Farnham Lab TF ChIP & Library Construction Protocol (2/1/13 edit)

*Use filtered tips throughout protocol to avoid DNA contamination in sequencing results.

A. Staph A Preparation: Day 0/1
Determine what to use for ChIP pull-down.

	Staph-Seq	Staph A	Magnetic beads
Pros	***Higher enrichment potential** ***Modified DNA ends eliminate Staph A DNA contamination in sequencing results**	***Higher enrichment potential*** *Does pellet tightly	***Quicker washes = less time** ***No need for secondary antibody if protein A/G** *Higher consistency
Cons	*Requires a rabbit secondary IgG for IgGs that don't bind protein A well *More time intensive washes *Does not pellet as tightly	*Requires a rabbit secondary IgG for IgGs that don't bind protein A well *Requires blocking & washing in advance *More time intensive prep & washes ***Staph DNA contamination is possible in sequencing results**	***Lower enrichment potential for some ChIPs** *Requires magnetic rack

Sigma-Staph: Must be prepared on Day 1 at the latest. Will be used on Day 2 of ChIP expt and can be used up to 2 weeks after being blocked.

Prepare BSA: Dissolve pure BSA powder (Sigma#A7638, off-white color) in cold mol. bio. grade (DNAse-, RNAse-, Protease- free) water at 20mg/mL and keep on ice. Make 10µL aliquots and freeze at -20ºC.

Block Staph-seq: Hydrate each vial of Staph-Seq with 200µL mol. bio. grade water, with 10µL of 20mg/mL BSA and 2µL of 100mM protease inhibitor cocktail. Block overnight rotating at 4ºC in a 0.5mL tube. Blocked Sigma-Staph can be stored at 4°C for up to 2 weeks.

Staph A: Washed Staph A should be prepared well in advance, made into 100µL aliquots and stored at -80ºC. Blocking of Staph A must be prepared on Day 1 at the latest. Prepare at least 5µL Staph A for every 1µg primary antibody. Will be used on Day 2 of ChIP expt and can be used up to 2 weeks after being blocked.

Washing Staph A cells:

1. Resuspend 1g lyophilized Staph A cells (Pansorbin®, Calbiochem Cat#507862) in 10mL **Dialysis Buffer without sarkosyl (DB–srk).** To aid in resuspension, allow 30 min for rehydration or use a pipetman with a small volume, then add remaining volume.
2. Transfer to a 15mL tube, centrifuge 5 min @ 6,000rpm @ 4°C, pour off supernatant.
3. Resuspend pellet in 10mL DB–srk.
4. Centrifuge 5 min @ 6,000rpm @ 4°C, pour off supernatant.
5. Resuspend in 3mL 1XPBS, 3% SDS, 10% BME in fume hood. (2.25mL 1XPBS, 450µL 20% SDS, 300µL BME).
6. Boil for 30 min in fume hood.
7. Centrifuge 5 min @ 6,000rpm @ RT, pour off supernatant into chemical waste.
8. Wash with 10mL DB–srk.
9. Centrifuge 5 min @ 6,000rpm @ RT, pour off supernatant.
10. Repeat steps 7-9.
11. Resuspend in 4mL DB–srk.
12. Aliquot to 100µL (40x 0.5 mL tubes), snap freeze, store at -80ºC.

Blocking Staph A cells:

1. Thaw necessary Staph A aliquots (100µL each).
2. Add 10µL of 10mg/mL BSA for each 100µL Staph A aliquot, mix by pipette.
3. For best results, incubate on rotating platform overnight (≥3 hrs) at 4°C, or 2 hrs at RT in case of time constraint.
4. Transfer to 1.5mL tube, microfuge 3 min @ 4°C @ top speed, remove supernatant.
5. Wash pellet by resuspending in 1mL DB–srk.
6. Microcentrifuge 3 min @ 4°C @ top speed, remove supernatant.

7. Repeat steps 5 and 6.
8. Resuspend pellet in 100µL DB–srk w/ 1mM PMSF (1µL of 100mM PMSF).
9. Washed and blocked Staph A Cells can be stored at 4°C for up to 2 weeks.

B. Cross-linking Cells: Day 0/1

Record cell information and details for future reference.

1. The amount of cells needed for ChIP-seq (excludes histones, see histone-specific protocol) is 1x10^8 cells which is equivalent to 10 ChIPs at 1x10^7 cells for each ChIP. In general, between 20 and 40µg of antibody are used for this number of cells, but optimization is recommended. For primary cells or tissues, the samples can be used immediately or snap frozen in liquid nitrogen and cross-linked at a later date. However, whenever possible it is recommended to cross-link cells, prepare the nuclear pellet and then freeze the samples. When cross-linking suspension cells, make sure the volume of solution you cross-link in is not too large. In a chemical hood add formaldehyde (from the 37% stock bottle) directly to the collection media or culture media to a final concentration of 1%. If using frozen cells, in a chemical hood prepare a 1% formaldehyde solution in media and add directly to frozen cell pellet; resuspend the cell pellet by pipetting up and down.
2. Rotate the cells in a tightly closed tube (for adherent cells use a shaking platform) 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.**
3. Stop cross-linking reaction by adding glycine to a final concentration of 0.125M. We use a 10X (1.25M) stock solution. Continue to agitate ≥5 min @ RT.
4. For cells cross-linked in suspension, centrifuge cells at 430 rcf for 5 min at 4ºC, discard solution, wash pellet twice with ice-cold 1XPBS (mix by pipetting, pellet cells by centrifugation at 430 rcf for 5 min at 4ºC and discard wash solution). For adherent cells, pour off media and rinse plates twice with ice-cold 1XPBS and pour off wash solution. Using a silicon stopper cut in half for large plates or a scraper for smaller plates, scrape cells in residual PBS and transfer cells from the culture dish to a tube on ice. Centrifuge the cross-linked cells at 430 rcf for 5 min at 4ºC. It is important to carefully aspirate supernatants so as to not lose cells. Note: Media containing formaldehyde should be treated as hazardous waste.
5. 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 results in a loss of chromatin, as some nuclei will inevitably rupture.

C. Chromatin Sonication: Day 1

Sonication conditions should be optimized for each cell type before processing large quantities of cells.

1. If using frozen cross-linked cells, thaw them on ice; keep all cells and chromatin samples on ice at all times. Prepare in advance 10mL of cell swelling buffer for 1x10^8 cells (exact amount is not important, but avoiding being too concentrated is): add Igepal (10µl per ml cell lysis buffer, agitate at 37°C to dissolve, cool on ice), then add protease inhibitors (see recipes for details on protease inhibitors). Resuspend cell pellet in freshly prepared cold cell lysis buffer by pipetting. Incubate on ice for 15 minutes to allow cells to swell.
2. Homogenize cells using a glass dounce homogenizer (type B) to break open the cells and release nuclei. Homogenize cells on ice with 20 strokes. *Skip this step if you are dealing with very low amounts of cells.*
3. Centrifuge the cross-linked cells at 430 rcf for 5 min at 4°C. Discard the 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.**

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4. Resuspend pellet in nuclei lysis buffer plus protease inhibitors with 5X the volume of the pellet present. (This dilution amount is good for most cells, except B lymphoblasts cells that do much better at 10X). 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). Incubate on ice for 30 minutes. An optional flash-freezing step may help break open nuclei more efficiently. This step is critical if the homogenizing in step 2 is omitted. Flash-freeze nuclei after 30 minutes of incubation in nuclear lysis buffer, thaw at room temperature. Once thawed, immediately transfer to ice (do not allow samples to warm up to room temperature) and proceed to sonication.

5. Sonicate cells in cold environment to achieve an average chromatin length of 200-500bp. Wear hearing protection! We use the BioRuptor on high setting for sonication. Volumes between 0.5mL and 2mL are sonicated in 15mL polystyrene tubes, volumes between 0.1 and 0.3mL are sonicated in 1.5mL Eppendorf tubes. The pulse duration, intensity, and number will vary depending on the sonicator, the extent of cross-linking, and cell type. Ideally the least amount of input energy that gives satisfactory fragmentation should be used. We commonly sonicate 30 minutes (pulses of 30 seconds at setting high, 1.5 minutes pause in between pulses). These settings have been used for hES cells and breast cells; T-cells and B-cells typically need extra sonication to achieve the desirable size range. Keep sonicated chromatin at 4°C while performing quantification and determining chromatin size.
6. 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.

D. Determining Chromatin Size & Quantification: Day 1

1. Take approximately 1x10^6 cells worth (or 10µl should work if you resuspended with nuclie lysis buffer that was 5X the volume of the pellet in previous step 4) of chromatin sample from step 6 above.
2. Add ChIP elution buffer to a total volume of 100µl, add 12µl of 5M NaCl, boil samples in water bath for 15 minutes to reverse cross-links.
3. Allow sample to cool down, add 1µl DNase-free RNase, incubate 10 minutes at 37ºC. This step is important because the presence of RNA results in false estimation of chromatin size.
4. Purify DNA using a PCR purification kit, elute in 30µl water. Measure DNA concentration by NanoDrop and calculate the chromatin yield. Cells typically yield 5-10µg of material per 1x10^6 cells.
5. Run 2-2.5µg of DNA on a 1.5% agarose gel to visualize average size of chromatin. If the chromatin is larger than ~600bp, adjust the sonication conditions (e.g. add more pulses after resuspending the cell pellet with the chromatin) and repeat steps 1-5 until desired range of 200 to 500bp is obtained. If samples are taking longer than an hour of sonication to achieve desired size, do not proceed. This is an indication that overcross-linking has occurred and your cross-linking protocol may need to be modified to decrease the amount of cross-linking.

E. Chromatin Immunoprecipitation: Day 1

For best results, proceed to ChIP assays on the same day as chromatin is prepared.

1. Use 100µg of chromatin for each small-scale ChIP and 500-1000µg for ChIP-seq scale.
2. Save 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 cross-links 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 TF 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.

F. Capture Antibody/Chromatin Complex and Reverse Cross-links: Day 2

STAPH Protocol (skip ahead for magnetic beads protocol)

1. For all mouse monoclonals or if the primary antibody is not from rabbit, add an appropriate secondary IgG. Use an equivalent quantity of rabbit secondary IgG as amount of primary antibody. Incubate on rotator at 4°C for 2 hours.
2. Add 5µL Staph-seq or Staph A cells per 1µg of primary antibody used.
3. Incubate on a rotating platform at RT for no longer than 15 min.
4. Microfuge at 14,000rpm for 3 min, remove supernatant.

------All the following steps are carried out at room temperature------

5. Wash pellets with 1mL of 1X Dialysis Buffer w/o sarkosyl by pipetting to resuspend.
6. Microfuge at 14,000rpm for 3 min, remove supernatant.
7. Repeat steps 4 and 5.
8. Wash pellets with 1mL of IP wash buffer 2 by pipetting to resuspend.
9. Microfuge at 14,000rpm for 3 min, remove supernatant.
10. Repeat steps 7 and 8.

11. Wash the pellet a third time with IP Wash Buffer 2 by repeating step 7, but transfer to a new tube for this 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.
12. Once the ChIP sample has been transferred to a new tube, microfuge at 14,000rpm for 3 min, remove supernatant.
13. Microfuge again by orienting the pellet on the tube hinge side to ensure visually locating the pellet and aspirate the last traces of wash buffer.
14. Elute antibody/chromatin complexes by adding 75µl IP elution buffer. Shake on vortexer for 30 minutes, setting 2.
15. Microfuge samples at 14,000rpm for 5 min. Carefully transfer supernatant containing antibody/chromatin complexes to a new tube without any Staph A.
16. Repeat steps 11 and 12, but combine the second 75µl elution with the first to obtain a total of 150µl of material.
17. Microfuge the 150µl samples at 14,000rpm for 5 min. Microfuge by orienting the pellet on the hinge side to ensure visually locating any trace amounts of the Staph A pellet and put supernatant in new tube. Repeating the spin may be necessary if the Staph pellet slips and is pipetted by accident. Trace amounts of Staph-Seq WILL NOT show up in sequencing data down the road, but regular Staph A will show up in sequencing data if any trace amounts are carried over.
18. 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.
19. Incubate all samples at 67°C overnight to reverse formaldehyde cross-links.

Magnetic beads protocol (DIFFERENT THAN PCR CLEAN UP AMPURE BEADS!)

1. ***Wash beads 1x in the same buffer solution it will be added to.*** Add 15µL magnetic protein A/G beads per small-scale ChIP sample (or 150µL per ChIP-seq sample) and incubate on a rotating platform for 2 hrs at 4°C. We use an equal mixture of magnetic protein A beads from Invitrogen DynaBeads Protein A (#10002D) and magnetic protein G beads from Invitrogen DynaBeads Protein G (#10004D), but others work just as well. Some examples are protein A magnetic beads from Cell Signaling Technology (#9006), magnetic protein G beads from Diagenode (#AIP-102-015), or a magnetic protein A/G mix from Pierce Protein AG (#88803). No carrier DNA should be added to beads.

------All the following steps are carried out at room temperature------

2. Allow beads to settle for 1 min in a magnetic separation rack, then carefully remove the supernatant.
3. Wash magnetic beads two times with IP wash buffer 1 (RIPA buffer without protease inhibitors); take tubes out of magnetic rack and mix by pipetting. Efficient washing is critical to reduce background. Avoid cross-contamination of samples and loss of magnetic beads.
4. Wash magnetic beads two or three times with IP wash buffer 2 (take tubes out of magnetic rack and mix by pipetting). ***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.***
5. *Optional step: May be too stringent for some antibodies.* Wash 1X with IP wash buffer 3.
6. Take care to discard all wash solution after final wash.
7. Elute antibody/chromatin complexes by adding 75µl IP elution buffer. Shake on vortexer for 30 minutes, setting 2.
8. Allow beads to settle for 1 min in a magnetic separation rack. Carefully transfer supernatant containing antibody/chromatin complexes to a new tube.
9. Repeat steps 11 and 12, but combine the second 75µl elution with the first to obtain a total of 150µl of material.
10. 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.
11. Incubate all samples at 67°C overnight to reverse formaldehyde cross-links.

G. DNA Purification: Day 3

1. Purify DNA with a PCR clean up kit, one column per sample. Elute each sample with 35µl EB.
2. Assess ChIP results by PCR or qPCR before proceeding to preparation of libraries. Use multiple negative and positive targets and properly dilute large scale ChIPs (for example, dilute 1:10 for a 10X ChIPs done with ~1000µg chromatin). Before proceeding to library making, verify positive site enrichments over negative sites.

ChIP Buffers List

Protease inhibitor stock solutions (stored at -20ºC) Note: Protease inhibitors and NP-40/Igepal are added at time of use only. Do not add protease inhibitors to IP wash and elution buffers

1) Aprotinin (10mg/mL), Leupeptin in water (10mg/mL), 100 mM PMSF in isopropanol.
   [Aprotinin (1µL/mL) and Leupeptin (1µL/mL), PMSF (10µL/mL)]
2) 200X Protease Inhibitor Cocktail, VWR catalogue #80053-852

Cell Lysis buffer
5mM PIPES pH 8.0
85mM KCl
Add Igepal (10µL/mL),
*agitate at 37°C to dissolve,
protease inhibitors immediately before use
cool on ice, add protease inhibitors

Nuclei Lysis buffer
50mM Tris-Cl pH 8.1
10mM EDTA
1% SDS
Add protease inhibitors immediately before use Add
cool on ice, add protease inhibitors

IP Dilution buffer (RIPA)
50mM Tris pH 7.4
150mM NaCl
1% (v/v) Igepal
0.25% Deoxycholic acid
1mM EDTA pH 8.0
Add protease inhibitors when cold

Dialysis Buffer (DB–srk)
2mM EDTA
50mM Tris-Cl pH 8.0

IP wash buffer 1
50mM Tris pH7.4
150mM NaCl
1% (v/v) Igepal
0.25% deoxycholic acid
1mM EDTA, pH 8.0

IP wash buffer 2
100mM Tris-Cl pH 9.0
500mM LiCl
1% Igepal/NP-40
1% deoxycholic acid

IP wash buffer 3
100mM Tris-Cl pH 9.0
500mM LiCl
1% Igepal/NP-40
1% deoxycholic acid
150mM NaCl

ChIP elution buffer
50mM $NaHCO_3$
1% SDS

Library Construction Protocol

This library protocol is based on the Illumina Sample Preparation Kit for Genomic DNA with some modifications. This protocol describes the preparation of libraries of ChIP DNA compatible with Illumina DNA sequencers. 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 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 magentic protein A/G beads used in the previous ChIP portion of the protocol.

ALL REAGENT ALIQUOTS ARE IN CLEARLY LABELED BOXES AND LOCATIONS ARE DESCRIBED BELOW.

Step 1: End-Repair Using the *End-It DNA Repair Kit* from Epicentre (Cat# ER0720)

BOX LABELED "LIBRARY STEP #1 & #2" ON THE BOTTOM SHELF OF THE -20 FREEZER

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

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

1-34µL	ChIP DNA (entire ChIPsample) -or- 100ng input DNA
5µL	10X End-Repair Buffer
5µL	10mM ATP
5µL	2.5M dNTP Mix
1µL	End-Repair Enzyme Mix
X µL	sterile water to a total volume of 50µL

B) Incubate 45 min @ RT

C) Purify with 90µL Ampure magnetic beads (1.8x volume). Beads may take several minutes to pellet with magnet during each step. During all pipetting, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping. Make **fresh** 70% ethanol and wash beads twice. **Do <u>not</u> allow the beads to dry.** Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.

D) Elute in 34µL EB.

E) Place tubes back in magnetic stands to collect the DNA and transfer liquid to new tubes.

Step 2: Addition of 'A' base to 3' Ends

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Make stocks of 1mM dATP using NEB 100mM dATP. For example, add 5µL 100mM dATP to 495µL sterile RNase/DNase-free Gibco water, split to 25µL aliquots, and freeze at -20ºC. Defrost only once.

A) Combine and mix the following in PCR tubes:

34µL	DNA from Step 1
5µL	Klenow buffer (NEB Buffer 2)
10µL	1 mM dATP (not included in NEB kit)
1µL	Klenow fragment (3'→5' exo, NEB cat# M0212s, 5,000 U/mL)
	Total reaction volume of 50µL

B. Incubate for 30 min @ 37°C in PCR machine

C. Purify with 90µL Ampure magnetic beads (1.8x volume). Beads may take several minutes to pellet with magnet during each step. During all pipetting, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping. Make **fresh** 70% ethanol and wash beads twice. **Do <u>not</u> allow the beads to dry.** Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.

D) Elute in 11µL EB.

E) Place tubes back in magnetic stands to collect the DNA and transfer liquid to new tubes.

Step 3: Illumina Barcode Adapter Ligation (BIOO adapters)
BOX LABELED "LIBRARY STEP #3 & #4" ON THE BOTTOM SHELF OF THE -20 FREEZER
Dilute Illumina 15μM adapters 1:10 with water (final concentration 1.5μM) to adjust for the smaller quantity of DNA. BIOO supplies their NEXTflex™ DNA Barcodes adapters at 25μM, so make a 1:17 dilution (final concentration 1.5μM). Excess adapters can interfere with sequencing.

A) Combine and mix the following components in a tube (total reaction volume = 30μL):
11μL DNA from Step2
15μL DNA ligase buffer
1μL Adapter mix (1.5μM)
3μL DNA ligase (Ligafast from Promega Cat# M8221)
Total reaction volume of 30μL
B) Incubate for 15 min at room temperature.
C) Purify with 24μL Ampure magnetic beads (0.8x volume). Beads may take several minutes to pellet with magnet during each step. During all pipetting, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping. Make **fresh** 70% ethanol and wash beads twice. **Do not allow the beads to dry.** Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.
D) Elute in 19μL EB.
E) Place tubes back in magnetic stands to collect the DNA and transfer liquid to new tubes.

Step 4: Size Selection & Gel Purification
BOX LABELED "LIBRARY STEP #3 & #4" ON THE BOTTOM SHELF OF THE -20 FREEZER
Size selection of the sample ensures removal of unused adapters and selection of proper fragment size for amplification and sequencing. We use pre-cast agarose gels (Invitrogen E-Gel EX 2% agarose; Cat# G4020-02) to minimize risk of contamination. Invitrogen Track-It Ladder and Loading Buffer is described in the steps below.

A) Dilute 10μL of 6X Cyan/Orange with 50μL EB buffer to obtain 1X Cyan/Orange buffer dye. Add 1μL of 1X Cyan/Orange buffer dye to eluted DNA from Step 3 above.
B) Load 20μL EB buffer in each of the empty wells. Make sure to skip a well between samples to avoid cross-contamination.
C) Load 2μL 50bp ladder and 18μL EB in the wells on the outer edge wells.
D) Load 20μL DNA with dye in each well.
E) Run the gel for ~10min with the newer version power base.
F) Image gel to visualize sample.
G) Size select using a clean razor blade but cutting out regions from 200-500bp. You may have to cut blindly due to low DNA concentrations.
H) Dissolve gels using QIAgen's gel extraction buffer at room temperature. Add 500μL QG buffer to each gel slice and shake on the vortexer for 30 min or until gel is dissolved.
I) Purify DNA using a QIAquick MinElute PCR Purification Kit. Elute in 24μL EB.

Step 5: Amplification of Adapter-Modified DNA Fragments
BOX LABELED "LIBRARY STEP #5" ON THE BOTTOM SHELF OF THE -20 FREEZER
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 1uL per 50uL PCR reaction (as outlined in step A below). For Illumina adapters, dilute Illumina paired-end primers (1.0 and 2.0) 1:4 using sterile water for use in the reactions. Paired end primers can be ordered via from Invitrogen and elsewhere. If possible, have primer purity checked by HPLC.

A) Combine and mix the following components in PCR tubes:
24μL DNA from Step 4
25μL KAPA HiFi NGS MM
1μL PCR primer mix (6.25uM)
50μL Total reaction volume

B) Amplify using the following PCR protocol:

- 2 min @ 98ºC
- 10-15 cycles : [30 sec @ 98ºC, 30 sec @ 65ºC, 1 min @ 72ºC]
- 4 min @ 72ºC
- Hold @ 10ºC

Make an educated guess on how many cycles of PCR to do based on your gel size selection image. Over-amplification should be avoided to reduce potential PCR artifacts. Input DNA typically requires ~10 cycles of PCR.

Step 6: Library Purification, Quality and Quantification
QUBIT HS DNA KIT IS IN THE BOTTOM DRAWER OF THE 4 DEGREE FRIDGE
GREEN AND WHITE KAPA BIOSYSTEMS KIT BOXES ARE LABELED "ILLUMINA DNA STANDARDS AND PRIMER PREMIX KIT" AND ARE LOCATED ON THE BOTTOM SHELF OF THE -20 FREEZER

A) Purify with 40µL Ampure magnetic beads (0.8x volume). Beads may take several minutes to pellet with magnet during each step. During all pipetting, keep beads stationary on the magnet (do not pipette up and down) and visually ensure that the beads aren't slipping. Make **fresh** 70% ethanol and wash beads twice. **Do <u>not</u> allow the beads to dry.** Do a final spin and hold tube against the magnet while pipetting off last remaining ethanol wash.
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. (Possible to exclude next step if this one is done).
E) Check concentration by Kappa Quant assay. (Possible to exclude prior step if this one is done, unless samples are being sent to Stanford!).
4) 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 7: Library Confirmation
SsoFast EVEGREEN SUPERMIX BY BIORAD IS LOCATED ON TOP SHELF OF THE -20 FREEZER
Check enrichment using same positive and negative control primers as used for original ChIP. Enrichment should show stronger enrichment than before if the library preparation was successful. Do not proceed with sequencing unless the positive targets are at least 10-fold enriched over input.

Enrichment of marks in the ChIP-seq libraries are determined by quantitative real-time PCR (qPCR). The input sample is 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 for 5% of the total number of reagents 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	undiluted ChIP Library Sample or diluted Input Sample (1ng/µl)
1µL	10µM target primer mix (containing both Forward and Reverse primers)
8µL	Nuclease-free H20
10µL	SsoFast Evagreen Supermix
20µL	Total reaction volume

Amplify with the following PCR protocol:

3 min @ 95ºC
40 cycles : 5 sec @ 95ºC, then 5 sec @ 60ºC
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.