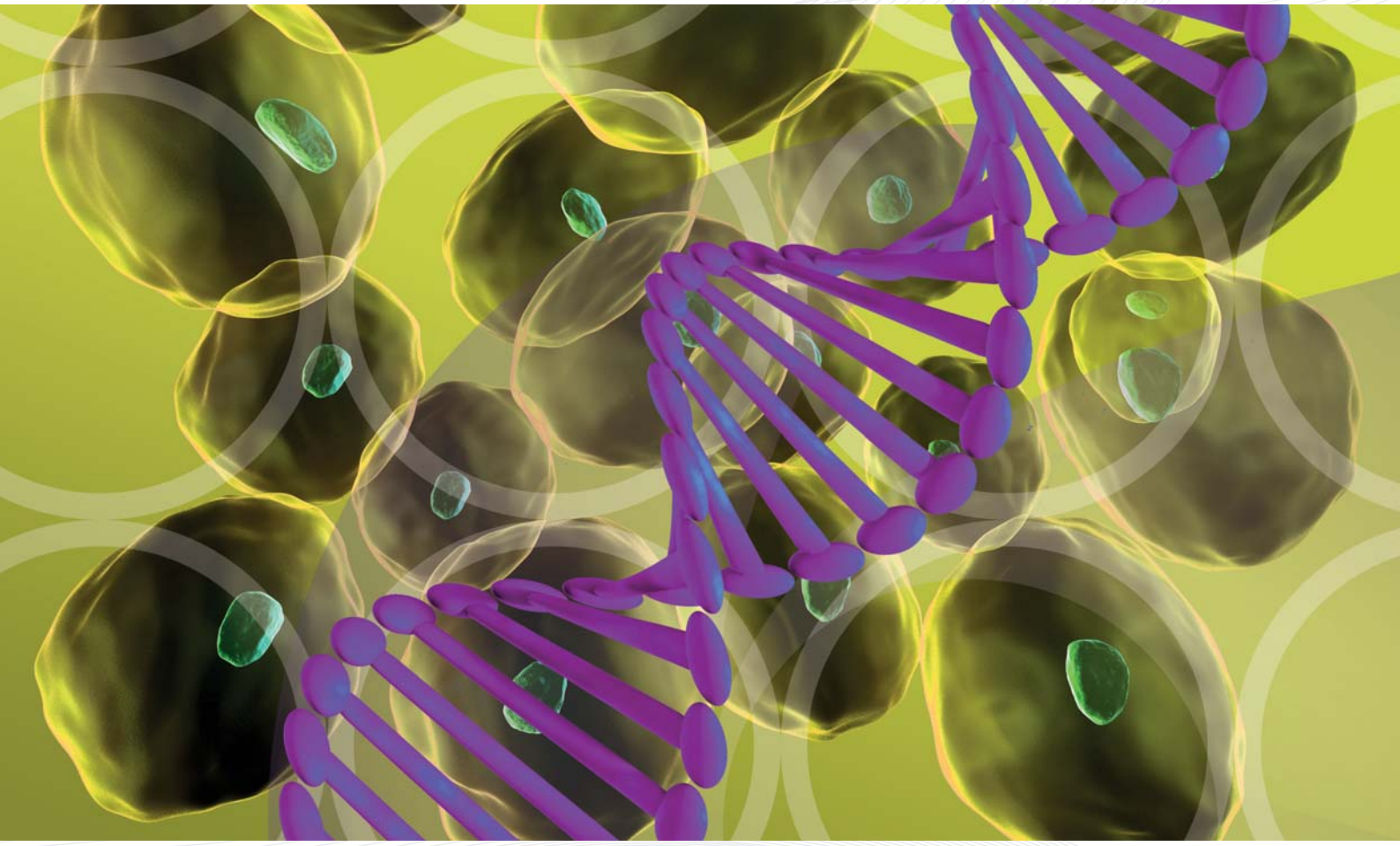
Chromatrap

V1.1

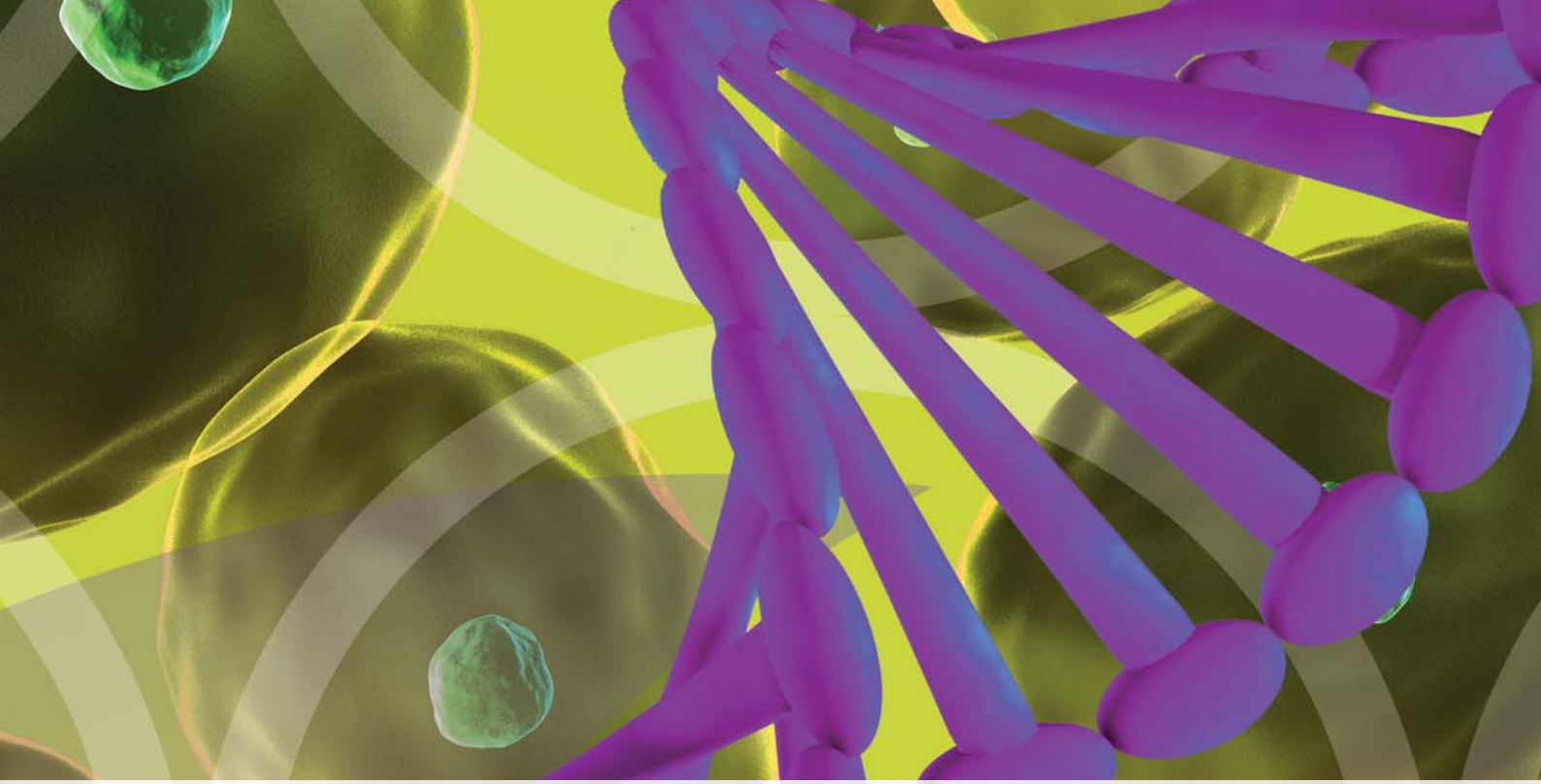
Chromatrap® Enzymatic Shearing Kit

A solid phase chromatin immunoprecipitation assay (ChIP)

Protocol v1.1



ADVANCEMENTS IN EPIGENETICS



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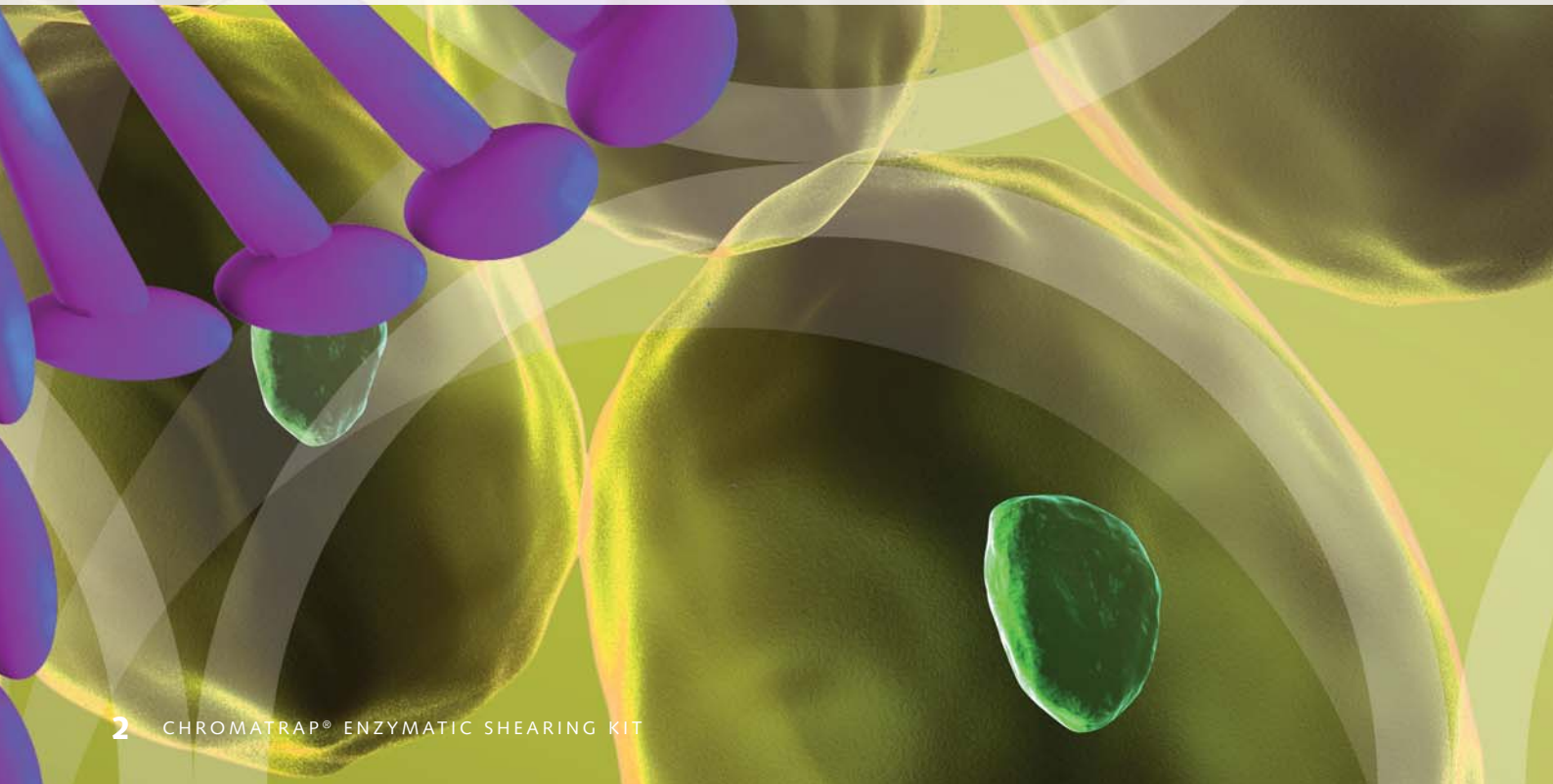


Kit components and storage

Kit Component	Qty	Storage Condition
1.3 M Glycine	20 mls	4°C (39°F)
Hypotonic Buffer	10 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)
5 M NaCl	500 µl	4°C (39°F)
1 M $NaHCO_3$	500 µl	4°C (39°F)
Shearing Cocktail	100 µl	-20°C (-4°F)
Protease Inhibitor Cocktail (PIC)	100 µl	-20°C (-4°F)
Proteinase K	50 µl	-20°C (-4°F)
Proteinase K Stop Solution	100 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 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 DNAprotein 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.

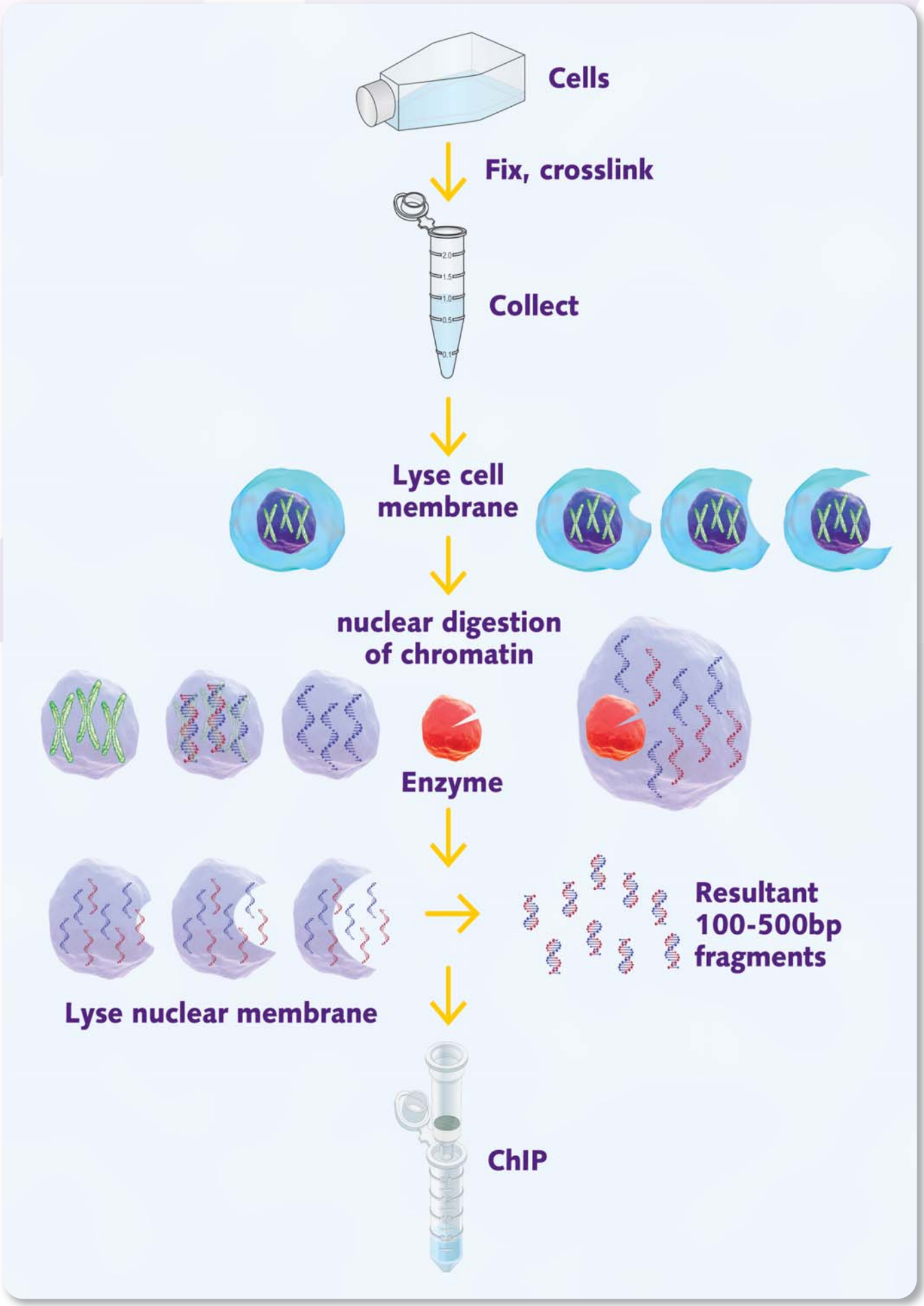
The success of a ChIP assay is highly dependent on the quality of chromatin prepared. Chromatrap® Enzymatic Shearing Kit supplies all the necessary reagents and buffers for up to 10 chromatin preparations. This allows you to determine optimal shearing conditions for your chromatin preparations and can 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.

Other products available from Chromatrap®

Product	ChIPs	Catalogue number
Chromatrap® Enzymatic Shearing kit	Up to 10 chromatin preps	500165
Standard Chromatrap® Pro A ChIP spin column kit for qPCR	24 IPs	500071
Standard Chromatrap® Pro G ChIP spin column kit for qPCR	24 IPs	500117
Premium Chromatrap® Pro A ChIP spin column kit for qPCR	24 IPs	500115
Premium Chromatrap® Pro G ChIP spin column kit for qPCR	24 IPs	500116
Standard Chromatrap® Pro A Enzymatic ChIP spin column kit for qPCR	24 IPs	500166
Standard Chromatrap® Pro G Enzymatic ChIP spin column kit for qPCR	24 IPs	500168
Premium Chromatrap® Pro A Enzymatic ChIP spin column kit for qPCR	24 IPs	500167
Premium Chromatrap® Pro G Enzymatic ChIP spin column kit for qPCR	24 IPs	500169
Chromatrap® Pro A high throughput ChIP microplate for qPCR	96 IPs	500161
Chromatrap® Pro G high throughput ChIP microplate for qPCR	96 IPs	500163
Chromatrap® Pro A enzymatic high throughput ChIP microplate for qPCR	96 IPs	500162
Chromatrap® Pro G enzymatic high throughput ChIP microplate for qPCR	96 IPs	500164
Chromatrap® Pro A ChIP-seq kit	24 IPs	500189
Chromatrap® Pro G ChIP-seq kit	24 IPs	500190
Chromatrap® Pro A ChIP-seq high throughput microplate	96 IPs	500214
Chromatrap® Pro G ChIP-seq high throughput microplate	96 IPs	500215
Chromatrap® Pro A Enzymatic ChIP-seq kit	24 IPs	500191
Chromatrap® Pro G Enzymatic ChIP-seq kit	24 IPs	500192
Chromatrap® Pro A Enzymatic ChIP-seq high throughput microplate	96 IPs	500216
Chromatrap® Pro G Enzymatic ChIP-seq high throughput microplate	96 IPs	500217

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*
- *Cell scrapers*
- *100 bp ladder*
- *Pipettes and tips (filter tips recommended)*

Experiment design, preparation and planning

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

** 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 (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.*

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 500xg for 5 minutes at 4°C.
2. Re-suspend in 1 ml pre-warmed PBS (perform cell count) and spin 500xg 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 500xg for 5 minutes at 4°C.
7. Re-suspend in 1 ml ice cold PBS.
8. Spin to collect cells at 500xg 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. 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. This protocol has been optimised for use with **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.

Step 2a: 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 2a, 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 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. 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.

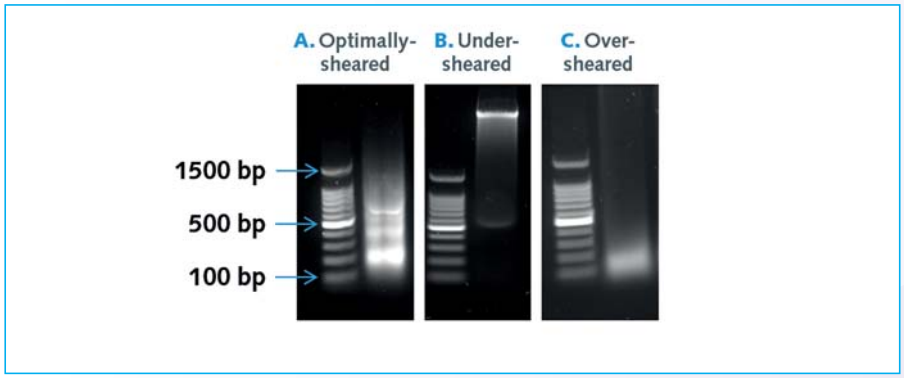
2. The protocol is based on using cultured cell lines; can the technique be used with cells from other sources?

This protocol has been optimised for 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.

3. 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 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?

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.

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

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

Chromatrap

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