Chromatrap

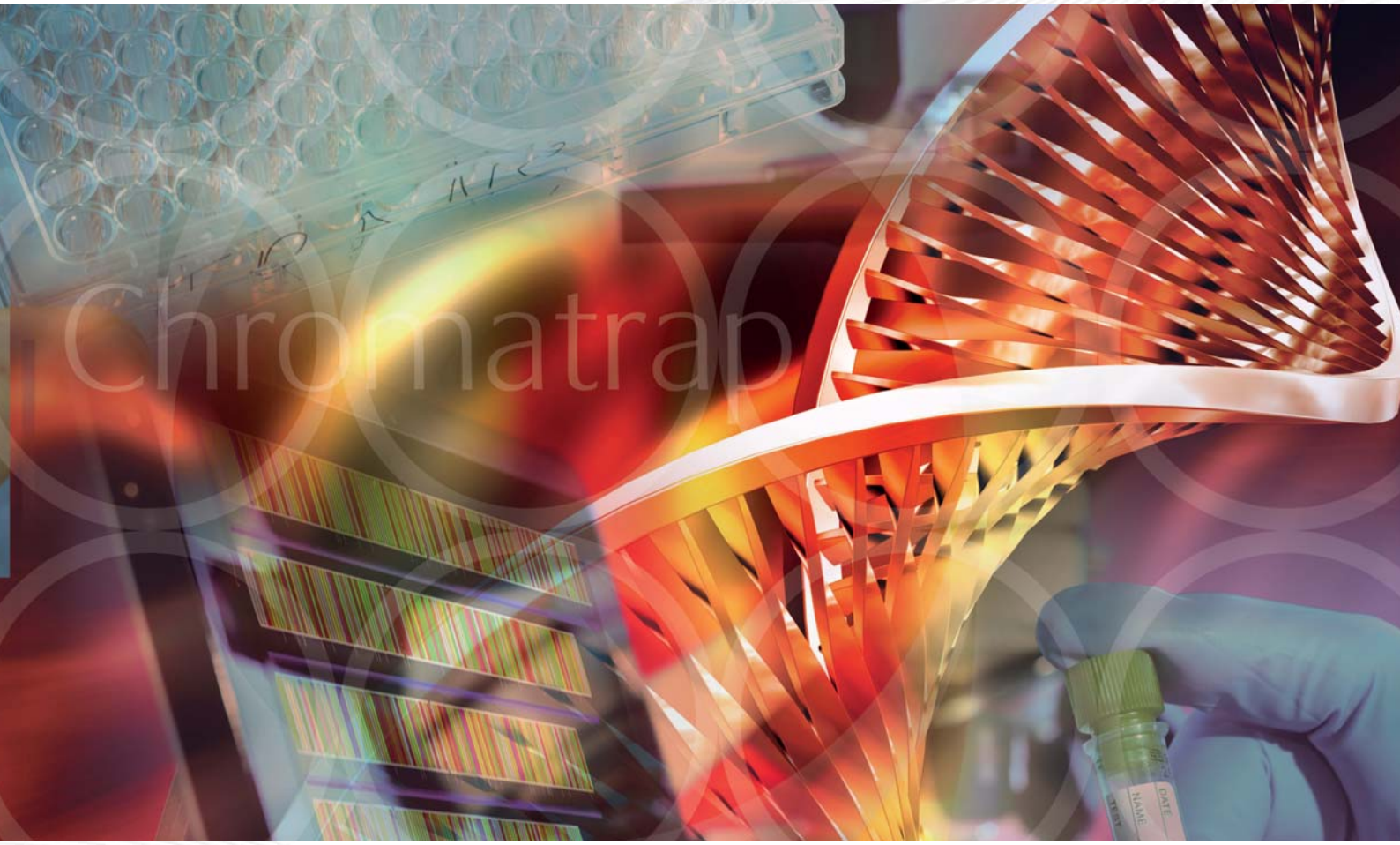
V 1.5

Chromatrap® ChIP-seq

A solid phase chromatin immunoprecipitation assay for next generation sequencing

Protocol v1.5

Catalogue no 500189, 500190, 500191, 500192



ADVANCEMENTS IN EPIGENETICS

Contents

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 an attractive alternative to ChIP-on-ChIP. With the ability to sequence 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 the leading technology for genome-scale analysis of protein-DNA interactions.

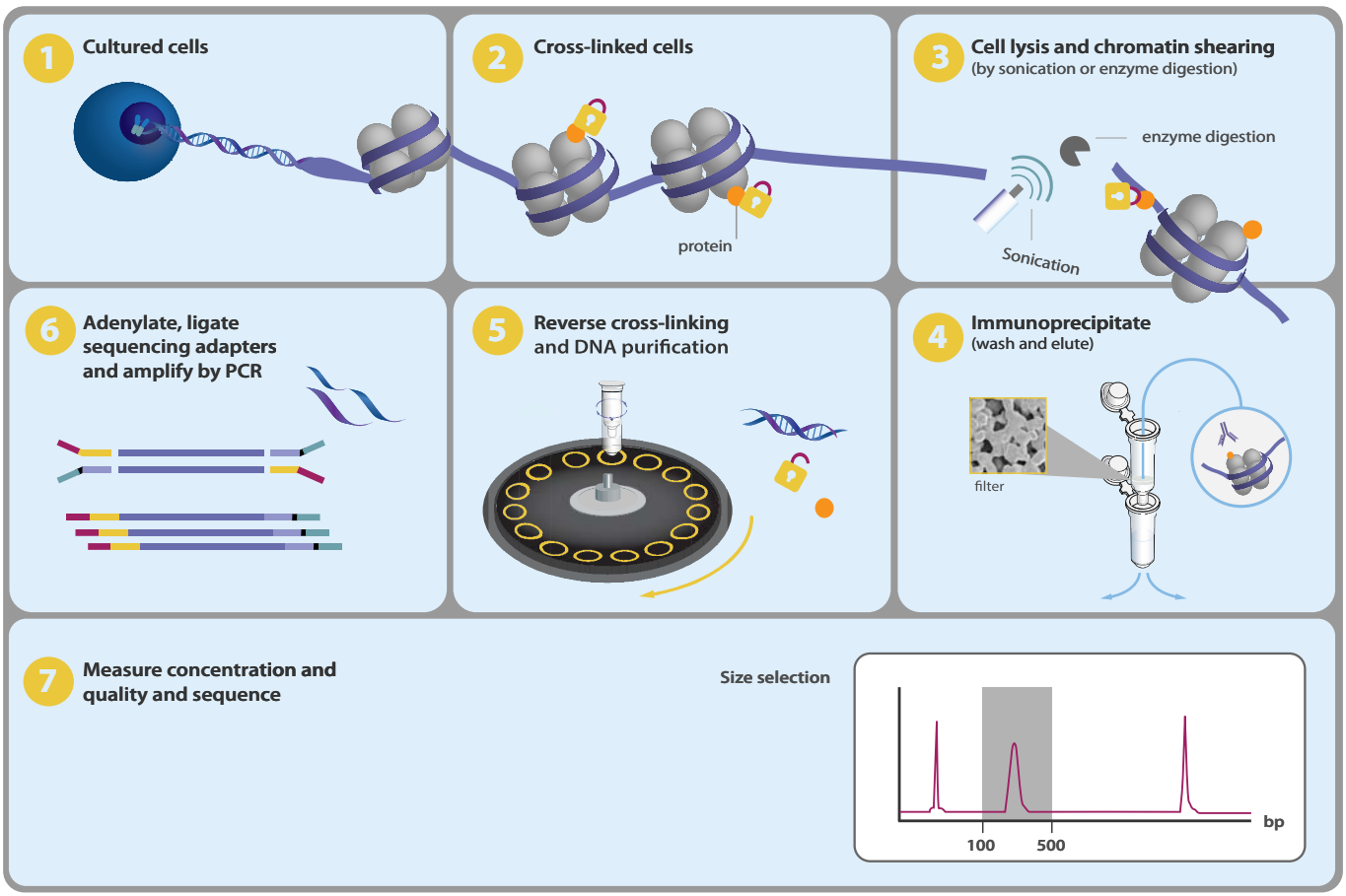


Figure 1: *Overview of ChIP-seq process*

Chromatrap® ChIP-seq

Chromatrap® is a new, quicker, easier and more efficient way of performing ChIP-seq assays (UK Patent No. GB2482209, US Patent No. 9523681, Chinese Patent No. ZL 2011 8 0067254.X, Japan Patent No. JP 6088434 and Australian Patent No AU 2011340263). 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.

ChIP-seq begins with a traditional ChIP assay involving cell fixation (cross-linking), chromatin shearing, immunoprecipitation (IP), reverse-crosslinking and DNA purification (Figure 1). 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. 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.

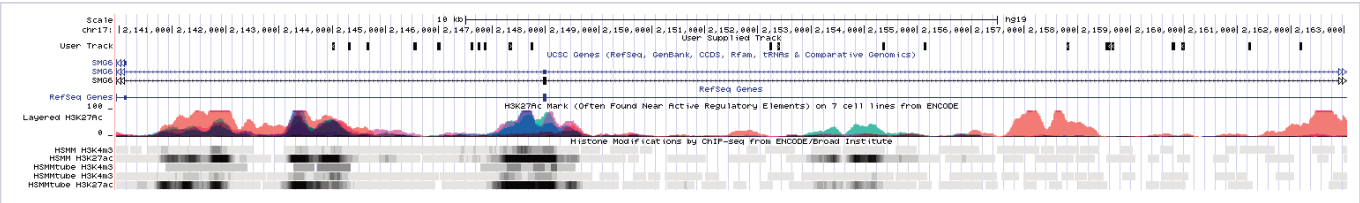


Figure 2: *An example of H3K4me3 sequence alignment using human HEC50 cells immunoprecipitated with the Chromatrap® ChIP-seq kit and sequenced using the Illumina® MiSeq sequencer.*

Advantages of Chromatrap® ChIP-seq:

- *ChIP-seq with as little as 1000 cells*
- *ChIP-seq from as low as 500 ng-50 µg of chromatin*
- *Enough DNA achieved from a single IP without the need to pool samples*
- *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 V1.5

- *ChIP-seq from as low as 500 ng chromatin*
- *Chromatin extraction from as little as 50 mg tissue*
- *Now compatible with downstream Mass Spectrometry*

Kit overview and timetable

The Chromatrap® ChIP-seq kit allows the user to perform up to 24 ChIP assays from cell collection 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. This protocol provides supporting information and tips for library synthesis, cluster generation and sequencing analysis using the Illumina® platforms.

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	*Chromatrap® NGS library synthesis	5 hours	2
7	Library quantification and quality analysis	2 hours	2

Table 1: *Chromatrap® ChIP-seq protocol overview.*
**When using the Chromatrap® UniqSeq library preparation kit (Cat no 500264/500265). Library preparation time with other kits will vary and may take up to 2 days.*

Kit components

Chromatrap® ChIP-seq kits (500189, 500190, 500191 and 500192) allow the user to perform up to 24 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® spin columns	24	4°C
Column Conditioning Buffer	60 ml	4°C
Wash Buffer 1	50 ml	4°C
Wash Buffer 2	50 ml	4°C
Wash Buffer 3	50 ml	4°C
1.3 M Glycine	20 ml	4°C
Lysis Buffer	10 ml	4°C
Digestion Buffer (only in 500191 and 500192)	10 ml	4°C
ChIP-seq Enzymatic Stop Solution (only in 500191 and 500192)	200 µl	4°C
ChIP-seq Elution Buffer	3 ml	4°C
5 M NaCl	500 µl	4°C
1 M $NaHCO_3$	500 µl	4°C
Hypotonic Buffer	10 ml	4°C
H3K4me3 antibody	20 µl (0.2 µg/µl)	-20°C
Immunoglobulin G	20 µl (0.2 µg/µl)	-20°C
Shearing Cocktail* (only in 500191 and 500192)	100 µl	-20°C
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C
Proteinase K stop solution	100 µl	-20°C
Proteinase K	50 µl	-20°C
Forward primer	50 µl (8 µM)	-20°C
Reverse primer	50 µl (8 µM)	-20°C
1.5 ml Collection tube	50	Room temperature
Chromatrap® DNA purification columns	24	Room temperature
DNA Binding Buffer	15 ml	Room temperature
DNA Wash Buffer	15 ml	Room temperature
DNA Elution Buffer	2 ml	Room temperature

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 24 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*
- *For enzymatic shearing 0.1% SDS solution*
- *Scalpel blade (for tissue)*

Equipment

- *Microcentrifuge (4°C)*
- *Agarose gel electrophoresis equipment*
- *Rocking platform for culture plates/flasks*
- *Spectrophotometer/fluorometer for DNA quantification*
- *Sonicator*
- *End to end rotator*

Additional materials required for sequencing using Illumina® platforms

- *Thermocycler*
- *Qubit® 2.0 fluorometer with dsDNA high sensitivity kit (or equivalent fluorometric quantification method)*
- *Agilent Technologies 2100 Bioanalyzer with high sensitivity DNA kit*
- *Library sample preparation kit*
- *Library quantification kit*
- *DNA purification kit with size selection, such as the Chromatrap® Size Selection kit (Cat. no. 500262)*

Optional materials

- *Phenol Chloroform*
- *3 M Sodium Acetate pH 5.2*
- *100% Ethanol*
- *70% Ethanol*
- *Linear Polyacrylamide (LPA)*

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.

Sample quantity

The amount of starting material required to obtain sufficient yields of enriched DNA to prepare a ChIP-seq library will depend on the quality of both chromatin and antibody, the abundance of the target protein and gene and the sensitivity and efficiency of the library preparation kit used. The Chromatrap® ChIP-seq kit has been optimised for library preparation with the Chomatrap® UniqSeq kit (cat. no. 500264/500265). High quality ChIP-seq data can be obtained from as little as 1000 cells using this workflow (see Troubleshooting for guidance on buffer volumes from small cell numbers). A minimum of 500 pg of DNA is required for library preparation using Chomatrap® UniqSeq. The input requirements of other library preparation kits will depend on the manufacturers' limits and will need to be optimised by the user. 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 primary cells, and provides enough reagents for up to 10 chromatin preparations ($15x10^6$ cells) and up to 24 ChIP assays. Lower cell numbers are possible, however, volumes of buffers will need to be adjusted accordingly (Table 3).

2. Shearing optimisation

The success of a ChIP assay is highly dependent on the quality of chromatin prepared. 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. 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 500 ng-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.

4. 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.

5. 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.

6. 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.

7. 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:

$$\% \text{ recovery} = 2^{(Ct_{input} - Ct_{sample})} * \text{dilution} * 100$$

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

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

Protocol

Step 1: Chromatin preparation; fixation and collection

The following section describes fixation for adherent cells (step 1a), suspension cells (step 1b), and fresh/frozen tissue (step1c). 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 1000-1.5x10^7 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 3 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 1 µl 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).

Table 3 (For buffer volumes for less than 1 million cells, please see page 23).

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.*

Step 1c: For fresh/frozen tissue
The following section describes fixation and chromatin preparation of 50-300 mg fresh/frozen tissue. Keep samples on ice at all times to minimise sample degradation, unless stated otherwise.

1. Thaw frozen tissue on ice.
2. Prepare 5-10 ml fixation solution per tissue sample in fume hood (1% formaldehyde in PBS). It is important all of the tissue is covered in order to fix the cells and cross-link DNA/protein complexes
3. Cut the tissue into small pieces (approximately 1 mm^3) in a petri dish using a scalpel blade.
4. Add the fixation solution to the tissue sample in the petri dish and incubate for 10-15 minutes at room temperature (RT) with gentle agitation on a rocking platform (if the tissue sticks to the petri dish, dislodge the tissue using a pipette tip ensuring the tissue is in solution).
5. Remove as much of the fixation solution as possible avoiding the tissue sample.
6. Add 0.65 M glycine solution to quench the fixation reaction (glycine is supplied as a 1.3 M solution and should be diluted 50:50 with PBS for use – refer to table 4 for optimum volumes) and incubate samples at RT for 5 minutes with gentle agitation on a rocking platform.
7. Collect tissue sample in glycine and transfer to a 15 ml centrifuge tube. If some tissue is left behind, add 5 ml PBS to the petri dish and collect the remaining sample and transfer to the 15 ml centrifuge tube.
8. Collect cells by centrifugation at 3500xg for 5 minutes at 4°C and discard supernatant.
9. Add 5 ml ice cold PBS and homogenise sample by pipetting up and down. If large pieces of sample remain, samples can be homogenised in alternative ways such as a hand held tissue homogeniser.
10. Collect cells by centrifugation at 3500xg for 5 minutes at 4°C and discard supernatant. Proceed to step 2.

Table 4: *Optimal buffer volumes for tissue*

Buffer	Tissue (mg)	Volume of buffer
0.65 M Glycine*	50-80 80-120 120-200	3 ml 5 ml 7 ml
Hypotonic buffer	50-80 80-120 120-200	1 ml 2 ml 2.5 ml
Lysis Buffer**	50-80 80-120 120-200	500 µl 700 µl 1 ml

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

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. Section 2a describes chromatin shearing by sonication for $1x10^4$-15×10^6 cell preparations and the buffer volumes required are outlined in Table 3, section 2b describes chromatin shearing by sonication for 50-300mg fresh/frozen tissue. 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 2c.

Step 2a: Cell lysis and chromatin shearing by sonication for adherent/suspension cells

1. Re-suspend the cell pellet in Hypotonic Buffer and incubate the samples at 4°C for 10 minutes (refer to Table 3 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 3) 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.

Step 2b Cell lysis and chromatin shearing by sonication for fresh/frozen tissue

1. Re-suspend cell pellet in Hypotonic Buffer (refer to Table 4 for optimal volumes) by pipetting and incubate sample for 10 minutes at 4°C. For efficient cell lysis, flick the tube every few minutes to prevent the cells settling at the bottom of the tube.
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 prewarmed prior to use to ensure all precipitates are fully dissolved, refer to Table 4) and incubate samples at 4°C for 10 minutes. Flick the tube every few minutes to prevent the cells settling at the bottom of the tube for efficient nuclear lysis.
5. Centrifuge the samples for 10 minutes at maximum speed at 4°C and transfer the supernatant to a clean dry 1.5 ml 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 (step 2d).

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 2d).*

Step 2c: 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 3 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 3 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 2d: Shearing efficiency

Chromatin shearing should be checked on a 1% 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 Immunoprecipitation

Step 3a: Slurry preparation and column activation

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. The DNA yield obtained from ChIP will depend on the quality of the chromatin, the affinity and avidity of the antibody and the abundance of the target. A minimum of 500 pg of DNA is required for preparation of high quality NGS sequencing libraries using the Chromatrap® Uniq-seq Kit (Cat. no. 500264/500265). As little as 1000 cells can yield this quantity of DNA from ChIP of an abundant target using a good quality antibody. As a starting point we would recommend using 10 µg chromatin per ChIP with 2-5 µg antibody. 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 5. 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 (1000 µl total volume)	Positive control (1000 µl 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 5: *Slurry preparation for spin column IP*

Step 3b: Chromatrap® spin column preparation

Chromatrap® spin columns are shipped in a storage solution, prior to use, columns must be washed and activated to remove any traces of shipping solution and to prepare them for slurry incubation.

1. Remove the spin column from the collection tube (save for later) and place in an empty 1 ml tip box rack (or alternative holder).
2. Add 600 µl Column Conditioning Buffer to each column 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 columns are 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 spin column 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 30 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. Load the entire 1ml slurry and allow to flow completely through the column at RT (approx 15-20min).
3. Position Chromatrap® spin columns back into the collection tubes provided and add 600 µl of Wash Buffer 1 to each column. Close the cap and centrifuge at 4000xg for 30 seconds at RT. Discard the flow through and repeat.
4. Add 600 µl of Wash Buffer 2 to each column and centrifuge at 4000xg for 30 seconds at RT. Discard the flow through and repeat.
5. Add 600 µl of Wash Buffer 3 to each column and centrifuge at 4000xg for 30 seconds at RT. Discard the flow through and repeat.
6. Spin dry at top speed for 30 seconds at RT to remove any remaining liquid from the spin column. The original collection tubes should be discarded at this point and columns transferred into clean dry 1.5 ml collection tubes (provided).
7. Add 50 µl **ChIP-seq Elution Buffer** to each column, cap and incubate at RT for 15 minutes.
8. Centrifuge the columns at top speed for 30 seconds to collect the eluted chromatin.

At this stage samples can also be analysed by Mass Spectrometry, for this Chromatrap® recommends pooling IP'd samples to ensure sufficient protein for sample complexity. For sequencing and qPCR analysis please proceed directly to reverse cross linking.

Step 4: Reverse cross-linking

Step 4a: Reverse cross linking

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 (>20 µl) 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. DNA purification columns and reagents are included in all Chromatrap® ChIP-seq kits to recover ultra pure DNA from ChIP samples'. 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.

Preparation of DNA Wash Buffer: Add 60 mL ethanol (95-100%) to the DNA Wash Buffer concentrate before first use and note on label that ethanol has been added.

Some of the components of this product are irritants, refer to MSDS sheet for more information and follow safety guidelines of your research facility.

1. Add 5 volumes of DNA Binding Buffer to 1 volume of sample and mix.

DNA Binding Buffer contains an integrated pH indicator. DNA adsorption requires a pH ≤7.5, and the pH indicator in the buffers will appear yellow in this range. If the pH is >7.5 the binding mixture will turn orange or violet and means that the pH of the sample exceeds the buffering capacity of the DNA Binding Buffer and DNA adsorption will be inefficient. In these cases add 10 µl 3M Sodium acetate, pH 5, to adjust the pH of the binding mixture, the colour of the mixture should turn yellow.

2. Place a Chromatrap® DNA purification column in collection tube provided and transfer sample onto column.
3. Centrifuge at 16,000xg for 60 seconds. Discard flow through.
4. Add 700 µl DNA Wash Buffer to each Chromatrap® DNA purification column and centrifuge at 16,000xg for 60 seconds to remove residual Wash Buffer. Discard flow through. Centrifuge the Chromatrap® DNA purification column once more at 16,000xg for 60 seconds to remove residual Wash Buffer.
5. Place the Chromatrap® DNA purification column in a clean 1.5 ml microcentrifuge tube.
6. To elute DNA, add 50 µl DNA Elution Buffer to the centre of the membrane and incubate for 1 minute, centrifuge at 16,000xg for 60 seconds.

Samples are now ready for validation by qPCR and library preparation.

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.

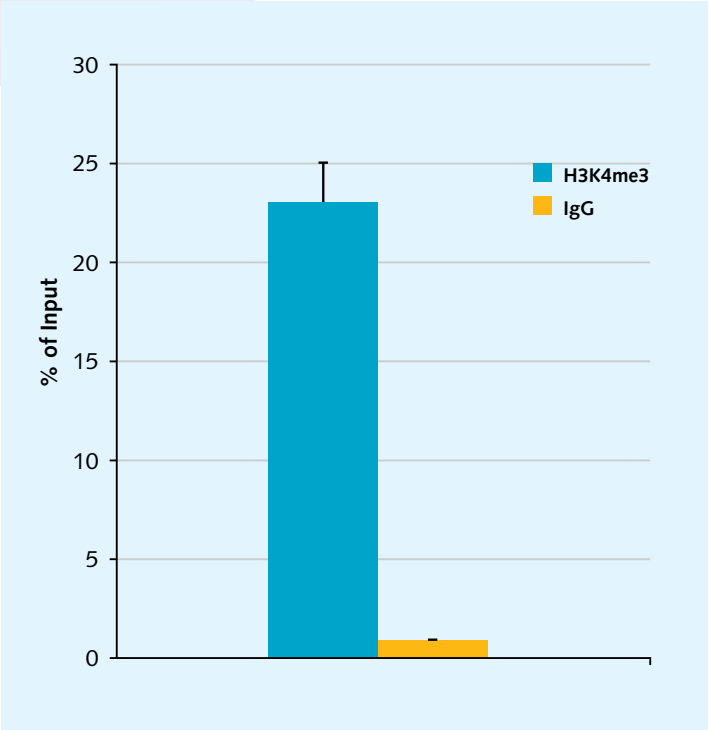


Figure 3: *ChIP was performed on human cancer cells using the Chromatrap® UniqSeq Kit (Cat. no. 500264/500265). IP was performed with the positive ChIP control antibody H3K4me3 using 2 µg antibody and 1 µg chromatin, and qPCR performed with positive control GAPDH primer set. The data is presented as mean % input (the relative amount of IPd DNA compared with input DNA after qPCR analysis).*

ChIP-seq guidelines for use with Illumina® platforms

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® sequencing platforms. The Chromatrap® UniqSeq Kit (Cat. no. 500264/500265) contains adapters and indices compatible with Illumina sequencing instruments only, such as the MiSeq, HiSeq and NextSeq. For all other sequencing platforms such as Ion Torrent, the SOLiD® system and Roche 454, the appropriate library kit will need to be provided by the user.

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 4). Therefore, the user should decide whether single or paired end reads would be most appropriate for mapping to a reference genome.

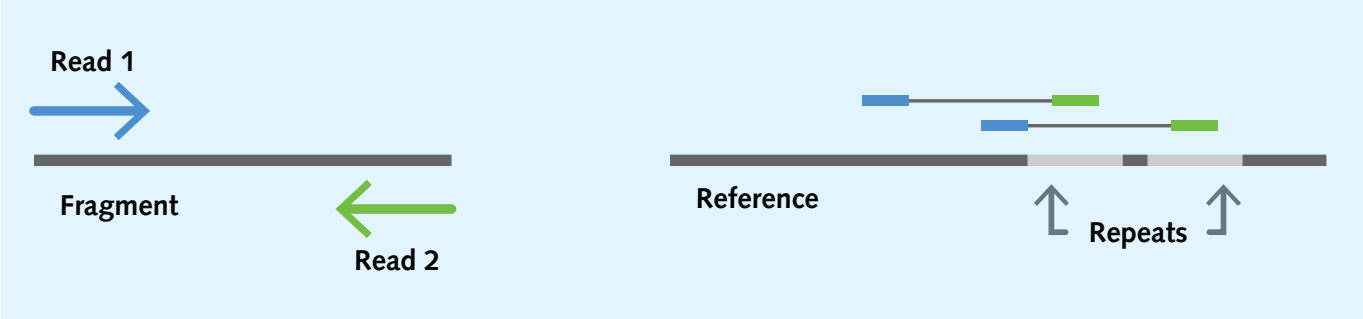


Figure 4: *Paired end sequencing allows both ends of the DNA fragment to be sequenced. The distance between each read is known, therefore, alignment algorithms can use this information to more accurately map the reads to the reference genome. In single end sequencing, the reads represented by the green 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 5). 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.

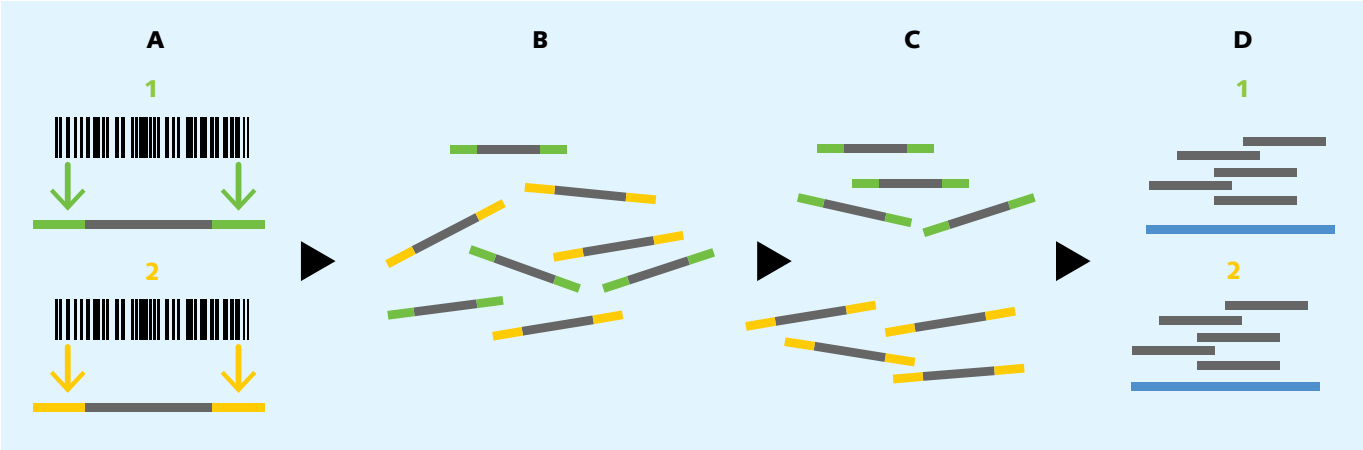


Figure 5: *Overview of sample multiplexing.* **A)** *Two DNA fragments from two unique samples are 'barcoded' with a specific index which identifies the sample from which it originated.* **B)** *Sample libraries are pooled and sequenced in parallel. Each new read contains both the specific index and 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.

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

Data analysis considerations

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 6).

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.

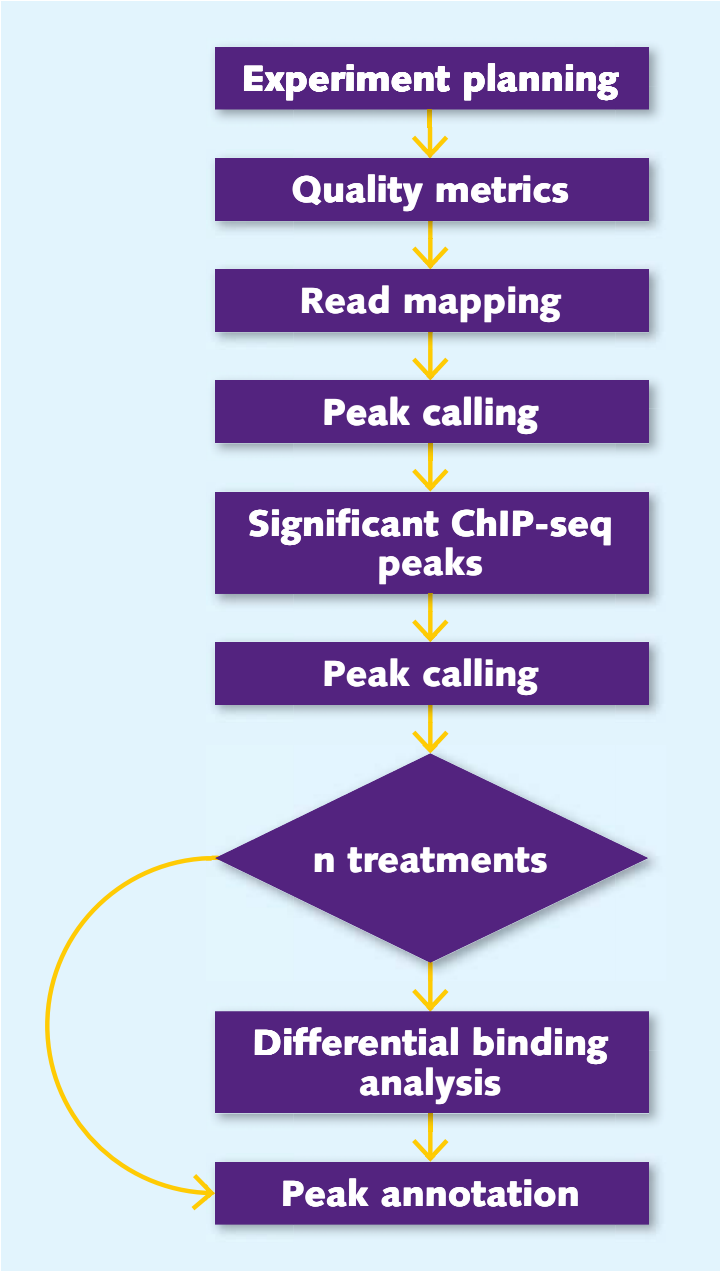
Peak calling – transcription factors

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 6 *ChIP-seq data analysis flow*

Troubleshooting and FAQs

Process	FAQ	Solution
Cross-linking and fixation	For how long should the cells be cross-linked?	Optimal cross-linking of DNA ensures that the chromatin structure is preserved during the isolation and ChIP procedure. Too little cross-linking will result in DNA loss, elevated background and reduced antigen availability. The optimal time for cross-linking will vary with cell and tissue type. 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. Ensure that adequate Lysis Buffer volume is used for the weight of the tissue being processed. During the two lysis steps (Hypotonic and Lysis Buffer) make sure the cells do not settle at the bottom of the tube by flicking the tube every few minutes to ensure efficient cell lysis.
	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 ChIP-seq Elution Buffer cloudy?	The Lysis and ChIP-seq 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?	This protocol has been optimised for both adherent/suspension cells and fresh/frozen tissue, careful planning for chromatin collection from different sources needs to be optimised by the user. If using FFPE tissue, refer to Chromatrap® FFPE ChIP protocol. The key requirement of working with tissue samples is to obtain a unicellular starting suspension before proceeding with any sonication steps. More stringent grinding and sonication steps to disaggregate the cells may be required if working with yeasts or plant tissues.

Table 6 *Buffer volumes for low cell numbers*

Buffer	Cell Count	Buffer volume (µl)
0.65M Glycine	1000 50,000 100,000	200 400 800
Hypotonic Buffer	1000 50,000 100,000	100 200 400
Lysis Buffer	1000 50,000 100,000	100 100 100
Digestion Buffer	1000 50,000 100,000	100 100 100
Enzymatic Stop Solution	1000 50,000 100,000	2.5 2.5 2.5

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. The DNA yield obtained from ChIP will depend on the quality of the chromatin, the affinity and avidity of the antibody and the abundance of the target. A minimum of 500 pg of DNA is required for preparation of high quality NGS sequencing libraries using the Chromatrap® ChIP-seq library preparation kit. As little as 1000 cells can yield this quantity of DNA from ChIP of an abundant target using a good quality antibody. As a starting point we would recommend using 10 µg chromatin per ChIP with 2-5 µg antibody.
	What buffer volumes should I use for low cell numbers?	The amount of cross-linking solution and 0.65M glycine required will depend on the size of the vessel the cells are contained in. Ensure sufficient solution is added to completely cover the cells. For example, 1ml of solution is sufficient for a 6 or 12 well plate and 500µl for a 48 well plate. It is recommended when using low cells numbers the cells and nuclei are lysed using 100µl Hypotonic Buffer and 100µl Lysis Buffer respectively. Chromatin should be sheared using the protocol optimised by the user and is not cell number dependent. For a general guide see Table 6 above. 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.
	How much tissue do I need for chromatin extraction?	This protocol has been optimized using 50 mg – 300 mg fresh/frozen tissue. Chromatin collection from less or more tissue needs to be optimised by the user.
	How small do I need to cut the tissue sample?	Chop the tissue into small pieces (approximately 1 mm^3) using a scalpel blade. The smaller the pieces of tissue, the more efficient the extraction.

	Some of my tissue sample remains in the petri dish, what should I do?	When transferring the sample from the petri dish to the 15 ml centrifuge tube, tilt the petri dish and wash the cells form the top of the petri dish to the bottom a few times to collect most of the tissue at the bottom of the petri dish. While still tilting the petri dish, transfer as much solution and tissue as possible to a 15 ml centrifuge tube. Cutting the end of a 1 ml pipette tip will help to transfer the tissue in solution. If tissue remains in the petri dish, add 5 ml cold PBS and transfer as much sample as you can to the 15 ml centrifuge tube.
Chromatin shearing	Why do I have a poor yield of sheared chromatin?	Cells may be over cross-linked, 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	Do the Chromatrap® spin columns require blocking?	There is no requirement to carry out a blocking step as the spin columns and buffers have been formulated to minimise non-specific binding.
	How much antibody should be used per ChIP?	This should be determined empirically and is dependent on the amount of chromatin used per IP and the quality of the antibody. We recommend using 1-10µg antibody per IP taking into account the amount of chromatin used and the quantity of DNA required downstream. 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 (Chromatrap® cat no. 700010), to validate the efficiency of the IP.
Reverse cross-linking	How long should samples be reverse cross-linked?	A minimum of 2 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?	The following SYBR® reagents have all been shown to produce optimal results with the positive and negative antibodies and GAPDH primers provided in the kit; iTaq™ Universal SYBR® Green Supermix, PerfeCTa SYBR® green supermix, SsoAdvanced™ SYBR® Green Supermix, IQ™ SYBR® Green Supermix.
	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. These primers are not compatible with chromatin from non-human species.
	What positive and negative controls should I use?	Use the kit supplied GAPDH primers as a positive control for ChIP using the supplied H3K4me3 antibody and a negative ChIP control using the supplied non-specific rabbit IgG (Chromatrap® cat. no. 700014). Alternatively, as a negative control, a gene locus not occupied by the target protein can be amplified in qPCR. The supplied GAPDH primers are only suitable for chromatin from human sources. For non-human chromatin it is recommended that the user design control primers around a region know to be occupied by H3K4me3, or another suitable target is used. In addition, it is important to amplify the Input with all primer sets 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?	The amount of DNA required to prepare an NGS library will depend on the library preparation kit used, check the manufacturers' guidelines. A minimum of 500pg of DNA is required to prepare a library of sufficient quality and complexity using the Chromatrap® UniqSeq kit. It is recommended that for optimal library complexity the user prepares libraries using as much ChIP DNA as possible.
	How many PCR cycles should I use for library enrichment?	The number of cycles required to amplify libraries to sufficient concentration depends on the quality and amount of input DNA. It is recommended that the minimum number of PCR cycles that yields sufficient library quantity for sequencing is used to maintain library complexity.
Sequencing and data quality	Why do I have adapter dimers in my Bioanalyser analysis and/or sequencing data?	It is essential that libraries are cleaned and size selected both before and after PCR enrichment to ensure unwanted DNA fragments such as unligated primers or primer dimers are not carried over into the sequencing reaction. Ensure the DNA clean up method used removes DNA fragments ≤200bp in length.
	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. Use the minimum number of PCR cycles necessary to achieve the desired library concentration to minimise PCR bias during enrichment. Loading more DNA generally allows the number of PCR cycles to be reduced and leads to improved duplication rates.
IP sequencing controls	What control should I use?	We recommend using an input as the 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.

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Other products available from Chromatrap®

ChIP products

Product	Quantity	Catalogue no.
Chromatrap® ChIP-seq Pro A	24	500189
Chromatrap® ChIP-seq Pro G	24	500190
Chromatrap® HT ChIP-seq Pro A	1 x 96	500214
Chromatrap® HT ChIP-seq Pro G	1 x 96	500215
Chromatrap® Enzymatic ChIP-seq Pro A	24	500191
Chromatrap® Enzymatic ChIP-seq Pro G	24	500192
Chromatrap® HT Enzymatic ChIP-seq Pro A	1 x 96	500216
Chromatrap® HT Enzymatic ChIP-seq Pro G	1 x 96	500217
Chromatrap® ChIP qPCR Pro A	24	500071
Chromatrap® ChIP qPCR Pro G	24	500117
Chromatrap® Premium ChIP qPCR Pro A	24	500115
Chromatrap® Premium ChIP qPCR Pro G	24	500116
Chromatrap® HT ChIP qPCR Pro A	1 x 96	500161
Chromatrap® HT ChIP qPCR Pro G	1 x 96	500163
Chromatrap® HT Enzymatic ChIP qPCR Pro A	1 x 96	500162
Chromatrap® HT Enzymatic ChIP qPCR Pro G	1 x 96	500164
Chromatrap® Enzymatic ChIP qPCR Pro A	24	500166
Chromatrap® Enzymatic ChIP qPCR Pro G	24	500168
Chromatrap® Premium Enzymatic ChIP qPCR Pro A	24	500167
Chromatrap® Premium Enzymatic ChIP qPCR Pro G	24	500169
Chromatrap® FFPE ChIP-seq Pro A	24	500235
Chromatrap® FFPE ChIP-seq Pro G	24	500236
Chromatrap® Native ChIP-seq Pro A	24	500237
Chromatrap® Native ChIP-seq Pro G	24	500238
Chromatrap® Sonication Shearing		500239
Chromatrap® Enzymatic Shearing		500165
Chromatrap® UniqSeq kit Pro A	24	500264
Chromatrap® UniqSeq kit Pro G	24	500265

DNA products

Product	Quantity	Catalogue no.
Chromatrap® DNA Purification	50	500218
Chromatrap® Gel Purification	50	500219
Chromatrap® HT DNA Purification	2 x 96	500220
Chromatrap® HT DNA Purify and Concentrate	2 x 96	500240
Chromatrap® DNA Extraction	50	500260
Chromatrap® HT DNA Extraction	2 x 96	500261
Chromatrap® Size Selection	50	500262
Chromatrap® HT Size Selection	2 x 96	500263

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