10 Tips for Positive ChIP Results

How to optimize your reagents and experiments for success

Chromatrap @ BitesizeBio

1 Chromatin quality is crucial for successful ChIP. 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 fully optimised. See below for details.

2 Keep chromatin on ice at all times

Chromatin degrades very quickly, especially when stored or handled at room temperature. For best results, ensure chromatin is kept on ice throughout your experiment. Freeze/thaw cycles should be avoided. Using small aliquot volumes of chromatin once stock is prepared will help prevent degradation.

3 Optimise cell fixation

Optimal cross-linking of proteins to DNA ensures that the chromatin structure is preserved during isolation and the ChIP procedure. Fixation times that are too short will result in poor cross-linking efficiency and resulting DNA loss following immunoprecipitation. If cells are fixed for too long they can become resistant to lysis and subsequent chromatin shearing. This overfixation can lead to elevated background, reduced antigen availability and reduced reverse cross-linking efficiency, limiting downstream signal clarity. The optimal time for cross-linking will vary with cell line.

4 Fixation solution must be fresh

Ensuring the formaldehyde is fresh for every chromatin preparation gives more reproducible results. Formaldehyde should be methanol-free, methanol increases the permeability of cell membranes leading to increased fixation.

5 Choose an appropriate shearing method for your cells either sonication or enzymatic

Both sonication (mechanical shearing using ultrasonics) and enzymatic (micrococcal nuclease digestion) shearing can be used. It is important to choose the appropriate method. 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 disrupt the protein/DNA complexes.

Certain cell types may be resistant to lysis resulting in poor enzymatic shearing efficiency, in this instance try sonication. Chromatrap® ChIP kits can be purchased with either sonication or enzymatic digestion reagents. Alternatively, to optimise chromatin preparation Chromatrap® offer kits containing sonication shearing reagents (Cat 500239) or enzymatic shearing reagents (Cat 500165).

	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.

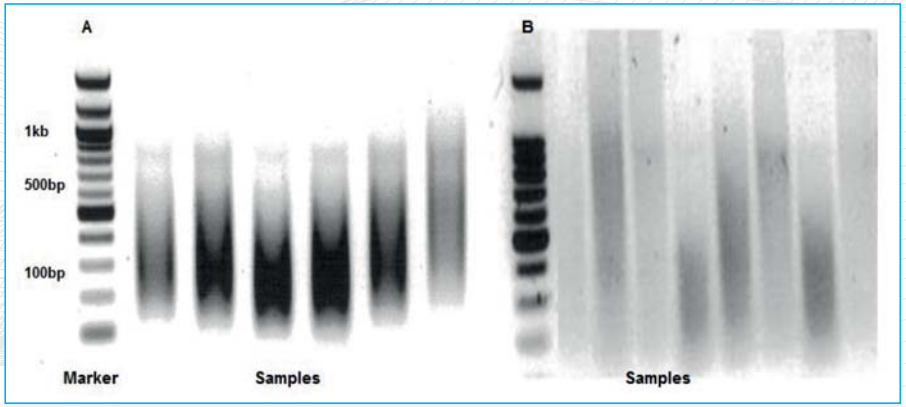
6 Ensure chromatin is sheared to between 100-500 bp

For every chromatin preparation it is essential to check the chromatin is sheared to fragments between 100-500 bp. Chromatin which is over- or under-sheared will reduce ChIP efficiency.

Quantitative analysis of chromatin is done with a spectrophotometer, fluorometer or microfluidics platform. Qualitative analysis requires an agarose gel or microfluidics platform. Chromatrap® recommends DNA quantification is done on a microfluidics platform which offers greatest accuracy and ideal compatibility with Chromatrap® buffer systems.

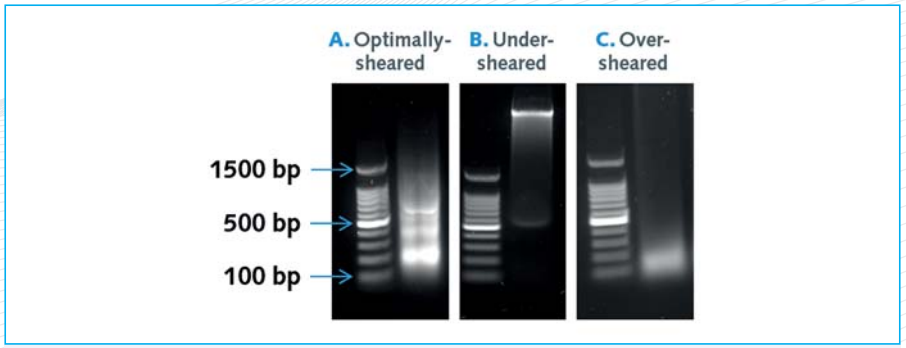
Over-shearing of chromatin will result in very small fragments which can reduce primer recognition and lower PCR efficiency. Over shearing by sonication can also increase the risk of damaging the protein epitopes. Under-shearing will produce larger fragments which will increase non-specific binding in 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. It is important to optimise sonication to ensure a successful ChIP result. Use sonication tubes which transfer ultrasonic waves more efficiently than standard plastic micro centrifuge tubes. 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 cycle number. When using Chromatrap® methodology, successful shearing of cell lines and primary cells to obtain 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 high power setting 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.



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)

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® 1 U per 5 µg 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.

If cell membranes have not been efficiently lysed, the enzyme has had only limited access to the chromatin. Check using a phase contrast microscope to ensure all the nuclei are released prior to proceeding to enzymatic digestion. Rectify this by incubating the samples for longer in the lysis buffer. After longer incubation, if cells are still resistant to lysis, convert to the sonication method.

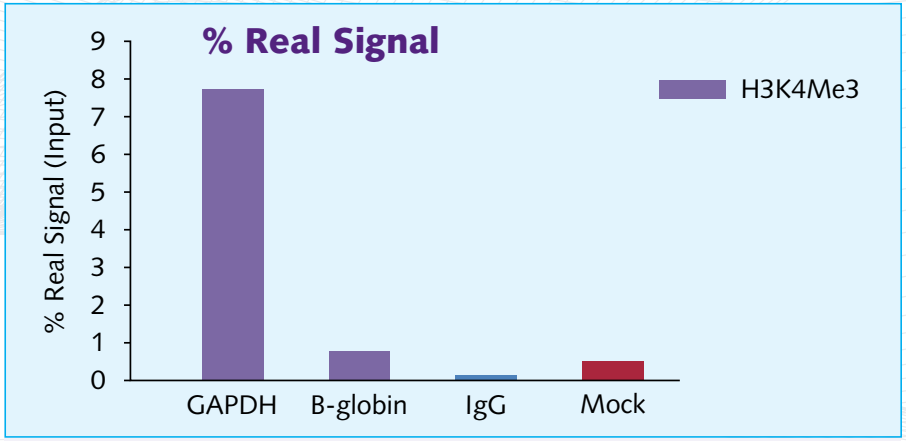
7 Always use a ChIP validated antibody

The use of high quality and specific ChIP validated antibodies is essential for the success of a ChIP assay, unvalidated antibodies do not always work well in ChIP. 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.

8 Always run a positive and negative antibody control

To indicate the efficiency of the immunoprecipitation and to ensure chromatin preparation is sufficient, positive and negative controls should be run alongside any test antibodies. Chromatrap® premium ChIP qPCR (Cat 500115/116) and Chromatrap® ChIP-seq (Cat 500189/190) supply a positive antibody control for a highly abundant histone mark and a negative antibody control IgG. Primers optimised for qPCR are also included. These recognise Glyceraldehyde 3-phosphate dehydrogenase (GAPDH), an ever present house-keeping 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, positive and negative gene targets are good controls to ensure antibody enrichment is selective.

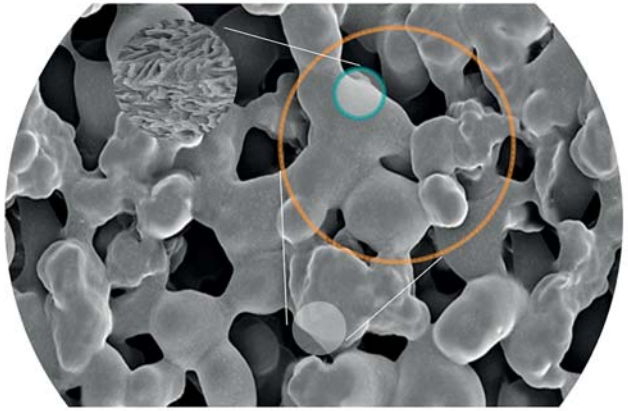


Graph illustrating high levels of enrichment (7.7% real signal) of H3k4Me3 onto a positive house-keeping gene, GAPDH, observed with Chromatrap®. Little to no enrichment is shown for H3k4Me3 on to the negative target gene B-globin (0.7% real signal). IgG and a no antibody (mock) control are also shown 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 Get the ratio of antibody to chromatin right

In a ChIP assay the antibody to chromatin ratio is important. An incorrect ratio 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 promotes molecular mixing and 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.

It is important to run an antibody dilution series before processing all samples in your ChIP assay to determine the optimum antibody: chromatin ratio. When using small concentrations of chromatin in the qPCR and sequencing kits, Chromatrap® recommend an optimal 2:1 antibody : chromatin ratio. When using higher concentrations (above 10 µg) for sequencing, Chromatrap® recommend using 5 µg antibody, allowing you to save on antibody usage.

10 Optimise lysis buffer

Lysis buffers usually contain a mild detergent which 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. 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 your ChIP assay. Chromatrap® kits have optimised volumes of lysis buffer to be used depending on starting cell number – see table below.

The fundamental requirement for optimal antibody binding is to ensure that the chromatin does not exceed more than 10% of the total slurry volume.
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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

Chromatrap

Worldwide Chromatrap® Technical Support Team
Tel: +44 (0) 7539 743216 support@chromatrap.com
Worldwide Sales and Customer Support Team
Tel: +44 (0) 1978 666222 sales@chromatrap.com
Clywedog Road South Wrexham Industrial Estate Wrexham LL13 9XS UK

www.chromatrap.com