Chromatrap

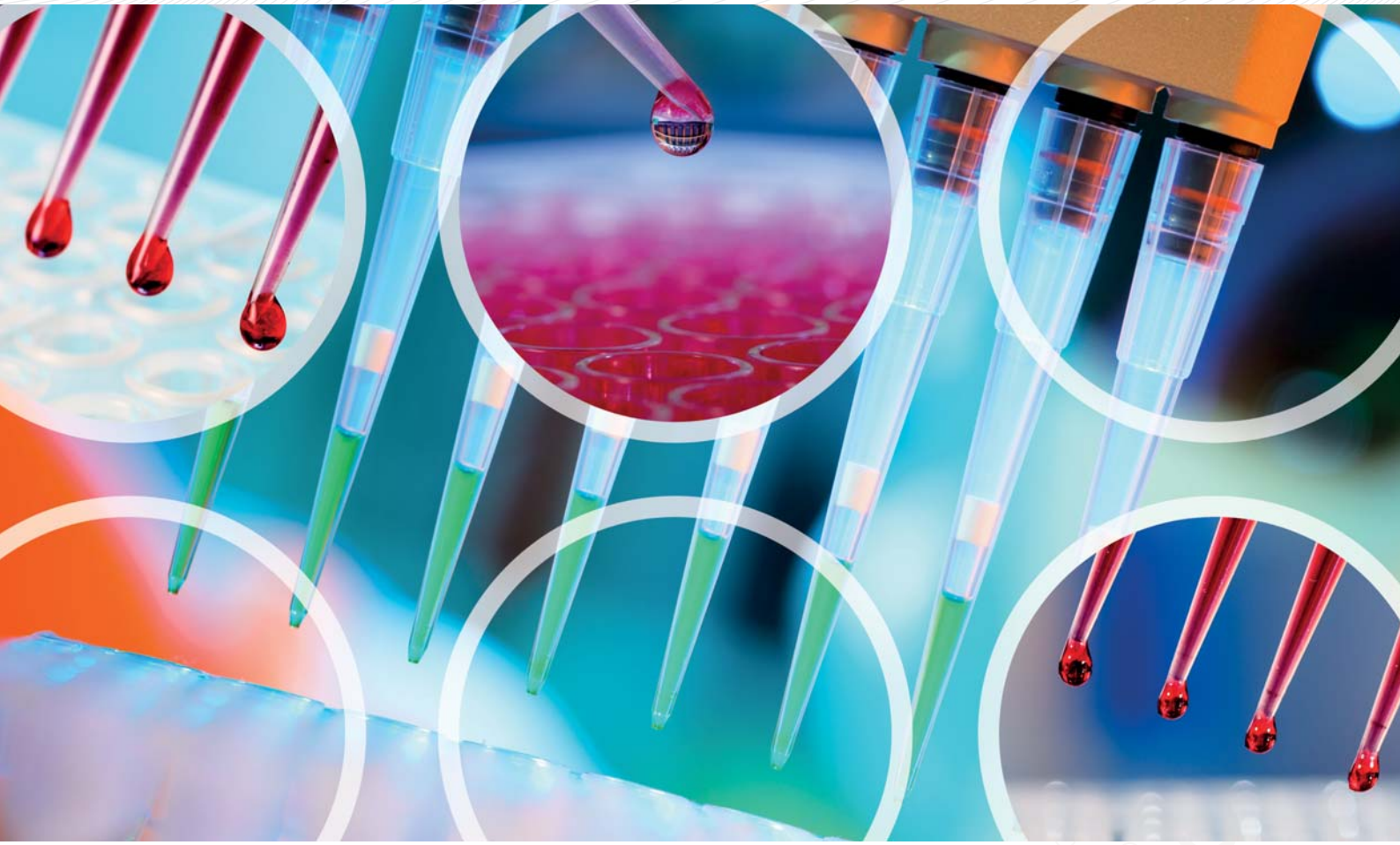
V1.4

Chromatrap® 96 ChIP kit for qPCR

A solid phase chromatin immunoprecipitation assay (ChIP)

Protocol v1.4

Catalogue no 500161, 500163, 500162, 500164



ADVANCEMENTS IN EPIGENETICS

Contents

Kit Components and Storage

For Standard Pro A (500161) **and Pro G** (500163) **high throughput ChIP kits for qPCR**

Kit Component	Qty	Storage Condition
Chromatrap® 96 HT	1	4°C (39°F)
Chromatrap® 96 HT balance plate	1	RT
96-well collection plate	1	RT
96-well balance collection plate	1	RT
96-well elution plate	1	RT
96-well balance elution plate	1	RT
Elution and balance plate strip caps	24	RT
Column Conditioning Buffer (3X)	60 mls	4°C (39°F)
Wash Buffer 1 (3X)	60 mls	4°C (39°F)
Wash Buffer 2 (3X)	60 mls	4°C (39°F)
Wash Buffer 3 (3X)	60 mls	4°C (39°F)
1.3 M Glycine	20 mls	4°C (39°F)
Lysis Buffer	10 mls	4°C (39°F)
Hypotonic Buffer	10 mls	4°C (39°F)
Elution Solution	10 mls	4°C (39°F)
0.1 M NaOH	10 mls	4°C (39°F)
5 M NaCl	500 µl	4°C (39°F)
1 M $NaHCO_3$	750 µl	4°C (39°F)
Protease Inhibitor Cocktail (PIC)	150 µl	-20°C (-4°F)
Proteinase K stop solution	300 µl	-20°C (-4°F)
Proteinase K	150 µl	-20°C (-4°F)

The kits are manufactured DNase free and when stored as directed are stable for at least 1 year.
Sufficient material is supplied for up to 96 ChIP assays and up to 10 chromatin sample preparations.

For Enzymatic Pro A (500162) **and Enzymatic Pro G** (500164) **high throughput ChIP kits for qPCR**

Kit Component	Qty	Storage Condition
Chromatrap® 96 HT	1	4°C (39°F)
Chromatrap® 96 HT balance plate	1	RT
96-well collection plate	1	RT
96-well balance collection plate	1	RT
96-well elution plate	1	RT
96-well balance elution plate	1	RT
Elution and balance plate strip caps	24	RT
Column Conditioning Buffer (3X)	60 mls	4°C (39°F)
Wash Buffer 1 (3X)	60 mls	4°C (39°F)
Wash Buffer 2 (3X)	60 mls	4°C (39°F)
Wash Buffer 3 (3X)	60 mls	4°C (39°F)
1.3 M Glycine	20 mls	4°C (39°F)
Digestion Buffer	10 mls	4°C (39°F)
Enzymatic Stop Solution	200 µl	4°C (39°F)
Lysis Buffer	10 mls	4°C (39°F)
Hypotonic Buffer	10 mls	4°C (39°F)
Elution Solution	10 mls	4°C (39°F)
0.1 M NaOH	10 mls	4°C (39°F)
5 M NaCl	500 µl	4°C (39°F)
1 M $NaHCO_3$	750 µl	4°C (39°F)
Shearing Cocktail	100 µl	-20°C (-4°F)
Protease Inhibitor Cocktail (PIC)	150 µl	-20°C (-4°F)
Proteinase K stop solution	300 µl	-20°C (-4°F)
Proteinase K	150 µl	-20°C (-4°F)

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 six months. Sufficient material is supplied for up to 96 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, which doesn't involve changes in the underlying DNA sequence.

Chromatin immunoprecipitation (ChIP) is a commonly used immunprecipitation technique for mapping the DNA-protein interactions in cells which are crucial for gene regulation. In a ChIP assay, DNA-protein complexes (chromatin) are fixed by the formation of cross links to preserve the interactions. The chromatin is then extracted and sheared either by sonication or enzymatic digestion into small fragments. The DNA/protein fragments are selectively immunoprecipitated using antibodies directed against the protein of interest and the resulting fractions treated to separate the DNA and protein components. Polymerase Chain Reaction (PCR), Real Time PCR, hybridization on microarrays, or direct sequencing are typically used to identify DNA fragments of defined sequence.

A 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*

Chromatrap® technology is a quicker, easier and more efficient way of undertaking ChIP (UK Patent No. GB2482209, US Patent No. 9523681, Chinese Patent No. ZL 2011 8 0067254.X). It uses discs of an inert, porous polymer to which Protein A or Protein G has been covalently attached to maximise the capture efficiency of the target chromatin/antibody complex.

The Chromatrap® 96 technology has the following benefits:
- *Up to* **96 ChIP** *assays can be performed in* **one** *day*
- *Multiple antibody target screening on a single plate using 50-3000 ng chromatin loadings*
- *Flexible experimental design possibilities (1 x 96; 8 x 12) making it ideally suited to robotic handling*
- *Robust reproducibility over a large subset of individual assays*
- *High signal to noise ratios*

Highlights of protocol v1.4
- *High quality of chromatin achieved via sonication or enzymatic digestion*
- *High and low abundant enrichment from small chromatin samples*
- *No DNA clean up is required prior to qPCR*
- *Preparation of columns by gravity for improved antibody binding*

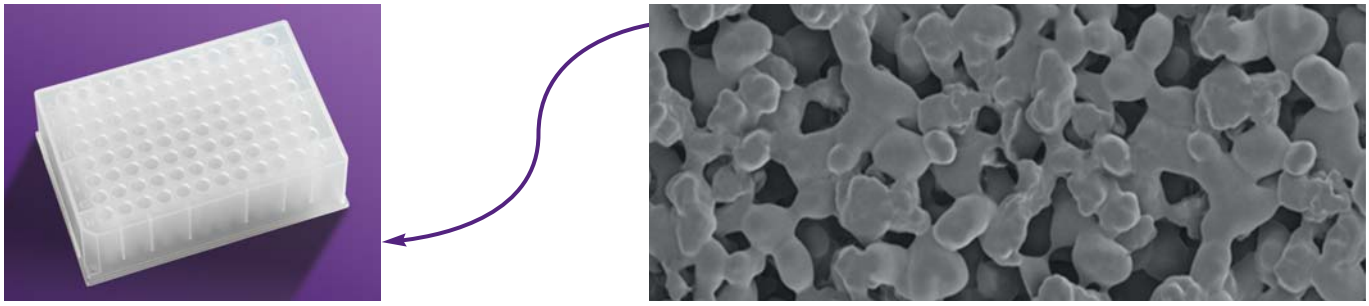
Wherever this 'pause point' symbol appears, it signifies that if required, the sample can be stored at -80°C.

Chromatrap®: an easier, more efficient ChIP assay

High throughput Chromatrap® 96 kits use revolutionary Chromatrap® 96-well plates which contain discs of an inert, porous polymer BioVyon™, to which protein A or protein G has been covalently attached. During an assay the chromatin/antibody complex is retained by the disc. Flushing with three buffers and an elution step are all that is required to obtain the DNA fragments of interest.

The Chromatrap® approach is preferable to other methods because:

- *Using Chromatrap® technology negates the need for micro beads*
- *BioVyon™ Protein A or Protein G discs have a proprietary internal structure, composed of pores that have inner surfaces specifically designed to maximise chromatin capture efficiency*
- *The disc base material is chemically inert, reducing incidences of non-specific binding*
- *All chemical reactions take place inside the disc, therefore there is no need for careful suspension or re-suspension by pipette*
- *The standard protocol does not require pre-blocking steps*
- *Flushing away unbound chromatin and other unwanted species is easily done using washing steps that are fast, efficient and less prone to error*



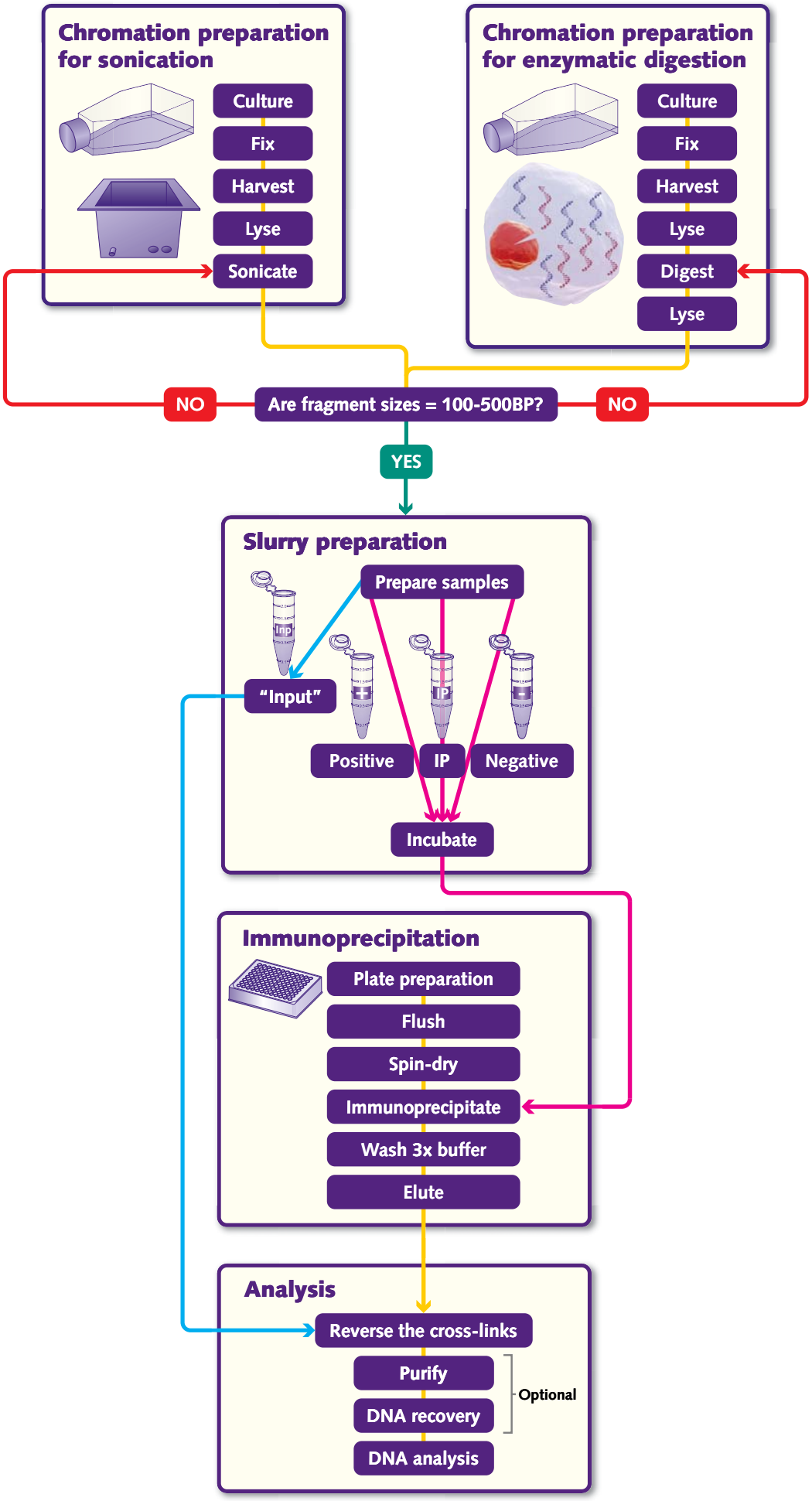
Protein A or G coated polymer matrix

Studies* have shown that:

- *Levels of DNA pull down are up to 25 times higher than with bead based procedures*
- *Excellent DNA enrichment with signal to noise ratios typically 2 to 3 times better than competing procedures*
- *Can be used for a wide range of chromatin loadings; 50 ng to 3,000 ng of chromatin per assay*
- *High performance with small sample sizes; as few as 7,500 cells per IP*

*** Sources:** *Case Study 1: Chernukhin et al., 2011; Analytical Biochemistry. Elsevier Inc. doi: 10.1016/j.ab.2011.01.036. Case Study 2: Francis et al., University of Swansea.*

ChIP Assay Overview



Additional materials required

Reagents and consumables

- *37% formaldehyde*
- *PBS*
- *Nuclease free water*
- *Microcentrifuge tubes*
- *Cell scrapers*
- *100 bp ladder*
- *Reservoirs*
- *Pipettes and tips (filter tips recommended)*
- *ChIP validated antibody*
- *qPCR primer pairs for gene of interest*
- *PCR plates*
- *For enzymatic shearing 0.1% SDS solution*

Equipment

- *Labware for preparation of 1X buffer solutions*
- *Rocking platform*
- *For sonication Sonicator*
- *Centrifuge with plate rotor (4°C)*
- *Microcentrifuge (4°C)*
- *Spectrophotometer/ fluorometer for DNA quanitification*
- *Multi channel pipette*
- *37°C waterbath*
- *65°C waterbath*

Optional Materials

- *PCR purification kit*
- *Phenol Chloroform*
- *3 M Sodium Acetate pH5.2*
- *100% Ethanol*
- *70% Ethanol*

Experiment design, preparation and planning

IT'S RECOMMENDED THAT YOU READ THROUGH THE ENTIRE PROTOCOL BEFORE STARTING.
This protocol has been optimised for **cell lines** and careful planning for chromatin collection from different sources needs to be optimised by the user.

Consider the following when planning your experiment:

Cell culture: When planning an experiment it's important to take into account the number of chromatin preparations and ChIP assays to be performed, including any control ChIP reactions and if looking at treatment effects be sure to prepare chromatin from untreated cells as controls. The Chromatrap® kit supplies enough reagents for up to 10 chromatin preparations ($15x10^6$ cells) and up to 96 ChIP assays.

Chromatin preparation: The success of a ChIP assay is highly dependent on the quality of chromatin prepared. The protocol described here is based on chromatin prepared from 1-15 million cells, however lower cell numbers are possible but volumes of buffers may need to be optimised by the user. 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).

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

Table 1

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

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

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

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

Column Conditioning Buffer (3X), 60 mls
Wash Buffer 1 (3X), 60 mls
Wash Buffer 2 (3X), 60 mls
Wash Buffer 3 (3X), 60 mls

To each 60 mls add 120 mls molecular biology grade water to achieve a 1X concentration solution (total volume 180 mls).

Slurry volume: A key difference in the Chromatrap® technique compared to conventional bead based assays is the volume of immunoprecipitation slurry used. **The fundamental requirement is to load 50-3000 ng chromatin in a total volume of 40 µl, taking into account all other components in the slurry**.

Antibodies: The use of ChIP validated antibodies is essential for the success of a ChIP assay. The antibody must recognise and bind to native protein that is bound to DNA. It is essential to include ChIP validated positive and negative antibody controls to ensure chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP. Chromatrap® offers an antibody validation service, please contact Chromatrap® customer support for more information.

Quantity of antibody: The user must determine the optimum antibody: chromatin ratio for use in ChIP. Optimal results have been achieved with a 2:1 antibody: chromatin ratio on the Chromatrap® 96-well plate.

Downstream analysis: This protocol has been optimised for q-PCR as a downstream detection method. For sequencing please refer to our Chromatrap® 96 ChIP SEQ kits (product codes 500214, 500215 500216, 500217).

Protocol

Step 1: Chromatin preparation; cell fixation and collection

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

Step 1a: For adherent cells

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

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

Step 1b: For suspension cells

1. Collect cells by centrifugation at 200xg for 5 minutes at 4°C.
2. Re-suspend in 1 ml pre-warmed PBS (perform cell count) and spin 200xg 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 200xg for 5 minutes at 4°C.
7. Re-suspend in 1 ml ice cold PBS.
8. Spin to collect cells at 200xg 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).

Step 2: Cell Lysis and Chromatin Shearing

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

Step 2a: Cell lysis and chromatin shearing by sonication

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

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

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

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

Determining DNA concentration

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

Example calculation

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

4. Add Shearing Cocktail to each stock nuclei suspension (from Step 2b, point 3) at a ratio of 1 U Shearing Cocktail:5 µg chromatin (Shearing Cocktail is supplied as 15 U/µl) and mix thoroughly.
5. Incubate for 5 minutes in a 37°C waterbath then immediately add Enzymatic Stop Solution (refer to Table 1 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 1 for optimum volume) and incubate the tubes on ice for 10 minutes to lyse the nuclei.
8. Centrifuge the samples for 10 minutes at maximum speed at 4°C and transfer the supernatant to a clean dry microcentrifuge tube.
9. Add 1 µl of PIC to the samples and mix.
10. Chromatin samples are now ready for IP. If samples are not to be used immediately, store at -80°C for a maximum of 2 months. It is recommended that the shearing efficiency of each chromatin stock is analysed at this stage.

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

Step 2c: Shearing efficiency

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

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

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

Step 3: Slurry preparation and immonoprecipitation

Step 3a: Slurry preparation

A key difference in the Chromatrap® technique compared to conventional bead based assays is the volume of immunoprecipitation slurry used. The Chromatrap® 96-well plate requires **40 µl** of IP slurry per well to completely fill the internal pores of its porous disc. **The fundamental requirement is to load 50-3000 ng chromatin in a total volume of 40 µl taking into account other components in the slurry**.

Plan how to achieve your **40 µl IP slurry volume**. In our experience a **2:1 antibody : chromatin** ratio produces optimal results. Refer to box 1 for an example of sample slurry calculation for achieving a 2:1 ratio. Prepare any positive and negative antibody controls along with your samples.

IP slurry

- *Add d.H_2O to a microcentrifuge tube so that the final slurry volume will be 40 µl.*
- *Add 5 µl of Wash Buffer 1*
- *Add 1 µl of PIC*
- *Transfer 50 ng-3 µg chromatin stock to the microcentrifuge tube*
- *Add ChIP certified antibodies of interest last (2:1 antibody:chromatin ratio)*

1. Thaw chromatin stocks on ice.
2. Chromatin stocks must be spun down after each subsequent thawing even if it's been spun down previously.
3. Spin down the sonicated chromatin, top speed for 10 minutes at 4°C, to minimise non-specific background interference from suspended solids. Use ONLY the clear supernatant for subsequent IP steps.
4. Prepare IP slurries in a fresh microcentrifuge tube as outlined in Box 1.
5. For every antibody IP set aside the equivalent amount of chromatin in a microcentrifuge tube and make up to 20 µl with water (if necessary), label as an input. These will be processed alongside the samples for reverse cross linking and proteinase K digestion at step 4 and will be used as controls in the PCR analysis.
6. Gently mix the IP slurries by pipetting. Briefly centrifuge to collect the slurry and keep at 4°C while the 96-well plate is activated.

Box 1

Example of 40 µl slurry calculation with 2:1 antibody : chromatin ratio, using 1 µg Chromatin

Chromatin stock = 628.5 ng/µl 1000/628.5= 1.6 µl	40 µl IP slurry calculation ● 22.4 µl d.H_2O ● 5 µl of Wash Buffer 1 ● 1 µl of PIC ● 1.6µl Chromatin stock (1 µg) ● 10 µl antibody (2 µg)

Step 3b: Chromatrap® spin column 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 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 (~5 minutes).
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 section 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. This allows for 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 elution buffer for maximal target recovery.

8. Transfer each **40 µl** IP slurry to a corresponding well on the Chromatrap® 96-well plate and incubate for 1 hour at 4°C on a rocking platform with gentle agitation.
9. Add 600 µl of Wash Buffer 1 to each well and centrifuge at 2000xg for 30 seconds at RT **(remember to add 600 µl d.H_2O to corresponding wells in 96-well balance plate)**. Discard the flow through and repeat.
10. Add 600 µl of Wash Buffer 2 to each well and centrifuge at 2000xg for 30 seconds at RT. Discard the flow through and repeat.
11. Add 600 µl of Wash Buffer 3 to each well and centrifuge at 2000xg for 30 seconds at RT. Discard the flow through and repeat.
12. Spin dry at top speed for 30 seconds at RT to remove any remaining liquid from the plate.
13. Transfer the Chromatrap® 96-well plate on to the supplied Elution plate.
14. Add 50 µl **Elution Solution** to each well, cap and incubate at RT for 15 minutes.
15. While incubating prepare a balance plate for the centrifuge, use the Chromatrap® 96-well balance plate with the balance elution plate provided and pipette 50 µl d.H_2O into the corresponding wells.
16. Centrifuge the plate at top speed for 1 minute to collect the eluted chromatin.

N.B. For difficult, low abundant targets up to 5 wash steps with each buffer can be performed to help improve target enrichment.

Step 4: Reverse cross-linking

The captured chromatin samples and inputs must be incubated with appropriate buffers to reverse the cross-links, this allows the release of the DNA from the protein bound complexes. Protein is then digested leaving the DNA ready for downstream processes. Inputs from Step 3a, point 5, must be re-introduced at this stage.

17. To each 20 µl input add **in the following order**
    a. 40 µl of Elution Solution
    b. 40 µl 0.1 M NaOH
    c. 5 µl 1 M $NaHCO_3$
18. To each 50 µl eluted sample in the elution plate add **in the following order**
    a. 50 µl 0.1 M NaOH
    b. 5 µl 1 M $NaHCO_3$
19. Mix thoroughly cap and incubate at 65°C for 2 hours to reverse the cross-linking.
20. Briefly centrifuge elution plate and inputs to remove any liquid from the caps.
21. Add 1 µl of Proteinase K solution (0.5 µg/µl) to each well and inputs, mix thoroughly and incubate for 1 hour at 37°C.
22. Return the elution plate and inputs to room temperature and add 2 µl of Proteinase K stop solution.

At this stage the DNA is ready for downstream processing, PCR, q-PCR. It is possible to go directly into q-PCR analysis without DNA clean-up. It is possible to go directly into q-PCR analysis without DNA clean up, for DNA clean up refer to p17.

DNA purification (optional)

The Chromatrap® 96-well plate technology has been designed so that DNA clean up is no longer required, therefore reducing the overall time of the ChIP assay. However, if you would still like to further purify your samples the following protocol provides an optimal methodology.

Either; Purify each DNA sample using a PCR purification protocol, e.g. QIAquick 96 PCR Purification kit (product code 28181)

Or; Perform phenol/chloroform extraction as follows.

Phenol/Chloroform Extraction

1. Add an equal volume of phenol: chloroform to the DNA containing reaction mixture (1:1) and vortex to mix thoroughly.
2. Centrifuge the samples at 4°C; maximum speed for 10 minutes to separate the aqueous phase from the organic phase.
3. Remove the aqueous phase with care into a fresh micro centrifuge tube.
4. Add 0.1 volume of 3 M sodium acetate pH 5.2 and 500 µl of ice-cold 100% ethanol to each sample and incubate for a minimum of 4 hours at -80°C or overnight at -20°C.

DNA recovery

5. Centrifuge at maximum speed for 10 minutes at 4°C.
6. Discard the supernatant and re-suspend the pellet in 500 µl of 70% ethanol. The pellet should be visible at this point.
7. Centrifuge at maximum speed for 5 minutes at 4°C.
8. Carefully remove and discard as much of the supernatant as possible.
9. Allow the pellet to air dry.
10. Re-suspend in 30 µl of deionised water.
11. The DNA samples are now ready for q-PCR, or other quantification method.

Troubleshooting guide and FAQs

1. Can the Chromatrap® 96 ChIP kit for qPCR be used for sequencing as a downstream process?
No, it is essential you use the specific Chromatrap® 96 ChIP SEQ kit for sequencing as a downstream process. The ChIP-seq kit contains columns with increased loading capacity and specific buffer compatibility for sequencing.

2. Should I use Protein A or Protein G Chromatrap® 96-well plate for 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.

3. Processing differences of the Chromatrap® 96-well plate
The Chromatrap® 96-well plate contains functionalised inert porous discs which have protein A or G covalently bound. The plates are designed for centrifugation with no difficult pipetting stages needed to separate supernatant liquids from retained chromatin complexes. The Chromatrap® technique therefore significantly reduces 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 discs 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.

The major difference in the Chromatrap® 96-well plate compared with conventional bead based assays is the volume in which the immunoprecipitation has to be performed. A 40 µl volume is the maximum bed volume of the discs and the immunoprecipitation slurry should not exceed this. The 40 µl volume ensures that the internal pores of the functionalised disc are completely filled thus providing a very efficient binding process and enabling shorter incubation times. This also results in excellent enrichment levels while minimising the amount of chromatin required.

4. How much chromatin is required per ChIP assay?
The Chromatrap® 96-well plate has been optimized to process between 50 and 3000 ng of chromatin (see Appendix B), with an antibody : chromatin ration of 2:1. Larger additions of chromatin can be used but may lead to a loss of efficiency depending on the percentage of lysis buffer within the chromatin stock. Sequential loading of the 40 µl slurry can also be done but this may prove time consuming. 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.

5. 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 300 µl following cross-linking and cell lysis. 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 1µg/IP, a total of 90 IP reactions could be performed from a 300 µl lysis buffer chromatin sample.

6. The Lysis buffer is cloudy, what do I do?
The Lysis buffer contains 1.0% wt/vol SDS. This is necessary to ensure the complete disruption of the nuclear membrane as well as the cell membrane. Assuming the Lysis buffer has been stored in a fridge it will need to be warmed up to room temperature to re-dissolve any precipitated SDS. This can occasionally take a long time so to speed up the process; stubborn precipitates can be re-dissolved by warming the buffer to 40°C in a water bath for 30 minutes with occasional shaking. The contents of 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. This makes sure that any precipitated SDS is not sticking to the walls of the bottle but rather is fully dissolved. Bring the buffer back to room temperature after the incubation by letting it sit on the bench for 5 minutes before adding it to the sample.

7. Why do I have a poor yield of sheared chromatin?
Cells could be over fixed making them resistant to lysis and shearing. Ensure cells are fixed for the appropriate time (10 minutes) if still obtaining a poor yield try reducing fixation time. Ensure formaldehyde is made up fresh for every chromatin preparation.

Buffers were not scaled proportionally to size of the sample, refer to Table 1 for optimum volume of buffer depending on starting cell number.

8. Does the Chromatrap® 96-well plate require blocking?
There is no requirement to carry out a blocking step to minimize non-specific binding as the composition of the Chromatrap® 96-well plate and the buffers provided in the kit have been formulated to ensure that non-specific binding is minimized.

9. Why do I have no enrichment with ChIP antibody?
The use of ChIP validated antibodies is essential for the success of a ChIP assay. The antibody must recognise and bind to native protein that is bound to DNA. It is essential to include ChIP validated positive and negative antibody controls to ensure chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP.

The antibody epitope may have been destroyed or masked during the cross-linking process or it may be hidden by other proteins in the chromatin complex. Reduce the cross-linking incubation time or find an antibody which has an alternative epitope.

There may be an incorrect addition of antibody to chromatin sample, carryout an antibody dilution series to determine the optimum ratio. Optimal results have been achieved with a 2:1 antibody: chromatin ratio in the Chromatrap® 96-well plate.

The antibody may be of low affinity and may require a longer incubation time than that suggested in the protocol, increase the ChIP reaction to an overnight incubation at 4°C.

For difficult low abundant targets too much SDS may interfere with antibody binding, ensure concentration of SDS in the slurry is below 0.2%.

10. What is the binding capacity of the plate?
We have measured the IgG binding capacity of the plate to be approximately 50 µg per well. The standard protocol uses only a small fraction of this capacity.

11. The protocol is based on using cultured cell lines; can the technique be used with cells from other sources?
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 yeasts or plant tissues.

12. What can be the causes of a 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. Inefficient sonication can produce incorrect DNA fragment sizes. Conditions will differ depending on cell line, cell density, and the extent of cross-linking. Ideally the chromatin should be 100-500 bp as optimised and checked on an agarose gel and visualised against a marker of known size DNA fragments. Sonication efficiency can also be compromised by excessive sample foaming and poor heat dissipation.

Inefficient washing 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 (section 3c).

An incorrect ratio of antibody to target protein can compromise the signal to noise ratio. We have found an antibody : Chromatin ratio of 2:1 to give optimal results (see Appendix A).

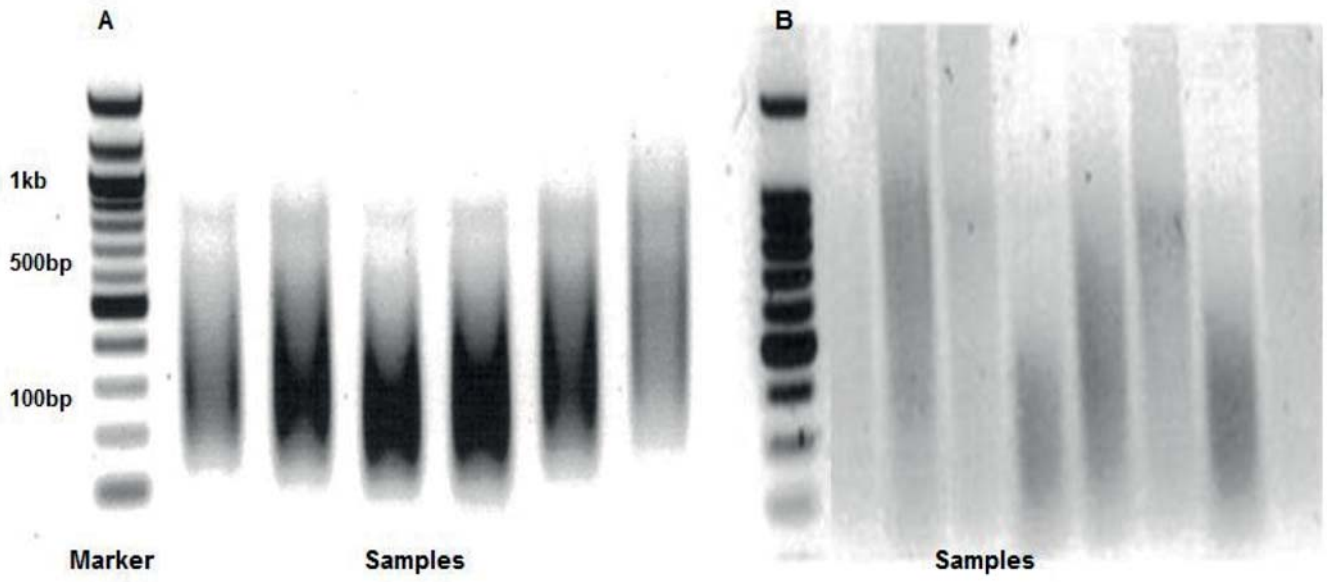
13. Should I use enzymatic digestion or sonication?

Sonication is a simple and effective method of chromatin shearing which provides randomly fragmented chromatin. Provided the temperature is controlled during the sonication process and emulsification is avoided good quality chromatin can be obtained from most cell types using this method.

Enzymatic shearing is useful if a sonicator is not available and is less disruptive to the epitopes of the protein of interest recognized by the specific antibody. Enzymatic shearing is essential when carrying out native ChIP (chromatin which has not been cross linked) as sonication can disrupts the protein/DNA complexes. Certain cell types may be resistant to lysis resulting in poor enzymatic shearing efficiency in this instance try sonication.

14. Have you got a suggestion for ideal sonication?

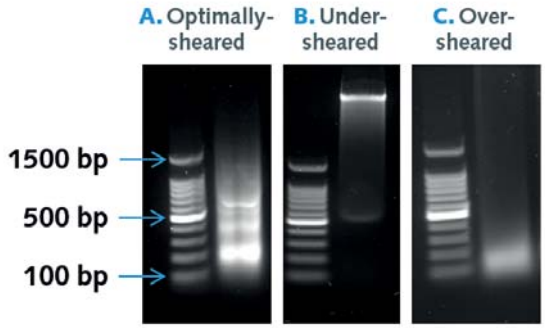
In our laboratory we have used a Bioruptor™ (Wolf Laboratories Limited, Pocklington, York, UK) to carry out the sonication step and found that 30 second bursts with 30 seconds intervals on ice, at a power setting of 3 for 15 minutes, produced chromatin of 100-500 bp. However the user of the kit will need to optimize their sonication method based on the chromatin they are investigating and the sonication equipment being used. The protocol provides a method of assessing the sonication process (see page 13, section headed *'Shearing Efficiency').*



Optimal sonication and chromatin fragment length. Following optimal sonication conditions, uniform chromatin fragment lengths between 100 and 500 base pairs should be visualised with agarose gel electrophoresis (a). Incorrect sonication will result in variable fragment lengths and diffuse smears with samples showing fragment sizes in the range of 100 to 1000 base pairs (b).

15. 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 optimised by the user. Refer to picture below for examples of chromatin sheared to desired fragment lengths.



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).

16. Do I need to clean up my samples for gel analysis?

No due to Chromatrap®'s unique buffer chemistry samples are compatible for use in gel analysis and ChIP without any need for clean up saving both time and cost.

17. 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. See Q18 for tips on under digested chromatin. Chromatin was completely digested to low molecular weight fragments see Q19 for tips on over digested chromatin. 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. Cell type is not suitable for enzymatic digestion in this instance try sonication refer to Chromatrap® spin column protocol.

18. Why is my chromatin under digested?

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 Coctail 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 (Step 10 Section B) 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 refer to Chromatrap® spin column protocol.

19. Why is my chromatin over digested?

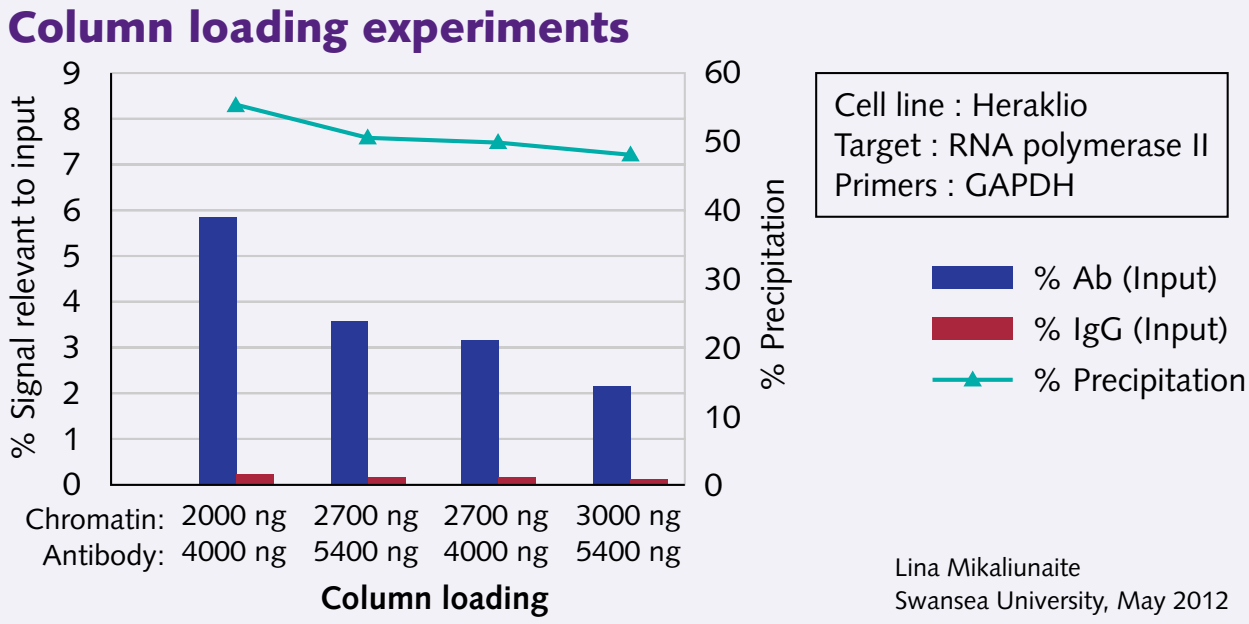
If chromatin is over sheared i.e. completely digested to mononucleosome fragments then the amount of Shearing Cocktail in the digestion may need to be reduced. Try reducing the U:chromatin ratio in the reaction (e.g. 1 U Shearing Coctail per 10 µg chromatin).

20. 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.

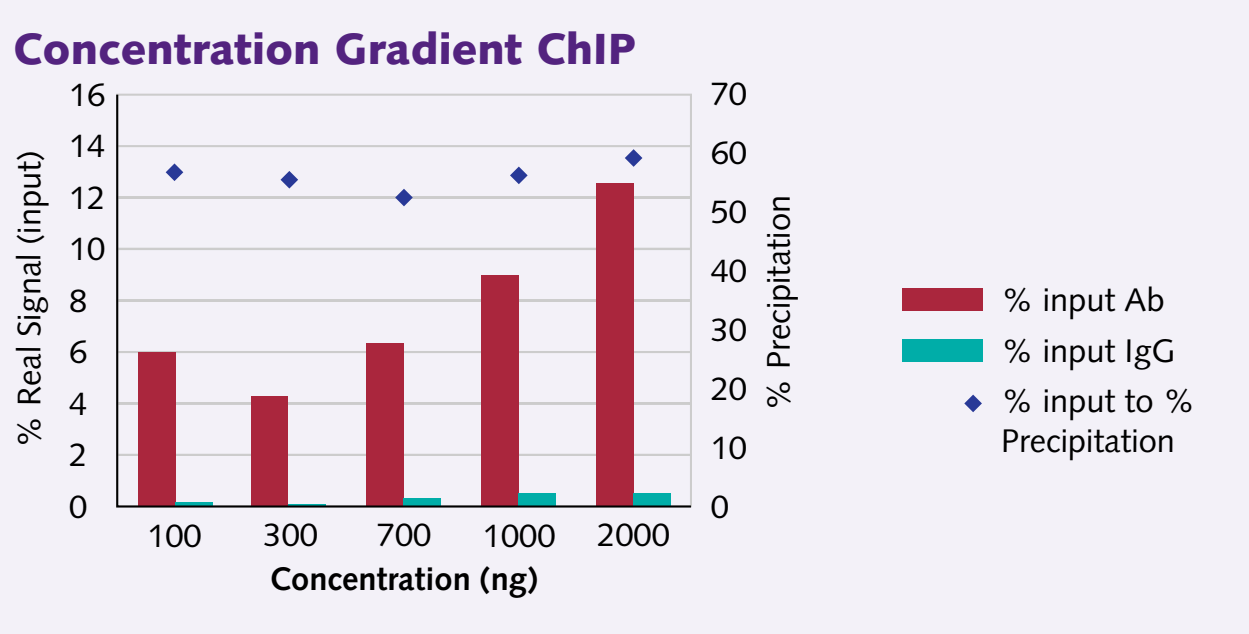
Appendix A

Plan how to achieve the chosen antibody : chromatin ratio in a 40 µl IP slurry volume. A 2: 1 ratio produces optimal results.



Appendix B

To emphasise the sensitivity of the Chromatrap® assay, concentration gradient of chromatin with a 2:1 antibody: chromatin ratio was applied to the wells. Excellent signal to noise ratio and % immunprecipitation, using as little as 100 ng chromatin for RNA Pol II pull down can be observed with the Chromatrap® assay.



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

A full step-by-step video of this protocol is available on YouTube to assist you in performing the assay;

youtu.be/mJXu3JBXWjs

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