ChIP-seq protocol

- Grow 2ml overnight culture.
- Next morning culture- subculture 1/100 (e.g. 400µl into 40ml). Grow to appropriate OD600 (0.3-0.6).
- Cross-link cells by adding formaldehyde to a final concentration of 1% (1ml of 37% stock to a 40ml culture). Swirl to mix and leave for 20 minutes.
- Quench formaldehyde by adding glycine to a final concentration to 0.5M (typically 10ml of 2.5M glycine per 40ml culture).

NOTE: if using a smaller culture than 40ml, change the amount of formaldehyde used. Keep the volume of glycine the same.

- Spin cells down for about 5 minutes at 4000rpm. Re-suspend in TBS (0.5-1 times the culture volume- resuspend in 1ml first, then add the rest)). Spin down again.
- Resuspend in 1ml TBS and transfer to eppendorf. Spin in microcentrifuge at max speed for minute.
- Discard supernatant. Cells can be frozen at this point.
- Resuspend cells in 1ml FA lysis buffer (150mM NaCl) containing 4mg/ml lysosome. Incubate at 37°C for 30 minutes to lyse cells. Crosslinked lysates can be frozen at this point.
- Chill on ice for >5 minutes. Sonicate as directed:

USING A TIP SONICATOR: 2 x 30s pulses, 50% output, keeping cells on ice.

USING A BIO-RUPTOR: Turn on pumps at least 45 minutes before use to allow water bath to chill to 4°C. Optimise pump speed to maintain marked water level. 2 x 15 minute cycles (check water level in between).

- Spin cell lysates in microcentrifuge at max speed for 5 minutes. Keep supernatant (about 1ml). If cultures >20ml in volume dilute in FA lysis buffer (150mM NaCl) so there is 1ml of lysate for each 20ml of original culture. (E.g. 40ml culture- dilute to 2ml).
- Set up cocktails containing 500µl lysate and 300µl FA lysis buffer (150mM NaCl). (4 per 40ml culture). Use 1 of these for each immunoprecipitation.
- OPTIONAL: save 20µl of lysate as 'input'. De-crosslink before you do anything with it.
- Cross-linked and sonicated lysates can be frozen at this point.
- Immunoprecipitation:

IMMUNOPRECIPITATION:

- **Use one of the 800µl cocktails as described in (11). i.e. 10ml culture sonicated lysate.**
- **Add 25µl of Protein A beads (50% slurry with TBS). Resuspend gently before use. BLUNT TIPS before use (use scissors).**
- **Add 1µl of Neoclone® antibody β/σ/CRP or 2µl of anti-FLAG (Sigma®) anti-H-NS.**
- **Rotate at room temperature for 90 minutes. Parafilm lid. For CRP, rotate at 4°C overnight.**

- Spin at 4000rpm for 1 minute, remove the supernatant carefully. Add 700µl of FA lysis buffer (150mM NaCl) and resuspend gently using a blunt tip. Transfer to a spin-X-column.
- Rotate for 3 minutes at room temp (check lids are closed before rotating), then spin at 4000rpm for 1 minute. Discard supernatant.

WASHES IN SPIN-X-COLUMN: With all wash steps, use 750µl unless indicated; check lids are closed and rotate at room temp for 3 minutes before spinning at 4000rpm for 1 minute. Discard supernatant unless directed otherwise. It is useful after a set of washes to briefly re-spin the spin-X-columns for a minute to remove any excess liquid left.

- Washes:
  - FA lysis buffer 150mM NaCl.
  - FA lysis buffer 150mM NaCl.
  - 10mM Tris-HCl, pH7.5.
  - 10mM Tris-HCl, pH7.5.

- Blunting: use NEB Blunting kit:

BLUNTING USING NEB® KIT (resuspend in buffer then add enzyme). Add:

- **10µl 10x quick blunting buffer.**
- **10µl dNTP mix (supplied in kit).**
- **80µl dH_2O.**
- **2µl blunt enzyme kit (T4 DNA polymerase).**

Parafilm lid and rotate (in such a way that doesn't invert) for 30 minutes at room temp.

- Washes:
  - FA lysis buffer 150mM NaCl.
  - FA lysis buffer 150mM NaCl.
  - 10mM Tris-HCl, pH8.
  - 10mM Tris-HCl, pH8.

- A tail: use NEB Klenow (3' -> 5' exo-) kit:

Resuspend beads in the following mixture:

- **10µl 10x NEB buffer 2**
- **2µl 100mM dATP (supplied).**
- **88 µl dH_2O.**
- **2µl Klenow.**

Parafilm lid. Incubate at 37°C rotating (not inverting) for 30mins).

- Washes:
  - FA lysis buffer 150mM NaCl.
  - FA lysis buffer 150mM NaCl.
  - 10mM Tris-HCl, pH7.5.
  - 10mM Tris-HCl, pH7.5.

- Ligate adaptors:

Resuspend beads in the following mixture:

- **100µl 1x ligase buffer (50µl dH_2O + 50µl 2x ligase buffer).**
- **1µl NEXTflex ChIP-seq barcoded adaptor (BioO) (have used 1µl non-diluted before).**
- **4µl quick ligase.**

Parafilm lid and rotate (not invert) at room temp for 15 mins.

- Washes:
  - FA lysis buffer 150mM NaCl.
  - FA lysis buffer 150mM NaCl.
  - FA lysis buffer 500mM NaCl.
  - ChIP wash buffer.
  - TE
  - Flush 300µl TE through the column after just to get rid of any bubbling.

- Transfer spin-X-columns to new dolphin-nosed tubes, add 100µl elution buffer and incubate at 65°C for 10 minutes.
- Elute by quickly transferring to a microcentrifuge: spin at 4000rpm for 1 minute, transfer flowthrough to an eppendorf.
- To de-crosslink: boil for 10 minutes, use a tube lock if possible. De-crosslink inputs at this point as well, if used.

- Phenol extract OR use Ampure® magnetic beads to clean up.

To phenol extract:

- Add 300µl dH_2O and 400µl PCIA (pH6.7/8.0), vortex.
- Take upper phase and transfer to new tube.
- Add 1µl 35 mg/ml glycogen, 40µl 3M Na acetate, and 1ml ethanol.
- Vortex and place at -20°C for > 2hours.
- Spin at max speed at 4°C for 15-20mins. Longer is better.
- Dump supernatant, add 1ml 80% ethanol.
- Spin again for 15mins at max speed at 4°C.
- Dump supernatant. Spin briefly again and remove any residual ethanol.
- Dry, either 10mins at 65°C with lids open or speedvac.

Ampure® magnetic beads:

- Warm up beads to room temperature, and resuspend gently.
- Add 1.1x volume beads to DNA solution.
- Mix gently 10x (blunt tips helpful), leave at room temperature for 5 minutes.
- Place on magnet for 2 minutes.
- Remove solution from beads.
- KEEP ON MAGNET. Add 200µl 70% ethanol for 30 seconds, and then remove ethanol. REPEAT.
- Air-dry for at least 5 minutes (65°C for 10 minutes works better).
- Off the magnet, resuspend beads in 11/12µl of water.
- Place on magnet for 1 minute, remove and keep liquid.

Amplifying library

- First run a real time- PCR, use 1µl of ChIP DNA as template- dilute 1/8, use 2µl each in three wells.
- OLIGOS= JW3764/3765 (primer mix for NEXTflex, 10µM each, 3µl of this mix per reaction.)
- Analyse on manual Ct set to 0.1.
- Cycle number at which sample 'appears'/ 'comes up'= X.

- Add 3 to X and run a PCR with this number of cycles. If using multiple samples, open up PCR machine after elongation cycle to take out tubes.
- Each mix (set up 4 reactions in strip PCR tubes):
    - 5µl 10x taq buffer.
    - 2µl DNA with ligated adaptors.
    - 1µl JW3764 100µM.
    - 1µl JW3765 100µM.
    - 0.4µl dNTP mix (25mM each).
    - 1.5µl taq.
    - 40.1µl dH_2O.
    - TOTAL= 50µL.

Clean and concentrate library

- Clean up with Ampure® magnetic beads, QIAgen MinElute®, or Zymogen clean and concentrate®. Elute in 15-25µl dH_2O (15µl better- then add 10µl loading dye.)
- Load onto a non-denaturing 8% acrylamide gel.

8% acrylamide gel:

- **7ml dH_2O.**
- **1ml 10xTBE.**
- **2ml 40% acrylamide.**
- **100µl 10% APS.**
- **10µl TEMED.**

- Run for 1 hour at 100V.
- Stain in Ethidium bromide for 5 minutes.
- On trans-illuminator, cut out a smear from 200-600bp, avoid the 150bp band- this is the adaptor-adaptor band.
- Pierce the bottom of a 0.7ml tube with a hot needle, place inside a 1.7ml tube.
- Place gel slice into 0.7ml tube and spin at max speed for 5 minutes to shred the gel.
- Remove 0.7ml tube, add 0.4ml acrylamide gel extraction buffer, parafilm lid and rotate overnight.
- Transfer gel and liquid to a spin-X-column (blunt tips helpful for removing the gel).
- Spin at max speed for 5 minutes.
- Precipitate DNA as follows:

Precipitation:

Add:

- **1ul 35mg/ml glycogen.**
- **0.4ml propan-2-ol/ 1ml cold ethanol.**

Vortex and place at -20°C for at least 2 hours.

- Spin for 15-30 mins at max speed at 4°C, dump supernatant.
- Add 1ml 80% ethanol, spin for 10-15 mins again.
- Remove supernatant, spin for 3-4mins, remove any remaining supernatant.
- Dry at 65°C for 10 minutes.
- Resuspend in 11-12µl dH_2O.

Quantify library:

- Use a Qubit® 2.0 Fluorometer - make up working solution in the following ratio:
  - 1µl 200x dye : 199µl dilution buffer.
- Dilute standards 1 & 2 as follows:
  - 10µl standard : 190µl working solution.
- Dilute samples 1µl in 199µl of working solution.
- MAKE UP ALL TUBES IN THE THIN-WALLED TUBES SPECIFIED.
- 0.5µg/L to 50µg/L is expected (when diluted 1:200).
- Multiply by 200, divide by 1000 to get ng/µl.
- Collate samples in an equimolar ratio, using the whole volume of the most dilute solution. Can concentrate sample in speedvac, 10µl works well (sent 6µl of this off for sequencing).

CAT. NUMBERS

Monoclonal Anti-FLAG (R) M2, antibody PR&	SIGMA	F3165-1MG
Spin-X .2CA, 25/BAG-N/S	VWR	83-8161-CS
2.0ml dolphin nosed tubes 250/bag Corning	VWR	29442-590
Quick Blunting ™ and Quick Ligation ™ Kits	NEB	E0542L
Klenow Fragment (3'→5' exo-)	NEB	M0212L
Phase Lock tubes GEL HVY 2ML 200PK	Fisher Sci	FP2302830
NEXTflex ChIP-seq Barcodes- 24	Bio0 Scientific	514122
Agencourt AMPure XP 5 ml	Beckman Coulter	A63880

kit, 139/278 preps		
DynaMag -2Magnet	Life Technologies	12321D
Qubit Fluorometer 2.0	Life Technologies	Q32866
Qubit® dsDNA HS assay Kit (for use with Fluorometer)	Life Technologies	Q32851

Illumina primer sequences (for library amplification)

AATGATACGGCGACCACCGAGATCTACAC	NEXTflex ChIP Primer 1
CAAGCAGAAGACGGCATACGAGAT	NEXTflex ChIP Primer 2

Buffers

FA lysis buffer

50mM Hepes-KOH, pH7

150/500mM NaCl

1mM EDTA

1% Triton X-100

0.1% Sodium deoxycholate

0.1% SDS

ChIP wash buffer

10mM Tris-HCl, pH 8.0

250mM LiCl

1mM EDTA

0.5% Nonidet-P40

0.5% Sodium Deoxycholate

ChIP elution buffer

50mM Tris-HCl, pH 7.5

10mM ETDA

1% SDS

TE

10mM Tris-HCl, pH 8.0

1mM EDTA

1x TBS

20mM Tris-HCl, pH7.4

0.9% NaCl