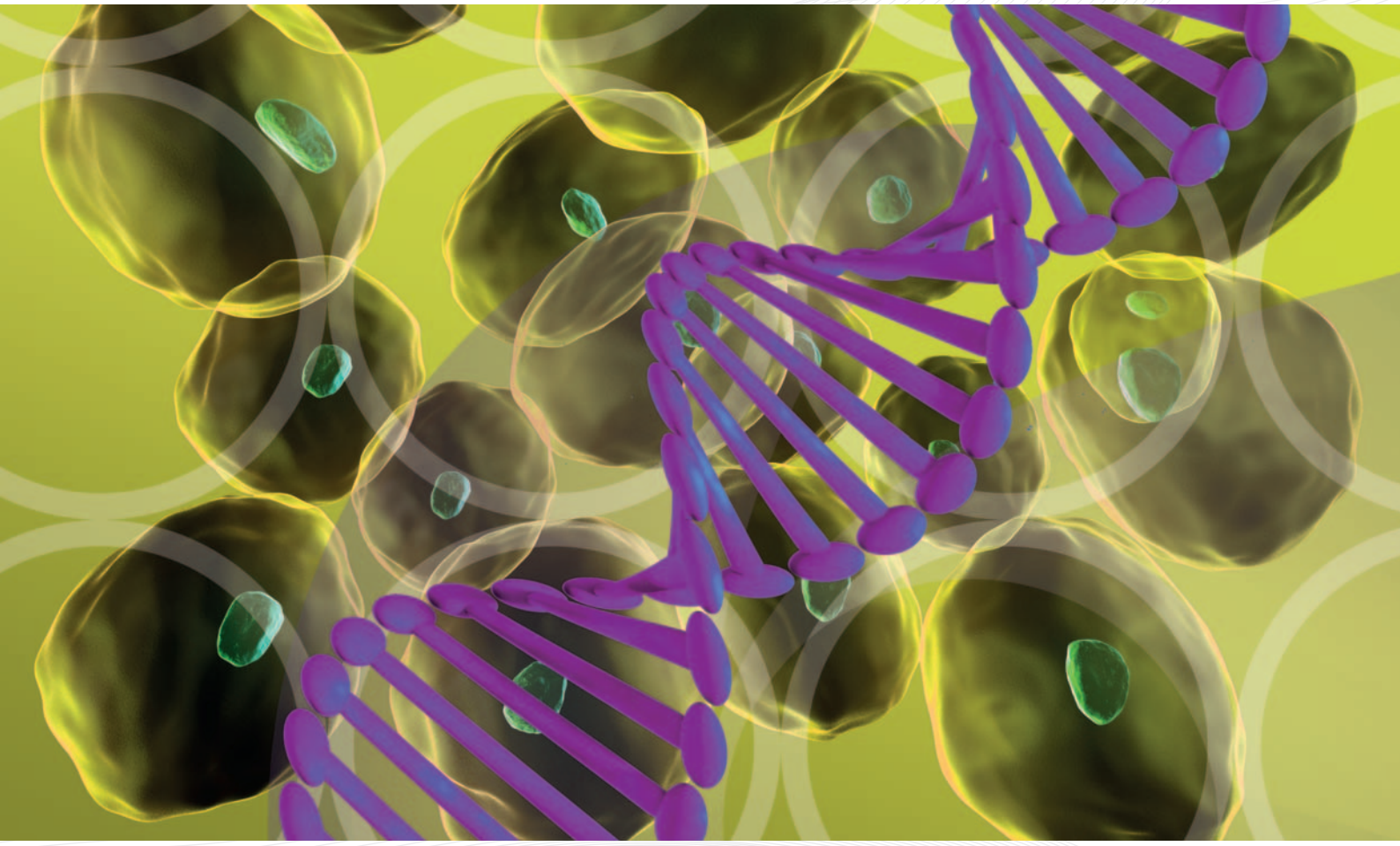
Chromatrap

V1.1

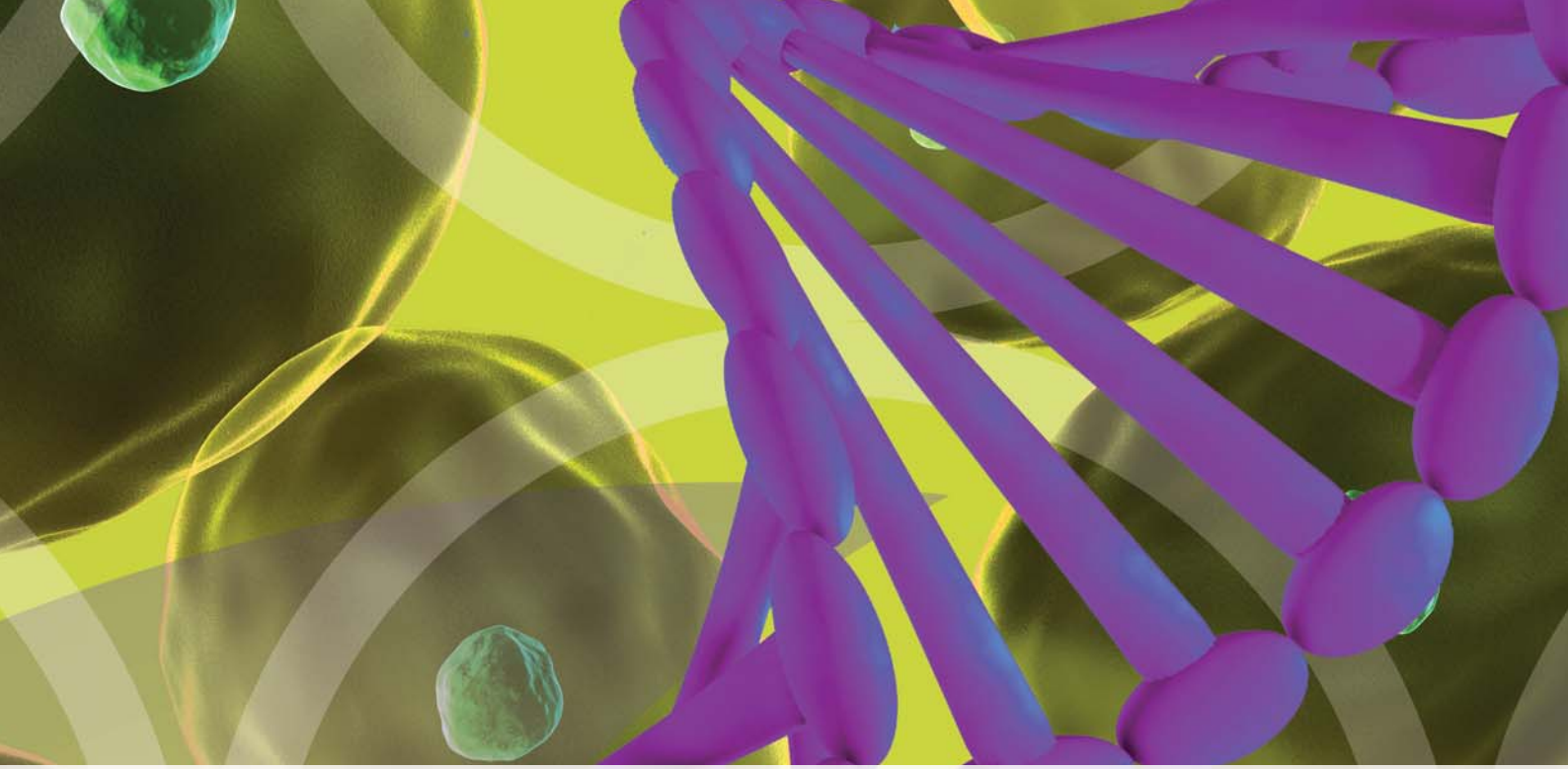
Chromatrap® Enzymatic Shearing Kit

A solid phase chromatin immunoprecipitation assay (ChIP)

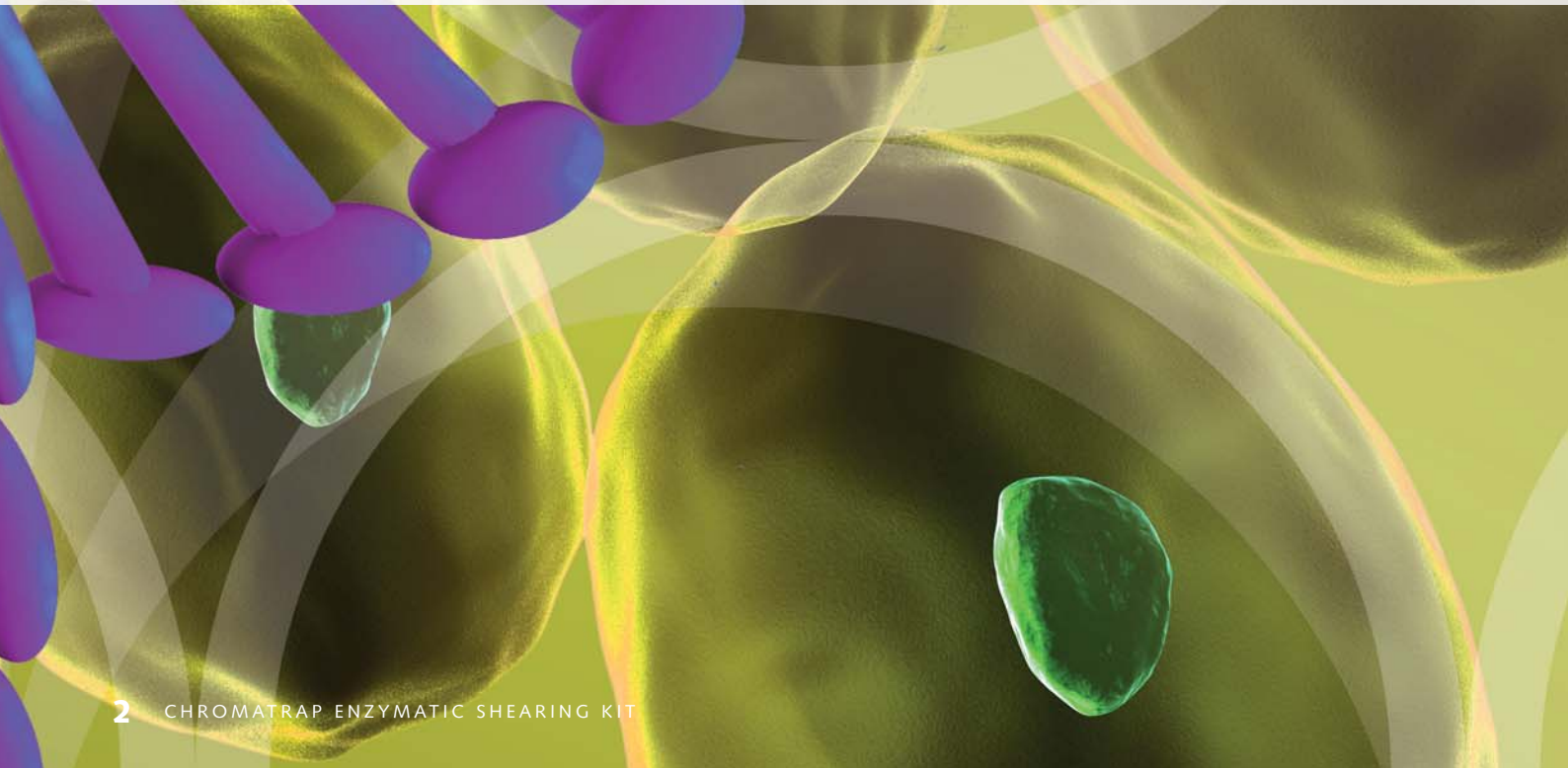
Protocol v1.1



ADVANCEMENTS IN EPIGENETICS



Contents



Kit components and storage

Kit Component	Qty	Storage Condition
1.3M Glycine	20mls	4°C (39°F)
Hypotonic Buffer	10mls	4°C (39°F)
Digestion Buffer	10mls	4°C (39°F)
Enzymatic Stop Solution	200µl	4°C (39°F)
Lysis Buffer	10mls	4°C (39°F)
5M NaCl	500µl	4°C (39°F)
1M $NaHCO_3$	500µl	4°C (39°F)
Shearing Cocktail	100µl	-20°C (-4°F)
Protease Inhibitor Cocktail (PIC)	100µl	-20°C (-4°F)
Proteinase K	50µl	-20°C (-4°F)
Proteinase K Stop Solution	100µl	-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 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 technique used to study the association of specific proteins, or their modified isoforms, with defined genomic regions.

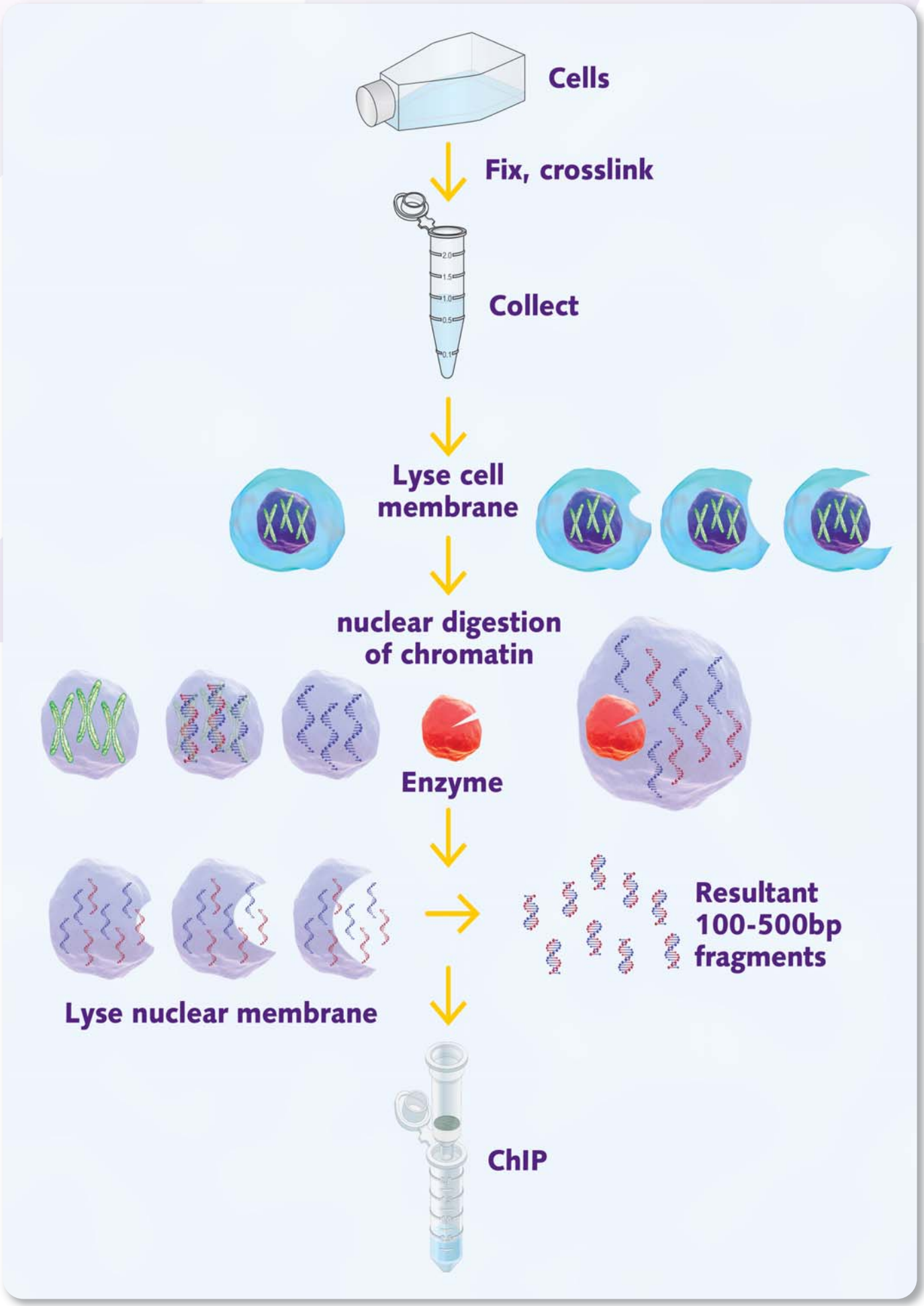
Chromatin Immunoprecipitation (ChIP) is a commonly used immunprecipitation technique for mapping the DNA-protein interactions in cells which are crucial for correct gene regulation. In a ChIP assay, DNA-protein complexes (chromatin) are fixed by the formation of cross links to preserve the interactions. 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® Enzymatic 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.

Product	ChIP's	Catalogue number
Chromatrap® Enzymatic Shearing Kit	Up to 10 chromatin preps	500165
Standard Chromatrap® Pro-A ChIP spin column kit	24 IP's	500071
Standard Chromatrap® Pro-G ChIP spin column kit	24 IP's	500117
Premium Chromatrap® Pro-A ChIP spin column kit	24 IP's	500115
Premium Chromatrap® Pro-G ChIP spin column kit	24 IP's	500116
Standard Chromatrap® Pro-A Enzymatic ChIP spin column kit	24 IP's	500166
Standard Chromatrap® Pro-G Enzymatic ChIP spin column kit	24 IP's	500168
Premium Chromatrap® Pro-A Enzymatic ChIP spin column kit	24 IP's	500167
Premium Chromatrap® Pro-G Enzymatic ChIP spin column kit	24 IP's	500169
Chromatrap® Pro-A High throughput ChIP microplate	96 IP's	500161
Chromatrap® Pro-G High throughput ChIP microplate	96 IP's	500163
Chromatrap® Pro-A Enzymatic high throughput ChIP microplate	96 IP's	500162
Chromatrap® Pro-G Enzymatic high throughput ChIP microplate	96 IP's	500164

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

Chromatin Preparation Overview



Additional materials and equipment required

- *37% formaldehyde*
- *PBS*
- *DNase free water*
- *Rocking platform*
- *Microcentrifuge (4°C)*
- *Microcentrifuge tubes*
- *0.1% SDS solution*
- *Nanodrop/spectrophotometer*
- *37°C waterbath*
- *65°C waterbath*

Experiment design, preparation and planning

IT IS RECOMMENDED THAT YOU READ THROUGH THE ENTIRE PROTOCOL BEFORE STARTING.
This protocol has been optimised for **adherent cells** 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® Enzymatic Shearing kit supplies enough reagents for up to 10 chromatin preparations (15 x10^6 cells).

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 adherent cells, however lower cell numbers are possible but volumes of buffers may need to be optimised by the user. Refer to section C for details on how to check the quality of chromatin.

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
0.65M Glycine*	1-5 5-10 10-15	3.0ml 4.0ml 5.0ml
Hypotonic Buffer	1-5 5-10 10-15	0.4ml 0.8ml 1.0ml
Digestion Buffer	1-5 5-10 10-15	0.3ml 0.4ml 0.5ml
Enzymatic Stop Solution	1-5 5-10 10-15	7.5µl 10µl 12.5µl
Lysis Buffer**	1-5 5-10 10-15	0.3ml 0.3-0.5ml 0.5-1.0ml

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

*** Lysis Buffer must be pre-warmed to 40°C (104°F) 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.*

A. Chromatin preparation

The following section describes cell culture fixation and collection of chromatin from **adherent cells**, chromatin from other sources will need to be optimised by the user. The protocol assumes your cell culture conditions and treatments have been optimised. Please remember to prepare enough chromatin for any biological IP controls along with chromatin from treated cells used to activate transcription factors targeted at the IP stage. If looking at treatment effects, chromatin from untreated cells must also be prepared at this stage to serve as a chromatin control.

1. Culture between 1-15 million cells.
2. Remove media and wash with warm PBS at room temperature (RT).
3. Decant off 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 whilst shaking on a rotating platform.
5. Decant off the fixation solution and add 0.65M glycine solution (glycine is supplied as a 1.3M 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 whilst shaking on a rotating platform.
7. Decant off glycine solution and collect the cells by scraping in 1ml of ice cold PBS. Transfer to a clean microcentrifuge tube and centrifuge at 3500xg for 5 minutes at 4°C (39°F).
8. Discard the supernatant.
9. At this point the protocol can be continued or the pellet can be frozen and stored at -80°C (-112°F), if freezing the pellet add 1µl Protease Inhibitor Cocktail (PIC).

Table 1

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

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

*** Lysis Buffer must be pre-warmed to 40°C (104°F) 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.*

B. Chromatin Shearing

Chromatin can be sheared either by a sonication or an enzymatic approach. This section describes chromatin shearing by enzymatic digestion for 1-15 x10^6 cell preparations and the buffer volumes needed are outlined in Table 1 section A. This protocol has been optimised for use with **adherent cell lines** however the user may find they need to try different conditions of shearing cocktail to achieve optimally sheared chromatin from their cells refer troubleshooting and FAQs.

10. Fully re-suspend the cell pellet in Hypotonic Buffer and incubate the samples on ice for 10 minutes (refer to Table 1 for optimum volume for starting cell number).
11. Centrifuge the hypotonic slurry at 5000xg for 5 minutes at 4°C (39°F) to collect the nuclei.
12. Discard the supernatant and re-suspend the pellet (nuclei) in Digestion Buffer by pipetting (refer to Table 1 for optimum volume for starting cell number), immediately add 2µl PIC to each stock nuclei suspension. Keep stock nuclei suspension on ice while determining DNA concentration.

Determining DNA concentration

- Remove a 10µl sample of each stock nuclei suspension and add to 490µl 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 9ng/µl.
9 (concentration) x 50 (dilution factor) x 400 (volume of Digestion Buffer)
= 180,000ng or 180µg total chromatin.
1U Shearing Cocktail per 5µg chromatin, therefore 180/5 **= 36U Shearing Cocktail**.
Shearing Cocktail is supplied as 15U per µl, therefore 36/15 **= 2.4µl Shearing Cocktail to be added**.

13. Add Shearing Cocktail to each stock nuclei suspension (**from step 12**) at a ratio of 1U Shearing Cocktail:5µg chromatin (Shearing Cocktail is supplied as 15U/µl) and mix thoroughly.
14. Incubate for 5 minutes in a 37°C (99°F) waterbath then remove, immediately add Enzymatic Stop Solution (refer to Table 1 for optimum volume) and place tubes on ice.
15. Centrifuge for 5 minutes at 12,000xg at 4°C (39°F) and discard the supernatant.
16. Fully resuspend 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.
17. Centrifuge the samples for 10 minutes at maximum speed at 4°C (39°F) and transfer the supernatant to a clean dry microcentrifuge tube.
18. Add 1µl of PIC to the samples and mix.
19. Chromatin samples are now ready for IP using Chromatrap® spin columns or Chromatrap® 96 HT. If samples are not to be used immediately store at -80°C (-112°F) until use.

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.

Checking Chromatin Shearing Efficiency

In order to check the quality and shearing efficiency of the stock chromatin, aliquots should be removed, reverse cross-linked to release the DNA and separated on an agarose gel. Prior to IP, 25µl aliquots should be used to quantify the chromatin and check that optimal shearing efficiency has been achieved as a standard quality control step at this stage.

1. Take a 25µl aliquot of sheared chromatin from each sample and place in a microcentrifuge capped tube.
2. Add 5µl of 1M $NaHCO_3$ and 5µl of 5M NaCl and make up to a final volume of 50µl with $d.H_20$, mix thoroughly.
3. Incubate the samples at 65°C (149°F) for 2 hours to reverse the cross-linking (samples can be left overnight if necessary).
4. Briefly centrifuge the samples to remove liquid from the caps.
5. Add 1µl of the Proteinase K solution (0.5µg/µl), mix thoroughly and incubate for 1 hour at 37°C (99°F).
6. Return the samples to room temperature and add 2µl Proteinase K stop solution.
7. Use the samples above to check the DNA concentration on a spectrophotometer. The concentration of chromatin needs to be multiplied by 2 in order to get a final concentration of chromatin. This is the concentration that will be used to calculate how much chromatin to add to the IP slurry.
8. To ensure that 100-500bp 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. 100bp ladder). A smear of DNA sequences 100-500bp 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 the amount of Shearing Cocktail added may need to be adjusted. Complete digestion of chromatin to mononucleosome fragments may affect the efficiency of downstream processing e.g. qPCR. See the relevant section of the troubleshooting guide and FAQs.

Troubleshooting Guide and FAQs

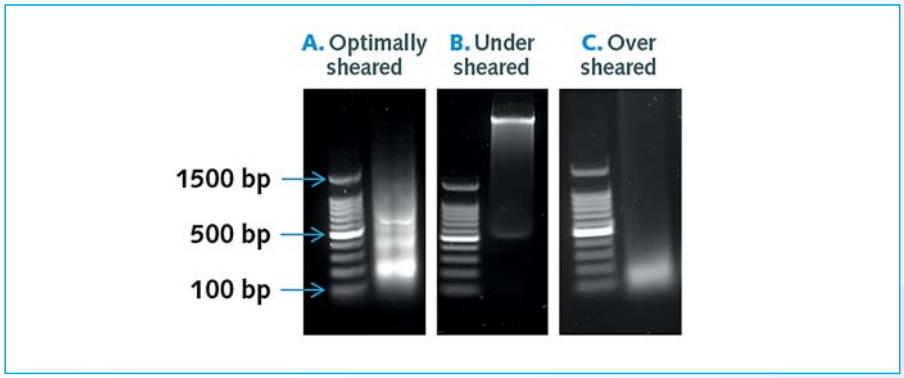
1. Why do I have poor yield of sheared chromatin?
Cells could be over fixed making them resistant to lysis and shearing. Ensure cells are fixed for the appropriate time (10 minutes) if still obtaining a poor yield try reducing fixation time. Ensure formaldehyde is made up fresh for every chromatin preparation.

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

2. 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 adherent cells and 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 shearing steps. More stringent grinding and sonication steps to disaggregate the cells may be required if working with yeasts or plant tissues.

3. Have you got a suggestion for ideal enzymatic shearing?
In our laboratory we have used our Shearing Cocktail at a ratio of 1U per 5µg 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 below picture 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 (C)

4. 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.

5. Should I use enzymatic digestion or sonication?
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 disrupt the protein/DNA complexes. Certain cell types may be resistant to lysis resulting in poor enzymatic shearing efficiency in this instance try sonication refer to Chromatrap® spin columns protocol.

6. 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 Q7 for tips on under sheared chromatin.

Chromatin was completely digested to low molecular weight fragments see Q7 for tips on over sheared 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 columns protocol.

7. Why is my chromatin under sheared?

If only larger bands (e.g. 400bp 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. 1U Shearing Coctail per 2µg 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 columns protocol.

8. 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. 1U Shearing Cocktail per 10µg chromatin).

9. What if the estimated total chromatin in my sample is less than 75ug (i.e. less than 1ul Shearing Cocktail is to be added to achieve 1U/5µG 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 1U/µl before adding it to the stock nuclei suspension.

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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