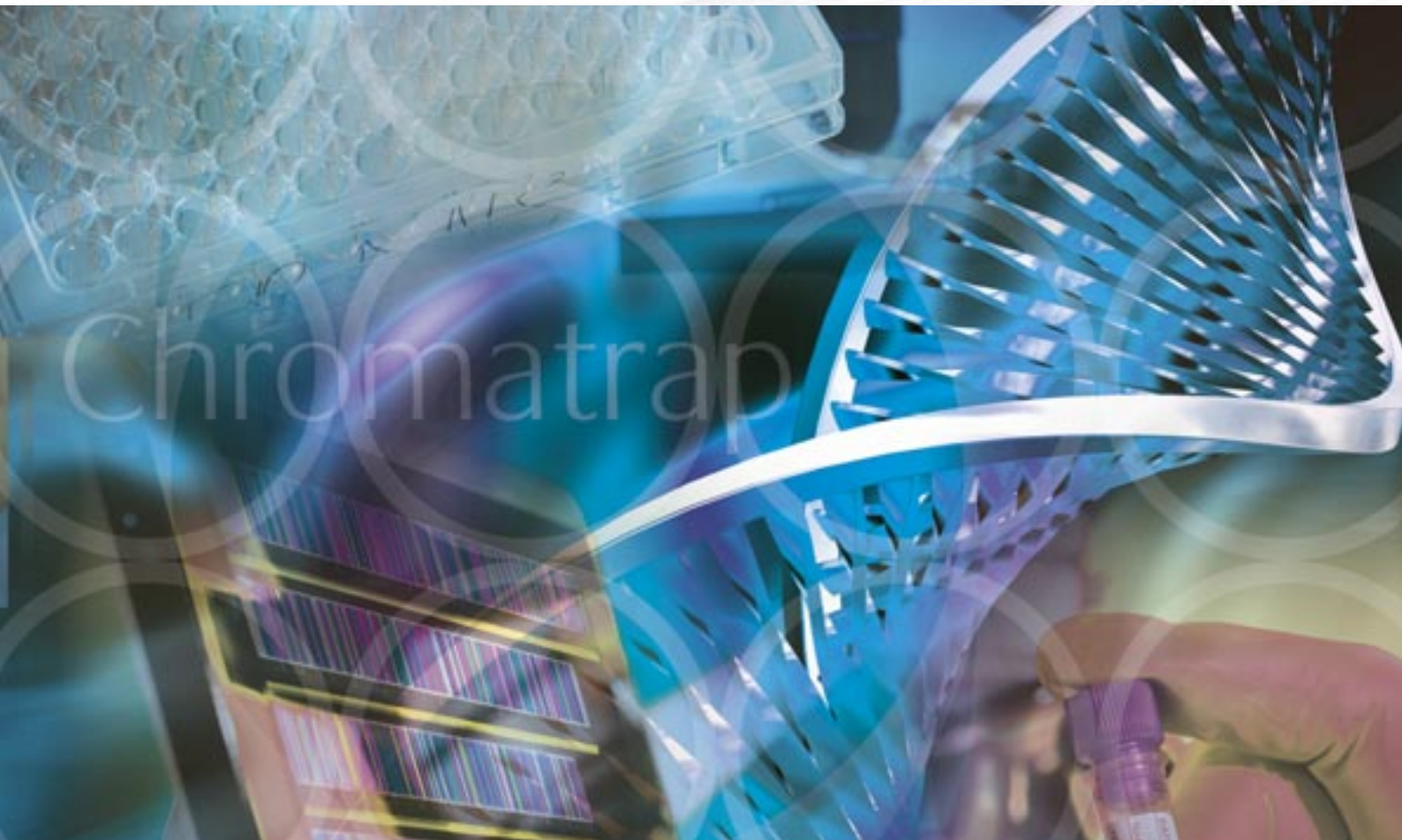


Chromatrap® 96 ChIP-seq

A solid phase chromatin immunoprecipitation assay for
next generation sequencing

Protocol v1.4

Catalogue no 500214, 500215, 500216, 500217



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Introduction

Chromatin Immunoprecipitation (ChIP) is a powerful tool in the study of epigenetics. The technique is used to study the association of specific proteins, or their modified isoforms, with defined genomic regions. In a ChIP assay, DNA-protein complexes (chromatin) are selectively immunoprecipitated using matching antibodies and the resulting fractions treated to separate the DNA and protein components. Polymerase Chain Reaction (PCR), Real Time PCR (qPCR), hybridization on microarrays (ChIP-on-chip), or Next Generation Sequencing (NGS) are typically used to identify DNA fragments of defined sequence.

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 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 ChIP followed by massively parallel sequencing (ChIP-seq). NGS of ChIP enriched DNA enables identification of target DNA sites that were in direct physical contact with regulatory mechanisms *in vivo*. Mapping of these sequenced fragments to whole genome sequence databases allows quick and efficient analysis of the DNA interaction pattern of any transcription factor or epigenetic modification.

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.

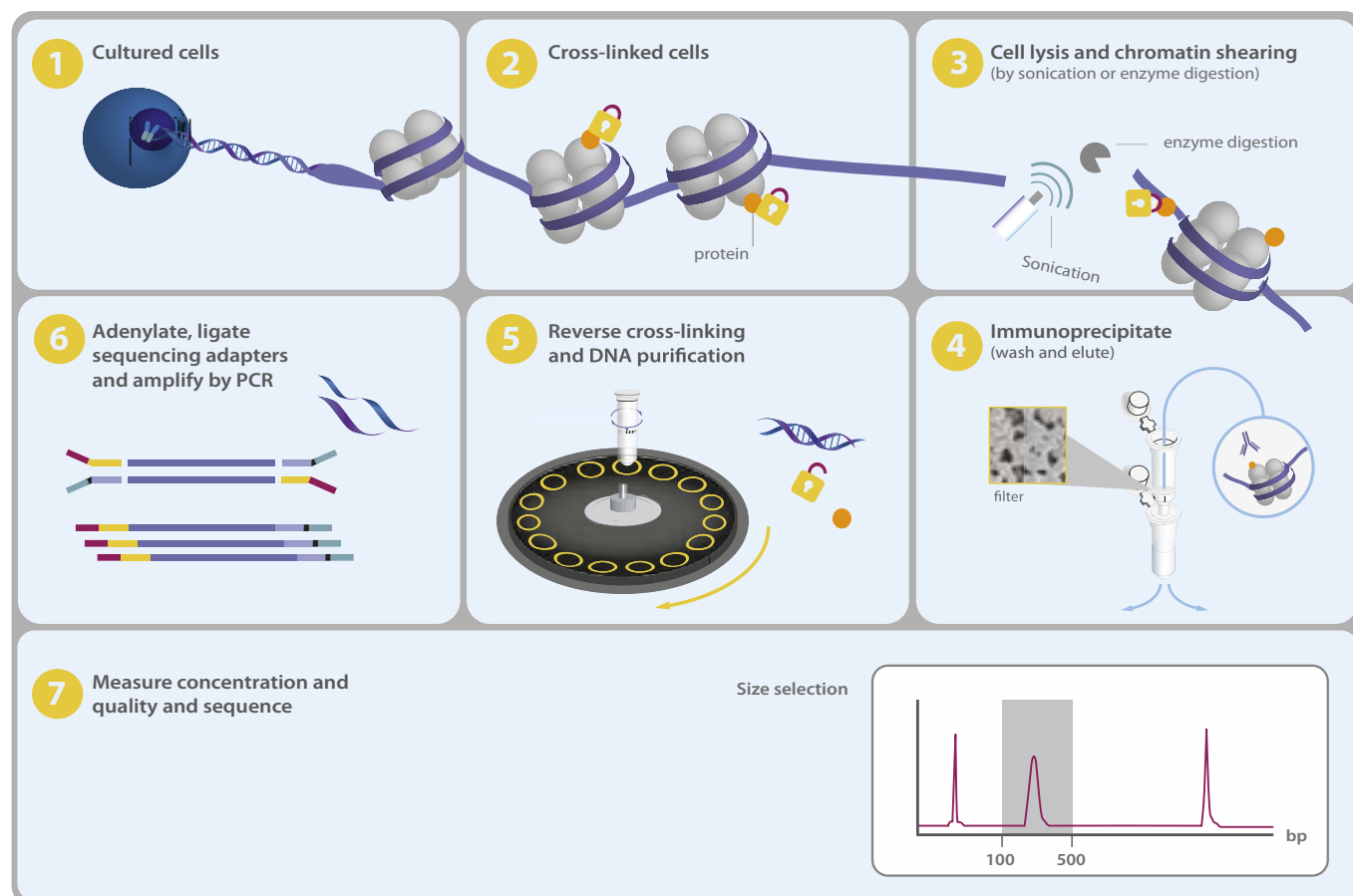


Figure 1: Overview of ChIP-seq process

Chromatrap® ChIP-seq

Chromatrap®'s unique patented* technology provides a quicker, easier and more sensitive way of performing. In this revolutionary system, filter discs of an inert porous plastic, Vyon®, replace magnetic or agarose beads. Protein A or Protein G has been attached in the correct orientation throughout the filter to maximise the capture efficiency of the target chromatin/antibody complex.

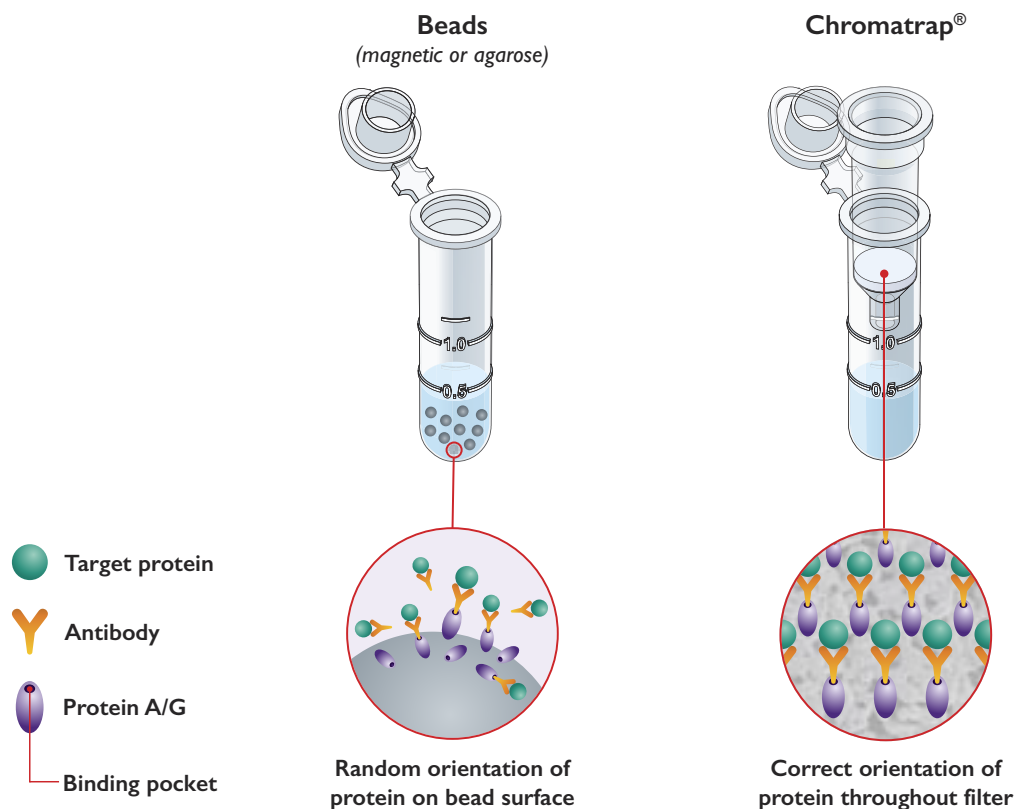


Figure 2: Chromatrap® ChIP technology

A Chromatrap® ChIP-seq assay using cell lines or tissue consists of five key steps:

1. Preparation of high quality chromatin using the reagents provided
2. Immunoprecipitation of chromatin using ChIP-validated antibody of interest specific to the target protein
3. Capture of the antibody-chromatin complex using the Chromatrap® spin column technology
4. Recovery of the enriched DNA using kit supplied reverse cross-linking, proteinase digestion and DNA clean-up reagents
5. DNA analysis

Chromatrap® utilises this solid state technology in 96-well microplate or column format, in parallel with Next Generation Sequencing (NGS) to deliver a precise ChIP-seq protocol from small cell numbers and low chromatin concentrations.

Advantages of Chromatrap® 96 HT ChIP-seq:

- ChIP-seq with as little as 1000 cells or 5 mg tissue (5 mg - 300 mg)
- ChIP-seq from as low as 50 ng up to 50 µg of chromatin
- Low background due to the inert filter technology
- Fast protocol – no blocking steps or overnight incubations
- NGS quality DNA from a single IP without the need to pool samples
- Compatible with qPCR, sequencing and mass spectrometry as downstream processes
- Process up to 96 samples in a single day

*UK Patent No. GB2482209, & GB2527623, US Patent No. 9523681 & 10,435,480, Chinese Patent No. ZL 2011 8 0067254.X, Japan Patent No. JP 6088434 and Australian Patent No. AU 2011340263.

Kit overview and timetable

The Chromatrap® 96 ChIP-seq kit allows the user to perform up to 96 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.

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, 500266 and 500267).*

Library preparation time with other kits will vary and may take up to 2 days.

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 plate	1	4°C
Chromatrap® 96 HT balance plate	1	RT
96-well collection plate	1	RT
96-well balance collection plate	1	RT
Elution plate	1	RT
96-well balance elution plate	1	RT
Elution and balance plate strip caps	24	RT
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	750 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
Chromatrap® 96 DNA Purification plate	1	RT
DNA Binding Buffer	50ml	RT
DNA Wash Buffer	15ml	RT
DNA Elution Buffer	5ml	RT
Chromatrap® 96 DNA Elution plate	1	RT

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
- 100 bp ladder
- Cell-scrapers
- Microcentrifuge tubes (0.5 ml and 1.5 ml)
- PCR plates
- Pipettes and tips (filter tips are recommended)
- ChIP validated antibody
- qPCR primer pairs for gene of interest
- For enzymatic shearing, 0.1% SDS solution
- Reservoirs
- 3 M Sodium acetate pH5.2
- Scalpel blade (for tissue)

Equipment

- Microcentrifuge (4°C)
- Agarose gel electrophoresis equipment
- Rocking platform for culture plates/flasks
- Spectrophotometer/fluorometer for DNA quantification
- 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 sequencing using Illumina® platforms

- 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
- 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
- 100% ethanol
- 70% ethanol
- Linear polyacrylamide (LPA)
- DNA LoBind tubes

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 Chromatrap® 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 Chromatrap® 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.

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.

Experimental planning

1. Cell culture

This protocol has been optimised for use with cell lines, tissue and primary cells, and provides enough reagents for up to 10 chromatin preparations (15×10^6 cells) and up to 96 ChIP assays. Lower cell numbers can be used, 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. 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 50 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.

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.

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:

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

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

Protocol

Step 1 – Chromatin preparation; 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 adherent cells (step 1a), suspension cells (step 1b), and fresh/frozen tissue (step 1c). 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 $1000-1.5 \times 10^7$ cells.
2. Remove media and wash with **pre-warmed 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.

N.B. Glycine is supplied as a 1.3 M solution and should be diluted 50:50 with PBS before 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 $3500 \times g$ 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 $500 \times g$ for 5 minutes at 4°C.
2. Re-suspend in 1 ml **pre-warmed PBS** (perform cell count) and spin $500 \times g$ 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%) 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 $500 \times g$ for 5 minutes at 4°C.
7. Re-suspend in 1 ml **ice cold PBS**.
8. Spin to collect cells at $500 \times g$ 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	Cell Count (Millions)	Buffer Volume (ml)
0.65 M Glycine*	1-5	3
	5-10	4
	10-15	5
Hypotonic Buffer	1-5	0.4
	5-10	0.8
	10-15	1.0
Lysis Buffer**	1-5	0.3
	5-10	0.3-0.5
	10-15	0.5-1.0
Digestion Buffer for enzymatic digestion	1-5	0.3
	5-10	0.4
	10-15	0.5
Enzymatic Stop Solution for enzymatic digestion	1-5	7.5 µl
	5-10	10 µl
	10-15	12.5 µl

Table 3 Optimal buffer volumes for cell lines (for buffer volumes for less than 1 million cells please see page 23).

Step 1c: For fresh or frozen tissue

The following section describes fixation and chromatin preparation of 5-300 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 mm³) 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 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.

N.B. 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 3500 x g 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 3500 x g for 5 minutes at 4°C and discard supernatant. Proceed to step 2.

Buffer	Tissue (mg)	Volume of buffer
0.65 M Glycine*	<80	3 ml
	80-120	5 ml
	120-200	7 ml
Hypotonic buffer	<50 mg	0.5 ml
	50-80	1 ml
	80-120	2 ml
	120-200	2.5 ml
Lysis Buffer**	<50 mg	300 µl
	50-80	500 µl
	80-120	700 µl
	120-200	1 ml

Table 4: Optimal buffer volumes for tissue

* 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 37°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), 60 mls

Wash Buffer 2 (3X), 60 mls

Wash Buffer 3 (3X), 60 mls

Add 160 mls molecular biology grade water to 80 mls 3x Column Conditioning Buffer (total volume 240 ml) and 120 mls molecular biology grade water to each of Wash Buffers 1, 2 and 3 (total volume 180 ml) to achieve a 1x final concentration of each solution.

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 1×10^4 - 15×10^6 cell preparations and the buffer volumes required are outlined in Table 3, section 2b describes chromatin shearing by sonication for 5 mg - 300 mg 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 5000 x g 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. Chromatin stocks can be stored 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 5000 x g 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.
4. Sonicate the 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 1.5 ml microcentrifuge tube.
6. Add 1 µl of PIC to the samples and mix.
7. Chromatin samples are now ready for IP. Chromatin stocks can be stored at -80°C for a maximum of 2 months.

N.B. It is recommended that the shearing efficiency of each chromatin stock is analysed at this stage. 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 5000 x g 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
 $9 \text{ (concentration)} \times 50 \text{ (dilution factor)} \times 400 \text{ (volume of Digestion Buffer)}$
= 180,000 ng or 180 µg total chromatin
1 U Shearing Cocktail per 5 µg chromatin therefore $180/5 = 36 \text{ U Shearing Cocktail}$
Shearing Cocktail is supplied as 15 U per µl therefore $36/15 = 2.4 \text{ µl Shearing Cocktail to be added.}$

4. Add Shearing Cocktail to each stock nuclei suspension (from step 2c, 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 12,000 x g 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 NaHCO₃ 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 Immunoprecipitation

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® UniqSeq 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)
Chromatin stock	Up to 100 µl
Antibody /IgG	Optimum addition rate
PIC	1 µl
Column Conditioning Buffer	Make up to final volume of 1000 µl

Table 5: 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 (~ 30 minutes).

N.B. Do not cap wells 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 **ChIP-seq Elution Buffer** then it should be warmed to 37°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 30-40 minutes).
3. Position the Chromatrap® 96 plate back on the collection plate and add 600 µl of Wash Buffer 1 to each well and centrifuge at 2000 x g for 30 seconds at RT (remember to add 600 µl d.H₂O 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 30 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 30 seconds at RT. Discard the flow through and repeat.
6. Centrifuge at max speed for 30 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 15 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.H₂O into the corresponding wells.
9. Centrifuge the plate at top speed for 1 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 NaHCO₃, 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 NaHCO₃ 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. If validating by qPCR, take a 10 µl aliquot of the input and dilute 1 in 10 or 1 in 100 with water and deduct 3.3/6.6 Cts respectively, with 100% primer efficiency. With the remaining 100 µl input please proceed to step 4b.

Step 4b: DNA purification

Chromatin must now be purified before proceeding with qPCR or library synthesis. DNA purification plate and reagents are included 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.

N.B. DNA Wash Buffer must be prepared **before first use**. Add 60 ml ethanol (95-100%) to the DNA Wash Buffer concentrate and note on the 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® 96 DNA Purification plate and position on to the 96-well collection plate provided.
3. Transfer each sample to a corresponding well on the Chromatrap® 96 DNA Purification plate.
4. Centrifuge at 3,000 x g for 60 seconds at RT. Discard the flow through.
5. Add 700 µl DNA Wash Buffer to each well and centrifuge at 3,000 x g for 60 seconds at RT. Discard the flow through.
6. Spin dry at 3,000 x g for 60 seconds at RT to remove any remaining liquid from the plate.
7. Transfer the Chromatrap® DNA Purification plate on to a clean supplied 96-well DNA Elution plate.
8. To elute DNA, add 50 µl DNA Elution Buffer to the centre of the membrane of each well and incubate for 1 minute. Centrifuge at 3,000 x g for 60 seconds to collect the eluted DNA.

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.

Prepare the qPCR reaction mix as follows:

- 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:

Two minutes at 95°C

10 seconds at 95°C

30 seconds at T_a

15 seconds at 72°C

} 40 cycles

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

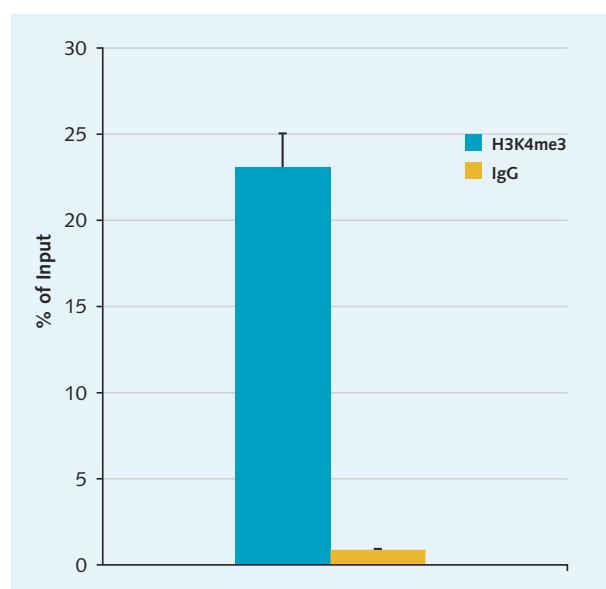


Figure 3: ChIP was performed on human cancer cells using the Chromatrap® ChIP-seq kit. IP was performed with the positive ChIP control antibody H3K4me3 using 2 µg antibody and 1 µg chromatin, and qPCR performed with positive control GAPDH primer set. The data is presented as mean % input (the relative amount of IPd DNA compared with input DNA after qPCR analysis).

Troubleshooting and FAQs

Process	FAQ	Solution
Crosslinking and fixation	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. Too much can result in 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.
	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.
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 Elution Buffer cloudy?	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. 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 of working with yeasts or plant tissues.
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.

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

Table 6: Buffer volumes for low cell numbers

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.
Chromatin shearing by sonication	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.

Chromatin shearing by enzymatic digestion	Why is my chromatin under sheared?	If only larger bands (e.g. 400 bp and above) are seen in the gel the amount of Shearing Cocktail in the digestion may need to be increased. Try increasing the U:chromatin ratio in the reaction (e.g. 1 U Shearing Cocktail per 2 µg chromatin). Cell membranes may not have been lysed efficiently in Hypotonic Buffer to allow the Shearing Cocktail access to the chromatin. Check cell lysis during Hypotonic Buffer incubation using a phase contrast microscope to ensure all the nuclei are released before resuspension in Digestion Buffer. If membranes are not efficiently lysed during the 10 minute incubation time in Hypotonic Buffer try incubating the samples for longer, monitoring the cell lysis using a phase contrast microscope to determine the optimum time for your cells. If membranes do not lyse following extended incubation in Hypotonic Buffer then cells may not be suitable for enzymatic shearing try sonication using step 2a, page 13 of this protocol.
	Why is my chromatin over sheared?	If chromatin is over sheared i.e. completely digested to mononucleosome fragments then the amount of Shearing Cocktail in the digestion may need to be reduced. Try reducing the U:chromatin ratio in the reaction (e.g. 1 U Shearing Cocktail per 10 µg chromatin).
	What if the estimated total chromatin in my sample is less than 75 ug (i.e. less than 1 ul Shearing Cocktail is to be added to achieve 1 U/5 µg chromatin)?	To minimise pipetting errors it is recommended that you make a dilution of Shearing Cocktail in the supplied digestion buffer to a final concentration of 1 U/µl before adding it to the stock nuclei suspension.
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 as the composition of the Chromatrap® 96-well plate and the buffers provided in the kit 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 1-10 µg 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.
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?	The following SYBR® reagents have all been shown to produce optimal results; iTaq™ Universal SYBR® Green Supermix, PerfeCTa SYBR® green supermix, SsoAdvanced™ SYBR® Green Supermix, IQ™ SYBR® Green Supermix.
	What positive and negative controls should I use?	Chromatrap have a wide range of ChIP validated antibodies with matching primer sets which can be used as controls, please visit www.chromatrap.com for more information. 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?	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 500 pg 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 ≤ 200 bp 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 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.

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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® 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® UniqSeq kit Pro A	24	500264
Chromatrap® UniqSeq kit Pro G	24	500265
Chromatrap® UniqSeq Enzymatic Pro A	24	500266
Chromatrap® UniqSeq Enzymatic Pro G	24	500267
Chromatrap® <i>Drosophila</i> ChIP-seq kit Pro A	24	500279
Chromatrap® <i>Drosophila</i> ChIP-seq kit Pro G	24	500275
Chromatrap® <i>Drosophila</i> UniqSeq kit Pro A	24	500276
Chromatrap® <i>Drosophila</i> UniqSeq kit Pro G	24	500277
Chromatrap® Sonication Shearing	–	500239
Chromatrap® Enzymatic Shearing	–	500165

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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