Chromatrap

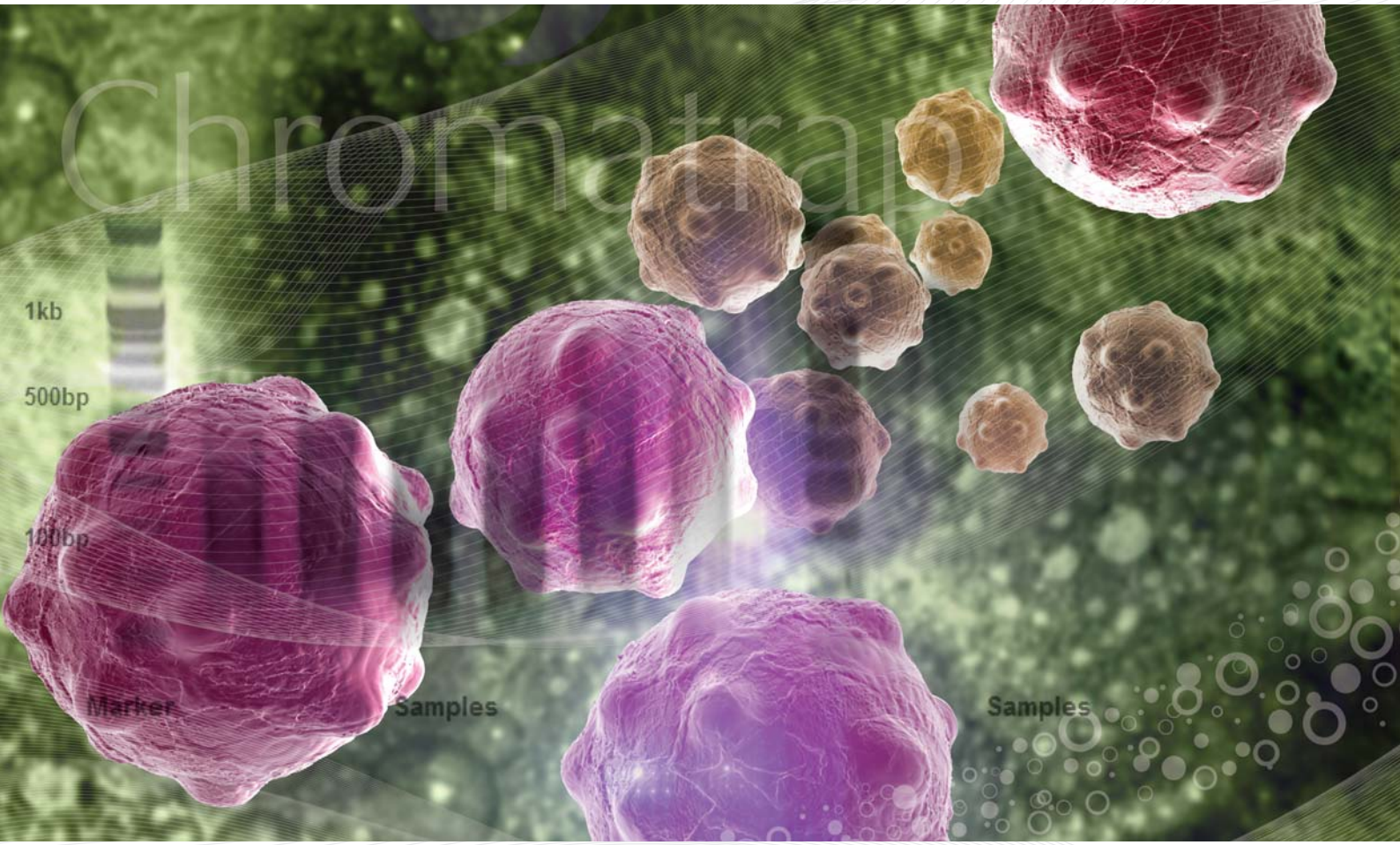
V1.1

Chromatrap® Sonication Shearing

A solid phase chromatin immunoprecipitation assay for next generation sequencing

Protocol v1.1

Catalogue no. 500239



ADVANCEMENTS IN EPIGENETICS

Contents

Kit Components and Storage

Kit Contents	Quantity	Storage temperature
1.3 M Glycine	20 mls	4°C (39°F)
Lysis buffer	10 mls	4°C (39°F)
5 M NaCl	500 µl	4°C (39°F)
1 M $NaHCO_3$	500 µl	4°C (39°F)
Hypotonic Buffer	10 mls	4°C (39°F)
Protease inhibitor cocktail (PIC)	100 µl	-20°C (-4°F)
Proteinase K stop solution	100 µl	-20°C (-4°F)
Proteinase K	50 µl	-20°C (-4°F)

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

Introduction

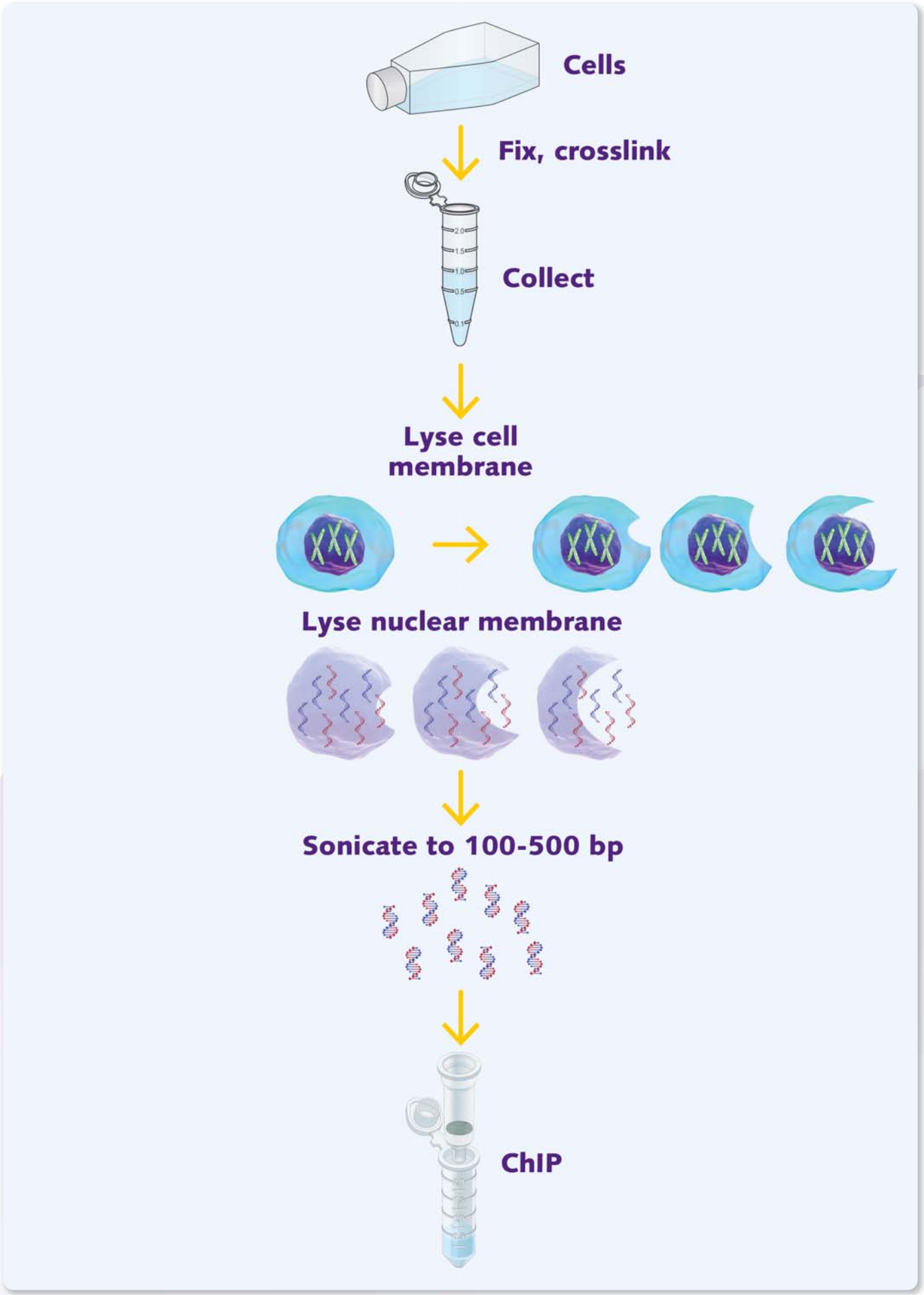
Epigenetics is the study of the molecular mechanisms which control gene expression in a potentially heritable way, which doesn't involve changes in the underlying DNA sequence.

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.

The success of a ChIP assay is highly dependent on the quality of chromatin prepared. Chromatrap® Sonication Shearing Kit supplies all the necessary reagents and buffers for up to 10 chromatin preparations. This allows you to determine optimal shearing conditions for your chromatin preparations and can supply you with enough chromatin to perform up to 24 ChIPs if using standard Chromatrap® ChIP spin column kit or up to 96 IP's if using Chromatrap® ChIP 96 High throughput microplate.

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

Assay Overview



Additional materials

Reagents and consumables

- *37% Formaldehyde*
- *PBS*
- *Nuclease free water*
- *Microcentrifuge tubes*
- *Cell scrapers*
- *100 bp ladder*
- *Pipettes and tips (filter tips recommended)*

Equipment

- *Sonicator*
- *Microcentrifuge (4°C)*
- *Spectrophotometer/fluorometer for DNA quantifications*
- *37°C waterbath*
- *65°C waterbath*

Assay preparation

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 24 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	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

** 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.*

Sonication Shearing Kit 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.

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: 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.*

Troubleshooting Guide and FAQs

1. 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 40ºC 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 on 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.

2. 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.

3. 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.

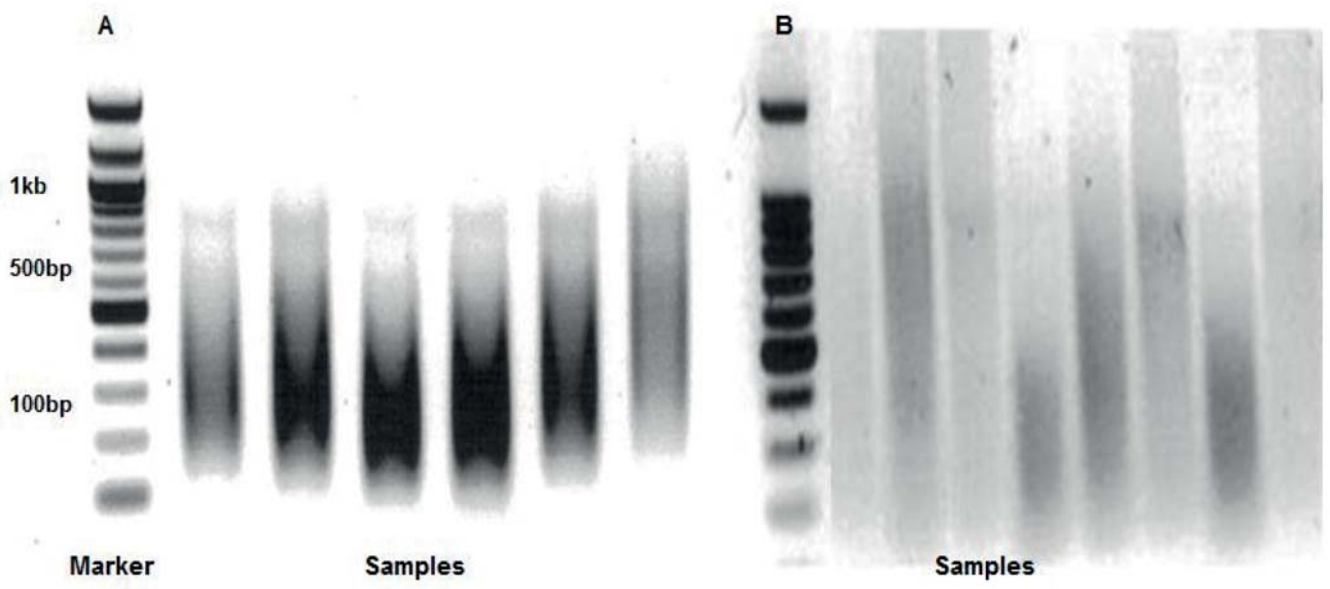
4. Should I use enzymatic digestion or sonication?

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.

5. 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 8) section headed '*Step 2b: 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 sizez in the range of 100 to 1000 base pairs (b).

6. 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.

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

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