beads with 20µL of EB and incubate for 2 min at room temperature to elute DNA off the beads.
P) Place tube on magnet and transfer clear solution to a new tube.

ONLY HALF OF LIGATED MATERIAL WILL BE USED IN NEXT STEP

Step 4: Amplification of Adapter-Modified DNA Fragments

For BIOO adapters, make sure to use the supplied BIOO primer mix with a 12.5µM conc. Dilute the mix 1:2 (final concentration 6.25µM) and use 1µL per 50µL PCR reaction (as outlined in step A below).

Use only half of the adapter-ligated material and proceed with 12 cycles of PCR for all ChIP-seq library samples and 10 cycles of PCR for all Input library samples. Depending on how 12 or 10 cycles of amplification works out, you have the option of using the other half of adapter ligated material if the sample is too overamplified. If 12 or 10 cycles is too low, simply take the same material and redo amplification with fresh reagents with an appropriate number of additional cycles.

A) Combine and mix the following components in PCR tubes:

10µL	Adapater Ligated Reaction Product
14µL	EB
25µL	KAPA HiFi HotStart Ready Mix
<u>1µL</u>	<u>BIOO PCR primer mix (6.25uM)</u>
50µL	Total Volume

B) Amplify using the following PCR protocol:

98ºC → 45 sec
10-15 cycles:
    98ºC → 15 sec
    60ºC → 30 sec
    72ºC → 30 sec
72ºC → 1 min
10ºC → ∞

Over-amplification should be avoided to reduce potential PCR artifacts.

Step 5: Library Purification, Quality and Quantification

A) Purify with 40µL Ampure magnetic beads (0.8x ratio volume).
B) Elute in 20µL EB.
C) Place tubes back in magnetic stands to collect the DNA and transfer liquid to new tubes.
D) Check concentration with High Sensitivity DNA Qubit.
E) Check quality by BioAnalyzer. If excessive amount of primer dimer (85bp) and/or adapters (120bp) are present, redo the Ampure bead clean up with a 0.8x volume ratio which allows for capturing larger size fragments and loss of smaller fragments, such as the primer and/or adapter dimers.

Step 6: Library Confirmation

Check enrichment using positive and negative target primers (**see Table below**). Enrichment of marks in the ChIP-seq libraries are determined by quantitative real-time PCR (qPCR). The ChIP-seq Library and Input Library are diluted with EB to give a final concentration of no more than 1 ng/µL and serves as a reference. Prepare a master reaction mix for each library with triplicate reactions per primer set. Add extra reagents to provide at least 5% more than the total number of reagents needed to account for loss of volume. Add 18µL of reaction mix to each PCR reaction well. Add 1µL primer mix to each well.

Recipe for one reaction:

1µL	ChIP-seq Library or Input Library (1ng/µl)
1µL	10µM Target Primer Mix (containing both Forward and Reverse Primers)
8µL	Nuclease-free H20
10µL	SsoFast Evagreen Supermix
20µL	Total Volume

Amplify with the following PCR protocol:

95ºC → 3 min
40 cycles:
95ºC → 5 sec
60ºC → 5 sec
Include a 65-95°C melting curve at the end of the qPCR program, reading every ≤0.5°C.

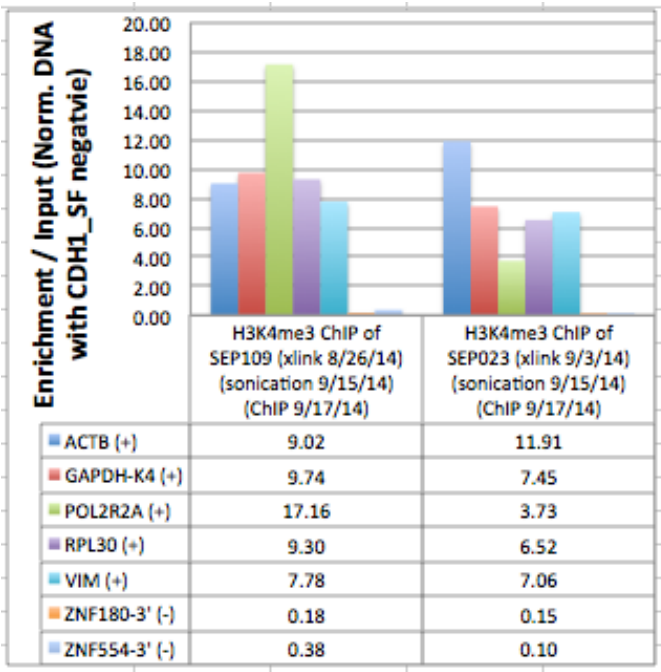
Analyze the qPCR results by first manually determining the cycle threshold for each reaction across the plate within the linear range of the amplification curve. Calculate the average cycle threshold for each triplicate reaction of each sample. The relative DNA amount is then calculated for any given primer set as 2 to the power of the cycle threshold (cT) difference between input chromatin and ChIP samples, where cT is the average value.

Ratio of sample DNA to Input DNA concentration = $2^{(cT_{INPUT}-cT_{SAMPLE})}$

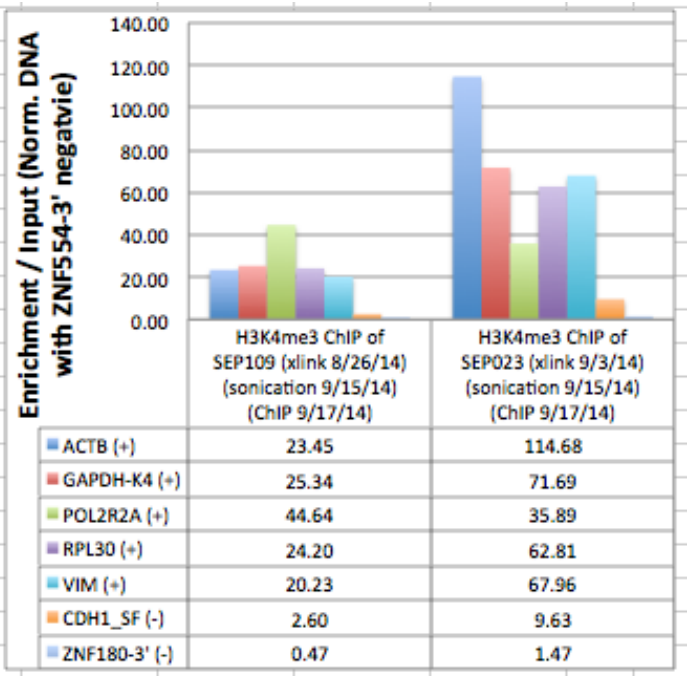
The values are then normalized with one of the negative controls. This is accomplished by dividing the relative DNA amount of each sample for a target primer set by the corresponding value for a negative control primer set. The resulting quotient represents the fold enrichment. Repeat normalization with the second negative control. **Do not proceed with sequencing unless the positive targets are at least 10-fold enriched over Input (with a possible exception for broad marks). See graphs below for examples of libraries that have produced good ChIP-seq data.**

Primer Name	Primer Sequence	hg19 Location	Size	Target
ACTB-F	AGGGTGAGGATGCCTCTCTT	chr7:5568955-5569056	102bp	H3K4me3 Positive
ACTB-R	GGGCTTCTTGTCCTTTCCTT			
GAPDH-K4_F	AAGCCTGCCGGTGACTAAC	chr12:6644505-6644614	110bp	H3K4me3 Positive
GAPDH-K4_R	CATCACCCGGAGGAGAAAT			
POLR2A-F	GAGGTGAACACGACAGACCA	chr17:7388594-7388741	148bp	H3K4me3 Positive
POLR2A-R	CTCCATGGGATTACCCCTTT			
RPL30_F	AACTGCCCAGCTTTGAGGTAATCG	chr8:99057027-99057187	161bp	H3K4me3 Positive
RPL30_R	ATGAAGGGCCAAACACCACAATCG			
VIM-F	CTCCTCTGTCCCCACACATT	chr10:17272185-17272300	116bp	H3K4me3 Positive
VIM-R	TTGGAGGACTGGCTCTCATT			
CDH1_SF-F	GTGAACCCTCAGCCAATCAG	chr16:68771118-68771224	107bp	H3K4me3 Negative
CDH1_SF-R	TCACAGGTGCTTTGCAGTTC			
ZNF180_3'-F	TGATGCACAATAAGTCGAGCA	chr19:44980743-44980879	137bp	H3K4me3 Negative
ZNF180_3'-R	TGCAGTCAATGTGGGAAGTC			
ZNF554_3'_F	CGGGGAAAAGCCCTATAAAT	chr19:2834442-2834559	118bp	H3K4me3 Negative
ZNF554_3'_R	TCCACATTCACTGCATTCGT			

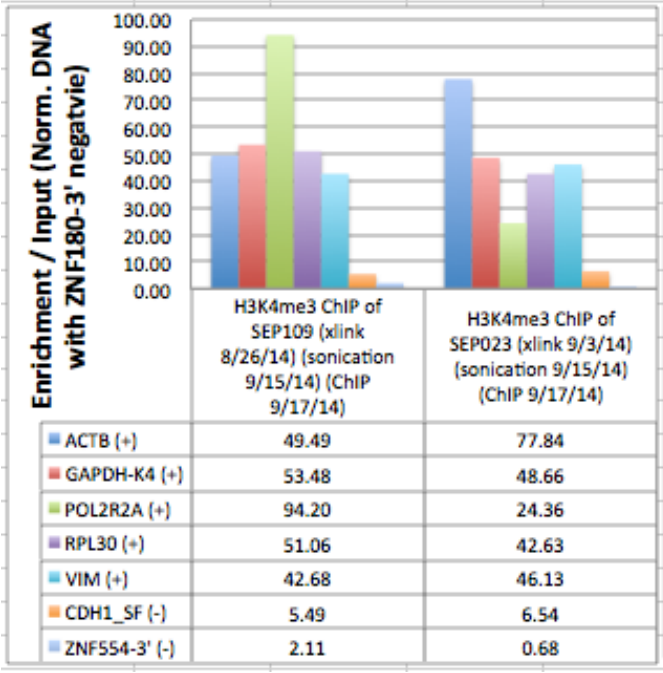
Example of H3K4me3 library enrichments



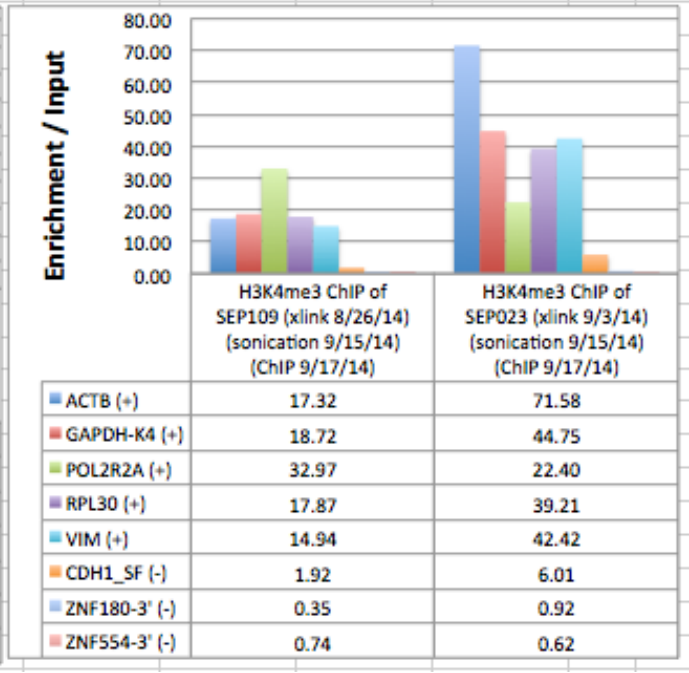
	H3K4me3 ChIP of SEP109 (xlink 8/26/14) (sonication 9/15/14) (ChIP 9/17/14)	H3K4me3 ChIP of SEP023 (xlink 9/3/14) (sonication 9/15/14) (ChIP 9/17/14)
ACTB (+)	9.02	11.91
GAPDH-K4 (+)	9.74	7.45
POL2R2A (+)	17.16	3.73
RPL30 (+)	9.30	6.52
VIM (+)	7.78	7.06
ZNF180-3' (-)	0.18	0.15
ZNF554-3' (-)	0.38	0.10



	H3K4me3 ChIP of SEP109 (xlink 8/26/14) (sonication 9/15/14) (ChIP 9/17/14)	H3K4me3 ChIP of SEP023 (xlink 9/3/14) (sonication 9/15/14) (ChIP 9/17/14)
ACTB (+)	23.45	114.68
GAPDH-K4 (+)	25.34	71.69
POL2R2A (+)	44.64	35.89
RPL30 (+)	24.20	62.81
VIM (+)	20.23	67.96
CDH1_SF (-)	2.60	9.63
ZNF180-3' (-)	0.47	1.47



	H3K4me3 ChIP of SEP109 (xlink 8/26/14) (sonication 9/15/14) (ChIP 9/17/14)	H3K4me3 ChIP of SEP023 (xlink 9/3/14) (sonication 9/15/14) (ChIP 9/17/14)
ACTB (+)	49.49	77.84
GAPDH-K4 (+)	53.48	48.66
POL2R2A (+)	94.20	24.36
RPL30 (+)	51.06	42.63
VIM (+)	42.68	46.13
CDH1_SF (-)	5.49	6.54
ZNF554-3' (-)	2.11	0.68



	H3K4me3 ChIP of SEP109 (xlink 8/26/14) (sonication 9/15/14) (ChIP 9/17/14)	H3K4me3 ChIP of SEP023 (xlink 9/3/14) (sonication 9/15/14) (ChIP 9/17/14)
ACTB (+)	17.32	71.58
GAPDH-K4 (+)	18.72	44.75
POL2R2A (+)	32.97	22.40
RPL30 (+)	17.87	39.21
VIM (+)	14.94	42.42
CDH1_SF (-)	1.92	6.01
ZNF180-3' (-)	0.35	0.92
ZNF554-3' (-)	0.74	0.62