Chromatrap

V1.2

Chromatrap® 96 ChIP-seq

A solid phase chromatin immunoprecipitation assay for next generation sequencing

Protocol v1.2

Catalogue no 500214, 500215, 500216, 500217

ADVANCEMENTS IN EPIGENETICS

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Introduction

Genome-wide mapping of protein-DNA interactions is essential for a complete understanding of gene regulation. A detailed map of epigenetic marks and transcription factor (TF) binding is necessary for deducing the regulatory networks that underpin gene expression in a variety of biological systems. The most widely used tool for examining these interactions is chromatin immunoprecipitation (ChIP) followed by massively parallel sequencing (ChIP-seq).

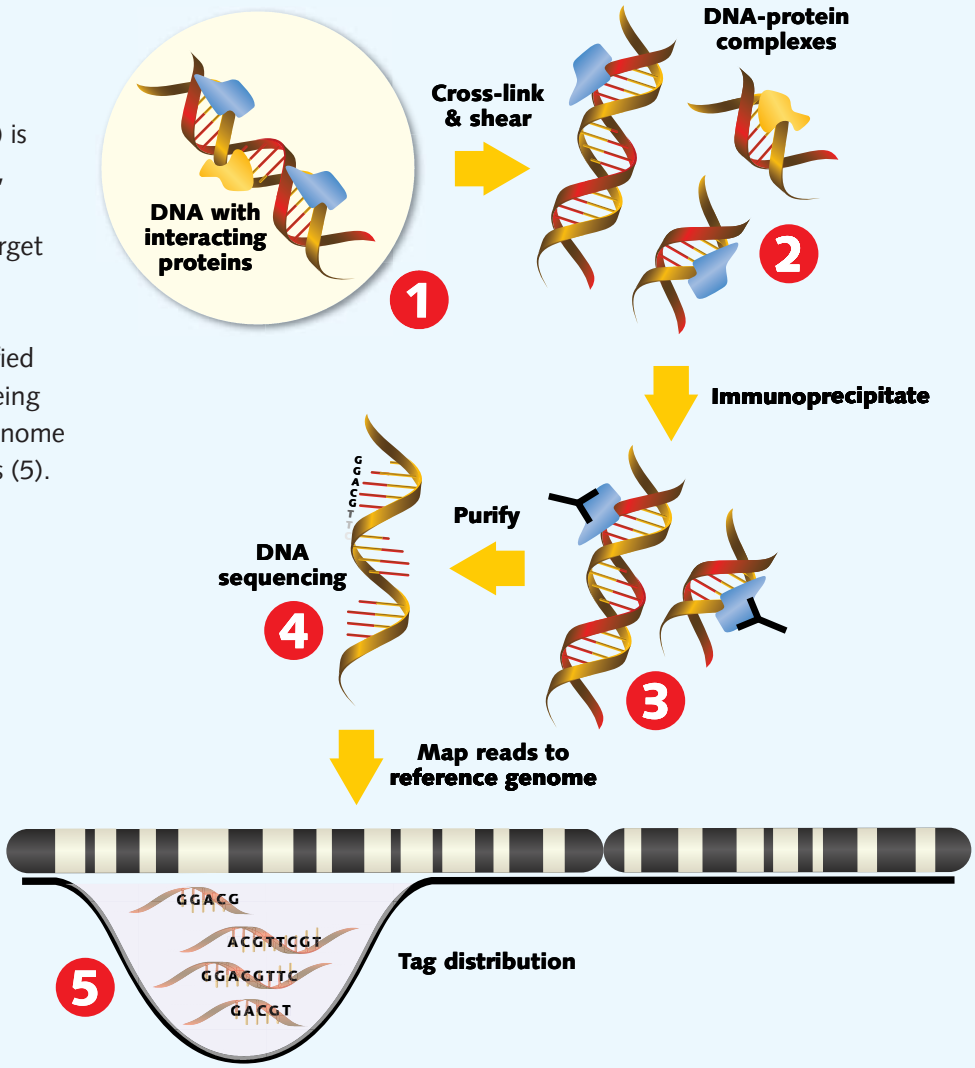
The ChIP assay is a technique that enriches DNA fragments to which a specific protein of interest or histone modification is bound. Sites enriched in this manner can be identified by qPCR, hybridisation to a microarray (ChIP-on-chip), or by sequencing, the latter two of which enable genome-wide analysis of protein-DNA interactions.

ChIP-seq is proving to be an attractive alternative to ChIP-on-chip. With the ability to sequence tens to hundreds of millions of DNA fragments in a single run, generating single-base pair resolution, fewer artefacts and greater coverage, ChIP-seq offers significantly improved data compared with previous technology. Many examples of ChIP-seq yielding mechanistic understanding of cellular regulatory processes can be found in the literature, including transcriptional regulation *(Lee 2002, Chen et al 2008, Nielsen 2008)* epigenetic regulation *(Barski 2007)* and nucleosome organisation *(Heintzman 2009, Tolstorukov 2009)*.

The short reads generated by next-generation sequencing (NGS) platforms are ideal for ChIP-seq and allow precise mapping of protein binding sites as well as improved identification of sequence motifs. Importantly, ChIP-seq allows the spatial resolution for profiling post translational modifications of chromatin and histone variants as well as nucleosome positioning. With the increasing performance of sequencing platforms, ChIP-seq is set to become the leading technology for genome-scale analysis of protein-DNA interactions.

Figure 1:
ChIP-seq overview

Chromatin in the nucleus (1) is cross-linked and sheared (2), followed by enrichment of complexes containing the target protein using immunoprecipitation (3). Enriched fragments are purified and sequenced (4) before being mapped onto a reference genome to yield a distribution of tags (5).



Szalkowski A M, and Schmid C D
Brief Bioinform 2011;12:626-633

Chromatrap® ChIP-seq

Chromatrap® is a new, quicker, easier and more efficient way of performing ChIP-seq assays (Patent No. GB2482209). It uses discs of an inert, porous polymer to which Protein A or Protein G has been covalently bound to maximise the capture efficiency of the target chromatin/antibody complex. Chromatrap® utilises the solid state technology in parallel with high throughput sequencing to deliver a precise ChIP-seq protocol from small cell numbers and low chromatin concentrations.

Specifically adapted for broader chromatin concentrations, Chromatrap® ChIP-seq now combines the dynamic range of Chromatrap® with the downstream analysis power of deep sequencing. This allows faster, more reproducible genome-wide identification of TF binding sites and specific DNA associated protein modifications. With no limitation in scale or resolution, NGS can elucidate the role of TFs and epigenetic marks on gene transcription and epigenetic chromatin status.

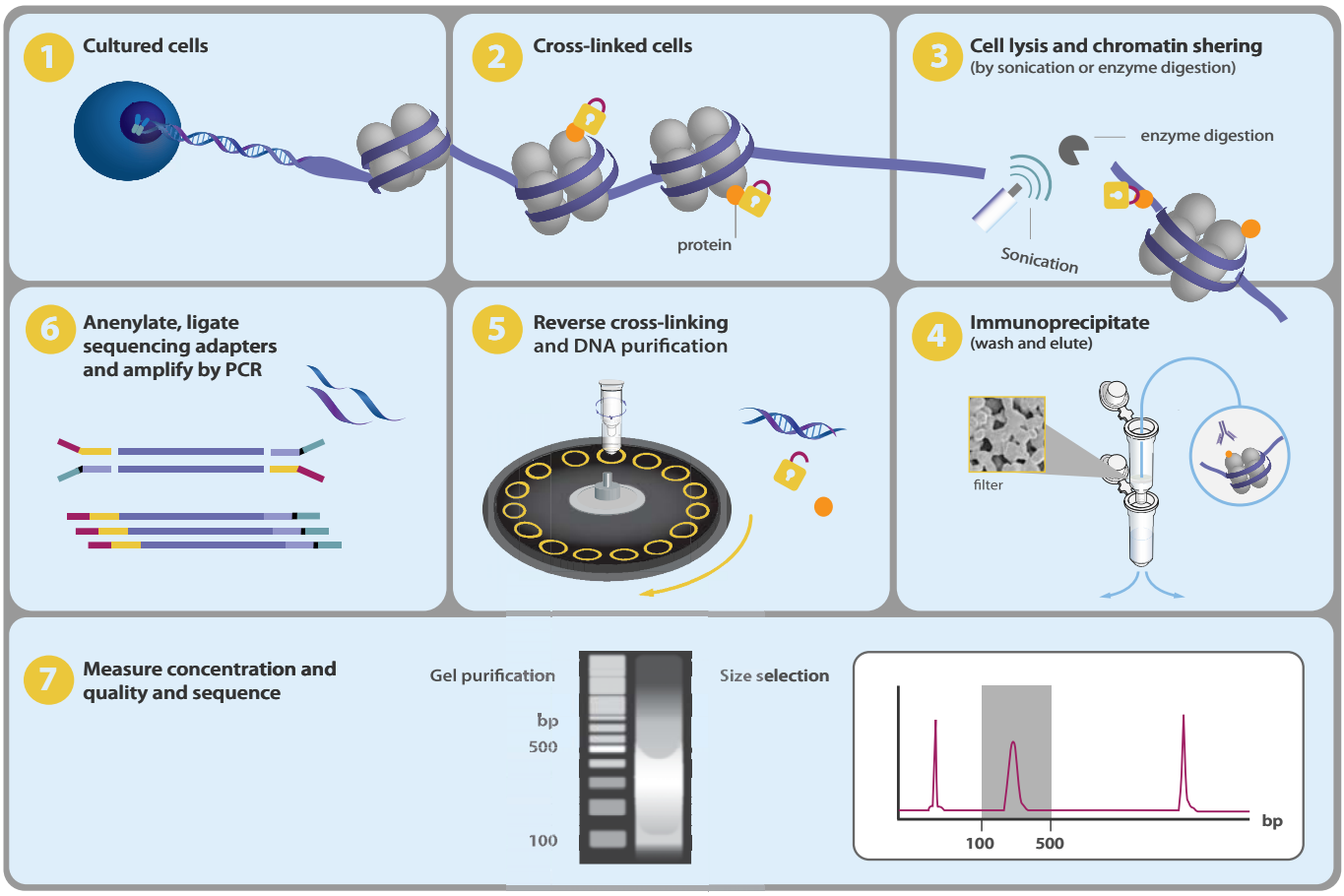


Figure 2: *Overview of ChIP-seq process*

ChIP-seq begins with a traditional ChIP assay involving cell fixation (cross-linking), chromatin shearing, immunoprecipitation (IP), reverse-crosslinking and DNA purification (Figure 2). Living cells are fixed with a reversible crosslinking agent to retain protein-DNA interactions at their natural sites before being lysed in order to release the chromatin for shearing. Following crosslinking, the chromatin is sheared to a specific size range (100-500 bp) for optimal IP and ChIP-seq results. Either sonication or enzymatic shearing can be used to achieve fragment sizes between 100-500 bp, the chromatin is then immunoprecipitated using an antibody of interest and isolated using the Chromatrap® spin column technology.

ChIP essentially produces a library of target DNA sites that were in direct physical contact with regulatory mechanisms *in vivo*. Oligonucleotide adapters are then added to the fragments of DNA that were bound to the protein of interest to enable massively parallel sequencing. After size selection, all the resulting ChIP DNA fragments are sequenced simultaneously, scanning for genome-wide associations with high resolution as opposed to large sets of tiling arrays required for lower resolution ChIP-on-chip. Mapping the sequenced fragments to whole genome sequence databases allows the DNA interaction pattern of any TF or epigenetic modification to be analysed quickly and effectively.

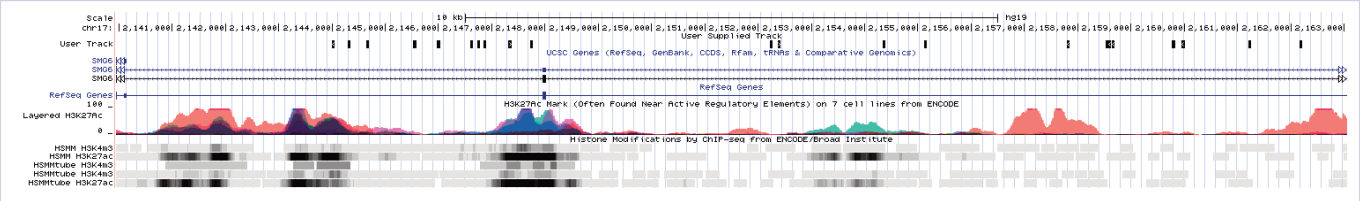


Figure 3: *An example of H3K4me3 sequence alignment using human HEC50 cells immunoprecipitated with the Chromatrap® ChIP-seq kit and sequenced using the Illumina MiSeq sequencer.*

Advantages of Chromatrap® ChIP-seq:

- *ChIP-seq from as little as 1-50 µg of chromatin*
- *Up to 10 library preparations from a single IP*
- *ChIP-seq assay completed in five days*
- *Sensitive and selective enrichment of low chromatin loading*
- *Fully compatible with next-generation sequencing technology*
- *Elution chemistry optimised for high quality and quantity of immunoprecipitated DNA*
- *The polymer disc is inert, reducing non-specific binding*
- *Reduced IP incubation times*

Highlights of protocol V1.2

- *High quality of chromatin achieved via sonication or enzymatic digestion*
- *High and low abundant enrichment from small chromatin samples*
- *Preparation of columns by gravity for improved antibody binding*
- *Extended chromatin loadings providing greater chromatin loading flexibility*
- *Increased slurry volumes for difficult samples*

Kit overview and timetable

The Chromatrap® ChIP-seq kit allows the user to perform up to 96 ChIP assays from cell collecton through to immunoprecipitation, including up to 10 chromatin sample preparations. The kit provides all of the major components required for performing ChIP assays to obtain adequate high quality DNA for NGS library preparation. Up to 10 libraries can be prepared from a single IP. This protocol provides supporting information and tips for library synthesis, cluster generation and sequencing analysis using the Illumina® MiSeq platform.

Step	Process	Time required	Day
1	Cell fixation and collection	0.5 hour	1
2	Cell lysis and chromatin shearing	0.5 hour	1
3	Immunoprecipitation	1 hour	1
4	Reverse cross-linking and DNA purification	3.5 hours	1
5	Quantitative PCR analysis	1 hour	1
6*	NGS library synthesis	Variable	2-4
7*	Cluster generation and sequencing	Variable	4-5
8*	Sequencing analysis	Variable	5-6

Table 1: *Chromatrap® ChIP-seq protocol overview. *The time required to complete process 6-8 is dependent on the library synthesis kit used, the number of samples being processed and the equipment used for sequencing. Days required are an approximation.*

Kit components

The Chromatrap® ChIP-seq kits (500214, 500215, 500216 and 500217) allow the user to perform up to 96 ChIP assays from cell collection through to immunoprecipitation. Upon receipt, please ensure the components are stored at the temperatures listed in Table 2.

Kit Component	Quantity	Storage
Chromatrap® 96 HT	1	4°C
Chromatrap® 96 HT balance plate	1	Room temperature
96-well collection plate	1	Room temperature
96-well balance collection plate	1	Room temperature
Elution plate	1	Room temperature
96-well balance elution plate	1	Room temperature
Elution and balance plate strip caps	24	Room temperature
Column conditioning buffer (3x)	80 ml	4°C
Wash Buffer 1 (3x)	60 ml	4°C
Wash Buffer 2 (3x)	60 ml	4°C
Wash Buffer 3 (3x)	60 ml	4°C
1.3 M Glycine	20 ml	4°C
Lysis Buffer	10 ml	4°C
Digestion buffer (only in 500216 and 500217)	10 ml	4°C
Enzymatic Stop Solution (only in 500216 and 500217)	200 µl	4°C
ChIP-seq Elution Buffer	10 ml	4°C
5 M NaCl	500 µl	4°C
1 M $NaHCO_3$	750 µl	4°C
Hypotonic Buffer	10 ml	4°C
Shearing cocktail (only in 500216 and 500217)	100 µl	-20°C
Protease Inhibitor Cocktail (PIC)	250 µl	-20°C
Proteinase K stop solution	300 µl	-20°C
Proteinase K	150 µl	-20°C
H3K4me3 antibody	20 µl (0.2 µg/µl)	-20°C
Immunoglobulin G	20 µl (0.2 µg/µl)	-20°C
Forward primer	50 µl (8 µM)	-20°C
Reverse primer	50 µl (8 µM)	-20°C

Table 2: *Chromatrap® ChIP-seq reagents and materials*

It is recommended that you aliquot Shearing Cocktail on receipt of the kit to minimise the number of freeze thaw cycles and maintain the activity of the cocktail.

The kits are manufactured DNase free and when stored as directed are stable for up to 6 months. Sufficient material is supplied for 96 ChIP assays and up to 10 chromatin sample preparations.

Additional materials required

Reagents and consumables

- *PBS*
- *37% formaldehyde, molecular biology grade*
- *Nuclease-free water*
- *100bp ladder*
- *Cell-scrapers*
- *Microcentrifuge tubes (0.5 ml and 1.5 ml)*
- *PCR plates*
- *DNA LoBind tubes (optional)*
- *Pipettes and tips (filter tips are recommended)*
- *ChIP validated antibody*
- *qPCR primer pairs for gene of interest*
- *PCR purification kit (e.g. Illustra GFX PCR DNA and Gel Band Purification Kit)*
- *For enzymatic shearing, 0.1% SDS solution*
- *Reservoirs*

Equipment

- *Microcentrifuge (4°C)*
- *Agarose gel electrophoresis equipment*
- *Rocking platform for culture plates/flasks*
- *Spectrophotometer/fluorometer for DNA quantification*
- *For sonication, Sonicator*
- *Labware for preparation of 1X buffer solutions*
- *Centrifuge with plate rotor (4°C)*
- *Multi channel pipette*
- *37°C waterbath*
- *65°C waterbath*
- *End to end rotator*

Additional materials required for Illumina® sequencing library synthesis

- *96-well PCR plate or tubes*
- *96-well magnetic stand or similar*
- *Thermocycler*
- *Qubit 2.0 fluorometer with dsDNA high sensitivity kit (or equivalent fluorometric quantification method)*
- *Agilent Technologies 2100 Bioanalyzer with high sensitivity DNA kit*
- *Ultrapure agarose (Inhibitor- and nuclease-free)*
- *SYBR® Gold nucleic acid stain*
- *Agencourt AMPure XP beads*
- *Library sample preparation kit (e.g. Illumina® TruSeq)*
- *Library quantification kit (e.g. KAPA library quantification kit)*

ChIP-seq considerations

Antibody quality

The success and value of any ChIP-seq experiment is dependent on the quality of the antibody used. A highly-specific antibody will increase the relative enrichment of the target compared with the background, making it easier to detect binding events during data analysis. Many commercially available antibodies are listed as ChIP-seq grade and, wherever possible, should be used for your experiments. However, lot to lot variations and variability in quality does occur and the antibodies of choice should be validated before use. Chromatrap® offers an antibody validation service, please contact Chromatrap® Customer Support for more information.

Sample quantity

A typical ChIP experiment yields between 10-200 ng of DNA, requiring approximately 10^7 cells. On the Illumina® platform the sample preparation guide recommends loading between 5-10 ng of ChIP DNA. However, if sequence duplication becomes an issue we would recommend increasing the quantity of starting material for library amplification to help minimise duplication levels. Additionally, the number of PCR cycles used during the enrichment step can also be reduced if duplication remains an issue.

Shearing

The experimental and processing steps in ChIP can introduce potential sources of artefacts. For example, chromatin shearing does not result in uniform fragmentation whether sheared mechanically through sonication or by enzymatic digestion. Open chromatin tends to shear more easily than closed regions, creating an uneven distribution of sequence fragments. Equally, nucleases used during enzymatic digestion exhibit a more pronounced sequence bias during cleavage. We find that once optimised, both sonication and enzymatic shearing generate fragment sizes ideal for ChIP-seq.

Control experiment

Peaks identified during sequencing analysis must be compared to the same region in a matched control sample in order to verify their significance. For example, a random region of repetitive sequences may appear enriched due to the number of copies of the region, creating a false-positive result. There are three commonly used controls: input DNA (DNA that has not been immunoprecipitated); mock IP (DNA treated the same but without antibody during the IP); and non-specific IP (IP with an antibody targeting a protein not known to be involved in DNA binding such as IgG). There is no consensus as to which control is most appropriate to use, however, input DNA and IgG controls are commonly used as they account for bias related to the shearing of DNA and amplification. We recommend using input as a control.

Experiment planning

1. Cell culture

This protocol has been optimised for use with cell lines and provides enough reagents for up to 10 chromatin preparations ($15x10^6$ cells) and up to 96 ChIP assays. Lower cell numbers are possible, however, volumes of buffers will need to be adjusted accordingly (Table 3). Up to 10 libraries (25 ng starting material each) can be prepared from a single IP.

2. Shearing optimisation

The success of a ChIP assay is highly dependent on the quality of chromatin prepared. Before starting the assay, chromatin should be sheared to fragments in the range of 100 to 500 bp. This kit has been optimised for chromatin shearing using both sonication and enzymatic digestion. The shearing conditions described within the protocol are suitable for a variety of cell types and may be taken as a guide. However, given the variations between cell types, we recommend optimising shearing conditions before progressing with ChIP (see Troubleshooting for more information).

3. Chromatrap® 96 plate

This kit includes a protein A- or protein G-coated Chromatrap® 96-well plate. Prepare only the required number of wells for your ChIP assay immediately prior to loading the slurry. Make sure that the wells do not dry out during the procedure as this may lead to reduced performance.

4. Slurry volume

A key advantage of the Chromatrap technique compared to conventional bead based assay is the flexibility in chromatin loading. The fundamental requirement for optimal antibody binding is to load 1-50 µg chromatin in a total volume of 1 ml ensuring that the chromatin does not exceed more than 10% (100 µl) of the total 1 ml slurry volume.

5. Quantification

For library synthesis, it is critical to determine the concentration of IPd DNA using a high sensitivity fluorescence based quantification method as UV-based spectrophotometers such as the NanoDrop are unreliable for quantification of low quantities of DNA (see Troubleshooting). The concentration of DNA will be influenced by a variety of factors including cell type, target abundance and antibody affinity.

6. Positive and negative IP controls

In addition to the ChIP validated antibody, we recommend the use of a positive and negative control antibody. We suggest including one negative IgG control antibody corresponding to the host species in which the antibody of interest was raised for each series of ChIP reactions. We provide a positive ChIP-seq grade control antibody, H3K4me3, and recommend using 1 µg chromatin and 2 µg H3K4me3 to validate successful IP. See Step 3a of the protocol for more information.

7. Quantitative PCR validation

Before beginning library synthesis for sequencing, we recommend analysing the IPd DNA using at least one positive and one negative control target of your choice. In order to have sufficient DNA for library preparation, it is recommended that not more than 10% of the total IPd DNA be used for qPCR. If necessary, DNA can be diluted 1:10 to provide an adequate volume for triplicate PCR reactions. Control targets for the antibody of choice should be analysed by the user as appropriate.

8. Quantitative PCR interpretation

The efficiency of immunoprecipitation provides an indicator of the relative success of a ChIP assay and requires the interpretation of qPCR data to determine which DNA fragments have been enriched. This can be expressed as the recovery of the locus calculated as a percentage of input as follows:

$$\% \text{ recovery} = 2^{(Ct_{input} - Ct_{sample})}$$

The ratio of the positive versus negative targets should be approximately five fold for a confident IP.

Protocol

Step 1: Chromatin preparation; cell fixation and collection

Wherever this 'pause point' symbol appears, it signifies that if required, the sample can be stored at -80°C.

The following section describes fixation for both adherent (step 1a) and suspension (step 1b) cells, chromatin extraction from other sources will require optimisation by the user. Remember to prepare enough chromatin for any biological IP controls.

Step 1a: For adherent cells

1. Culture between 1-15 million cells.
2. Remove media and wash with warm PBS at room temperature (RT).
3. Remove the PBS and add basic cell culture media (this should not contain any serum or large molecular weight proteins) containing 1% formaldehyde, ensure all cells are covered in order to fix the cells and cross link the DNA/protein complexes.
4. Incubate for 10 minutes at RT with gentle agitation on a rocking platform.
5. Remove the fixation solution and add 0.65 M glycine solution to quench the reaction (glycine is supplied as a 1.3 M solution and should be diluted 50:50 with PBS for use, refer to Table 1 for optimum volume for starting cell number).
6. Incubate for 5 minutes at RT with gentle agitation on a rocking platform.
7. Remove the glycine solution and collect the cells by scraping in ice cold PBS (ensure sufficient PBS to cover the surface of the cells). Collect cells by centrifugation at 3500xg for 5 minutes at 4°C.
8. Discard the supernatant. Proceed to step 2.

At this point the protocol can be continued or the pellet can be frozen and stored at -80°C, if freezing the pellet add 1µl Protease Inhibitor Cocktail (PIC).

Step 1b: For suspension cells

1. Collect cells by centrifugation at 500xg for 5 minutes at 4°C.
2. Re-suspend in 1 ml pre-warmed PBS (perform cell count) and spin 500xg for 5 minutes at RT.
3. Re-suspend pellet in 1 ml PBS then add 27 µl 37% formaldehyde (to give final concentration of 1%) in order to cross link DNA/protein complexes.
4. Incubate for 10 minutes at RT on an end to end rotator.
5. Add 1.3 M Glycine (114 µls / ml of sample) and incubate 5 minutes at RT on an end to end rotator.
6. Spin to collect cells at 500xg for 5 minutes at 4°C.
7. Re-suspend in 1 ml ice cold PBS.
8. Spin to collect cells at 500xg for 5 minutes at 4°C and discard the supernatant. Proceed to step 2.

At this point the protocol can be continued or the pellet can be frozen and stored at -80°C, if freezing the pellet add 1 µl Protease Inhibitor Cocktail (PIC).

Buffer preparation: The volume of buffer required for chromatin preparation is dependent upon the starting cell number. Use the following table to determine the optimum volume for each buffer.

Table 3

Buffer	Cell Count (Millions)	Buffer Volume (ml)
0.65 M Glycine*	1-5 5-10 10-15	3 4 5
Hypotonic Buffer	1-5 5-10 10-15	0.4 0.8 1.0
Lysis Buffer**	1-5 5-10 10-15	0.3 0.3-0.5 0.5-1.0
Digestion Buffer for enzymatic digestion	1-5 5-10 10-15	0.3 0.4 0.5
Enzymatic Stop Solution for enzymatic digestion	1-5 5-10 10-15	7.5 µl 10 µl 12.5 µl

** Glycine is supplied as 1.3 M, please dilute 50:50 with PBS buffer to reach a working concentration of 0.65 M for adherent cells only.*

*** Lysis Buffer must be pre-warmed to 40°C in a water bath for 30 minutes with occasional shaking before use, to remove any precipitates. The contents of the bottle should be mixed by inverting it a couple of times before putting it into the water bath and (at least) once half-way through the incubation. Bring the buffer back to room temperature when ready to use.*

Preparation of 1X buffers: The following buffers are provided at a 3X concentration and require dilution to a 1X working stock prior to performing IP.

Column Conditioning Buffer (3X), 80 mls
Wash Buffer 1 (3X), 80 mls
Wash Buffer 2 (3X), 80 mls
Wash Buffer 3 (3X), 80 mls

To each 80 mls add 160 mls molecular biology grade water to achieve a 1X concentration solution (total volume 240 mls).

Step 2: Cell Lysis and Chromatin Shearing

Chromatin can be sheared either by a sonication (mechanical using ultrasonic sound waves) or an enzymatic (micrococcal nuclease digestion) approach. It is important to choose the appropriate method of shearing for cells. Section 2a describes chromatin shearing by sonication for 1-15 x 10^6 cell preparations and the buffer volumes required are outlined in Table 1. The protocol assumes shearing conditions have been optimised by the user, if this is not the case please refer to Troubleshooting for optimal shearing conditions. For enzymatic shearing please refer to section 2b.

Step 2a: Cell lysis and chromatin shearing by sonication

1. Re-suspend the cell pellet in Hypotonic Buffer and incubate the samples at 4°C for 10 minutes (refer to Table 1 for optimum volume from starting cell number).
2. Centrifuge the hypotonic slurry at 5000xg for 5 minutes at 4°C to collect the nuclei.
3. Discard the supernatant and re-suspend the pellet in Lysis Buffer (ensure the Lysis Buffer has been pre-warmed prior to use to ensure all precipitates are fully dissolved, refer to Table 1) and incubate samples at 4°C for 10 minutes.
4. Sonicate samples until the desired lengths of DNA fragments are achieved (100-500 bp).
5. Centrifuge the samples for 10 minutes at maximum speed at 4°C and transfer the supernatant to a clean dry microcentrifuge tube.
6. Add 1 µl of PIC to the samples and mix.
7. Chromatin samples are now ready for IP, if samples are not to be used immediately store at -80°C for a maximum of 2 months. It is recommended that the shearing efficiency of each chromatin stock is analysed at this stage.

N.B. *Shearing efficiency varies greatly and will need to be optimised and confirmed separately, checking the size of the fragments on an agarose gel such as described in the following quantification section (Step 2c).*

Step 2b: Cell lysis and chromatin shearing by enzymatic digestion

1. Re-suspend the cell pellet in Hypotonic Buffer and incubate the samples at 4°C for 10 minutes (refer to Table 1 for optimum volume from starting cell number).
2. Centrifuge the hypotonic slurry at 5000xg for 5 minutes at 4°C to collect the nuclei and discard the supernatant.
3. Re-suspend the pellet (nuclei) in Digestion Buffer by pipetting (refer to Table 1 for optimum volume for starting cell number), immediately add 2 µl PIC to each stock nuclei suspension. Keep stock nuclei suspensions on ice while determining DNA concentration.

Determining DNA concentration

- Remove a 10 µl sample of each stock nuclei suspension and add to 490 µl 0.1% SDS, mix well and incubate on ice for 10 minutes.
- Estimate the concentration of DNA on a spectrophotometer and use this to calculate the total amount of chromatin in each stock nuclei suspension in order to determine volume of Shearing Cocktail to be used (eg. Nanodrop reading x 50 x total volume of stock nuclei suspension).

Example calculation

Sample measures 9 ng/µl
9 (concentration) x 50 (dilution factor) x 400 (volume of Digestion Buffer)
= 180,000 ng or 180 µg total chromatin
1 U Shearing Cocktail per 5 µg chromatin therefore 180/5 **= 36 U Shearing Cocktail**
Shearing Cocktail is supplied as 15 U per µl therefore 36/15 **= 2.4 µl Shearing Cocktail to be added**.

4. Add Shearing Cocktail to each stock nuclei suspension (from step 2b, point 3) at a ratio of 1 U Shearing Cocktail:5 µg chromatin (Shearing Cocktail is supplied as 15 U/µl) and mix thoroughly.
5. Incubate for 5 minutes in a 37°C waterbath then immediately add Enzymatic Stop Solution (refer to Table 3 for optimum volume) and place tubes on ice.
6. Centrifuge for 5 minutes at 12,000xg at 4°C and discard the supernatant.
7. Re-suspend the pellets (nuclei) in Lysis Buffer (ensure the Lysis Buffer has been pre-warmed prior to use to ensure all precipitates are fully dissolved, refer to Table 3 for optimum volume) and incubate the tubes on ice for 10 minutes to lyse the nuclei.
8. Centrifuge the samples for 10 minutes at maximum speed at 4°C and transfer the supernatant to a clean dry microcentrifuge tube.
9. Add 1 µl of PIC to the samples and mix.
10. Chromatin samples are now ready for IP. If samples are not to be used immediately, store at -80°C for a maximum of 2 months. It is recommended that the shearing efficiency of each chromatin stock is analysed at this stage.

N.B. *Shearing efficiency varies greatly and will need to be optimised and confirmed separately, checking the size of the fragments on an agarose gel such as described in the following quantification section.*

Step 2c: Shearing efficiency

Chromatin shearing should be checked on an agarose gel to ensure that the appropriate fragment sizes have been generated during shearing. Prior to immunoprecipitation, aliquots of stock chromatin are also used for DNA quantification in order to determine the volume of DNA required for slurry preparation in step 3.

1. Take a 25 µl aliquot of sheared chromatin from each sample and place in a microcentrifuge capped tube.
2. Add 5 µl of 1 M $NaHCO_3$ and 5 µl of 5 M NaCl and make up to a final volume of 50 µl with nuclease free water and mix thoroughly.
3. Incubate the samples at 65°C for 2 hours to reverse the cross-linking. If required samples can be left overnight.
4. Briefly centrifuge the samples to remove any liquid from the caps.
5. Add 1 µl of the Proteinase K solution and mix thoroughly. Incubate for 1 hour at 37°C.
6. Return the samples to room temperature and add 2 µl Proteinase K stop solution.
7. Quantify the DNA in the samples using a spectrophotometer at 260 nm. Multiply the reading by 2 to account for the dilution during the reverse cross-linking. This will be used to determine the volume of chromatin to load in Step 3; Slurry Preparation and Immunoprecipitation.
8. To ensure that 100-500 bp fragments have been obtained during shearing the DNA should be run on an agarose gel and visualised against a marker of known size DNA fragments (e.g. 100 bp ladder). A smear of DNA fragments 100-500 bp in length is ideal, fragments of smaller or greater length may affect the efficiency of the ChIP reaction.

N.B. *If chromatin is over- or under-sheared refer to the relevant section of the troubleshooting guide and FAQs.*

Step 3: Slurry preparation and column activation

Step 3a: Slurry preparation and plate activation

This section has been optimised for 30 µg chromatin per ChIP. Although it is possible to use more, or even less chromatin, this will require optimisation by the user to ensure an adequate quantity of IPd DNA for library preparation is acquired at the end of the assay. The initial recommendation would be to load **30 µg chromatin and 5 µg antibody** in a total volume of **1 ml** including other components of the slurry. The ratio of antibody:chromatin will need to be optimised by the user. For the positive control for qPCR analysis, prepare the slurry in a 1 ml volume with a 2:1 antibody : chromatin ratio. Remember to prepare negative controls for both antibody validation and the standard assay described in Table 4.

1. Thaw chromatin stocks at 4°C.
2. Centrifuge sheared chromatin at max speed for 10 minutes at 4°C, even if previously centrifuged.
   NOTE: Use only the clear supernatant for subsequent steps.
3. Prepare IP slurries in a fresh microcentrifuge tube according to Table 4. For every antibody IP set aside the equivalent amount of chromatin in a microcentrifuge tube and make up to 100 µl with Column Conditioning buffer (if necessary), label as an input. These will be processed alongside the samples for reverse cross-linking and proteinase K digestion at step 4a and will be used as controls in the downstream analysis.
4. Mix well and incubate the IP slurries on an end to end rotor for 1 hour at 4°C.

Reagent	Immunoprecipitation Slurry (1000µl Total Volume)	Positive Control (1000µl Total Volume)
Chromatin stock	Up to 100 µl	1 µg
Antibody /IgG	Optimum addition rate	10 µl (2 µg)
PIC	2 µl	2 µl
Column Conditioning Buffer	Make up to final volume of 1000 µl	Make up to final volume of 1000 µl

Table 4: *Slurry preparation for IP*

Step 3b: Chromatrap® 96-well plate preparation

The Chromatrap® 96-well plate is shipped in a storage solution, prior to use, the plate must be washed and activated to remove any traces of shipping solution and to prepare them for slurry incubation.

1. Remove the top and bottom seals of the Chromatrap® 96-well plate and position on to the 96-well collection plate provided.
2. Add 600 µl of Column Conditioning Buffer to each well of Chromatrap® 96-well plate and allow to flow through under gravity (~ 15 minutes).

N.B. do not close caps when flow is under gravity.

3. Discard the flow through and repeat this conditioning step a second time.
4. Discard the flow through. The plate is now ready for the addition of the IP slurries proceed to step 3c.

Step 3c: Immunoprecipitation

The immunoprecipitation step involves the binding of the antibody of interest to the protein A/G attached to the Chromatrap® 96-well plate frit. This allows the selective enrichment of the target protein/DNA complex and allows any non-specific complexes to be washed away. Target chromatin is then eluted using a specially formulated ChIP-seq elution buffer for maximal target recovery.

N.B. *If precipitates have formed in the elution buffer then it should be warmed to 40°C in a water bath for thirty minutes with regular shaking until precipitates have dissolved* **before use**.

1. Remove slurries from the end-to end rotator following 1 hr pre-incubation and briefly spin down to remove residual liquid from the caps.
2. **Remove the Chromatrap® 96-well plate from the collection plate (save for later)** and place in an empty 1 ml tip box rack (or alternative holder), load the entire 1 ml slurry and allow to flow completely through the wells of the plate at RT (approx 15-20 minutes).
3. Add 600 µl of Wash Buffer 1 to each well and centrifuge at 2000 x g for thirty seconds at RT (remember to add 600 µl d.H20 to corresponding wells in 96-well balance plate). Discard the flow through and repeat.
4. Add 600 µl of Wash Buffer 2 to each well and centrifuge at 2000 x g for thirty seconds at RT. Discard the flow through and repeat.
5. Add 600 µl of Wash Buffer 3 to each well and centrifuge at 2000 x g for thirty seconds at RT. Discard the flow through and repeat.
6. Centrifuge at max speed for thirty seconds. This step removes any remaining liquid from the plate.Transfer the Chromatrap® 96-well plate on to the supplied Elution plate.
7. Add 50 µl **ChIP-seq Elution Buffer** to each well, cap and incubate at RT for fiveteen minutes.
8. While incubating prepare a balance plate for the centrifuge, use the Chromatrap® 96-well balance plate with the balance elution plate provided and pipette 50 µl d.H_2O into the corresponding wells.
9. Centrifuge the plate at top speed for one minute to collect the eluted chromatin.

Step 4: Reverse crosslinking

Step 4a: Reverse crosslinking

Chromatin samples must be reverse cross-linked to release the DNA from protein bound complexes. Protein is then degraded by Proteinase K digestion before being purified in Step 4b of the protocol. Input controls which have not been through the IP process (step 3a.3) must be reintroduced at this stage and treated as per the sample.

1. To each eluted sample add 5 µl of 1 M $NaHCO_3$, 5 µl of 5 M NaCl and make up to a final volume of 110 µl with water. To each input add 5 µl of 1 M $NaHCO_3$ and 5 µl of 5 M NaCl for a final volume of 110 µl. Mix thoroughly and incubate for 2 hours at 65°C. If required, the incubation at 65°C can be performed overnight.
2. Add 1 µl Proteinase K to each IP and input sample. Vortex briefly and perform a short spin. Incubate for one hour at 37°C.
3. Add 2 µl Proteinase K Stop Solution to each IP and input sample. Vortex briefly and perform a short spin.

N.B. *When using larger concentrations of chromatin it may be necessary to dilute the input prior to qPCR to prevent any inhibition in the PCR reaction. Do not clean up the inputs if diluting. Chromatrap® recommends diluting inputs 1 in 10 or 1 in 100 and deducting 3.3/6.6 Cts respectively, with 100% primer efficiency.*

Step 4b: DNA purification

Chromatin must now be purified before proceeding with qPCR or library synthesis. IPd DNA can be purified using a commercially available DNA purification kit. We recommend the Illustra GFX PCR DNA and Gel Band Purification Kit (GE Healthcare Life Sciences, 28 9034 70). Alternatively, DNA can be purified by phenol/chloroform extraction using an inert carrier such as linear polyacrylamide (LPA). The use of glycogen as a carrier is not recommended due to potential contamination with nucleic acids from a biological source.

Extraction and ethanol precipitation of IPd DNA

1. Add an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) to the IPd DNA. Vortex thoroughly.
2. Centrifuge the samples at 4°C, max speed for ten minutes to separate the aqueous phase from the organic phase.
3. Carefully remove as much of the top (aqueous) phase as possible and transfer to a new 1.5 ml tube.
4. Add 0.1 volume 3 M sodium acetate pH 5.2 and 500 µl ice-cold 100% ethanol.
   Optional – add 1 µl LPA (25 µg/µl) to each sample.
5. Incubate for a minimum of four hours at -80°C or overnight at -20°C.
6. Centrifuge at max speed for ten minutes at 4°C. Discard the supernatant.
7. Resuspend the pellet in 500 µl 70% ethanol. Centrifuge at max speed for five minutes at 4°C.
8. Without disturbing the pellet, carefully remove and discard the supernatant.
9. Allow the pellet to air dry and resuspend in 30 µl nuclease-free water or 1x TE buffer pH 8.0 for long-term storage.

Samples are now ready for validation by qPCR.

Step 5: Quantitative PCR analysis

Step 5: Quantitative PCR analysis

Prior to sample sequencing, we recommend analysing the IPd DNA by qPCR using at least one positive and one negative control to validate the IP. The Chromatrap® Chip-seq kit contains positive control primers for human GAPDH.

1. Prepare the qPCR reaction mix as follows for a 10 µl reaction volume:

- 5 µl of a 2x SYBR Green qPCR mix
- 2.5 µl primer mix (combine primers 1:1)
- 2.5 µl IPd or input DNA

Primer concentrations may need to be adjusted but we recommend a final concentration of 1 µM in the reaction mix for each primer.

Program the thermal cycler as follows for the positive and negative primers supplied.

Two minutes at 95°C
10 seconds at 95°C
30 seconds at 60°C
15 seconds at 72°C
} 40 cycles

These conditions may require optimisation depending on the primer, qPCR mix and qPCR system used.

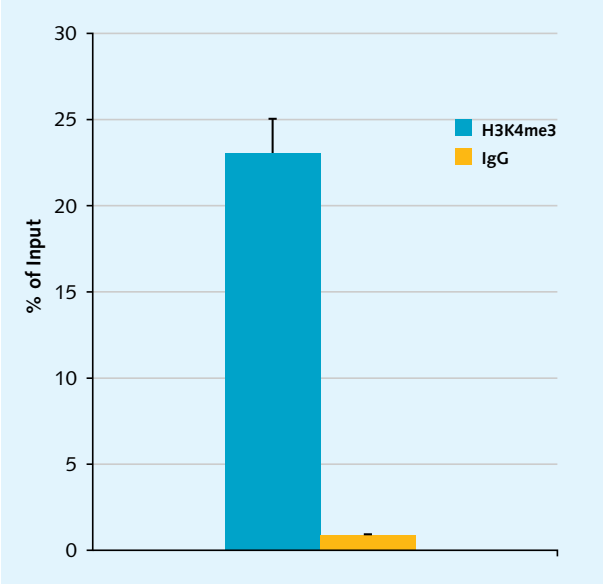


Figure 4: *ChIP was performed on human cancer cells using the Chromatrap® ChIP-seq kit. IP was performed with the positive ChIP control antibody H3K4me3 using 2 µg antibody and 1 µg chromatin, and qPCR performed with positive control GAPDH primer set. The data is presented as mean % input (the relative amount of IPd DNA compared with input DNA after qPCR analysis).*

ChIP-seq guidelines for use with the Illumina® MiSeq

Reaction conditions

ChIP-seq requires careful optimisation of numerous reaction conditions from the number of cells used in culture to the number of fragment clusters for optimal sequencing analysis. Chromatrap® ChIP-seq reduces the number of optimisation steps required and has been tailored for use with Illumina® Miseq sequencing platforms. However, the kit is also compatible with other sequencing platforms such as the Illumina® HiSeq, the SOLiD system and Roche 454.

Sequencing coverage

Before starting a sequencing experiment, you should consider the depth of sequencing you want to achieve. It is recommended that a minimum of 20 million mapped reads are achieved for transcription factors and that two biological replicates are used for site discovery. The greater the depth of coverage i.e. the number of times a base in the genome is sequenced, the greater the confidence that the base called was correct. Ultimately the standard is set by journal publications although we suggest approximately 30x coverage as a benchmark.

Paired end or single read

Generally, ChIP fragments are sequenced at the 5′ end as a single read which extends in one direction. Although, they can also be sequenced at both ends, as is frequently used to detect structural variations in the genome such as insertions, deletions and inversions. Paired-end reads can be used in conjunction with ChIP for additional specificity when mapping as paired end reads are more likely to align unambiguously to a reference genome, especially in repetitive regions (Figure 5). Therefore, the user should decide whether single or paired end reads would be most appropriate for mapping to a reference genome.

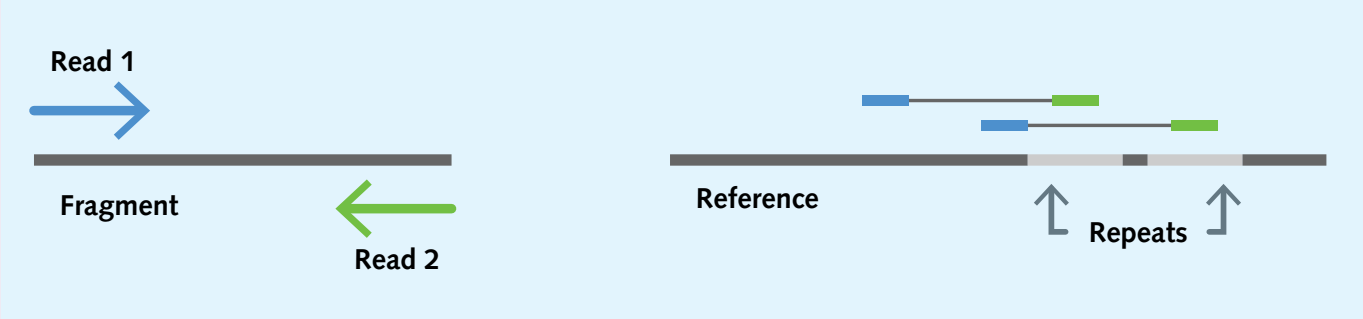


Figure 5: *Paired end sequencing allows both ends of the DNA fragment to be sequenced. The distance between each read is known, therefore, alignment algorithms can use this information to more accurately map the reads to the reference genome. In single end sequencing, the reads represented by the green bars would not be uniquely mapped due to the repetitive sequences in the reference genome.*

Multiplexing

Sequencing samples individually can be a laborious and expensive endeavour, making a sequencing run more cost-effective is highly beneficial to the user. The number of reads generated by sequencing one sample at a time may be several times greater than what is needed for adequate coverage. As such, the ability to sequence multiple samples simultaneously becomes desirable. Samples can be labelled with a unique barcode or index that allows multiple samples to be pooled for sequencing in a single run. A short string of bases provides the distinguishing identifier that, once sequenced, allows the user to isolate those reads that belong to a specific sample (Figure 6). For example, it may be desirable to pool ChIP DNA and IgG control for a single sequencing run. We recommend multiplexing a limited number of samples in order to retain sufficient depth of coverage for each sample, or increasing the number of sequencing runs to obtain sufficient coverage.

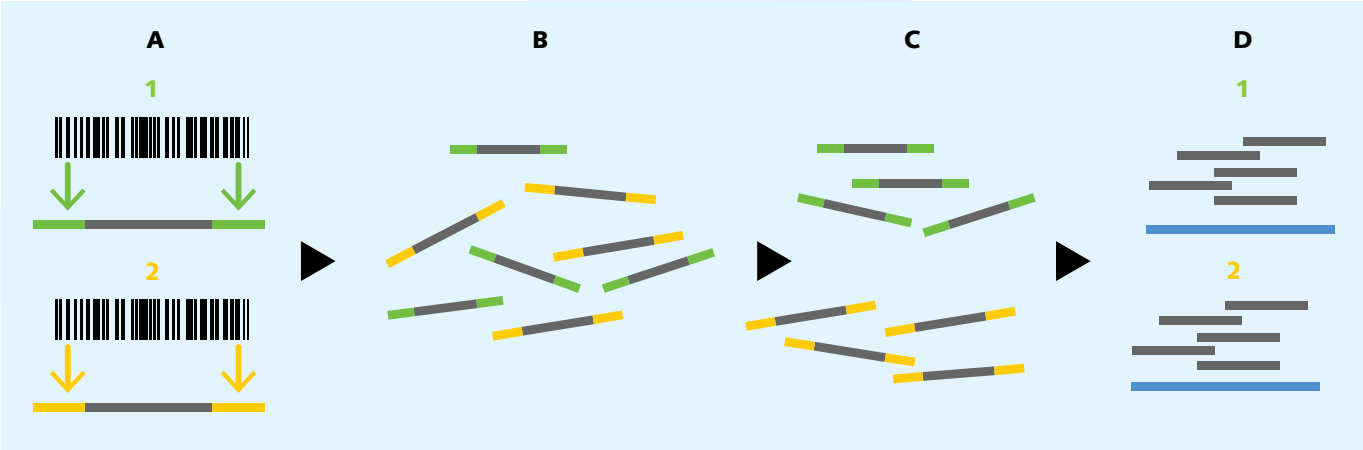


Figure 6: *Overview of sample multiplexing. A) Two DNA fragments from two unique samples are 'barcoded' with a specific index which identifies the sample from which it originated. B) Sample libraries are pooled and sequenced in parallel. Each new read contains both the specific index and insert sequence. C) Samples are de-multiplexed using software to differentiate reads obtained for each sample. D) Each set of reads is aligned to the reference genome and produces two unique data sets.*

Cluster generation

During cluster generation DNA fragments are hybridised to the surface of the flow cell and are bridge-amplified to form clusters. Millions of single-molecule clusters can be achieved per square centimetre. The recommended cluster density is between 800 K-1200 K/mm^2. Over-clustering can cause an overlap between clusters, making them indistinguishable from each other, resulting in a loss of data. Similarly, under-clustering can result in reduced data output by not maximising the number of potential clusters that could be sequenced. Density should be optimised by the user before deciding on the final library concentration to use for sequencing.

Handling magnetic beads

Magnetic beads should be brought to room temperature before use and should not be reused.

- *Immediately prior to use, vortex the beads until they are well dispersed.*
- *Once added to the reaction mixture, pipette up and down gently to ensure beads are dispersed homogenously.*
- *Take care not to disturb the beads during aspiration.*
- *Gently pipette the clear solution with the pipette tip facing the opposite side wall to which the beads are attached.*
- *Perform these steps with the plate on the magnetic stand throughout.*
- *When performing ethanol wash steps ensure that all ethanol is left to evaporate by air-drying at room temperature.*
- *Do not over-dry the beads as this can make resuspension difficult.*

DNA quantification

The accurate quantification of IPd DNA for library preparation is critical. The success of library preparation depends on accurately quantified DNA. It is difficult to accurately quantify the IPd DNA using spectrophotometers due to its low yield and concentration.

- Quantify DNA using a fluorometric-based quantification method such as the Qubit or PicoGreen.
- The NanoDrop is not recommended for quantification as it is not sufficiently sensitive at lower concentrations and is influenced by RNA, dsDNA, ssDNA, and free nucleotides.

DNA quality

The quality of DNA is commonly assessed by absorbance measurements at 260 nm. The ratio of absorbance at 260 nm to 280 nm is used as an indication of sample purity.

- A ratio of 1.8 is typically considered "pure" DNA.
- Further validation is recommended using the Agilent Bioanalyzer High Sensitivity DNA kit to ensure ChIP samples are of the expected size range and concentration.

Ligation product purification

When purifying adapter-ligated products, SYBR® Gold is recommended as the nucleic acid stain due to its sensitivity.

- Follow the instructions for SYBR® Gold use according to the sequencing library synthesis guide, (not the manufacturer's). Any deviation from this procedure may result in incorrect size excision.

Additional protocols

Bioanalyzer 2100

For validation of IPd DNA prior to library preparation, we recommend diluting your sample to 5 ng/µl, if necessary. For post library synthesis follow the sequencing library manufacturer's instructions.

1. Allow the High Sensitivity DNA dye concentrate (blue) and High Sensitivity DNA gel matrix (red) to equilibrate to room temperature for thirty minutes.
2. Add 15 µl of High Sensitivity DNA dye concentrate (blue) to a High Sensitivity DNA gel matrix vial (red).
3. Vortex solution well and spin down. Transfer to spin filter.
4. Centrifuge at 2240 g ± 20% for ten minutes. Protect solution from light. Store at 4°C.

Loading the Gel-Dye Mix

1. Allow the gel-dye mix equilibrate to room temperature for thirty minutes before use.
2. Put a new High Sensitivity DNA chip on the chip priming station.
3. Pipette 9.0 µl of gel-dye mix in the well marked **G**.
4. Make sure that the plunger is positioned at 1 ml and then close the chip priming station.
5. Press plunger until it is held by the clip.
6. Wait for exactly one minute then release clip.
7. Wait for five seconds, then slowly pull back the plunger to the 1 ml position.
8. Open the chip priming station and pipette 9.0 µl of gel-dye mix in the other wells marked G.

Loading the Marker

1. Pipette 5 µl of marker (green) in all sample and ladder wells. Do not leave any wells empty.

Loading the Ladder and the Samples

1. Pipette 1 µl of High Sensitivity DNA ladder (yellow) in the well marked ladder.
2. In each of the 11 sample wells pipette 1 µl of sample (used wells) or 1 µl of marker (unused wells).
3. Put the chip horizontally in the adapter and vortex for one minute at the indicated setting (2400 rpm).
4. Run the chip in the Agilent 2100 Bioanalyzer within five minutes.

Evaluating ChIP-seq data

Data Analysis Considerations

Histone modifications alter the chromatin environments in cells by changing the DNA binding patterns or providing recognition sites for other chromatin effector modules to ensure complex mechanisms of gene regulation and cellular function. Epigenome profiles generated from ChIP-seq provide increased understanding of these relationships between specific histone modifications and gene regulation outcomes. Interestingly, histone modification and TF ChIP-seq data generated using the same technique generate vastly different profiles and read map distributions. TFs, for example, provide discrete, sharp peak distributions along the genome, whilst histone modification reads have continuous properties due to the homogenous epigenetic status of nearby nucleosomes, resulting in broad peak marks.

Despite the widespread use of ChIP-seq, there are considerable differences in how these experiments are conducted, how the results are evaluated for quality and how the data and meta data are archived for public use. It is important to consider the common issues such as IP specificity and quality, impact of DNA sequencing depth, scoring and evaluation of data sets, appropriate control experiments, biological replication and data reporting (Figure 7).

Sequencing depth

The quality of individual ChIP-seq experiments can vary considerably and can be especially difficult to assess when new antibodies are being tested or when very little is known about the TF and its binding motif. Effective analysis of ChIP-seq data requires sufficient sequencing depth which can be calculated using the Lander/Waterman equation:

$C = LN/G$

C: coverage; L: read length; N: number of reads, G: haploid genome length

The required depth depends mainly on the size of the genome and size of the binding sites of the protein. Proteins that associate at specific localised sites typically have narrower peaks on the order of thousands of binding sites. However, broader factors such as histone modifications have far more binding sites and will require a greater number of reads to achieve adequate coverage. Saturation analysis is built into some peak calling algorithms and allows the user to determine if the sequencing depth was adequate. If the number of reads is not adequate then the reads from technical replicates can be combined to generate adequate depth. For example, if 20 million reads are required for adequate depth but only 10 million reads are obtained from a sequencing experiment, two technical replicates can be combined to generate the 20 million reads required.

Read mapping and quality metrics

Before mapping the reads to a reference genome the quality of the raw reads should be filtered by applying a cut-off value. FastQC software provides an overview of the data quality to identify possible sequencing errors or biases. Phred scores are used to assign a quality metric to describe the confidence of each base call in each sequence tag. This can be used to filter low quality reads which are more likely to have been called incorrectly. It may also be necessary to trim the ends of the reads as the harsh chemicals used in the sequencing process cause the quality of reads to decrease further along the fragment.

The remaining reads should then be mapped using mapping tools such as Bowtie, BWA or MAQ. The percentage of uniquely mapped reads should be above 70%, whereas < 50% suggests an anomaly during the ChIP-seq procedure. Excessive amplification during PCR can lead to a low percentage of uniquely mapped reads and high sequence duplication. Multi-mapping reads may also be caused by proteins binding to repetitive regions of the genome. In this case, paired end sequencing can help to reduce the mapping ambiguity.

Once mapped, the signal-to-noise ratio of the ChIP-seq experiment should be assessed by using software such as CHANCE, which assesses IP strength by comparing the IP reads pulled down by the antibody with the background.

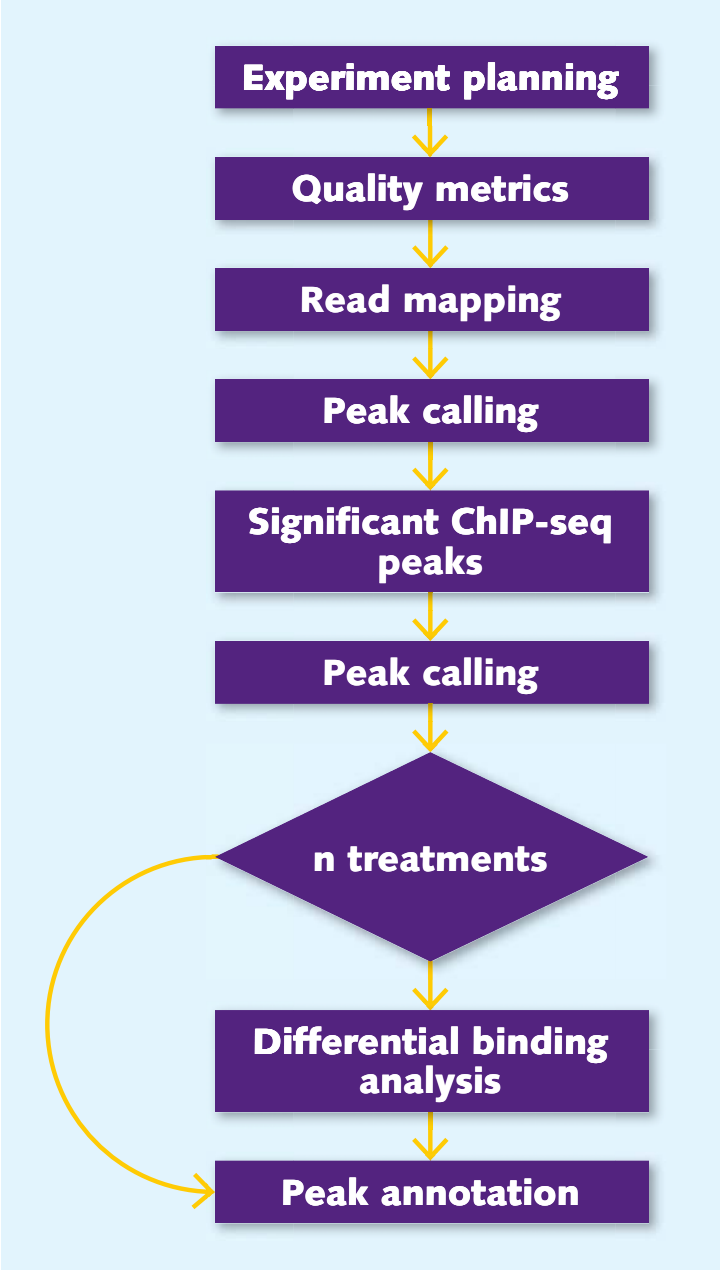
Peak calling – transcription factors

Most peak callers are designed for TFs with limited binding regions that generate a narrow peak. There is a variety of peak calling software available, most of which differ in the algorithms used for background modelling. Software such as MACS is commonly used to predict the protein-DNA interacting regions. Background models are then used to remove noise and peaks are finally called above a user-defined signal to noise ratio.

Most algorithms use a window of a given size to identify the enriched regions and then assessed by enrichment over the control. Statistical significance is commonly measured by false discovery rate, the expected proportion of incorrectly identified sites among those found to be significant. Many nearby binding events may be merged as a single peak, therefore it is advisable to use an R package such as NarrowPeaks to re-rank and narrow-down the final peak list after general peak calling.

Peak calling – broad regions from histone marks

Histone marks tend to have broad spacing and narrow peak callers are not suitable for analysis of such data. Several peak callers specifically designed for predicting broad regions include SICER, CCAT and ZINBA among others. There are no clearly defined peaks when analysing broad marks, therefore the pattern of enrichment is typically described as a domain. Narrow peak algorithms can be used for certain marks enriched at narrow genomic regions (e.g. H3K4me3). MACS2 can actually be used to analyse narrow peaks within broad regions associated with domain boundaries. However, a broad-peak algorithm should be used if possible.



Normalisation

When comparing one ChIP sample with another, e.g. IgG control, there are linear and non-linear normalisation methods to make samples comparable. Commonly, sequencing depth normalisation is used where the number of reads in a sample is multiplied by a scale factor to make the total reads between samples the same. Although normalisation issues are currently not fully exploited, they could be important factors influencing the results.

Duplicated reads

Duplicate reads can arise from independent DNA fragments or can be introduced by amplification of a single fragment by PCR. In the latter case, the signal is an unwanted artefact introduced during library enrichment. Duplicates can be filtered in a well-designed peak calling algorithm to call confident peaks.

Peak annotation

Peaks can be annotated such that they can be located in relation to known genomic features such as transcriptional start sites or exon/intron boundaries. This type of data can be used to generate a genomic landscape, identifying possible associations where significant peaks are generally ranked more consistently across replicates. The irreproducible discovery rate is used to classify peaks into reproducible and irreproducible groups, providing significance criteria that reflect the probability of a peak belonging to the irreproducible group.

Figure 7 *ChIP-seq data analysis flow*

Troubleshooting and FAQs

Process	FAQ	Solution
Crosslinking and fixation	For how long should the cells be crosslinked?	Optimal crosslinking of DNA ensures that the chromatin structure is preserved during the isolation and ChIP procedure. Too little crosslinking will result in DNA loss, elevated background and reduced antigen availability. The optimal time for crosslinking will vary with cell line. Short incubations may improve shearing efficiency whilst over-incubation can cause inhibition, hampering the ChIP assay.
Cell lysis	How do I ensure cells are completely lysed?	Ensure that adequate Lysis Buffer volume is used for the number of cells being processed. Check Lysis under a light microscope.
	How do I prevent protein degradation?	Add protease inhibitors to the chromatin at the appropriate step. Proteases can degrade proteins crosslinked to the DNA, resulting in less efficient IP. If protein degradation is a problem, 1 µl PIC can be added to the ice-cold PBS before collecting the cells for chromatin extraction. Ensure that chromatin extraction steps are performed at 4°C and always keep the samples on ice when processing.
	Why is the Lysis and/or Elution Buffer cloudy?	The Lysis and Elution Buffers contain detergents which precipitate at 4°C. Warm the buffer to 37°C in a water bath for 30 minutes or until fully redissolved. Return to room temperature before use.
Cell type	What cell types have been validated for use with this protocol?	Adherent human cancer cell-lines have been used to validate this ChIP-seq protocol. Other cell types may require optimisation.
Number of cells	How do I determine the optimal number of cells for ChIP-seq?	An important consideration when performing ChIP-seq is the amount of chromatin that will need to be loaded to the column in order to elute sufficient IPd DNA for library synthesis. Sufficient cell numbers should be used so that at least 10 µg of chromatin can be used per IP. We recommend initially extracting chromatin from 90% confluent cells cultured on a 175 cm^2 surface area.

Chromatin shearing	Why do I have a poor yield of sheared chromatin?	Cells may be over crosslinked, making them resistant to lysis and shearing. Ensure cells are fixed for the appropriate time or reduce incubation with formaldehyde. Make sure that the appropriate buffer volumes have been used.
	What sonication conditions should I use?	We have found that 30 second ON/OFF pulses for 15 minutes at a high power setting produces chromatin fragments of 100-500 bp. Ensure that the sample is kept at 4°C during the OFF phase. Shearing conditions may need optimisation by the user.
	Can I use enzymatic shearing?	Yes, enzymatic digestion of chromatin is an ideal method of shearing DNA if a sonicator is not available. Shearing conditions should be optimised to ensure 100-500 bp fragments are generated.
Shearing efficiency	How much chromatin should I load into the gel?	Adequate chromatin should be loaded into the gel for visualisation against the ladder. Do not over- or under-load as this may hinder visualisation. Typically 15-30 µl of the reverse crosslinked stock is adequate for analysis.
	What percentage of agarose should I use?	Use a 1-2% agarose gel.
	What buffer should I use?	Prepare a 1x TAE or TBE buffer for electrophoresis.
	What electrophoretic conditions should I use?	Run the gel slowly at 100-120 V until the dye-front has migrated at least 2/3 the length of the gel.
Chromatin IP	Does the Chromatrap® 96-well plate require blocking?	There is no requirement to carry out a blocking step to minimise non-specific binding as the composition of the Chromatrap® 96-well plate and the buffers provided in the kit have been formulated to ensure that non-specific binding is minimised.
	How much antibody should be used per ChIP?	This should be determined empirically. Abundant targets such as histones will require less antibody per IP compared with low abundant targets such as transcription factors. Use 5-10 µg antibody for low abundant targets. Insufficient antibody may result in poor IP whereas excess can cause non-specific binding and lower specificity.
	What is causing high background?	The quality of the ChIP antibody has a major impact on the success of the assay. Use only ChIP-seq validated antibodies. Inefficient wash steps can also leave traces of non-specific chromatin alongside enriched DNA. If background remains high include an additional wash step during the IP protocol.
	Why do I not have any enrichment?	The antibody used must be ChIP validated. It is essential to include ChIP validated positive and negative antibody controls. Antibodies from other applications may not work in ChIP. The ChIP-Seq kit contains a positive control antibody, H3K4me3, to validate the efficiency of the IP.

Reverse crosslinking	How long should samples be reverse crosslinked?	A minimum of two hours at 65°C. Although, samples can be left overnight if necessary. We recommend the use of DNA LoBind tubes to minimise sample loss during heating.
qPCR	What SYBR® reagents can I use?	iTaq™ Universal SYBR® Green Supermix, PerfeCTa SYBR® green supermix, SsoAdvanced™ SYBR® Green Supermix and IQTM SYBR® Green Supermix have all been shown to produce comparable results with GAPDH primers included in the kit.
	What primers are included in the kit?	The kit contains GAPDH primers for human cells. The forward primer sequence is TCGACAGTCAGCCGCATCT, the reverse primer is CTAGCCTCCCGGGTTTCTCT and the amplicon is 69 bp. If you are working with cells from non-human species these primers will not work.
	What positive and negative controls should I use?	Use the GAPDH primers supplied with the kit for a positive control using H3K4me3 IP. Negative PCR controls: PCR DNA from samples IPd using IgG using GAPDH primers. Alternatively, PCR a region of DNA to which your antibody does not bind. Always include input PCR for data interpretation.
DNA profiling	Why are the NanoDrop and Qubit readings so dissimilar?	The NanoDrop cannot accurately quantify the typically low concentration of IPd DNA. Use a fluorometer such as the Qubit to accurately quantify DNA before library preparation.
	What concentration of DNA should I use for the Bioanalyzer?	The quantitative range of the Bioanalyzer high sensitivity kit is 5-500 pg/µl. IPd DNA may not require diluting; see library synthesis guide for instructions. Load a maximum of 5 ng/µl of sample for analysis using the Bioanalyzer pre-library synthesis.
Library synthesis	How much DNA is required for library synthesis?	We recommend multiplexing a limited number of samples in order to retain sufficient depth of coverage for each sample.
Sequencing parameters	Why do I have high levels of duplication?	ChIP-seq enriches specific fragments of DNA associated with a protein of interest. Therefore high duplication levels in the IP are not unusual. However, if the control sample also has high levels of duplication then we recommend loading more starting material during library preparation to reduce PCR sequence bias introduced during library enrichment.
IP sequencing controls	What control should I use?	We recommend using input as background control.
Sample storage	How should I store my IPd DNA?	Ideally at -80°C for a maximum of three months. We recommend the use of DNA LoBind tubes to minimise sample loss during storage.

Other products available from Chromatrap®

Product	ChIPs	Catalogue number
Chromatrap® Enzymatic Shearing kit	Up to 10 chromatin preps	500165
Standard Chromatrap® Pro A ChIP spin column kit for qPCR	24 IPs	500071
Standard Chromatrap® Pro G ChIP spin column kit for qPCR	24 IPs	500117
Premium Chromatrap® Pro A ChIP spin column kit for qPCR	24 IPs	500115
Premium Chromatrap® Pro G ChIP spin column kit for qPCR	24 IPs	500116
Standard Chromatrap® Pro A Enzymatic ChIP spin column kit for qPCR	24 IPs	500166
Standard Chromatrap® Pro G Enzymatic ChIP spin column kit for qPCR	24 IPs	500168
Premium Chromatrap® Pro A Enzymatic ChIP spin column kit for qPCR	24 IPs	500167
Premium Chromatrap® Pro G Enzymatic ChIP spin column kit for qPCR	24 IPs	500169
Chromatrap® Pro A high throughput ChIP microplate for qPCR	96 IPs	500161
Chromatrap® Pro G high throughput ChIP microplate for qPCR	96 IPs	500163
Chromatrap® Pro A enzymatic high throughput ChIP microplate for qPCR	96 IPs	500162
Chromatrap® Pro G enzymatic high throughput ChIP microplate for qPCR	96 IPs	500164
Chromatrap® Pro A ChIP-seq kit	24 IPs	500189
Chromatrap® Pro G ChIP-seq kit	24 IPs	500190
Chromatrap® Pro A ChIP-seq high throughput microplate	96 IPs	500214
Chromatrap® Pro G ChIP-seq high throughput microplate	96 IPs	500215
Chromatrap® Pro A Enzymatic ChIP-seq kit	24 IPs	500191
Chromatrap® Pro G Enzymatic ChIP-seq kit	24 IPs	500192
Chromatrap® Pro A Enzymatic ChIP-seq high throughput microplate	96 IPs	500216
Chromatrap® Pro G Enzymatic ChIP-seq high throughput microplate	96 IPs	500217

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