10 Optimise lysis buffer for cells, too much lysis buffer can interfere with antibody binding

Lysis buffer usually contains a mild detergent and when stored at cold temperatures can precipitate out of solution. To ensure the lysis buffer is optimal for lysing of cells for the ChIP assay always pre-warm solution to 40°C with occasional mixing or inverting before use to remove any precipitates. Ensure the buffer is returned to room temperature for the lysis step and all precipitates are re-dissolved.

Volume of lysis buffer is important when performing chromatin preparation. Lower cell numbers (1-5 million) require less lysis buffer than greater cell numbers (10-15 million). Too much lysis buffer will result in excess detergent which could have an inhibitory effect on antibody binding and some downstream analysis e.g. qPCR. Excess lysis buffer will also result in a less concentrated chromatin preparation. Ensure lysis buffer volumes are optimised for each chromatin preparation before proceeding with ChIP assay. Chromatrap® has optimised volumes of lysis buffer to be used depending on starting cell number, see table below.

Buffer	Cell count (millions)	Buffer volume (ml)
0.65M 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

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Top 10 tips for positive ChIP results

Chromatrap

1 Quality of chromatin is the most important aspect of a ChIP assay. Poor chromatin = poor ChIP result

The success of a ChIP assay is highly dependent on the quality of chromatin prepared. The 3 most important aspects of chromatin preparation are **lysis**, **fixation** and **shearing**. Each step needs to be optimised individually to best represent the biological scenario being investigated. See below tips for each stage of chromatin preparation.

2 Keep chromatin on ice at all times

Chromatin degrades very quickly, especially when stored or handled at room temperature. To prevent rapid degradation and for reproducible results ensure chromatin is kept on ice throughout experiment. Freeze thaw cycles should be avoided, aliquoting chromatin in small volumes once stock is prepared should help prevent degradation. In Chromatrap® very small volumes of chromatin are required per IP therefore a single aliquot provides many ChIP reactions.

3 Do not over fix cells this will make them resistant to lysis and shearing

As mentioned, the quality of chromatin going in to a ChIP assay is key for a great result. To ensure chromatin quality is good, fixation of cells must be optimised. It is important not to over fix as cells can become resistant to lysis and shearing. Over fixation will also affect the reverse cross-linking efficiency (cross links cannot efficiently be removed) which will affect downstream processes (eg. qPCR).

Chromatrap® recommends cell fixation with 1% formaldehyde in either media or PBS for 10 minutes at room temperature on a rotating platform, if still obtaining a poor yield try reducing fixation time to 5 minutes.

4 Make sure fixation solution is made up fresh every time

Fixing of cells will affect quality of chromatin which will in turn affect the result of the ChIP assay. Ensuring the formaldehyde is fresh for every chromatin preparation gives more reproducible results. Ensure formaldehyde is methanol free as methanol can disrupt cell membranes altering the fixation conditions.

5 Choose an appropriate shearing method for your cells; sonication/enzymatic. Some cells are resistant to enzymatic digestion and require sonication

Chromatin can be sheared either by a sonication (mechanical using ultra sonic sound waves) or by an enzymatic (micrococcal nuclease digestion) approach. It is important to choose the appropriate method of shearing for cells. Chromatrap® offers both a standard ChIP kit for use with sonication and an enzymatic ChIP kit. We also provide an enzymatic shearing kit for use in optimisation of chromatin.

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. However, this method can create bias as nuclease exhibits sequence specific cleavage. 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 which aids in both shearing and lysis of the cells.

See over for optimisation of shearing via both sonication and enzymatic digestion.

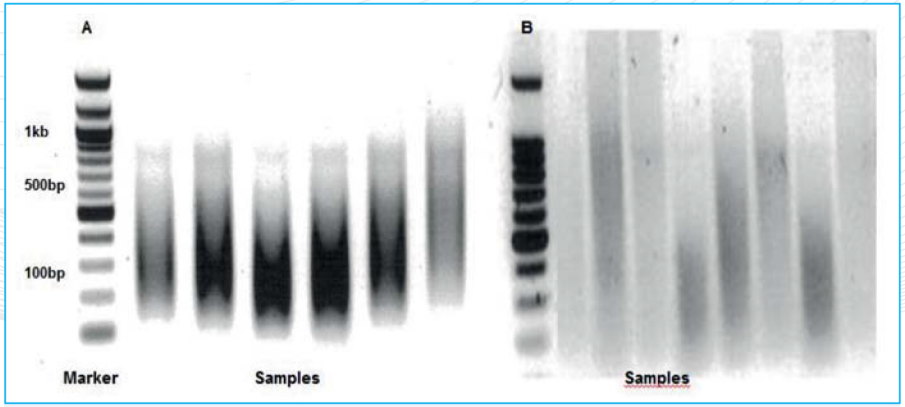
	Advantages	Disadvantages
Sonication	Random fragmentation. Suitable for difficult to lyse cell types.	Potential antigenic epitope damage through emulsification or overheating. Requires expensive equipment. Cannot be used for Native chromatin preparation (non cross-linked).
Enzymatic	Milder treatment, less damaging to epitopes of interest. Does not require any expensive equipment. Suitable for native chromatin preparation.	Restriction enzymes may exhibit some sequence bias during fragmentation. Not suitable for some difficult to lyse sample types.

6 Make sure chromatin is sheared to between 100-500bp, chromatin which is over or under sheared will reduce ChIP efficiency

For every chromatin preparation it is essential to check the chromatin is sheared to fragments between 100-500 bp. Chromatin analysis can be carried out quantitatively with a spectrophotometer, fluorometer or microfluidics platform. Samples can be qualitatively assessed using an agarose gel or microfluidics platform. Chromatrap® would recommend DNA quantification on a microfluidics platform as the most accurate measure of DNA and is highly compatible with Chromatrap® buffer systems.

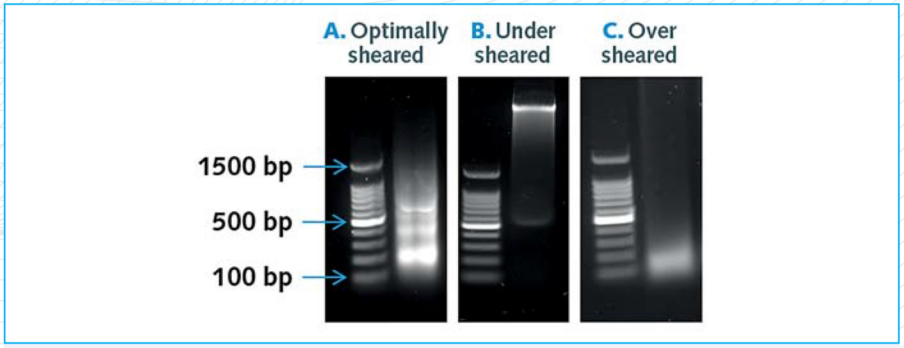
Problems with chromatin include over shearing which will result in very small fragments which can be detrimental for downstream processes e.g. qPCR where the small fragments can reduce primer recognition and thus reduce PCR efficiency. Over shearing by sonication also increases the risk of damaging the protein epitopes. In comparison, under shearing will produce fragments of larger size which will result in an increase of non-specific binding in the ChIP assay.

Sonication efficiency will vary between cell types and can be affected by the extent of cross linking, heating and emulsification of the sample. These will all reduce shearing efficiency. It is important to optimise sonication to ensure a successful ChIP result. Ensure sonication tubes are used when shearing your chromatin as these are made of harder plastic which transfer ultra sonic waves more efficiently than softer plastic tubes. A good idea is to start with optimising a cell line or tissue sample which is abundant. This allows optimisation of the different parameters of your sonicator, including power setting and number of cycles. When using Chromatrap® methodology, successful shearing of cell lines and primary cells to convert fragment size (100-500 bp) has been observed using a water bath (4°C) sonicator with 30 second bursts with 30 second intervals, at a power setting of 3 for 15 minutes.



Optimal sonication and chromatin fragment length. Following optimal sonication conditions, uniform chromatin fragment lengths between 100 and 500 base pairs should be visualised with agrose 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).

For enzymatic shearing the most important factors are; lysis of cell membranes and the concentration of enzyme. It is important to optimise the chromatin ratio in order to achieve optimal fragment lengths of between 100-500 bp. Perform a dilution series of enzyme to chromatin to determine the optimum ratio in your sample. Alternatively choose a concentration of enzyme and adjust digestion times. In Chromatrap® 1U per 5µg chromatin for 5 minutes provides optimal fragment lengths. For under sheared chromatin (400 bp and above) try increasing the U:chromatin ratio in the reaction. If chromatin is over sheared i.e. completely digested to mononucleosome fragments then the amount of enzyme to chromatin ratio should be reduced.



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)

Alternatively cell membranes may not have been efficiently lysed, limiting access of enzyme to chromatin, check cells using a phase contrast microscope to ensure all the nuclei are released prior to proceeding to enzymatic digestion. If membranes are not efficiently lysed try incubating the samples for longer in lysis buffer. After longer incubation if cells are still resistant to lysis convert to sonication method.

7 Ensure you use a ChIP validated antibody, antibodies from other applications do not always work well in ChIP

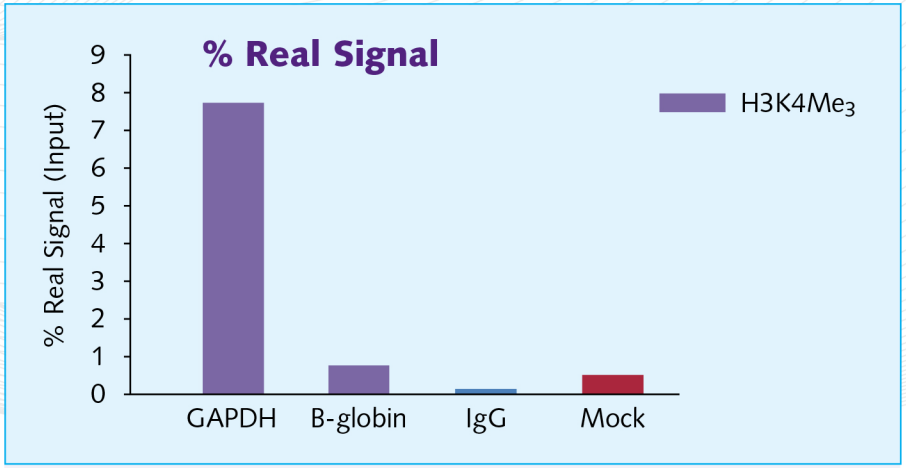
The use of high quality and specific ChIP validated antibodies is essential for the success of a ChIP assay. The antibody must recognise and bind to native protein that is bound to DNA. It is essential to include ChIP validated positive and negative antibody controls to ensure chromatin preparation and ChIP methodology are appropriate. Antibodies from other applications do not always work well in ChIP.

8 Always run a positive and negative antibody control

To indicate the efficiency of the immunoprecipitation step and to ensure chromatin preparation is sufficient, positive and negative antibody controls should be run alongside any test antibodies. In the Chromatrap® premium ChIP kit a polyclonal antibody for the highly abundant histone mark H3 as a positive control and a negative control, IgG, are supplied. Primers optimised for qPCR are included, these recognise Glyceraldehyde 3- phosphate dehydrogenase (GAPDH), an ever-present housekeeping gene (Barber et al., 2005), to ensure chromatin preparation and methodology are appropriate.

Alternatively, a 'mock' ChIP reaction, containing no primary antibody, can be used as a control to determine background levels.

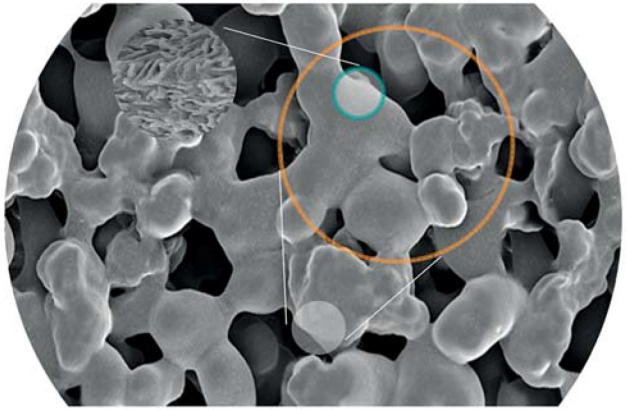
In addition to antibody controls a positive and negative gene target are good controls to ensure antibody enrichment is selective.



Graph illustrating high levels of enrichment (7.7% real signal) of H3k4Me$_3$ onto a positive house keeping gene GAPDH observed with Chromatrap®. Little to no enrichment is shown for H3k4Me3 on to the negative target gene B-globin (0.7% real signal). IgG and a no antibody (mock) control are also shown with 0.15 and 0.38 % real signal respectively highlighting specificity and sensitivity of Chromatrap® ChIP assay. The percentage of real signal was calculated as a proportion of the input chromatin, normalised using the signal generated by non-specific binding of unspecific IgG

9 Determining quantity of antibody to be used in the ChIP assay, usually an excess of antibody to chromatin is required

In a ChIP assay the antibody to chromatin ratio is important, an incorrect ratio of antibody to target protein can compromise the signal to noise ratio. Too much antibody can saturate the ChIP assay leading to unspecific binding. Too little antibody will not be able to bind to all of the chromatin that is present and therefore won't provide a good representation of antibody enrichment in your sample. Magnetic and agarose beads are known to vary in their antibody binding efficiency. Due to Chromatrap's® unique solid phase platform we provide a much greater surface area for antibody binding capacity which minimises non specific background.



Chromatrap® offers an inert solid phase scaffold which increases the surface area for greater antibody binding, allowing for better immunoprecipitation and reducing non specific binding.

In Chromatrap® technology very small concentrations of chromatin are used per IP (50ng-7µg). When lower chromatin loadings are used a 2:1 antibody : chromatin ratio is optimal, at higher chromatin loadings (5µg and above) a 1 : 1 antibody : chromatin ratio is optimal- allowing you to save on antibody. It is important to run an antibody dilution series before processing all samples in your ChIP assay to determine the optimum ratio.