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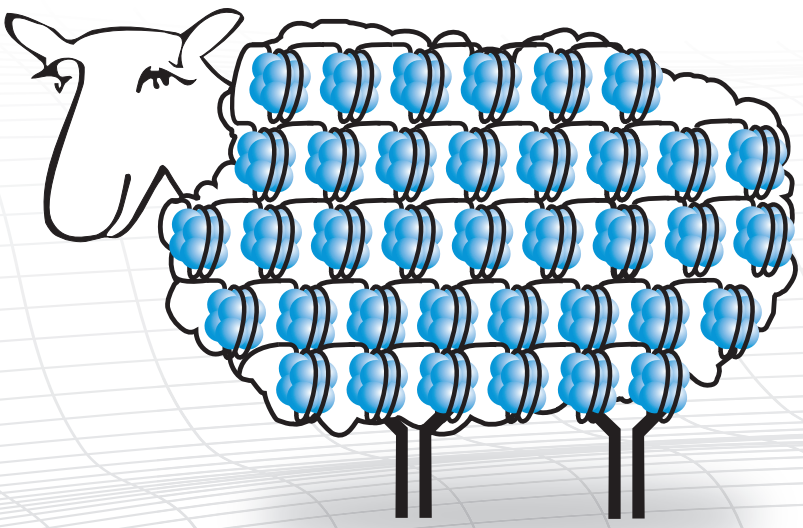
A Beginner's Guide to ChIP



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Preface

The purpose of this book is to demonstrate the powerful tool of **Ch**romatin **I**mmuno**p**recipitation (ChIP) and how it can be easily adapted or incorporated into your own research. Within these pages you will find an overview of ChIP, suggested protocols and troubleshooting tips as well as contact details for scientific support should a question come to mind.

Why ChIP?

ChIP is a tool that has unlocked the mysteries of chromatin and revolutionized our understanding of the science behind it. The ChIP technique can be used in any area of research to further elucidate gene function and regulation in their native state.

When to ChIP?

ChIP is a technique that may sound intimidating, but with the right tools can certainly be mastered. The principle of ChIP is simple: the selective enrichment of a chromatin fraction containing a specific antigen. Antibodies that recognize a protein or protein modification of interest can be used to determine the relative abundance of that antigen at one or more locations in the genome *in vivo*. ChIP is extremely versatile; it may be used to compare the enrichment of a protein/protein modification at different loci (Figure 1a), to map a protein/protein modification across a locus of interest (Figure 1b) or to quantify a protein/protein modification at an inducible gene over a time course. Basically, if you're looking to observe your protein of interest and its interactions with the genome in its 'natural' state ChIP is a great choice.

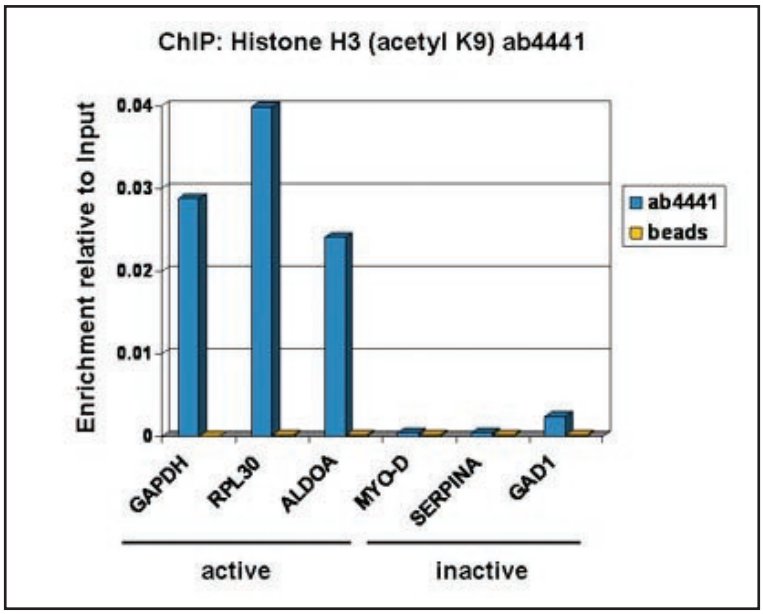
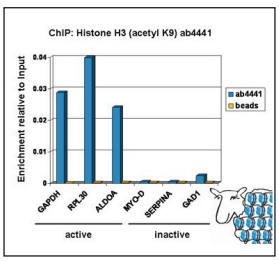


Figure 1a.
ChIP performed with an antibody specific for Histone H3 acetylated at K9 (ab4441). The immunoprecipitated DNA was analyzed with primers and probes for active (GAPDH, RPL30, ALDOA) and inactive (MYO-D, SERPINA, GAD1) loci by real-time PCR. The results show that Histone H3 is acetylated at K9 on actively transcribed loci.

Control antibodies for transcriptionally active genes



Tri-methylation of Histone H3 at lysine 4 and acetylation of Histone H3 at lysine 9 are hallmarks of transcriptionally active genes. Not only do antibodies to these histone modifications serve as useful reagents in checking the transcriptional activity at a locus of interest, they also can be used as good controls to ensure that the ChIP experiment is working.

Try:
Mouse monoclonal [mAbcam1012] to Histone H3 (tri methyl K4) – ChIP Grade ab1012
Rabbit polyclonal to Histone H3 (acetyl K9) – ChIP Grade ab4441

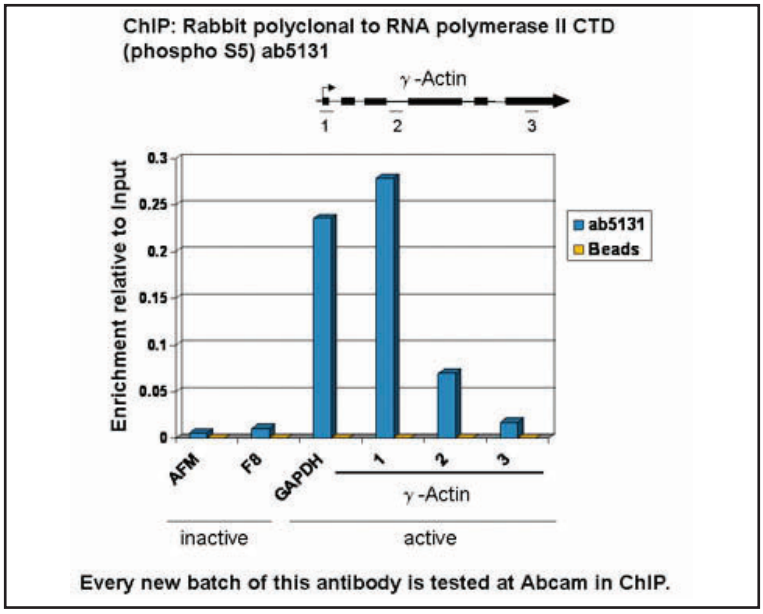
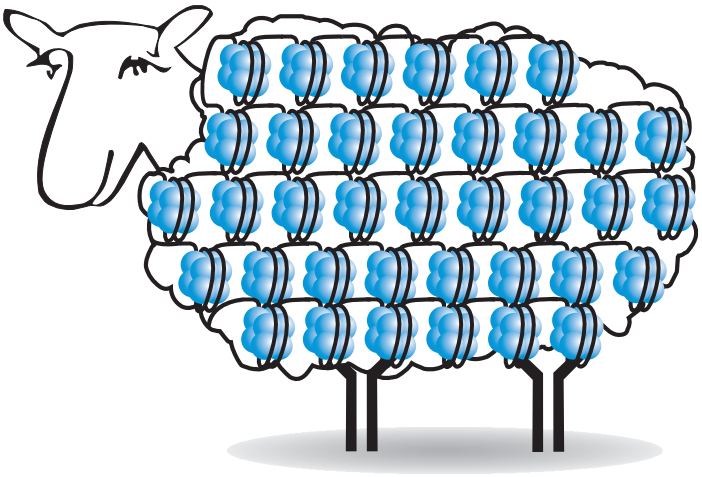


Figure 1b.
ChIP performed with an antibody specific for the RNA polymerase II CTD repeat phosphorylated at S5 (ab5131). The immunoprecipitated DNA was analyzed with primers and probes for the active GAPDH locus (promoter proximal), the inactive AFM and F8 loci and primers across the active γ-Actin gene. RNA polymerase II CTD phosphorylated at S5 is enriched close to the promoters of the active GAPDH and γ-Actin genes (1), the signal decreases within the γ-Actin gene (2) and gets very low close to the 3'end (3).



Introduction to Chromatin

Before we begin describing how to actually *do* ChIP, it helps to have some background on chromatin and where this technique originated.

Structure and function of chromatin
In eukaryotes DNA is found *in vivo* in complex with proteins and RNA. It is divided between heterochromatin (highly condensed) and euchromatin (less extended). The major components of chromatin are DNA and histone proteins, although many other chromosomal proteins have prominent roles too. The fundamental unit of chromatin is the nucleosome, which consists of 2 copies each of H2A, H2B, H3 and H4 histones, and approximately 147 bp of DNA wrapped almost two times around the octamer.

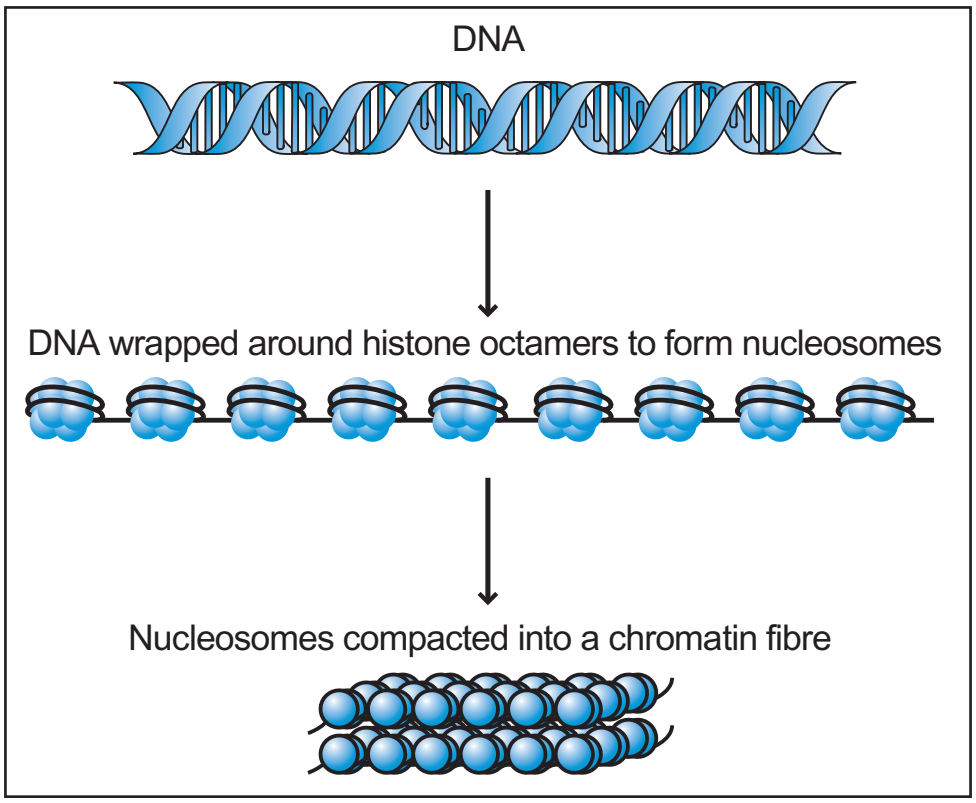


Figure 2.
DNA wraps around histone proteins to form nucleosomes; these in turn couple to become the chromatin fiber.

The function of chromatin is to package DNA to enable it to fit in the cell, strengthen DNA to assist with mitosis and meiosis, and serve as a mechanism to control gene expression, DNA repair, and DNA replication. Histone proteins play an important role in the regulation of these processes. A large number of residues found on the histones can be covalently modified with chemical groups by processes such as acetylation, methylation, phosphorylation, ubiquitylation, sumoylation, ADP ribosylation and deimination (Kouzarides *et al*, 2007). Furthermore, cis-trans proline isomerisation while not strictly a modification results in a conformational change (Nelson *et al*, 2006). These modifications function either by disrupting chromatin contacts or by affecting the recruitment of non-histone proteins to chromatin (Kouzarides *et al*, 2007). A number of enzymes have been identified which catalyze the addition of these modifications to histone proteins.

History
The technique now referred to as ChIP has been continually refined since the seminal publications in the 1980's by Gilmour and colleagues. They demonstrated an association of RNA polymerase II and topoisomerase I with active genes in Drosophila cells (Gilmour and Lis, 1985; Gilmour *et al*., 1986). The first account of an antibody against a histone modification being used in ChIP was in 1988 by Hebbes *et al*. An antibody recognizing N-acetyl-lysine was used to immunoprecipitate nucleosomes containing acetylated histones from 15-day-old chicken erythrocyte nuclei. The precipitated chromatin was then probed with β-globin and ovalbumin DNA sequences. Specific enrichment of the ß-globin locus, but not the ovalbumin gene, demonstrated a link between histone acetylation and an active transcriptional state *in vivo* (Hebbes *et al*., 1988).

What ChIP is right for me?

Native ChIP (N-ChIP) and cross-linking ChIP (X-ChIP)
There are two general procedures for carrying out ChIP experiments, native ChIP (N-ChIP) and cross-linking ChIP (X-ChIP). The choice between N-ChIP and X-ChIP is dependent on your experimental aims and the starting material used:

(i) N-ChIP: Native chromatin is used as the substrate, which means that proteins are not cross-linked to the DNA. Fragmentation of the chromatin is achieved by micrococcal nuclease digestion, resulting in a nucleosome based resolution. N-ChIP is restricted to proteins that are very tightly associated with chromatin, typically limiting this type of ChIP to histones and their modifications (O'Neill and Turner, 2003).

(ii) X-ChIP: Proteins are cross-linked to the DNA. Cross-linking is typically achieved using formaldehyde and chromatin is fragmented by sonication, creating random fragments. As the proteins are cross-linked to the DNA a broad range of chromatin associated factors can be analyzed using this technique.
Detailed ChIP protocols can be found in the Appendix (see page 16).

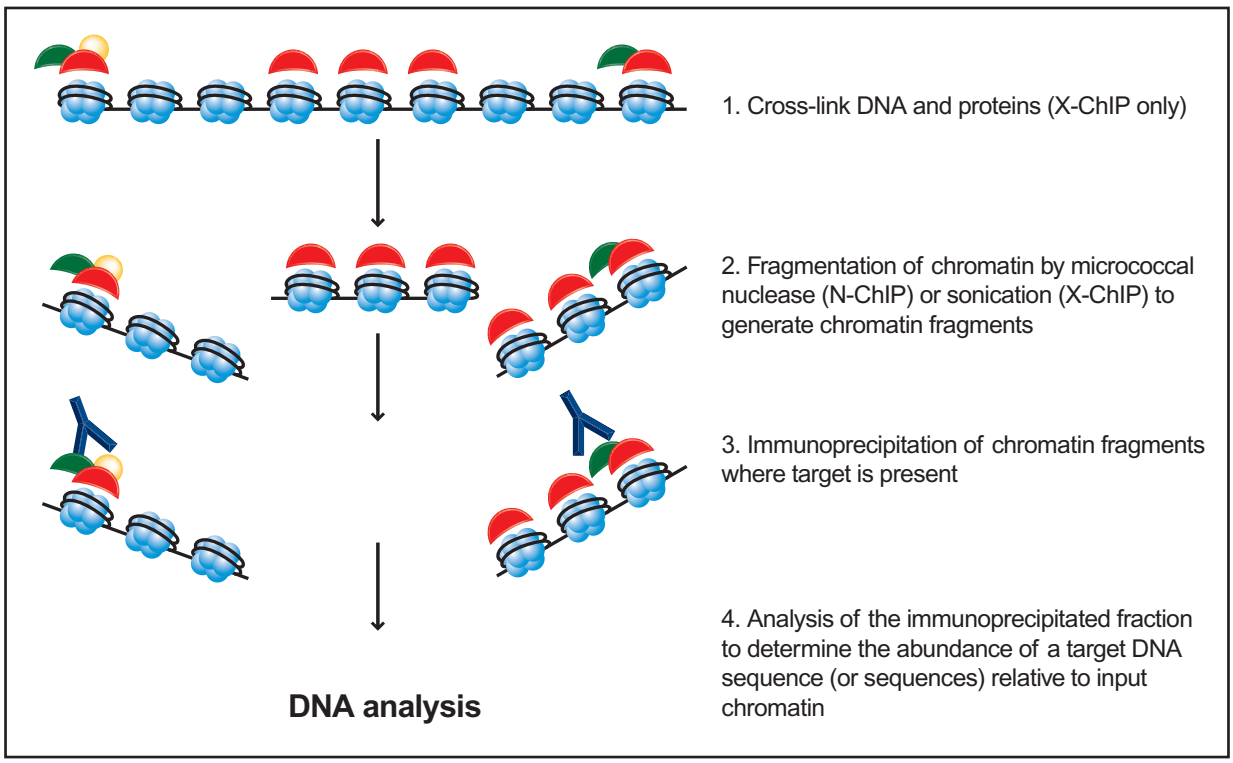


Figure 3.
Schematic overview of the N-ChIP and X-ChIP protocols.

What do I do with my DNA to get Data?

Analysis of the isolated DNA can be performed in a number of ways and allows one to analyze the enrichment of the target**.** The combination of ChIP with whole genome analyses such as microarray (ChIP-chip) and next generation sequencing (ChIP-seq) has permitted the mapping of a protein or a protein modification over the entire genome. Alternatively PCR analyses of a specific region may be performed.

DNA Quantification
Enrichment of a target is not solely dependent on the quantity of antigen associated with it. Immunoprecipitation will be affected by the accessibility of that antigen in that particular chromatin environment, the affinity of the antibody and the precise conditions of the immunoprecipitation. For this reason, the level of enrichment is always expressed as a ratio of bound (or precipitated) sequence over input. This also means that absolute levels of different antigens present at the same sequence cannot be directly compared.

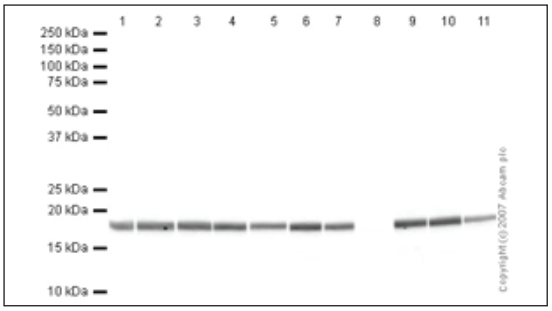
Variations of ChIP

ChIP (PCR)
The isolated DNA can be quantified by Real Time PCR (RT-PCR). RT-PCR typically uses TaqMan® or Sybr Green® technologies to amplify and simultaneously quantify a targeted DNA molecule by measuring changes in fluorescence. This allows the analysis of a specific region in multiple samples and can be quicker and more cost effective when compared to whole genome sequencing.

ChIP-chip
Microarray technology allows the generation of high resolution genome-wide maps of protein/protein modifications. DNA purified from the immunoprecipitated chromatin and input are labeled with fluorescent dyes using ligation mediated PCR. The fluorescently-labeled DNA is applied to the microarray and after subsequent image analysis, the enrichment of the regulatory protein relative to the input is recorded at each genomic locus.

ChIP-seq
Direct sequencing of the DNA isolated, generates genome wide profiles. ChIP-seq combines ChIP and direct sequencing technology for genome-wide analysis of antigen distribution. Immunoprecipitated DNA is sequenced and mapped to the genome (Barski *et al*., 2007). With newly developed sequencing technology researchers are able to sequence large amounts of DNA in a matter of days.

Control antibody for heterochromatic genes



Western blot - Histone H3 (tri methyl K9) antibody -ChIP Grade (ab8898)

All lanes : Histone H3 (tri methyl K9) antibody - ChIP Grade (ab8898) at 1 µg/ml

Lane 1 : Calf Thymus Histone Preparation Nuclear Lysate
Lane 2 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - unmodified (ab7228) at 0.5 µg/ml
Lane 3 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - mono methyl K4 (ab1340) at 0.5 µg/ml
Lane 4 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - di methyl K4 (ab7768) at 0.5 µg/ml
Lane 5 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - tri methyl K4 (ab1342) at 0.5 µg/ml
Lane 6 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - mono methyl K9 (ab1771) at 0.5 µg/ml
Lane 7 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - di methyl K9 (ab1772) at 0.5 µg/ml
Lane 8 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - tri methyl K9 (ab1773) at 0.5 µg/ml
Lane 9 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - mono methyl K27 (ab1780) at 0.5 µg/ml
Lane 10 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - di methyl K27 (ab1781) at 0.5 µg/ml
Lane 11 : Calf Thymus Histone Preparation Nuclear Lysate with Histone H3 peptide - tri methyl K27 (ab1782) at 0.5 µg/ml

Lysates/proteins at 0.5 µg per lane.

Tri-methylation of Histone H3 at lysine 9 is associated with transcriptional silencing. Histone H3 that is tri methylated at lysine 9 is recognized and bound by the chromo domain of Heterochromatin Protein 1, an abundant component of heterochromatin that is associated with silencing at both euchromatic and heterochromatic loci. Antibodies to this modification can be used to study the transcriptional silencing of a gene of interest, and can also be used as a good control to check whether the ChIP experiment is working (by looking at known inactive loci).

Try:
Rabbit polyclonal to Histone H3 (tri methyl K9) -ChIP Grade ab8898

ChIP: Getting Started

This section describes in detail the process by which you can learn how to perform a successful X-ChIP, from choosing an antibody to validating results.

1. How to choose an antibody for ChIP?

In the ChIP assay, the antibody is used to capture a specific DNA - associated protein. The success of this protocol is entirely dependent on the quality of the antibody used. There are many ChIP-tested antibodies available. Below we describe a few tips on how to select an antibody for ChIP if you can't find a ChIP-tested antibody to your protein modification of interest.

1.1 Antibody should be specific

The antibody should be specific and efficient in the immunoprecipitation of a specific protein. Ideally, antibodies for ChIP should be affinity-purified. Antibodies raised against histone modifications should be tested in peptide inhibition Western blot or ELISA for specificity.

Note: Even characterization in a standard IP assay will not tell you whether or not an antibody will function in X-ChIP, as the effects of cross-linking can be dramatic to the extent that different epitopes may be generated and specific epitopes may be lost.

1.2 Immunoprecipitation / immunohistochemistry / immunocytochemistry are good indicators of success in ChIP

Successful use of an antibody in immunoprecipitation (IP), immunohistochemistry (IHC) or immunocytochemistry (ICC) are good indicators of success in ChIP. In all three techniques, the antibody recognizes an epitope in its native conformation and within the context of any complexes that the epitope may be found in. This differs from the use of antibodies in western blotting, where the epitope that is recognized by the antibody is denatured. Therefore, if an antibody works in IP, IHC or ICC, there is a good chance that the epitope will also be recognized in ChIP (See figure 4).

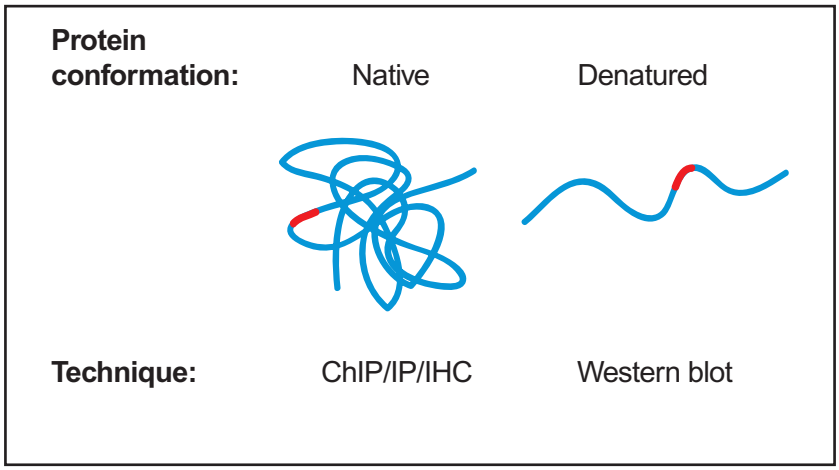


Figure 4.
Schematic representation of protein conformation (native-denatured) recognized by different techniques.

1.3 Polyclonal or monoclonal?

As the success of an antibody in ChIP is dependent on which epitope(s) it recognizes (see Section 1.2), there is a higher probability that a polyclonal antibody will work in ChIP when compared to a monoclonal antibody. A polyclonal antibody is derived from many different B cells similar to the mixture of antibodies to a given antigen found in sera. Therefore, a polyclonal antibody population contains antibodies that recognize a number of different epitopes on the same protein (Figure 5). In contrast, a monoclonal antibody is derived from a single clone and only recognizes a single epitope. Monoclonal antibodies do have an advantage – they show much less batch-to-batch variation than polyclonal antibodies as making them again is independent of generating an immune response.

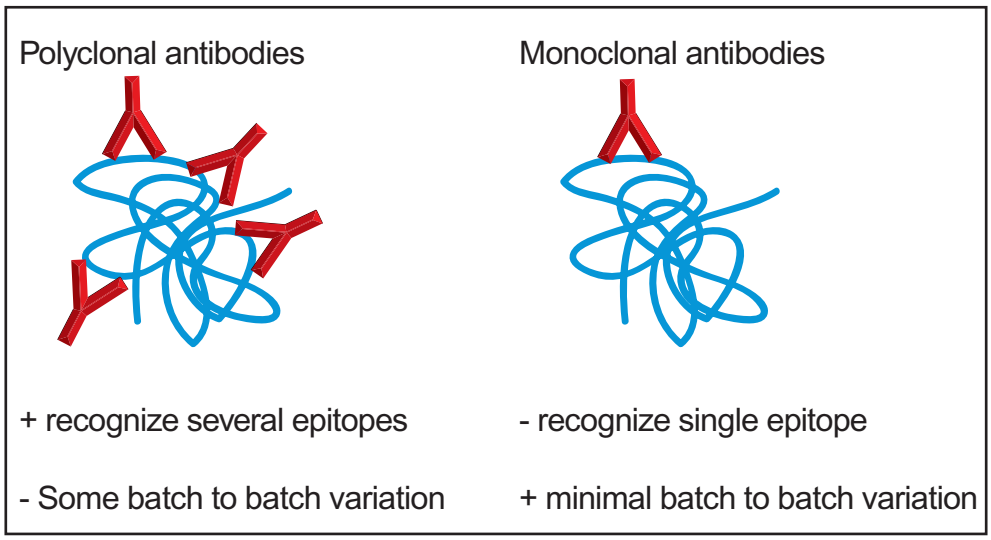


Figure 5.
Pros and cons for using polyclonal vs monoclonal antibodies for ChIP.

2. How to validate an antibody for ChIP

There are many ChIP tested antibodies available but if the antibody you have chosen is not ChIP grade – what should you do?

2.1 Perform standard ChIP experiment

Once you have selected an antibody for ChIP as decribed in Section 1, you can perform a standard ChIP experiment. You will need a positive and a negative control locus, *i.e.* a locus where you know your protein/modification of interest is present and one where you know it is absent. It is also worth including a positive control antibody to test if all the steps of the technique work *e.g.* antibodies directed against Histone H3 (tri methyl K4) or H3 (acetyl K9), both of which are present at the promoters of actively transcribed genes. As the pass rate for antibodies in ChIP is low, it is worth testing several antibodies in parallel.

2.2 Determine the optimal antibody concentration

Determining the optimal antibody concentration can significantly improve the signal to background ratio. Titrate the amount of antibody required by performing a ChIP experiment using a range of antibody concentrations. The amount of antibody required per ChIP typically ranges from 1–10 µg of antibody for every 25 µg of chromatin.

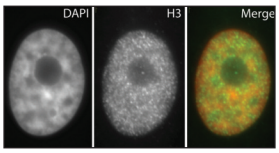
2.3 Test different washing conditions

Some antibodies have a low affinity for the target; it is therefore worth testing different washing conditions. The stringency of the last wash can be varied from 250-500 mM salt (usually NaCl or LiCl).

2.4 Western blot can be used to test whether the target has been immunoprecipitated

To test if the antibody pulls down the target of interest you can perform ChIP up to the final wash after the IP and then boil the beads in loading buffer for 10 min. You can then confirm the presence of your protein in the IP by Western blot.

Control antibody for ChIP experiments



As Histone H3 is abundant and found throughout the genome, antibodies to this protein can be used as general controls for ChIP experiments.

Try:
Rabbit polyclonal to Histone H3 – ChIP Grade ab1791

ChIP - Histone H3 antibody - ChIP Grade (ab1791)
Hela cells cultured on coverslips were fixed with 4% paraformaldehyde and then stained with ab1791 (green) at a working dilution of 1/500. The DNA stained with DAPI is shown in red. (100x magnification).
Image courtesy of Kirk McManus, University of Manitoba

Note: Even if the antibody is able to immunoprecipitate the protein of interest in formaldehyde fixed chromatin this does not mean that the ChIP experiment has worked, as it is possible that your protein of interest is not cross-linked to the DNA. Formaldehyde is a very good DNA – protein cross-linker but due to its small size (2 Å) it is not a very efficient protein – protein cross-linker. It is therefore often difficult to ChIP proteins that do not bind directly to the DNA.

3. Controls

Several controls should be included in each ChIP experiment.
We have listed the most important controls below.

3.1 Negative control
As a negative control, use `beads only` or beads with an isotype matched control immunoglobulin (Ig), this will give you the background of the assay. For example, ab5408, a RNA polymerase II CTD repeat YSPTSPS-ChIP grade antibody, is a **mouse** monoclonal **IgG1**. A suitable isotype control would be ab18443, **mouse IgG1**, Kappa monoclonal isotype control.

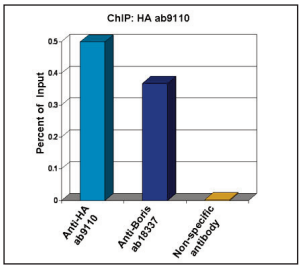
3.2 Positive and negative control loci
Primers for a locus where you know your protein or modification of interest is present (positive control locus) and one where it is absent (negative control locus) should both be tested in RT-PCR. This will tell you whether the observed enrichment is specific. It is important to include these controls as some antibodies result in non-specific enrichment.

3.3 Non-template control
A non-template control should always be included in the PCR reaction. This will help you spot any contamination.

3.4 Positive control antibody
Antibodies specific for Histone H3 (tri methyl K4) (ab8580) and H3 (acetyl K9) (ab4441), both enriched on actively transcribed regions, are good controls to ensure that each step of your experiment is working.

Note: Remember that these antibodies are not positive controls for the success of the ChIP experiment per se - this depends on the locus you are studying. For example, if there is no Histone H3 tri methylated at K4 at the particular locus of interest, even the best ChIP antibody in the world would not be able to achieve any immunoprecipitation.

ChIP using tagged proteins



It may be useful to perform ChIP using an antibody to the tag on a protein of interest (for example, if the protein is expressed at very low levels or if existing antibodies to the protein do not ChIP). Care must be taken to include appropriate controls if the tag is derived from a protein that may also be endogenous to the cells (e.g., a myc tag).

Try:
Rabbit polyclonal to HA tag ab9110

Xenopus laevis oocytes were injected with mRNA for HA-tagged human BORIS. Chromatin was prepared according to the Abcam X-ChIP protocol. Oocytes were fixed with formaldehyde for 10min. The ChIP was performed with 25µg of chromatin, 20µl of Protein A/G sepharose beads, and 3µg of ab9110 (anti-HA, light blue) or 3µg of ab18337 (anti-Boris, dark blue). A non-specific antibody was used as a control (yellow). The immunoprecipitated DNA was quantified by real time PCR (Taqman approach).

4. Optimizing ChIP experiments

4.1 Cross-linking
The aim of cross-linking is to fix the antigen of interest to its chromatin binding site.

How do I cross-link?
Use formaldehyde, as the links it forms are reversible and the DNA is easily purified. UV cross-linking is not appropriate as it is irreversible.

Is it possible to cross-link too much?
Yes. Cross-linking is a time-critical procedure. Excessive cross-linking can lead to a decrease in the amount of protein bound to the DNA, reduction in the availability of epitopes/changes in epitopes for antibody binding and, in turn, reductions in the material bound/antigen availability in your sample.

Tip: Always perform a time course experiment to optimize cross-linking conditions (2-30 min).

4.2 Sonication
Sonication of the chromatin is a key step in the ChIP protocol as it renders the chromatin soluble and dictates the resolution of the assay. The extent to which one can fine-map the location of a specific protein in the genome depends on the extent of DNA fragmentation. The ideal DNA fragment size after sonication is 200 – 1000 bp.

Sonication conditions should be optimized for different cell lines and experimental settings; a typical time course experiment is shown in Figure 6. The sonication efficiency depends on the cell line, the cell density, and the extent of cross-linking. For reproducible results these parameters should be kept constant.

Note: Avoid foaming as this results in a decrease of energy transfer within the solution and will decrease the sonication efficiency.

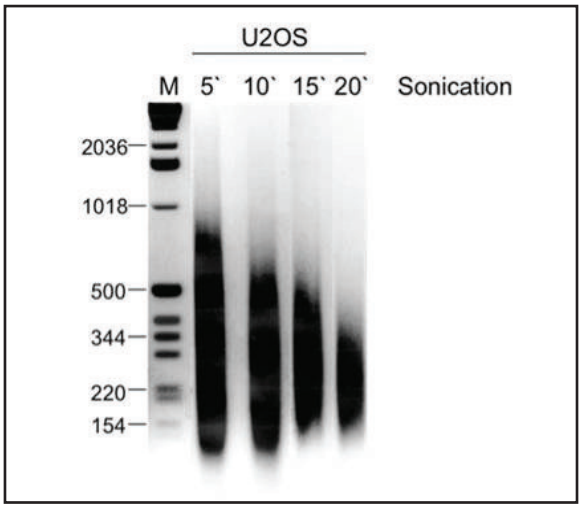


Figure 6.
Example of sonication time course experiment. U2OS cells were sonicated for 5, 10, 15 and 20 min. The cross-links were reversed and the purified DNA was resolved on a 1.5% agarose gel. The fragment size decreases during the time course. The optimal fragment size is observed at 15 min.

4.3 Immunoprecipitation and Washes
Antibodies are used in ChIP to capture the protein of interest and the interacting DNA.

How much antibody should I add?
The optimal antibody concentration should be calibrated for each antibody as this can improve the signal to noise ratio of your experiment. Approximately 1-10 µg of antibody per 25 µg of chromatin are good starting conditions.

How should I wash the beads?
Different antibodies have different affinities for their target. Low affinity antibodies may not be able to bind the protein of interest in very stringent buffers. It is therefore worth trying different salt concentrations in the final wash buffer (*i.e.* 250 and 500 mM salt). Be aware though that you want to keep the stringency as high as possible as reducing it can increase the background and affect the signal to noise ratio.

Note: Do not centrifuge the sepharose beads at high rpm (do not exceed 6,000 rpm) as this will compact the beads and damage them.

4.4 Primer design for proper DNA analysis

How should I design and test the PCR primers?
Design primers that have a Tm of 58-60°C and a GC content of 30-80%. Avoid runs of an identical nucleotide, especially guanine. The five nucleotides at the end should have not more than two G and/or C bases. Always run the primers through BLAST to make sure they are unique and test the primers on genomic/input DNA.
Certain areas of the genome will purify better than others, and sonication does not always create breaks at random. As a result, it is important to generate PCR primers to several regions in the starting material (input), as well as the purified/ChIP'ed material as controls for spurious results. Generate input and take a sample for simple PCR control regions in parallel with ChIP.
It is important to perform a primer matrix with the forward and reverse primers to determine the optimal primer concentration for RT-PCR. The optimal concentration for Sybr green experiments is the minimum primer concentration that gives the lowest threshold cycle (Ct) and maximum ΔRn while minimizing non-specific amplification. A typical primer matrix is shown in Table 1.
Data should always be normalized for the amount of input in order to remove errors introduced due to uneven sample quantities. To normalize your data, take the final amplicon value and divide it by the amplicon value of input material.

Tip: Measuring the amounts (and quality) of starting material is the key to interpreting your results effectively.

Table 1. Primer Design Matrix

	Forward primer (nM)			
Reverse primer (nM)		50	300	900
	50	50/50	300/50	900/50
	300	50/300	300/300	900/300
	900	50/900	300/900	900/900

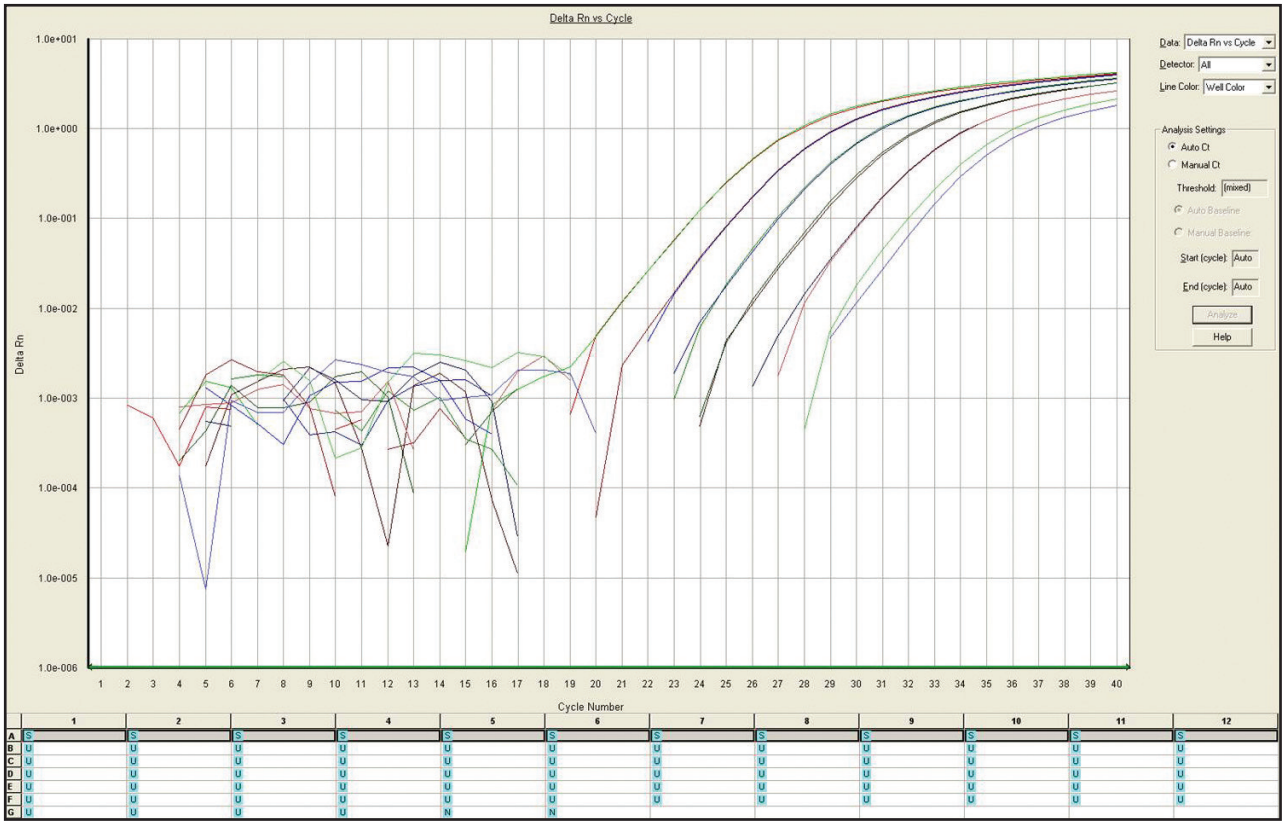
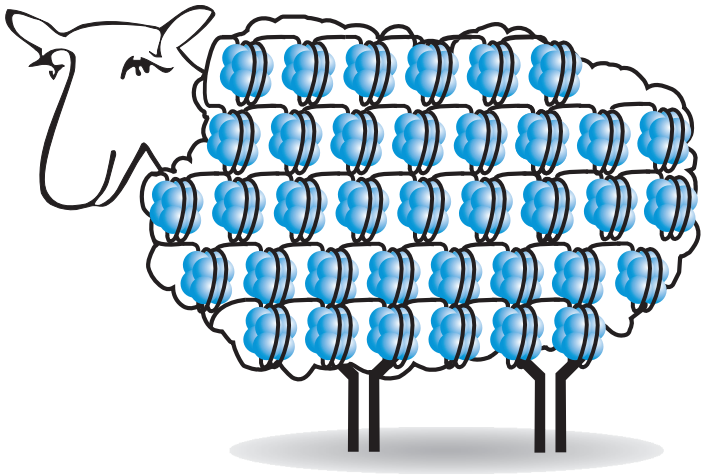


Figure 7.
The plot displays Real Time PCR amplification data in a plot of Delta Rn (normalized reporter signal minus the baseline signal) (y axis) vs. Cycle Number (x axis). For each sample, the threshold cycle (Ct) can be calculated as this represents the PCR cycle in which an increase in reporter fluorescence above the baseline signal is detected.



5. Troubleshooting

5.1. High background in non-specific antibody controls

Non-specific binding to Protein A or G beads
Include a pre-clearing step, whereby the lysed sample is mixed with beads alone for 1 hr that are removed prior to adding the antibody.

The ChIP buffers may be contaminated
Prepare fresh lysis and wash solutions.

Protein A or G beads give high background
Some Protein A or G beads can give high background levels. Find a suitable supplier that provides the cleanest results with low background in the non-specific control.

5.2. Low resolution with high background across large regions

DNA fragment size may be too large
Perform a time course experiment to optimize sonication conditions. We would suggest a DNA fragment size of 200-1000 bp.

5.3. Low signal

The chromatin size may be too small
Do not sonicate chromatin to a fragment size of less than 200 bp. Sonication to smaller fragment size can result in breaks within the amplicon.

Cells are not effectively lysed
We would suggest using IP Dilution buffer to lyse cells. The composition can be found in our X-ChIP protocol.

The cells may have been cross-linked for too long
Excessive cross-linking can reduce the availability of epitopes and thus reduce antibody binding. Cross-link with formaldehyde for 10-15 min and wash well with PBS. Cells may need to be treated with glycine to quench the formaldehyde.

Not enough starting material
We would suggest using 25 µg of chromatin per immunoprecipitation.

Not enough antibody included in the immunoprecipitation
We would suggest using between 1-10 µg of antibody in the first instance.

Specific antibody binding is being eliminated
Do not use higher than 500 mM salt (usually NaCl or LiCl) in the wash buffers as this may be too stringent and remove specific antibody binding. The salt concentration in the last wash can be reduced to 250 mM.

No antibody enrichment at region of interest
The target may not be present at the region analyzed, therefore design primers for a different location. Include a positive control antibody to confirm the procedure is working well *e.g.* H3K4me3 (ab8580)/ H3k9ac (ab4441) antibody at active/inactive promoters.

The wrong antibody affinity beads were used
Protein A and G are bacterial proteins that bind various classes of immunoglobulins with varying affinities. Use an affinity matrix that will bind your antibody of interest. We would suggest using a mix of protein A and protein G that have been coupled to sepharose.

The antibody may not be suitable for ChIP
The epitope may be masked in X-ChIP. Use a different antibody if one is available.

5.4. Problems with PCR amplification on immunoprecipitated DNA

High signal in all samples after PCR, including no template control
Contamination in real-time PCR solutions, we suggest preparing new solutions from stocks.

No DNA amplification in samples
Include standard/input DNA to confirm the primers are working well.

References

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Hebbes TR, Thorne AW, Crane-Robinson C. A direct link between core histone acetylation and transcriptionally active chromatin. EMBO J. 1988 May;7(5):1395-402.

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Nelson CJ, Santos-Rosa H, Kouzarides T. Proline isomerization of histone H3 regulates lysine methylation and gene expression. Cell. 2006 Sep 8;126(5):905-16.

O'Neill LP, Turner BM. Immunoprecipitation of native chromatin: NChIP. Methods. 2003 Sep;31(1):76-82.

Useful reagents for ChIP

Try:
Herring sperm ssDNA ab46666
Proteinase K (Active) ab51501
Ribonuclease A (Active) ab52579
ChIP Kit (ab500)

Protocols Appendix

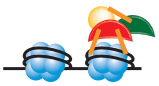
Cross-linking Chromatin Immunoprecipitation (X-ChIP) Protocol Overview

Map proteins/histone modifications to genomic loci

Chromatin and associated proteins.

Cross-link

Start with 2 X 150 cm^2 dishes of confluent cells
(1 X 10^7 - 5 X 10^7 cells per dish)
Add formaldehyde to a final conc of 0.75%
Incubate 2 - 30 min RT
Add glycine to a final conc of 125 mM
Shake gently for 5 min



Cross-linking fixes proteins to DNA.

Cell collection

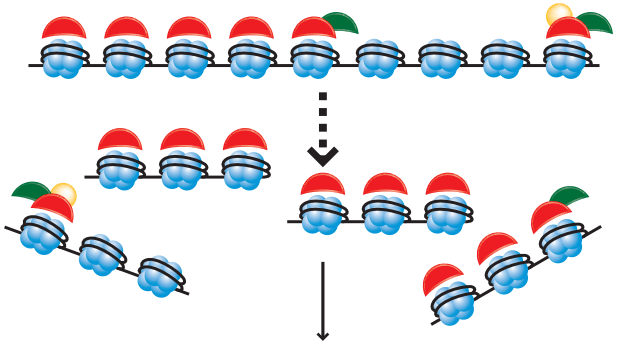
Wash cells with ice cold PBS
Scrape and collect cells into 5 ml ice cold PBS and transfer to a new tube
Wash dishes with 3 ml PBS to ensure all cells are collected

Cell lysis

Centrifuge 5 min 1,000 g and remove supernatant
Add FA lysis buffer to cell pellet (750 µl per 1 X 10^7 cells)

Sonication

Sonicate to give a fragment size of 200 - 1000 bp



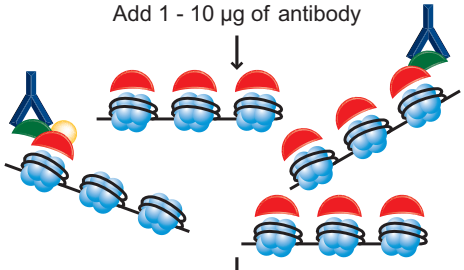
Sonication generates sheared, soluble chromatin. Optimize by performing a time course. Purify DNA, and analyse fragment size on 1.5 % agarose gel.

Centrifuge 30 sec 4˚C 8,000 g to pellet cell debris
Transfer supernatant containing chromatin to a new tube

Remove 50 µl (INPUT) and purify DNA to calculate the DNA concentration

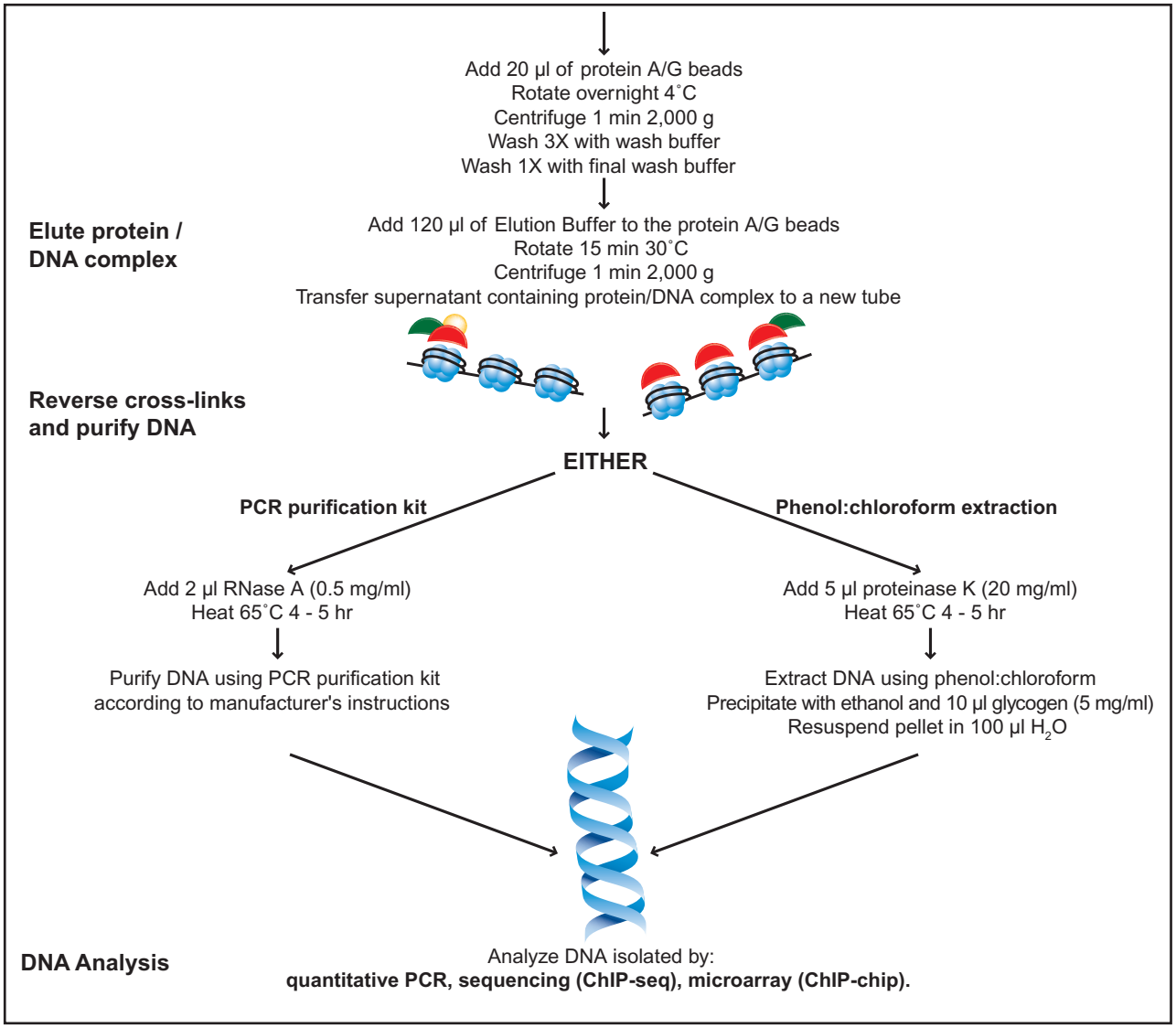
Immunoprecipitation

Use an amount of chromatin equivalent to approximately 25 µg of DNA per IP
Dilute 1:10 with IP Dilution buffer
Add 1 - 10 µg of antibody

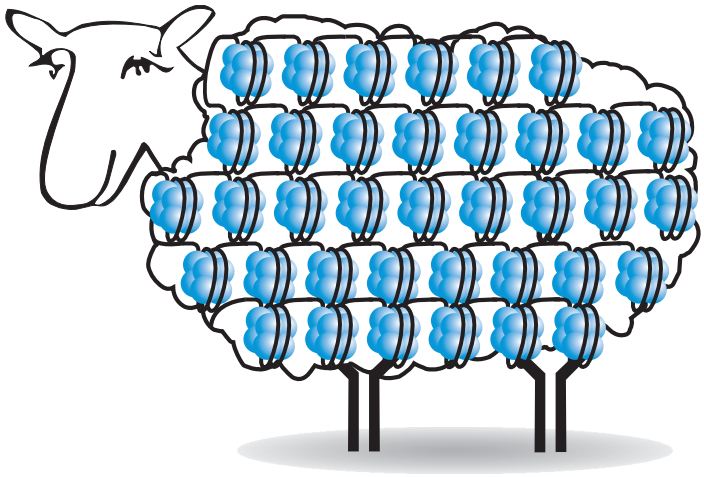


Antibody binds to target and associated DNA is isolated. DNA fragments not associated are removed during washes.

(Continued Overleaf)



This protocol provides specific details of how a cross-linking ChIP (X-ChIP) experiment can be performed.



1. Cross-linking and Cell Harvesting

Formaldehyde is used to cross-link the proteins to the DNA. Cross-linking is a time dependent procedure and optimization will be required. We would suggest cross-linking the samples for 2-30 min. Excessive cross-linking reduces antigen accessibility and sonication efficiency. Epitopes may also be masked. Glycine is added to quench the formaldehyde and terminate the cross-linking reaction.

1.1. Start with 2 confluent 150 cm^2 dishes ($1x10^7$- $5x10^7$ cells per dish). Cross-link proteins to DNA by adding formaldehyde drop-wise directly to the media to a final concentration of 0.75% v/v and rotate gently at room temperature (RT) for 10 min.

1.2. Add glycine to a final concentration of 125 mM to the media and incubate with shaking for 5 min at RT.

1.3. Rinse cells 2 X with 10 ml cold PBS.

1.4. Scrape cells into 5 ml cold PBS and transfer into 50 ml tube.

1.5. Add 3 ml PBS to dishes and transfer the remainder of the cells to the 50 ml tube.

1.6. Centrifuge for 5 min, 1,000 g.

1.7. Carefully aspirate off supernatant and resuspend pellet in ChIP Lysis Buffer (750 µl per $1x10^7$ cells).

When using suspension cells, start with $1x10^7$- $5x10^7$ cells and treat with both 0.75% v/v formaldehyde and glycine as described above (Section 1). Pellet cells by centrifugation (5 min, 1,000 g). Wash 3X with cold PBS and resuspend pellet in ChIP Lysis Buffer (750 µl per $1x10^7$ cells). Proceed to Step 2.1.

Sonication

When using tissue (Yeast see page 19 or plant see page 21) as a starting material, generate a unicellular suspension and start the protocol here at the sonication stage.

1.8. Sonicate lysate to shear DNA to an average fragment size of 200-1000 bp. This will need optimizing as different cell lines require different sonication times.

The cross-linked lysate should be sonicated over a time course to identify optimal conditions.Samples should be removed over the time course and DNA isolated. The fragment size should be analyzed on a 1.5 % w/v agarose gel.

1.9. After sonication, pellet cell debris by centrifugation for 30 sec, 4°C, 8,000 g. Transfer supernatant to a new tube. This chromatin preparation will be used for the immunoprecipitation (IP) in Step 4.

1.10. Remove 50 µl of each sonicated sample. This sample is the INPUT and is used to quantify the DNA concentration (see Step 3) and as a control in the PCR step.

The sonicated chromatin can be snap frozen in liquid nitrogen and stored at -80°C for up to 2 months. Avoid multiple cycles of freeze thaw.

Determination of DNA concentration

1.11. The INPUT samples are used to calculate the DNA concentration for subsequent IPs. Purify the DNA using either a PCR purification kit (add 70 µl of Elution Buffer and proceed to Step 3.2a) or phenol:chloroform (add 350 µl of Elution Buffer and proceed to Step 3.2b).

1.12. Add 2 µl RNase A (0.5 mg/ml). Heat with shaking at 65°C for 4-5 hr (or overnight) to reverse the cross-links. Purify the DNA using a PCR purification kit according to the manufacturer's instructions. The samples can be frozen and stored at -20°C.

Samples are treated with RNase A as high levels of RNA will interfere with DNA purification when using the PCR purification kit. Yields can be severely reduced as the columns become saturated.

1.13. Add 5 µl proteinase K (20 mg/ml). Heat with shaking at 65°C for 4-5 hr (or overnight) to reverse the cross-links. Extract the DNA with phenol:chloroform followed by ethanol precipitation in the presence of 10 µl glycogen (5 mg/ml). Resuspend in 100 µl water. The samples can be frozen and stored at -20 °C.

Samples are treated with proteinase K, which cleaves peptide bonds adjacent to the carboxylic group of aliphatic and aromatic amino acids. Cross-links between proteins and DNA are disrupted to aid DNA purification.

1.14. To determine the DNA concentration, transfer 5 µl of the purified DNA into a tube containing 995 µl TE to give a 200 fold dilution and measure the OD_{260}. Calculate the DNA concentration of the chromatin preparation in µg/ml.

Immunoprecipitation

1.15. Use the chromatin preparation from Step 2.2. Chromatin containing approximately 25 µg of DNA per IP is recommended. Dilute each sample 1:10 with IP Dilution Buffer. You will need one sample for the specific antibody and one sample for the control (beads only).

1.16. Add primary antibody to all samples except the control. The amount of antibody to be added should be determined empirically. 1-10 µg of antibody per 25 µg of DNA often works well.

1.17. Add 20 µl of protein A/G beads (pre-adsorbed with sonicated single stranded herring sperm DNA and BSA, see step 4.3a) to all samples and IP overnight with rotation at 4°C.

1.18. Preparation of protein A/G beads with single-stranded herring sperm DNA. If using both Protein A and Protein G beads, mix an equal volume of Protein A and Protein G beads and wash 3 X in IP Dilution Buffer. Aspirate IP Dilution Buffer and add single stranded herring sperm DNA to a final concentration of 75 ng/µl beads and BSA to a final concentration of 0.1 µg/µl beads. Add IP Dilution Buffer to twice the bead volume and incubate for 30 min with rotation at RT. Wash once with IP Dilution Buffer and add IP Dilution buffer to twice the bead volume.

1.19. Centrifuge the protein A/G beads for 1 min, 2,000 g and remove the supernatant.

1.20. Wash beads 3 X with 1 ml Wash Buffer. Centrifuge for 1 min, 2,000 g.

1.21. Wash beads 1 X with 1 ml Final Wash Buffer. Centrifuge 1 min, 2,000 g.

If high background is observed, additional washes may be needed. Alternatively, the sonicated chromatin may also be pre-cleared by incubating with the Protein A/G beads for 1 hr prior to Step 4.2. Any non-specific binding to the beads will be removed during this additional step. Transfer the supernatant (sonicated chromatin) to a new tube and incubate with the antibody and beads as described in Step 4.2 onwards.

Elution and reverse cross-links

1.22. Elute DNA by adding 120 µl of Elution Buffer to the protein A/G beads and rotate for 15 min, 30 °C.

1.23. Centrifuge for 1 min 2,000 g, and transfer the supernatant into a fresh tube. The samples can be stored at -20 °C.

1.24. The DNA can be purified using a PCR purification kit (proceed with Step 3.2a) or phenol:chloroform (add 280 µl of Elution Buffer and proceed with Step 3.2b).

1.25. DNA levels are quantitatively measured by real time PCR. Primers and probes are often designed using software provided with the real time PCR apparatus. Alternatively, online design tools may be used.

See Buffers (page 25).

2. ChIP using yeast samples

This protocol describes how chromatin for ChIP is prepared from Saccharomyces cerevisiae yeast cells.
This section was adapted from a protocol kindly provided by Dr. Jessica Downs, University of Sussex.

2.1. Chromatin cross-linking and cell harvesting.

2.2. Grow strains overnight in appropriate selective media.

2.3. Use the overnight culture to inoculate 100 ml fresh media to an OD600 of 0.2. Grow cells 4 h (or into mid-log phase, dependent on strain growth rate), 30°C, shaking.

2.4. Cross-link proteins to DNA by adding formaldehyde drop wise directly to the media to a final concentration of 1% v/v and rotate gently at room temperature (RT) for 15 min.

Cross-linking is a time dependent procedure and optimization will be required. We would suggest cross-linking the samples for up to 2h for yeast samples. However, excessive cross-linking may reduce antigen accessibility, sonication efficiency and epitopes may also become masked.

2.5. Add glycine to a final concentration of 125 mM to the media and incubate with shaking for 5 min at RT.

Glycine is added to quench the formaldehyde and terminate the cross-linking reaction.

2.6. Pellet cells by centrifugation for 5 min at 1,000 g. Wash the cells in 1x PBS (50 ml).

Store pellet at -20°C if not proceeding with sonication.

2.7. Centrifuge again for 5 min at 1,000 g and resuspend cells in 400 µl cold Lysis buffer in screw cap tubes. Add equal amount of glass beads and lyse in Mini-bead beater 2 x 45 sec at 4°C.

Optimization of lysis with glass beads will be required. From here on the steps need to be at 4°C.

2.8. Transfer supernatant to a new tube. Centrifuge for 15 min, max speed, 4°C. Move supernatant to new tube.

2.9. Sonication

2.10. Sonicate lysate to get the desired average fragment size 200- 1000 bp (average 500 bp).

The cross-linked lysate should be sonicated over a time-course (e.g. 5-30 min) to identify optimal conditions as sonication times vary among strains. Samples should be removed over the time-course and DNA isolated as described in Section 3/DNA Determination of DNA concentration. The fragment size should be analyzed on a 1.5% agarose gel as demonstrated in Figure 6.

2.11. Spin lysate for 10 min, 4°C, maximum speed (microfuge). Transfer supernatant with sheared chromatin to a new tube. Remove 10 µl for the input sample (add to 90 µl Lysis buffer and store on ice while finishing), and remove 5 µl to analyze the DNA fragment size range.

The sonicated chromatin can be snap frozen in liquid nitrogen and stored at -80°C for up to 2 months. Avoid multiple cycles of freeze thaw.

2.12. Please continue with the X-ChIP protocol from stage three (Determination of DNA concentration, page 19).

Lysis Buffer
50 mM HEPES, pH 7.4
140 mM NaCl
1 mM EDTA
1 % Triton-X-100
Protease inhibitors (added fresh each time)

See Buffers (page 25).

3. Chromatin preparation from tissues for ChIP

This protocol describes how chromatin is prepared from tissue, which can subsequently be used for ChIP. It is recommended that 30 mg of liver tissue is used for each ChIP/antibody. However, this amount may vary for other tissues. The exact amount of tissue depends upon protein abundance, antibody affinity and the efficiency of cross-linking. The protocol was optimized using 5-15 µg chromatin for each ChIP assay. The exact chromatin concentration should be determined for each tissue type before starting the cross-link ChIP (X-ChIP) assay. Our X-ChIP protocol should be used after the chromatin preparation detailed below. Protease inhibitors should be included in all solutions used, including PBS [PMSF (10 µl/ml), aprotinin (1 µl/ml) and leupeptin (1 µl/ml)].

This section was adapted from protocols kindly provided by Henriette O'Geen, Luis G. Acevedo and Peggy J. Farnham.

Cross-linking

Frozen tissues should be thawed on ice. (This process could take hours depending on the amount of tissue). It is important that the frozen tissue samples do not reach high temperatures, in order to prevent sample degradation by proteases. Samples should be kept on ice at all times and all steps performed quickly to minimize thawing. Tissue should be cut in a petri dish resting on a block of dry ice.

3.1. Chop frozen or fresh tissue into small pieces using 2 razor blades (between 1-3 mm^3).

3.2. Determine the weight of an empty 15 ml conical tube, transfer tissue into the tube and weigh again to calculate the amount of tissue.

3.3. Prepare cross-linking solution in fume hood. Use 10 ml PBS per gram of tissue. Add formaldehyde to a final concentration of 1.5 % v/v and rotate tube at RT for 15 min.

3.4. Stop the cross-linking reaction by adding glycine to a final concentration of 0.125 M. Continue to rotate at RT for 5 min.

3.5. Centrifuge tissue samples for 5 min, 720 rpm, 4°C.

3.6. Aspirate media off and wash with 10 ml ice cold PBS. Centrifuge for 5 min, 720 rpm, 4°C and discard wash buffer.

The tissue may be snap-frozen at this stage in liquid nitrogen and stored at -80°C. Avoid multiple freeze thaws.
If using immediately, resuspend tissue in 10 ml cold PBS per gram of starting material. Place on ice.

Tissue disaggregation

The Medimachine from Becton Dickinson may be used to obtain a single cell suspension. Use 2 medicones (50 µm) per gram of tissue to process.

3.7. Cut the end off a 1 ml pipette tip to make the orifice larger.

3.8. Add between 50-100 mg (3-4 chunks) of tissue resuspended in 1 ml of PBS.

3.9. Add this solution to the medicone and grind tissue for 2 min.

3.10. Collect cells from the medicone by inserting an 18 gauge blunt needle attached to a 1 ml syringe. Transfer cells to a conical tube on ice.

3.11. Repeat step 2.2 until all the tissue is processed.

3.12. Check the cell suspension using a microscope to ensure a unicellular suspension has been obtained. If more grinding is necessary, add more PBS to the tissue and repeat steps 2.2 to 2.5 until all tissue is ground into a homogeneous suspension.

3.13. Centrifuge cells for 10 min, 1000 rpm, 4°C. Measure/estimate cell pellet volume for next step.

3.14. Carefully aspirate off supernatant and resuspend pellet in FA Lysis Buffer (750 µl per $1x10^7$ cells).

3.15. Continue with x-ChIP protocol from stage 1 (Cross-linking, pg 18).

See Buffers (page 25).

4. ChIP using plant samples - Arabidopsis

Detailed procedure and tips for ChIP using plant samples

This protocol describes how chromatin is prepared from Arabidopsis, which can subsequently be used for ChIP. The exact chromatin concentration should be determined before starting the X-ChIP assay. View our cross-linking chromatin immunoprecipitation (X-ChIP) protocol, which should be used after the chromatin preparation detailed below.

Procedure
Arabidopsis seeds are stratified for 48 hours in 0.1% Phytablend w/v at 4°C and then sown onto soil. 1.5 g of whole, three to four week-old seedlings, are used per chromatin preparation. It is imperative to avoid contamination with soil as much as possible during harvest.

Cross-linking

4.1. Harvest 1.5 g seedlings and place them into a 50 ml tube.

4.2. Rinse seedlings twice with 40 ml double distilled water. Remove as much water as possible after second rinse.

4.3. Add 37 ml 1% w/v formaldehyde solution. Gently submerge seedlings at the bottom of the tube by stuffing the tube with nylon mesh. Screw on cap and poke cap with needle holes. Put in exsiccator and draw vacuum for ten minutes.

4.4. Release vacuum slowly and shake exsiccator slightly to remove air bubbles. Seedlings should appear translucent.

4.5. Add 2.5 ml 2 M glycine to quench cross-linking. Draw vacuum for five minutes.

4.6. Again, release vacuum slowly and shake exsiccator slightly to remove air bubbles.

4.7. Remove nylon mesh, decant supernatant and wash seedlings twice with 40 ml of double distilled water. After second wash, remove as much water as possible and put seedlings between two layers of kitchen paper. Roll up paper layers carefully to remove as much liquid as possible.

At this step, plant material can be snap-frozen in liquid nitrogen and stored at -80°C.

Chromatin preparation

4.8. Pre-cool mortar with liquid nitrogen. Add two small spoons of white quartz sand and plant material. Grind plant material to a fine powder.

4.9. Use cooled spoon to add powder to 30 ml of Extraction Buffer 1 stored on ice. Vortex to mix and keep at 4°C until solution is homogenous.

4.10. Incubate for 30 min at 4°C with gentle agitation.

4.11. Filter extract into a new, ice-cold 50 ml conical tube. Press to recover extract from solid material.

4.12. Repeat step 4.11.

4.13. Centrifuge extract at 4000 rpm for 20 minutes at 4°C.

4.14. Gently pour off supernatant and resuspend pellet in 1 ml of Extraction Buffer 2 by pipetting up and down. Transfer solution to Eppendorf tube.

4.15. Spin in cooled benchtop centrifuge at 13000 rpm for ten minutes.

4.16. Remove supernatant and resuspend pellet in 300 µl of Extraction Buffer 2 by pipetting up and down.

4.17. Add 300 µl of Extraction Buffer 3 to fresh Eppendorf tube. Use pipette to carefully layer solution from step nine onto it.

4.18. Spin in cooled benchtop centrifuge at 13000 rpm for one hour. In meantime, prepare 10 ml Nuclei Lysis Buffer. Put buffers in coldroom.

4.19. Remove supernatant and resuspend pellet in 300 to 500 µl of cold Nuclei Lysis Buffer. Resuspend by pipetting up and down and by vortexing. Keep solution cold between vortexing. Incubate for 20 minutes on ice.

4.20. Remove 10 µl to run on an agarose gel.

4.21. Sonicate for ten minutes at 4°C.

Make sure that the solution does not foam during sonication, e.g., by cooling the tubes with a mix of 100% w/v ethanol in ice during the sonication step.

4.22. Spin in cooled benchtop centrifuge at 13000 rpm for ten minutes. Add supernatant to new Eppendorf tube.

4.23. Repeat step 21. Remove 10 µl to run on an agarose gel.

4.24. Separate aliquots from steps 12 and 15 on 1.5% w/v agarose gel. In the sonicated samples, DNA should be shifted and more intense compared to untreated samples and range between 200-2000 bp, centering around 500 bp.

4.25. Please continue with the X-ChIP protocol from stage three (Determination of DNA Concerntration, page 19).

Following step 23, the chromatin samples can be "snap-frozen" in liquid nitrogen and stored at -80°C. Repeated freezing/thawing cycles, however, should be avoided.

Buffers

Extraction Buffer 1
0.4 M Sucrose
10 mM Tris-HCl, pH 8.0
10 mM MgCl2
5 mM beta-mercaptoethanol
Protease inhibitors

Extraction Buffer 2
0.25 M Sucrose
10 mM Tris-HCl, pH 8.0
10 mM MgCl2
1% w/v Triton X-100
5 mM beta-mercaptoethanol
Protease inhibitors

Extraction Buffer 3
1.7 M Sucrose
10 mM Tris-HCl, pH 8.0
2 mM MgCl2
0.15% w/v Triton X-100
5 mM beta-mercaptoethanol
Protease inhibitors

Nuclei lysis Buffer
50 mM Tris-HCl, pH 8.0
10 mM EDTA
1% w/v SDS
Protease inhibitors

References:

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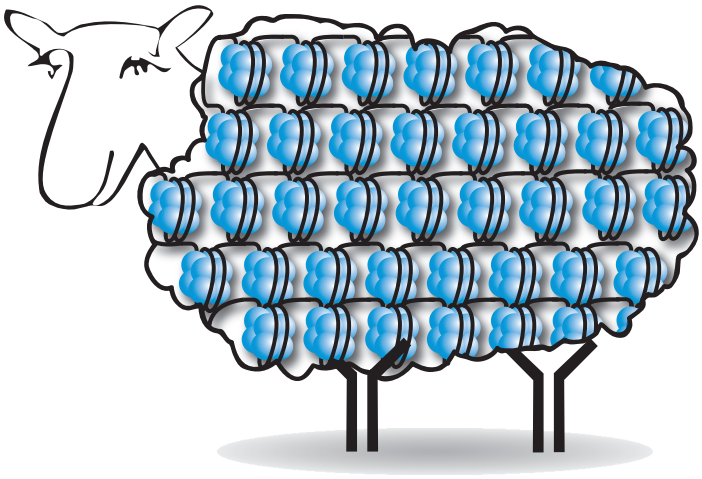

ChIP Lysis Buffer	50ml	Stock Solution
50mM HEPES-KOH pH 7.5	2.5ml	1M
140mM NaCl	1.4ml	5M
1mM EDTA pH 8	0.1ml	0.5M
1% Triton X-100	2.5ml	20%
0.1% Sodium Deoxycholate	0.5ml	10%
0.1% SDS	0.25ml	20%
Protease Inhibitors	(added fresh each time)	-

Dilution Buffer	50ml	Stock Solution
1% Triton X-100	2.5ml	20%
2mM EDTA pH 8	0.2ml	0.5M
20mM Tris-HCL pH 8	1ml	1M
150mM NaCl	1.5ml	5M
Protease inhibitors	(added fresh each time)	-

Wash Buffer	500ml	Stock Solution
0.1% SDS	2.5ml	20%
1% Triton X-100	25ml	20%
2mM EDTA pH 8	2ml	0.5M
20mM Tris-HCL pH 8	10ml	1M
150mM NaCl (add last)	15ml	5M

Final Wash Buffer	500ml	Stock Solution
0.1% SDS	2.5ml	20%
1% Triton X-100	25ml	20%
2mM EDTA pH 8	2ml	0.5M
20mM Tris-HCL pH 8	10ml	1M
500mM NaCl (add last)	50ml	5M

Elution Buffer	10ml	(make fresh every time)
1% SDS	0.5ml	20%
100mM NaHCO3	1ml	1M

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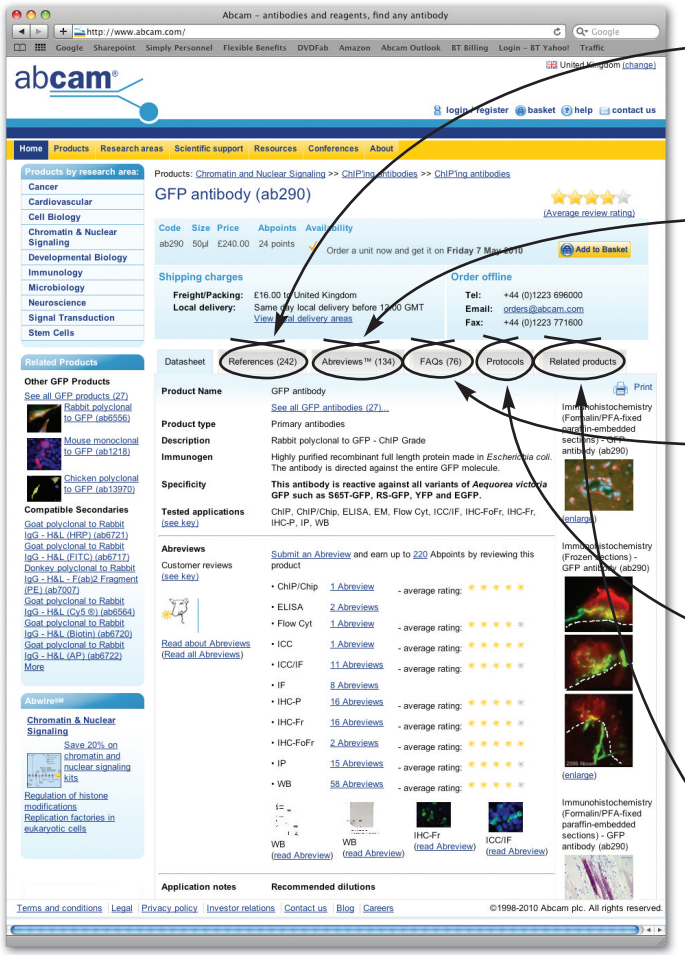

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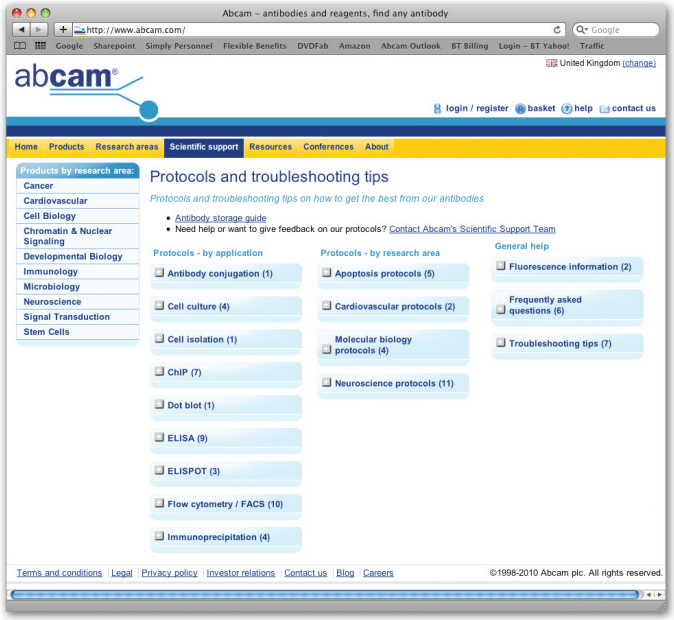

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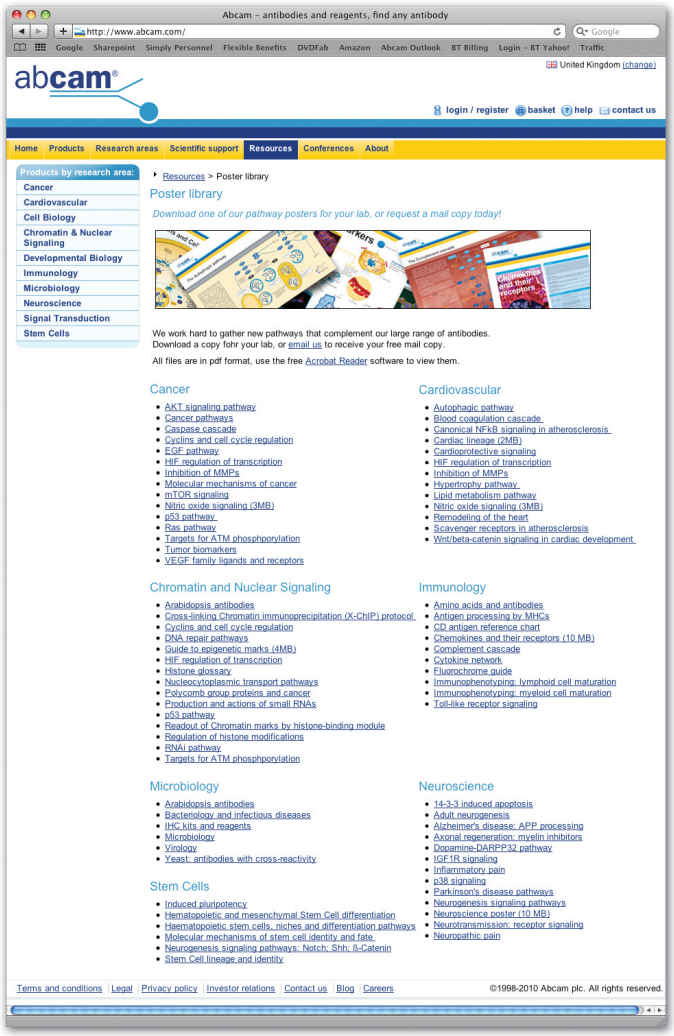

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Ordering and contact details

Ordering online

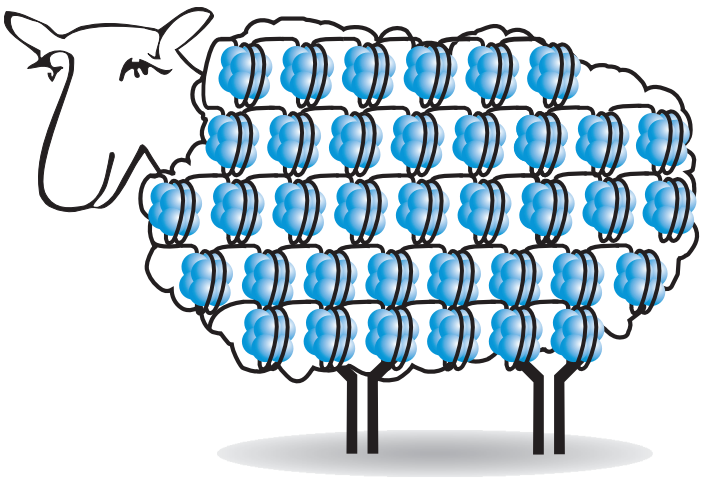
- Shipping address and billing address.
- Purchase order number* or credit card details.
- Your telephone number (so that we, or our delivery company, can phone you if there is a query).
- Your VAT number, if you are in the European Union. If you are in the UK and are VAT exempt, you will need to fax your exemption certificate.

* A purchase order number is a unique order reference number that your purchasing department will issue.

Ordering offline - Extra details

If you are not placing your order online then please also specify the following:

- Product code(s) of the item(s) you require (*e.g.* ab1).
- Your email address and/or fax number (so that we can confirm the details of the products you have ordered and our team can keep you continuously informed of the planned delivery date of your products).



Abcam's Terms and Conditions

In these Terms and Conditions 'Abcam' shall mean Abcam Limited, 330 Science Park, Cambridge CB4 0FL, United Kingdom. The Terms and Conditions set out below shall apply to Abcam branded products and services and to products distributed by Abcam on behalf of other suppliers.

Ordering details

All orders are subject to availability. Prices of products do not include freight and packing, VAT or import duties where these are applicable.

Price and other information provided is subject to change without notice, and prices may be changed up to the time of delivery. While every effort is made to keep information provided by Abcam up to date, Abcam will not be liable to you or anyone else if errors should occur in information provided on this website.

When your order is received Abcam will confirm receipt. However, formal acceptance of the order will only take place when the goods are despatched. If prices should be changed between the time of receipt of an order and despatch, Abcam will contact you in advance.

Invoices should be paid no later than 30 days after the invoice date, and customers must themselves pay any bank charges that are incurred in making the payment. Full payment instructions are set out on the invoice.

Receipt of products

Packaging and products should be inspected immediately upon receipt. Notification of damage, shortages or defects should be given immediately by e-mail, fax or phone.

Return & replacement policy

Quality is important to Abcam. If a product does not perform as described on the datasheet notify us within 120 days of delivery and our dedicated team of scientific experts will examine details of your protocol to determine whether the problem is protocol related or product related:

- For protocol-related problems, our experts will give you independent professional advice.
- For product- related problems, a replacement or refund will be offered.
- If you do not provide details of your protocol or follow the recommendations of our experts, Abcam will not issue a replacement or refund.

If items are ordered incorrectly by the customer Abcam will consider taking them back. This will normally be subject to a 20% restocking charge on the items plus any shipping, handling & packaging costs. Requests for returns must have prior authorization (contact returns@abcam.com) and must be made within 7 days of receipt of the items. Items must be returned in the same or equivalent packaging (i.e. cold and insulated) as originally despatched by Abcam.

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General

Contracts and agreements with Abcam shall in all respects be governed by and interpreted in accordance with English law.

All information correct at time of printing.

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