General Schedule

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1 Practical Day One

1.1 Antibody-Bead Binding

Prerequisites: Small ice bucket and work on ice throughout the entire process – may be practical to work in teams.

1.1.1 Take a 300 µl PCR tube containing 50 µl Protein-A Magnetic Beads
1.1.2 Place the tube on a magnet rack
1.1.3 Discard the supernatant
1.1.4 Take off magnet
1.1.5 Add 5 µl 20 mg/ml BSA (20% $^{w}/_{v}$)
1.1.6 195µl Chromatin Dilution Buffer and mix by gently pipetting

Note: Final BSA = 0.5% $^{w}/_{v}$

1.1.7 Place the tube on a magnet rack
1.1.8 Discard the supernatant
1.1.9 Take off magnet
1.1.10 Add 5µl 20 mg/ml BSA
1.1.11 Add 195 µl Chromatin Dilution Buffer and mix by gently pipetting
1.1.12 Place the tube on a magnet rack
1.1.13 Discard the supernatant
1.1.14 Take off magnet
1.1.15 Add 5µl 20 mg/ml BSA
1.1.16 Add 175 µl Chromatin Dilution Buffer
1.1.17 Add 20 µl 2% $^{w}/_{v}$ SDS
1.1.18 Mix by pipetting
1.1.19 Aliquot 80 µl into 2x 1.5ml tubes
1.1.20 Label each tube: IgG Control and H3K36me3
1.1.21 Add 3 µl of IgG antibody to the IgG tube

Note: Rabbit IgG (Diagenode, C15410206)

1.1.22 Add 3 µl of H3K36me3 antibody to the H3K36me3 tube

Note: Tri-Methyl-Histone H3 (Lys36) (D5A7) XP Rabbit mAb (Cell Signaling Technology, 4909)

1.1.23 Put the samples on the rotator and leave rotating at 2-8°C for 2 hours

Note: Alternatively incubate on ice and occasionally pipette slowly to mix

INFORMATION
Antibodies can be against whole proteins (e.g. CTCF) against modifications on protein (e.g. H3K36me3 (Histone 3 Lysine 36 Tri-methylation), H2AXγ (phosphorylated histone 2AX variant) etc), or even against nucleic acids (e.g. Anti-DNA-RNA Hybrids, Anti-5-methylcytosine (DNA methylation on Cytosines), etc).
The quality of antibody makes the ChIP experiment, if the antibody is unspecific then it does not matter how well you do the experiment the data will be erroneous or of poor quality.
The ENCODE project, defined a set of criteria for ChIP-grade antibodies (Landt et al 2012), but most companies do not follow the same quality criteria so purchase with caution – no company is not guilty of producing a bad antibody. Some companies have better reputations than others…
Top tip, if designing your own experiment, you should ALWAYS use the same Catalogue and Lot Numbers for the entire experiment; this is especially important for non-recombinant antibodies (which is most of them).

Landt et al. ChIP-seq guidelines and practices of the ENCODE and modENCODE consortia. Genome Research: 22:1813-1831 (2012)

1.2 Chromatin Pre-clearing

Note: All chromatin aliquots have been prepared and have been sonicated in the following buffer (10 mM Tris-HCl, pH 8.0, 0.1% $^{w}/_{v}$ SDS, 1 mM EDTA)
Important: Work on ice throughout the practical

1.2.1 Take a 300 µl PCR tube containing 50 µl Protein-A Magnetic Beads
1.2.2 Place the tube on a magnet rack
1.2.3 Discard the supernatant
1.2.4 Take off the magnet
1.2.5 Add 5 µl 20 mg/ml BSA (20% $^{w}/_{v}$)
1.2.6 195µl Chromatin Dilution Buffer and mix by gently pipetting

Note: Final BSA = 0.5% $^{w}/_{v}$

1.2.7 Place the tube on a magnet rack
1.2.8 Discard the supernatant
1.2.9 Take off magnet
1.2.10 Add 5µl 20 mg/ml BSA
1.2.11 Add 195 µl Chromatin Dilution Buffer and mix by gently pipetting
1.2.12 Place the tube on a magnet rack
1.2.13 Discard the supernatant
1.2.14 Take off magnet
1.2.15 Take a thawed 310 µl Chromatin sample

Note: 4M (4 x 10^6) cells per 120 µl

1.2.16 Add the entire chromatin sample to the washed Protein-A Magnetic Beads
1.2.17 Put the samples on the rotator and leave rotating at 2-8°C for 1 hour

Note: Alternatively incubate on ice and occasionally invert slowly to mix

INFORMATION
Pre-clearing is necessary to remove pieces of sticky cell debris and chromatin. This step should never been avoided, even if it is one of the more expensive steps when scaling up the amount of different samples being processed.

LUNCH BREAK

1.3 Recover Pre-cleared Chromatin

1.3.1 Label a 1.5ml tube and put on ice (e.g. Chromatin Master Mix)
1.3.2 Take the pre-clearing chromatin sample and pulse centrifuge
1.3.3 Place sample on the magnet
1.3.4 Transfer the supernatant to the labelled tube and place on ice

Note: The magnet pellet can now be discarded

Note: If doing outside the practicals, the Pre-cleared Chromatin can be stored a -80°C for 12 months (the shorter the time to processing the better)

1.3.5 Add 150 µl Chromatin Dilution Buffer
1.3.6 Add 12.5 µl 20mg/ml BSA
1.3.7 Add 2.5 µl Proteinase Inhibitor (Diagenode, C12010011)
1.3.8 Add 35 µl 2% SDS

Note: Final SDS concentration adjusted to 0.2% (if you want a challenge, determine why this volume)

1.3.1 Mix thoroughly and by gently pipetting
1.3.2 Keep Chromatin Master Mix sample on ice

1.4 Chromatin Immunoprecipitation (ChIP) Set-up

1.4.1 Take two tubes: IgG Control and H3K36me3

1.4.2 Pulse centrifuge the tubes

1.4.3 Place sample on the magnet

1.4.4 Discard the supernatants

1.4.5 Take off the magnet

1.4.6 Add 50 µl Chromatin Dilution Buffer and mix by gently pipetting

1.4.7 Place sample on the magnet

1.4.8 Discard the supernatants

1.4.9 Take off the magnet

1.4.10 Add 50 µl Chromatin Dilution Buffer and mix by gently pipetting

1.4.11 Place sample on the magnet

1.4.12 Discard the supernatants

1.4.13 Add 200 µl of Chromatin Master Mix (Step 1.3.2) to each tube

Important: The remainder of the Chromatin Master Mix must be keep, so label (initials at a minimum) and store at -20°C

1.4.14 Put the samples on the rotator and leave rotating at 2-8°C overnight

END OF PRACTICAL DAY 1

2 Practical Day Two

2.1 ChIP Wash Steps

Prerequisites: Small ice bucket and work on ice throughout the entire process.

2.1.1 Take the two ChIP samples (IgG and H3K36me3) from the 2-8°C coldroom
2.1.2 Pulse centrifuge to collect contents
2.1.3 Place sample on the magnet
2.1.4 Discard the supernatants
2.1.5 Take off the magnet
2.1.6 Add 200 µl Low Salt Wash Buffer and mix by gently pipetting
2.1.7 Place samples on ice and pipette slowly to mix the samples every minute for a total of 10 min

Note: generally the better the mixing the better the ChIP quality

2.1.1 Pulse centrifuge to collect contents
2.1.2 Place sample on the magnet
2.1.3 Discard the supernatants
2.1.4 Take off the magnet
2.1.5 Add 200 µl High Salt Wash Buffer and mix by gently pipetting
2.1.6 Place samples on ice and pipette slowly to mix the samples every minute for a total of 10 min

Note: generally the better the mixing the better the ChIP quality

2.1.7 Pulse centrifuge to collect contents
2.1.8 Place sample on the magnet
2.1.9 Discard the supernatants
2.1.10 Take off the magnet
2.1.11 Add 200 µl LiCl Wash Buffer and mix by gently pipetting
2.1.12 Place samples on ice and pipette slowly to mix the samples every minute for a total of 10 min

Note: generally the better the mixing the better the ChIP quality

2.1.13 Pulse centrifuge to collect contents
2.1.14 Place sample on the magnet
2.1.15 Discard the supernatants
2.1.16 Take off the magnet
2.1.17 Add 200 µl TE Wash Buffer and mix by gently pipetting
2.1.18 Place samples on ice and pipette slowly to mix the samples every minute for a total of 10 min

Note: generally the better the mixing the better the ChIP quality

2.1.19 Pulse centrifuge to collect contents
2.1.20 Place sample on the magnet
2.1.21 Discard the supernatants
2.1.22 Resuspend the bead pellets in 96 µl SDS Elution Buffer
2.1.23 Add 4 µl 5M NaCl to each tube
2.1.24 Leave samples on ice (whilst preparing Input Sample (2.2))

2.2 Input Preparation

2.2.1 Take the remaining Chromatin Master Mix from the -20°C
2.2.2 Label a 1.5 tube (e.g. Input)
2.2.3 Add 1 µl of the Chromatin Master Mix

Note: Was stored at -20C

2.2.4 Add 95 µl SDS Elution Buffer
2.2.5 Add 4 µl 5M NaCl
2.2.6 Leave samples on ice (If still preparing the ChIP Samples)

INFORMATION
The Input is included in ChIP-seq experiments as a control to normalise the data generated by next generation sequencing (NGS). There are some inherent biases in NGS platforms (e.g. Illumina HS4000) and distinct variations from sample to sample (mainly due to samples sonication).
For larger cohort studies, it is common to save money by generating “pooled” Inputs that are composed of

several chromatin samples – these are typically divided into cell type specific Inputs.

2.3 Reverse Crosslink

2.3.1 Using a hot block, incubate both the ChIP (2.1.24) and Input (2.2.6) samples at 65°C for 4 hours

LUNCH BREAK and JOURNAL CLUB

2.4 RNase and Proteinase K Digestions

2.4.1 Add 2 µl RNase Cocktail to all ChIP/Input samples
2.4.2 Incubate at 65°C for 30 min
2.4.3 Add 2 µl Proteinase K to all ChIP/Input samples
2.4.4 Incubate at 65°C for 30 min

2.5 Recover DNA

2.5.1 Pulse centrifuge the PCR tubes
2.5.2 Place sample on the magnet
2.5.3 Transfer the supernatants to different 1.5ml tubes
2.5.4 Recover ChIP/Input DNA using Zymo DNA Clean & Concentrator 5 columns (See Attached Manufacturers Protocol)

Note: Use a "DNA Binding Buffer : Sample" ratio of "7 : 1"
Note: Elute DNA in a total volume of 12 µl H_2O (ideally elute twice with 6 µl); the small volume is necessary as we will have very little amounts of DNA.

2.6 Quantify DNA

2.6.1 Quantify the recovered DNA samples using the Qubit (See Attached Manufacturers Protocol)

Note: Prepare samples using 1 µl of each sample
Note: You want the final concentration in ηg/µl
Warning: Be careful with the units and the dilution factor

2.6.2 Store samples at -20°C

Note: If doing this experiment with your own samples, store ChIP DNA at -80°C and make into NGS libraries in 1-2 weeks; ChIP DNA is inherently unstable due to the damage caused by the sonication.

2.7 Determine Success…

Think about what you expect. Compare the IgG vs. H3K36me3; which should have the highest amount? Should the Input and ChIP samples be comparable?

INFORMATION
A single Nanogram (ηg) of good quality ChIP DNA is sufficient to make a NGS library. The Diagenode Microplex DNA Library Preparation Kit if recommended if sequencing on Illumina technology (Hiseq 2000/2500/4000, Nova-seq, Miseq, Nextseq). This library prep method is extremely easy but long, so is guaranteed to work if sufficient DNA is acquired (User manual: www.diagenode.com/files/products/kits/MicroPlex-Libary-Prep-Kit-v2-manual.pdf). However, we will not do this in these practical due to the lack of useful utility and time.
Other kits can be used, but this one is very convenient although is one of the more expensive on the market; other kits include: NEBNext Ultra II DNA Library Prep Kit (NEB), TruSeq ChIP Library Prep Kit (Illumina), KAPA HyperPrep Kit (Roche), etc.

END OF PRACTICAL DAY 2

3 Practical Day Three

3.1 NGS Library Size Selection

Important: Work at RT throughout the process, the Ampure XP Beads do not work efficiently when cooled.
Tip: Pipetting accuracy is vital for good size selection

3.1.1 Take a 50 µl Post-PCR NGS Library aliquot (in PCR Tube)
3.1.2 Warm the sample to approximate RT
3.1.3 Pulse centrifuge to collect contents
3.1.4 Add 30 µl Ampure XP Beads

Note: this has to be at warmed to RT, if necessary warm a little in the hand.
Note: Ensure the beads are completely homogenous before adding to the sample

3.1.5 Incubate at RT for 10 min
3.1.6 Place sample on the magnet until clear
3.1.7 Transfer the supernatant to a new PCR tube

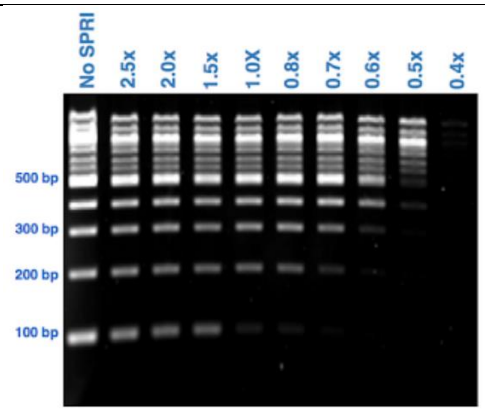
Note: Magnet pellet contain the larger fragments of DNA and can now be discarded

3.1.8 Add 20 µl Ampure XP Beads to the supernatant
3.1.9 Incubate at RT for 10 min
3.1.10 Place sample on the magnet until clear
3.1.11 Discard the supernatant
3.1.12 Add 200 µl 80% Ethanol
3.1.13 Keep on the magnet for 30s
3.1.14 Discard the supernatant
3.1.15 Add 200 µl 80% Ethanol
3.1.16 Keep on the magnet for 30s
3.1.17 Discard the supernatant
3.1.18 Use pipette tip to remove any residual ethanol globules
3.1.19 Take off the magnet
3.1.20 Allow the beads to dry

Note: the beads need to be dry, but should not be over-dried. The beads will crack across the entire surface when they are ready – depending on the air conditioning it will be 2-10 minutes

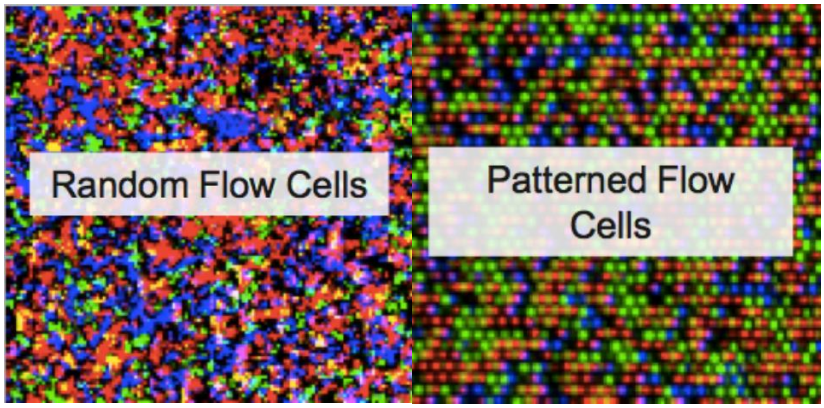
3.1.21 Resuspend the dried beads in 10 µl H_2O
3.1.22 Incubate at RT for 3 min
3.1.23 Place sample on the magnet until clear
3.1.24 Transfer the supernatant to a 1.5ml tube (labelled pleased)
3.1.25 Place the samples in the 2-8°C refrigerator

INFORMATION
The size selection functions by using different Ampure Ratios. The ratio is the volume of Ampure Solution:Sample, the ratio at step 3.1.4 was 0.6x (30 µl:50 µl) but the ratio at step 3.1.8 is 1.0x (50 µl:50 µl). It is important to consider that the volume of Ampure XP Bead solution is the important factor in calculating the ratio not the amount of the beads themselves; the beads have a very high capacity so are rarely the limiting factor. The Ampure XP solution is probably 20% PEG-8000, 2.5M NaCl (proprietary information, so only probably).
The different Ampure XP ratios can be used to bind for different molecular weights, this is a general binding demonstration:



http://core-genomics.blogspot.com/2012/04/how-do-spri-beads-work.html

Size selection is becoming more and more important for NGS sequencing. This is due to Illumina altering their technology to increase the throughput; they transitioned from a Random to a Patterned flow cells and this means there is less tolerance for longer fragments. For example:



http://core-genomics.blogspot.com/2016/01/almost-everything-you-wanted-to-know.html

3.2 Library Final QC (Run by Instructors)

3.2.1 Samples will be run on a Agilent Bionalayzer using High Sensitivity DNA Chips

Note: Due to time limitations only 11 samples will be run during Talk 1.

INFORMATION

The bioanalyzer works by capillary electrophoresis and creates a profile useful for both molecular weight range and the concentration, although, a ±20% error is quoted for DNA quantification.

END OF PRACTICALS

4 Appendix

4.1.1 Sonication Shear Buffer

10 mM Tris-HCl, pH 8.0, 0.1% $^{w}/_{v}$ SDS, 1 mM EDTA

4.1.2 Chromatin Dilution Buffer

20 mM Tris-HCl, pH 7.9, 1% $^{v}/_{v}$ Triton X-100, 2 mM EDTA, 150 mM NaCl

4.1.3 Low Salt Wash Buffer

20 mM Tris-HCl, pH 7.9, 0.1% SDS, 1% $^{v}/_{v}$ Triton X-100, 2 mM EDTA, 150 mM NaCl

4.1.4 High Salt Wash Buffer

20 mM Tris-HCl, pH 7.9, 0.1% SDS, 1% $^{v}/_{v}$ Triton X-100, 2 mM EDTA, 500 mM NaCl

4.1.5 LiCl Wash Buffer

100 mM Tris-HCl, pH 7.5, 0.25 M LiCl, 0.5% $^{v}/_{v}$ NP-40, 0.2% $^{w}/_{v}$ Sodium Deoxycholate

4.1.6 TE Wash Buffer

10 mM Tris-HCl, pH 8.0, 0.1 mM EDTA

4.1.7 SDS Elution Buffer

10 mM Tris-HCl, pH 8, 300mM NaCl, 5 mM EDTA, 1% $^{w}/_{v}$ SDS