Other products available from Chromatrap®

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® DNA purification HT	2 x 96	500220
Chromatrap® DNA clean and concentrate HT	2 x 96	500240

Chromatrap

Worldwide Chromatrap® Technical Support Team
support@chromatrap.com

Worldwide Sales and Customer Support Team
Tel: +44 (0) 1978 666222 sales@chromatrap.com
Clywedog Road South Wrexham Industrial Estate Wrexham LL13 9XS UK

www.chromatrap.com

Chromatrap

V1.2

Chromatrap® Native ChIP kit

A solid phase native chromatin immunoprecipitation assay (N-ChIP)

Protocol v1.2

Catalogue no. 500237 & 500238

ADVANCEMENTS IN EPIGENETICS

Contents

Kit components and storage

Chromatrap® Native ChIP Pro A (500237) and ProG (500238) kits allow the user to perform upto 24 ChIP assays from cell collection through to imuunoprecipitation and DNA purification. Upon receipt, please ensure the components are stored at the temperature indicated in Table 1.

Kit Component	Qty	Storage Condition
Chromatrap® ChIP-seq Spin Columns	24	4°C
Hypotonic Buffer	10 ml	4°C
N-ChIP Digestion Buffer	5 ml	4°C
ChIP Dialysis Buffer	22 ml	4°C
Resuspension Buffer	22 ml	4°C
Separating Solution 1	12 ml	4°C
Separating Solution 2	12 ml	4°C
Enzymatic Stop Solution	200 µl	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
ChIP-seq Elution Buffer	3 ml	4°C
Shearing Cocktail*	100 µl	-20°C
Protease Inhibitor Cocktail (PIC)	50 µl	-20°C
Proteinase K	50 µl	-20°C
Proteinase K Stop Solution	100 µl	-20°C
1.5 ml Collection Tube	50	4°C
Chromatrap® DNA Purification Columns	24	RT
DNA Binding Buffer	15 ml	RT
DNA Wash Buffer	15 ml	RT
DNA Elution Buffer	2 ml	RT

Table 1: *Chromatrap® Native ChIP kit 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 at least 6 months. Sufficient material is supplied for 24 ChIP assays and up to 10 chromatin sample preparations.

Introduction

Epigenetics is the study of the molecular mechanisms which control gene expression in a potentially heritable way, that do not involve changes in the underlying DNA sequence.

In native chromatin the DNA, histones and DNA-binding proteins are naturally linked. Traditional methods of mechanical shearing can easily disrupt these DNA-protein complexes if they are not chemically fixed in place prior to shearing. Therefore, in the Chromatrap® Native ChIP process the DNA is sheared by enzymatic digestion of the chromatin. Native ChIP is primarily used for the study of histone modifications and certain abundant transcription factors that are tightly bound to DNA.

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.

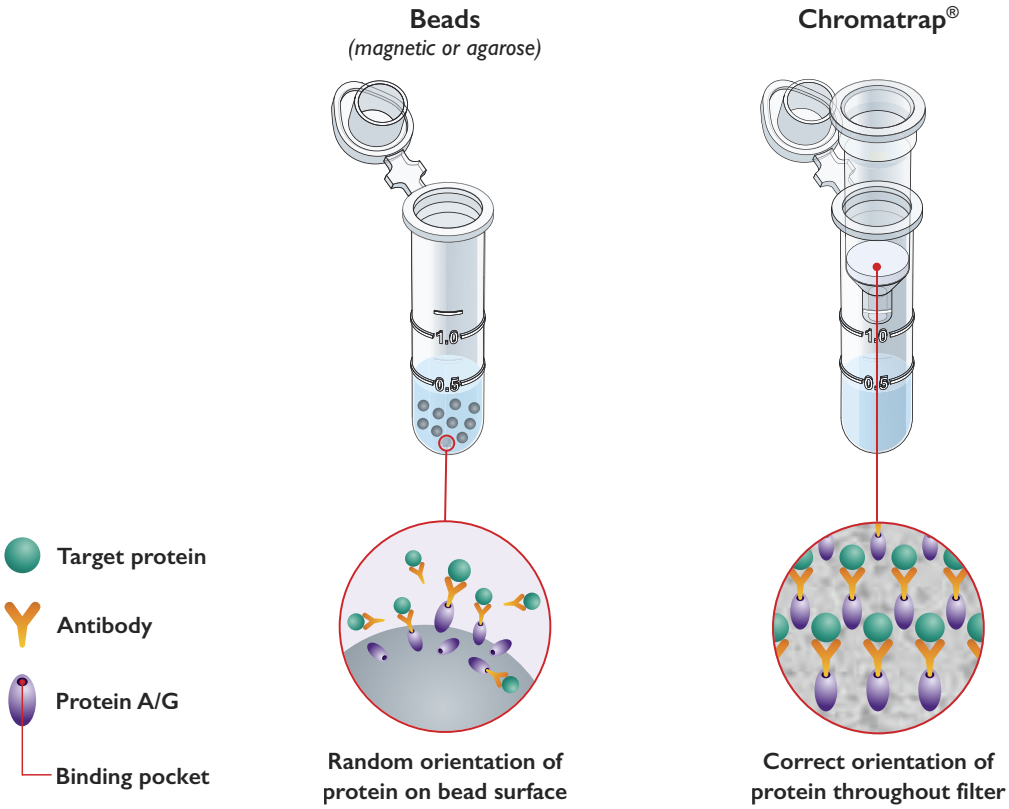

Figure 1: *Chromatrap® ChIP technology*

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 parallel with Next Generation Sequencing (NGS) to deliver a precise ChIP-seq protocol from small cell numbers and low chromatin concentrations for native chromatin.

Advantages of Chromatrap® Native ChIP:

- 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

* 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.

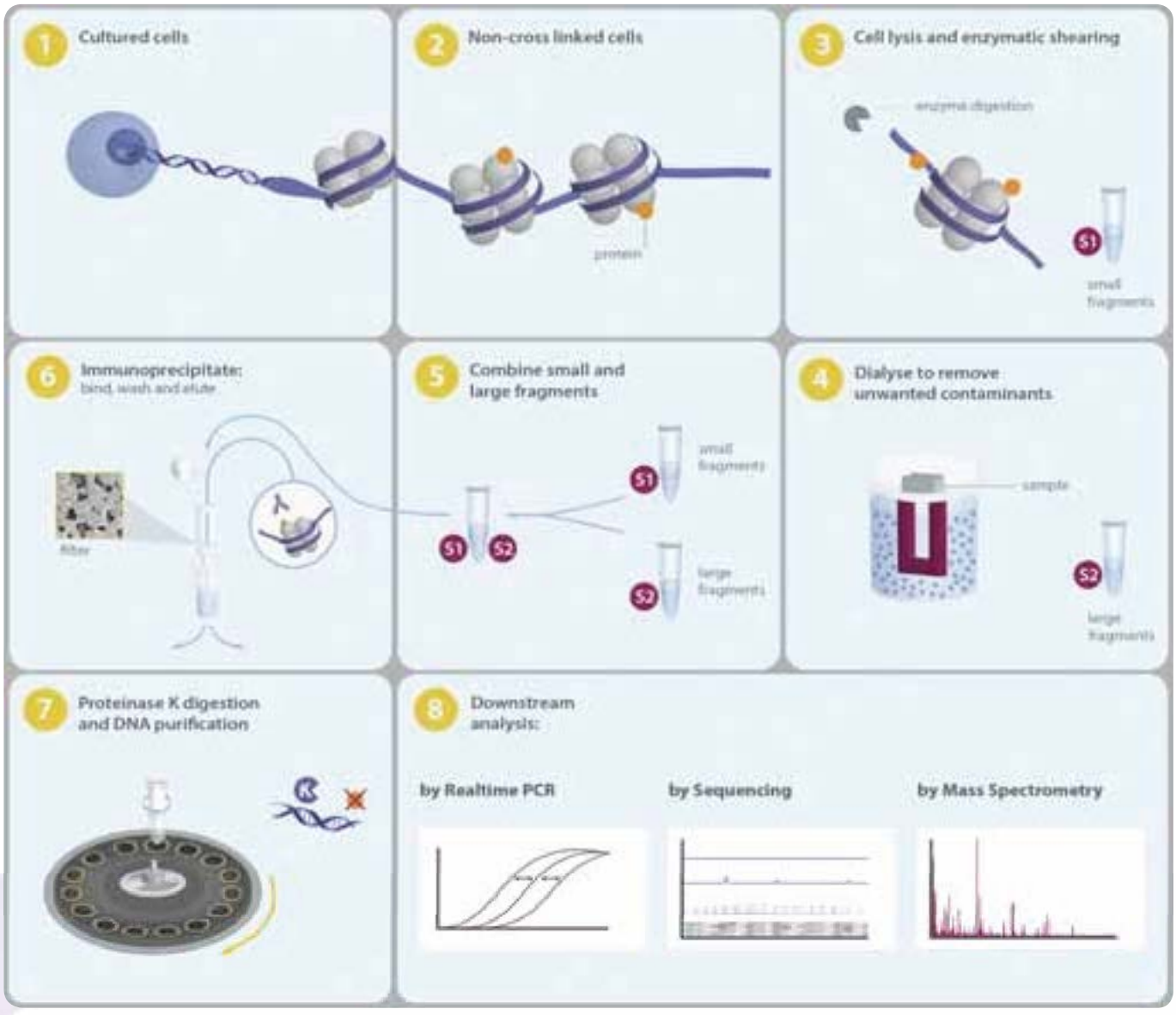

Figure 2: *Overview of Chromatrap® Native ChIP process*

A native ChIP assay normally involves five key steps:

- *Preparation of chromatin to be analysed from cells*
- *Immunoprecipitation of chromatin using a high quality ChIP-validated antibody specific to the target protein*
- *Isolation of the precipitated chromatin fragments*
- *DNA recovery from the precipitated product*
- *DNA analysis*

Additional materials and equipment required

Reagents and consumables

- *PBS*
- *Nuclease- free water*
- *100 bp ladder*
- *Cell scrapers*
- *Microcentrifuge tubes*
- *PCR plates*
- *Pipettes and tips (filter tips recommended)*
- *ChIP validated antibody*
- *qPCR primer pairs for gene of interest*
- *0.1% SDS solution*
- *Ethanol (95-100%)*

Equipment

- *End-to-end rotator*
- *Microcentrifuge (4°C)*
- *Agarose gel electrophoresis equipment*
- *Dialysis equipment*
- *37°C waterbath*
- *Spectrophotometer/fluorometer for DNA quantification*

Optional materials

- *Phenol Chloroform*
- *3M Sodium Acetate, pH 5.2*
- *Linear Polyacrylamide (LPA)*

Experiment design, preparation and planning

Cell culture
This protocol has been optimized for use with cell lines and primary cells and provides enough reagents for up to 10 chromatin preparations and up to 24 native ChIP assays. Volumes of buffers will need to be adjusted according to cell numbers (Table 1).

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

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).

Antibody Quality
The success and value of any ChIP 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 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.

Quantity of Antibody
The user must determine the optimum antibody: chromatin ratio for use in native ChIP. Optimal results have for native ChIP have been achieved with a 5: 2 µg chromatin: antibody ratio.

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.

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:

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

Native ChIP –Seq
Chromatrap®'s Native ChIP kit allows enough DNA to be eluted from an IP'd sample to be processed via NGS.

Protocol

Wherever this 'pause point' symbol is displayed it signifies that, if required, the sample can be stored under the designated conditions and the protocol continued at a later time.

Step 1 – Chromatin Preparation

Buffer	Cell Count (Millions)	Buffer Volume (µl)
Hypotonic Buffer	1-5 5-10 10-15	400 800 1000
N-ChIP Digestion Buffer	1-5 5-10 10-15	300 400 500
Enzymatic Stop Solution	1-5 5-10 10-15	7.5 10 12.5

Table 2: *Optimal buffer volumes*

1. Culture cells to ~80% confluency.
2. Remove media and wash with warm PBS at room temperature (RT).
3. Collect the cells by scraping in ice cold PBS (ensure sufficient PBS to cover the surface of the cells).
4. Transfer to a new microcentrifuge tube and centrifuge at 3500xg for 5 minutes at 4°C.
5. Discard the supernatant.
6. 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 2 – Cell Lysis and Chromatin Shearing

Step 2a: Cell Lysis and Chromatin Shearing

1. Fully re-suspend the pellet in 800 µl Hypotonic Buffer and incubate the sample on ice for 10 minutes.
2. Centrifuge the hypotonic slurry at 5000xg for 5 minutes at 4°C to collect the nuclei. Discard the supernatant.
3. Add 1 ml of Separating Solution 1 to a new microcentrifuge tube. Re-suspend the nuclei pellet in 1 ml Separating Solution 2 and layer on top of the 1 ml separating solution 1.
4. Centrifuge at 845xg for 10 minutes at 4°C.
5. Discard the supernatant and re-suspend the pellet (nuclei) in 400 µl N-ChIP Digestion Buffer. Immediately add 2 µl PIC to stock nuclei suspension. Keep stock nuclei suspension 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 (e.g. 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**.

6. Add shearing cocktail to each stock nuclei suspension at a ratio of 1 U Shearing Cocktail: 5 µg chromatin (Shearing Cocktail is supplied at 15 U/µl) and mix thoroughly.
7. Incubate for 5 minutes in a 37°C waterbath then remove, immediately add Enzymatic Stop Solution and place tubes on ice.
8. Leave tubes on ice for 5 minutes, then centrifuge at full speed for 10 minutes at 4°C. The resultant supernatant is the S1 fragment containing smaller fragments of DNA and can now be stored at -80°C with the addition of 1 µl of PIC whilst the protocol is continued.

9. Re-suspend the pellet in 1 ml of Resuspension Buffer.
10. **Take 2 ml of ChIP Dialysis Buffer and make up to a working solution with 2 L dH_20.**
11. Dialyse the resuspended pellet overnight (12 hours) in 2 L of ChIP Dialysis Buffer (working solution) at 4°C. The dialysis process removes any unwanted components such as small ions and detergents.
12. Transfer the sample to a new microcentrifuge tube.
13. Centrifuge at top speed for 10 minutes at 4°C. The resultant supernatant is the S2 fraction containing larger fragments of DNA. S2 fraction can now be stored at -80°C with the addition of 1 µl of PIC, until use.
14. Re-suspend the pellet (P fraction) in 1 ml ChIP Dialysis Buffer and store at -80°C. The P fraction is not needed for the IP as it contains chromatin that is longer than 5 nucleosomes in length but it can be added into 'Checking Chromatin Shearing Efficiency'. The Chromatrap® native extraction method efficiently produces chromatin that is 100-500 bp in length so very little of the sample should be in this P fraction.

Step 2b: Shearing efficiency

1. Take 25 µl S1 fraction and 25 µl S2 fraction in a clean microcentrifuge tube and mix thoroughly.
2. Add 1 µl Proteinase K to each sample. Vortex briefly and perform a short spin. Incubate for 1 hour at 37°C.
3. Add 2 µl Proteinase K Stop Solution to each aliquot to stop the reaction. Vortex briefly and perform a short spin.
4. Estimate the concentration of DNA in the samples using a spectrophotometer at 260 nm. This will be used to determine the volume of chromatin to load when preparing your slurries for immunoprecipitation.
5. To ensure that 100-500 bp fragments have been obtained during enzymatic shearing the DNA should be run on a 1% agarose gel and visualized 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.

Step 3 – Slurry Preparation and Immunoprecipitation

Step 3a: Slurry preparation

In our experience a 5 µg: 2 µg chromatin: antibody ratio produces optimal results in native ChIP. The flexibility of the Chromatrap® native ChIP assay means that a maximum of 100 µl of chromatin in a total 1 ml slurry can be added to each column. The ratio of chromatin: antibody will need to be optimised by the end user. Ensure to measure the concentration of both fractions and use the lowest concentration as a minimum volume. An example calculation is described in Table 3.

Chromatin stock = 500 ng/µl 5000/500 = 10 µl	*1 ml IP slurry calculation:* ● 10 µl chromatin (S1 and S2 combined) ● 2 µl of antibody (2 µg) ● 1 µl PIC ● 987 µl Column Conditioning Buffer

Table 3: *Example of a 1 ml slurry calculation with 5:2, chromatin: antibody ratio*

1. Thaw chromatin stocks (S1 and S2 fractions) at 4°C.
2. Centrifuge sheared chromatin at max speed for 10 minutes at 4°C, even if previously centrifuged.
   N.B. Use only the clear supernatant for subsequent steps.
3. Add an equal volume of both fractions (S1 and S2) and use this to prepare your slurries. Use the lowest concentration fraction as a minimum volume.
4. Prepare IP slurries in a fresh microcentrifuge tube using Table 2 as a guide. 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 proteinase K digestion and will be used as controls in the downstream analysis.
5. Mix well and incubate the IP slurries on an end-to-end rotor for 1 hour at 4°C.

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 (~ 30 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 Immunoprecipitation.

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.

N.B. If precipitates have formed in the **ChIP-seq Elution Buffer** then it should be warmed to 37°C for 30 minutes, inverting regularly, until precipitates have dissolved **before use**.

1. Remove slurries from the end-to-end rotator following 1 hour pre-incubation and briefly spin down to remove residual liquid from the caps.
2. Load the entire 1 ml slurry and allow to flow completely through the column at RT (~30-40 minutes).
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 the columns transferred into a clean dry 1.5 ml collection tube.
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 protein digestion.

Step 4 – Protein Digestion and DNA Purification

Step 4a: Protein digestion

Chromatin samples are subjected to Proteinase K digestion before DNA purification and validation. Input controls which have not been through the IP process must be reintroduced at this stage and treated as per the sample.

1. Make each IP and input sample to 100 µl with dH_20.
2. Add 1 µl Proteinase K to each IP and input sample. Vortex briefly and perform a short spin. Incubate for 1 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.

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 kits to ensure recovery of 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 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 ul 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 Chromatrap® DNA purification column and centrifuge at 16,000xg for 60 seconds. Discard flow through. Centrifuge Chromatrap® DNA purification column once more at 16,000xg for 60 seconds to remove residual wash buffer.
5. Place 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.

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 IP'd 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.

Instructions of Real Time PCR instruments should be followed and amplification for each target locus should be optimized.

A suggested protocol is as below:

95°C 2 minutes – 1 cycle
95°C 10 seconds
T_a°C 30 seconds } 40 cycles
72°C 15 seconds
Melt curve

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

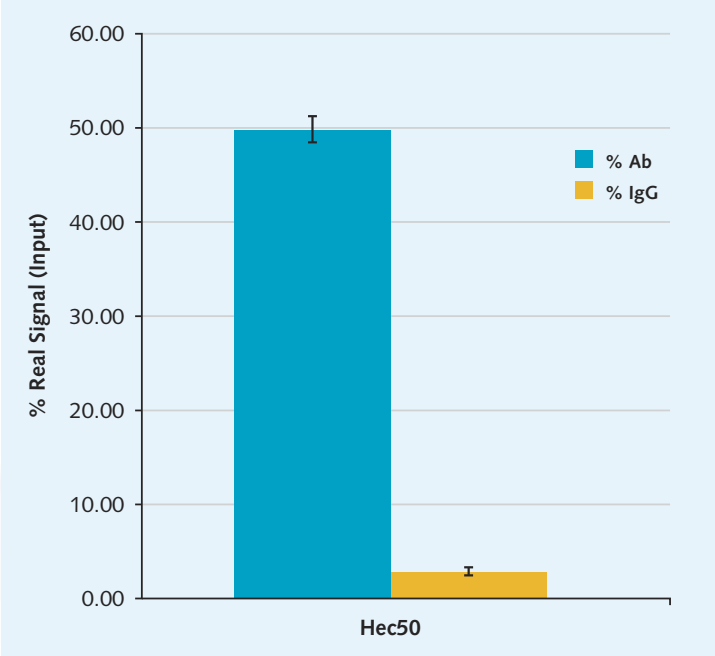

Figure 3: *ChIP was performed on human endometrial cancer cells using the Chromatrap® Native-ChIP kit. IP was performed with 2 µg H3K27me3 antibody and 5 µg chromatin and qPCR was perfomed with positive control Myt1 primer set. The data is presented as mean % input (the relative amount of IP'd DNA compared with input DNA after qPCR analysis).*

Troubleshooting Guide and FAQs

1. Have you got a suggestion for ideal enzymatic shearing?
In our laboratory we have used our Shearing Cocktail at a ratio of 1 U per 5 µg chromatin to achieve optimal fragment lengths of between 100-500 bp on adherent cell lines. However, different cell types may require different ratio of Shearing Cocktail, which must be optimized by the user.

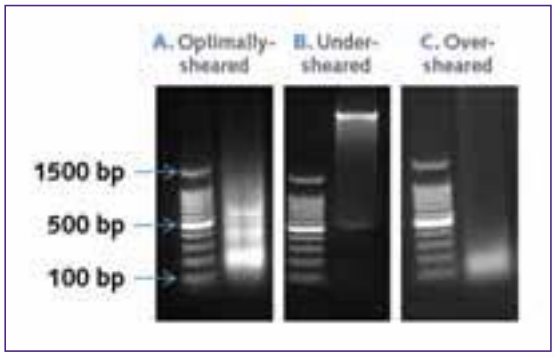

Figure 2: *Optimal enzymatic digestion for chromatin fragment length. Optimal fragment sizes of 200-600 bp (A). Under digestion will result in large fragment lengths greater than 500 bp size (B), over digestion will result in complete fragmentation to 200 bp, reducing IP specificity and PCR efficiency, respectively, (C).*

2. Why can I not see my band on the gel?
Chromatin was not sufficiently digested. Larger fragments of DNA are less soluble and may be spun out following nuclear lysis. Chromatin was completely digested to low molecular weight fragments and is therefore oversheared. Cell membranes were not efficiently disrupted prior to digestion. Cell membranes must be completely lysed in the Hypotonic Buffer to enable permeation of the nuclear membrane by the Shearing Cocktail. To improve the quality of bands on a gel, clean up your samples using a commercial gel purification kit prior to running on the agarose gel.

3. Why is my chromatin under digested?
If only large 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 ug chromatin). Cell membranes may not have been lysed efficiently in Hypotonic Buffer to allow the Shearing Cocktail access to the chromatin. Check cell lysis using a phase contrast microscope after lysis in Hypotonic 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 either the S1 or S2 fragment is individually under sheared add more shearing cocktail during the enzymatic shearing step.

4. 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).

5. What if the estimated total chromatin in my sample is less than 75 µg (i.e. less than 1 µl 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.

6. Can I increase the volume of the Separating Solutions?
Yes, this is possible but Chromatrap® has found that increasing the volumes of the Separating Solutions can result in a reduction in the concentration of your end sample.

If you need to concentrate your sample further you can reduce the volume of the Separating Solutions, however care must be taken not to reduce the volume too much as this will affect the separation of your samples.

7. I do not obtain a pellet after centrifuging the layered separating solutions what should I do?
It is advisable for you to break the surface tension between Separating Solutions 1 and 2 by pushing your pipette tip, or a Pasteur pipette, to the bottom of the microcentrifuge tube after layering the solutions. This can help to prevent the nuclei becoming trapped at the interface between the two solutions.

8. When do I combine my S1 and S2 fractions?
You should combine an equal volume of each fraction before you make up your slurries; use this combined S1/S2 fraction for the IP.

9. Can I use N-ChIP for the pull down of transcription factors?
Native ChIP is best used to analyse histone proteins but can be used to pull down some abundant transcription factors. They must be transcription factors that bind to DNA in order to be extracted as part of the DNA-protein complex (chromatin) without the need for fixation.

10. How long should I dialyse my S2 fraction for?
You should dialyse the S2 fraction overnight for at least 12 hours. This can be increased to 16 hours for maximum removal of unwanted compounds e.g. small ions and detergents that can affect how clean the chromatin sample is.

11. How should I check my native chromatin fractions?
Chromatrap® advise to check the combined S1 and S2 fractions on a 1% agarose gel. However, it is possible to check each fraction individually to ensure you have consistent shearing of both fractions.

12. Should I use Protein A or Protein G Chromatrap® spin columns for my Native ChIP?
Different antibody sources have different affinities to protein A or G, check the affinity of the host species of your antibody. Protein G has the widest range of antibody affinities.

13. Processing differences of the Chromatrap® spin columns.
Chromatrap® spin columns use protein A or G functionalized inert porus discs, and are designed for centrifugation with no difficult pipetting stages needed to separate supernatant liquids from retained chromatin complexes. The Chromatrap® technique therefore significantly reduced the chance of pipetting errors normally associated with separating beads from solutions. The format supports ease of handling and speed of assay development. Antibody/chromatin complexes are retained on the column and can be very effectively rinsed to remove any unwanted background. Complexes can then be released at the appropriate time by a change in pH provided by the elution step.

14. How much chromatin is required per native ChIP assay?
Addition rates may need to vary due to antibodies used and each ChIP experiment should be optimized by the end-user for their specific application. It should be noted that there is a limit of 100 µl and a maximum of 50 µg of chromatin per slurry.

15. How many ChIP assays will I be able to perform from 1-15 million cells?
The standard protocol uses 1-15 million cells where the chromatin is present in a volume of 800 µl following cell collection and re-suspension with Hypotonic Buffer. The chromatin addition rate per ChIP will depend on the concentration of chromatin present in the chromatin stock solution. For example if the chromatin stock is 300 µg/µl and ChIP is being performed at 1 µg/IP, a total of 90 IP reactions could be performed from a 800 µl native chromatin suspension.

16. Do the Chromatrap® spin columns require blocking?
There is no requirement to carry out a blocking step to minimize non-specific binding as the composition of the Chromatrap® spin columns and the buffers provided in the kit have been formulated to ensure that non-specific binding is minimized.

17. Why do I have no enrichment with my ChIP antibody?
The use of ChIP validated antibodies is essential for the success of a ChIP assay. The antibody must recognize and bind to native protein that is bound to DNA. It is essential to validate with antibody controls to ensure native chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP. The antibody epitope may be hidden by other proteins in the chromatin complex and you may need to find an antibody which has an alternative epitope. There may be an incorrect addition of antibody to chromatin samples, carry out an antibody dilution series to determine the optimum ratio. Optimal results have been achieved with 5:2 chromatin: antibody ratio with the Chromatrap® spin columns. The antibody may be of low affinity and may require a longer incubation time than suggested in the protocol, increase the ChIP reaction to overnight incubation at 4°C.

18. What controls should I use?
To check the quality of chromatin a ChIP can be with appositive and negative control antibodies. An IP omitting the primary antibody can also be used as negative control. Alternatively, as a negative control, a gene locus not occupied by the target protein can be amplified in qPCR. In addition, it is important to amplify the Input with all primer sets for data interpretation.

19. What is the binding capacity of the column?
We have measured the IgG binding capacity of the columns to be approximately 50 µg per spin column. The standard protocol uses only a small fraction of this capacity.

20. What cell types have been validated for use with this protocol?
This protocol has been optimised for both adherent and suspension cells, 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 if working with tissues.

21. What can be the cause of high background?
The quality and selectivity of the antibody has a major influence on the success of the ChIP assay, ensure the antibody is ChIP validated. The success of a ChIP assay is highly dependent on the quality of chromatin prepared. Insufficient enzymatic shearing can produce incorrect DNA fragment sizes. Conditions will differ depending on cell line and cell density. Ideally the chromatin should be 100-500 bp as optimized and checked on an agarose gel and visualized against a marker of known size DNA fragments. Insufficient wash steps can leave traces of unwanted chromatin alongside that selected by the antibody. Chromatrap® is intrinsically better than beads for performing these wash steps, however if background continues to be high increase the number of wash steps for each buffer. An incorrect ration of antibody to target protein can compromise the signal to noise ratio. We have found a chromatin: antibody ratio of 5:2 to give optimal results.

22. Should I use Native or Cross-linked ChIP?
This is dependent on your research. Native ChIP removes the need for chemically fixing your cells prior to extraction so if you would prefer your chromatin sample in a more natural state, native ChIP may be the best option. Native ChIP is best used for histone marks and abundant transcription factors, so if your research is investigating low abundant targets, cross-linked ChIP may be a better option.

23. What are the advantages of N-ChIP?
The benefits of N-ChIP include: not chemically fixing your cells with formaldehyde so the cells remain in a more natural, 'native' state; in some cases there is increased affinity of antibody binding to antigens on native chromatin as it is more accessible.

24. What is the best method of dialysis?
It is important to ensure that the same buffer is used inside the dialysis equipment and outside so the osmosis can occur. The volume of buffer outside the dialysis equipment needs to be in excess for efficient movement of sample, hence Chromatrap® use a 2 litre volume of ChIP Dialysis Buffer (working solution). Chromatrap® advise the use of Slide-A-Lyzer™ Dialysis Cassettes from Thermo Fisher Scientific.

25. Can I reduce or increase my cell numbers for extraction?
This protocol is based on using 1-15 million cells, any further differentiation in cell numbers needs to be optimised by the user.