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How to BECOME A CHIP EXPERT in 4 days

CHIP 101

Comprehensive ChIP guide for beginners

- Overview of different types of ChIP
- How to choose & validate antibodies for ChIP
- What ChIP controls to include
- ChIP protocol with optimization tips
- Troubleshooting guide & FAQs

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PREFACE

Chromatin Immunoprecipitation (ChIP) is a versatile, powerful tool that has revolutionised our understanding of protein-genome interactions. Through this eBook, we aim to inform scientists and academics about the principles and protocols underlying ChIP. We will also highlight how this technique can be incorporated into your studies to dissect gene function and regulation *in vivo*.

The book will describe the two main types of ChIP, including brief descriptions of the latest advancements in ChIP protocols. Recent advances in downstream analysis methods using high throughput sequencing will also be described. Finally, the book will also provide our readers with ways to troubleshoot any difficulties that may arise in this protocol.

INTRODUCTION

In eukaryotes, genomic DNA is packed into compact, dense structures called chromatin. This complex organisation is achieved by the binding of double helix DNA with basic proteins called histones. Further, genomic DNA binds to a variety of other proteins and protein complexes including transcription factors, chromatin modifiers, and nuclear factors. These interactions play a crucial role in the regulation of gene expression, DNA replication and repair (Quina et al., 2006). The elucidation of the spatial and temporal dynamics of these protein-DNA interactions will help understand gene expression and regulation in both normal physiological and diseased pathological contexts.

The chromatin immunoprecipitation assay (ChIP) is a multi-faceted powerful tool to map protein-DNA interactions at a specific genomic locus or at multiple sites across an entire genome. The assay allows for a minute-by-minute detection of enrichment for a specific protein at a genomic site in vivo. The fundamental principle of the ChIP assay is the selective enrichment of a protein or protein complex bound to chromatin. The assay involves fragmentation of chromatin following cell lysis. The chromatin-bound protein fragment is then immunoprecipitated using corresponding antibodies. Recovered DNA can then be amplified and sequenced to determine protein interaction dynamics.

The assay provides scope for multiple applications, depending on the availability of an antibody for the targeted protein, stability of the protein epitope, and integrity of the chromatin structure.

ChIP was first successfully used to investigate the binding of RNA polymerase II and topoisomerase I to the bacterial genome in 1984 (Gilmour and Lis, 1984). The first antibody against a histone modification was developed in 1988, facilitating the discovery of histone acetylation (Hebbes et al., 1988). Subsequently, in 1993, ChIP was used to dissect the function of polycomb repressors in Drosophila (Rastelli et al., 1993). Since then, ChIP has been adapted for use in a variety of cell and tissue types including humans and plants.

What is ChIP used for?

The ChIP assay can be applied to study multiple facets of protein-chromatin interactions including the binding of histones, chromatin modifiers, transcription factors and regulators in cell differentiation, proliferation, and development.

Many in vitro assays, such as electrophoretic mobility shift assays (EMSA), are used to investigate DNA-protein binding. However, in vitro conditions may not always resemble the native state of the protein accurately. Therefore, ChIP provides a valuable alternative to detect enrichment of protein in the genome invivo. Further, it can be also used to measure the real-time effect of a stimulus on gene regulation (Kuo and Allis, 1999).

The dynamics of RNA polymerases in gene transcription and regulation were also demonstrated using ChIP (Sainsbury et al., 2015). Furthermore, with the proposal of the histone code hypothesis, which refers to a barcode of modifications in the tails of histone proteins that regulate gene transcription, genome-wide mapping of histone PTMs has been carried out using ChIP-Seq (Janssen et al., 2017). ChIP has facilitated the discovery of transcription factors as well as their binding and dysregulation in many diseases including cancer, resulting in the identification of novel drug targets (Barski et al., 2007).

The human ENCODE and NIH roadmap databases now contain a comprehensive list of the functional elements in the human genome thanks to the combination of ChIP assay with high throughput sequencing technologies (Qu and Fang, 2013).

How does ChIP work?

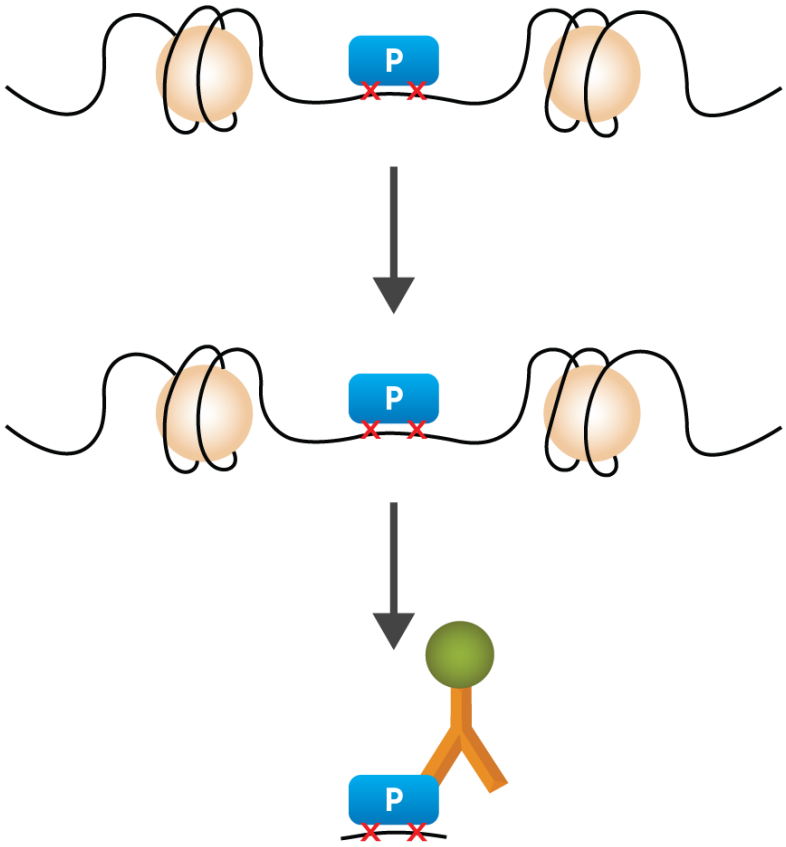
ChIP is generally used in the study of euchromatin which maintains an open structure with activated genes. Heterochromatin, which is more densely packed with inactive genes, is harder to analyze.

In brief, the general procedure of ChIP entails crosslinking of the targeted protein to DNA, followed by cell lysis and chromatin fragmentation. The protein-chromatin complexes are then captured by immunoprecipitation using specific antibodies. Crosslinking is then reversed using proteinase K while isolated DNA fragments are amplified and sequenced using an array of downstream technologies including qPCR and microarray. The efficiency of ChIP assay depends on a variety of factors including antibody specificity, protein complex stability, and quality of chromatin fragmentation.

TYPES OF CHIP

There are two main types of ChIP assays based on the process used to prepare chromatin fragments before immunoprecipitation.

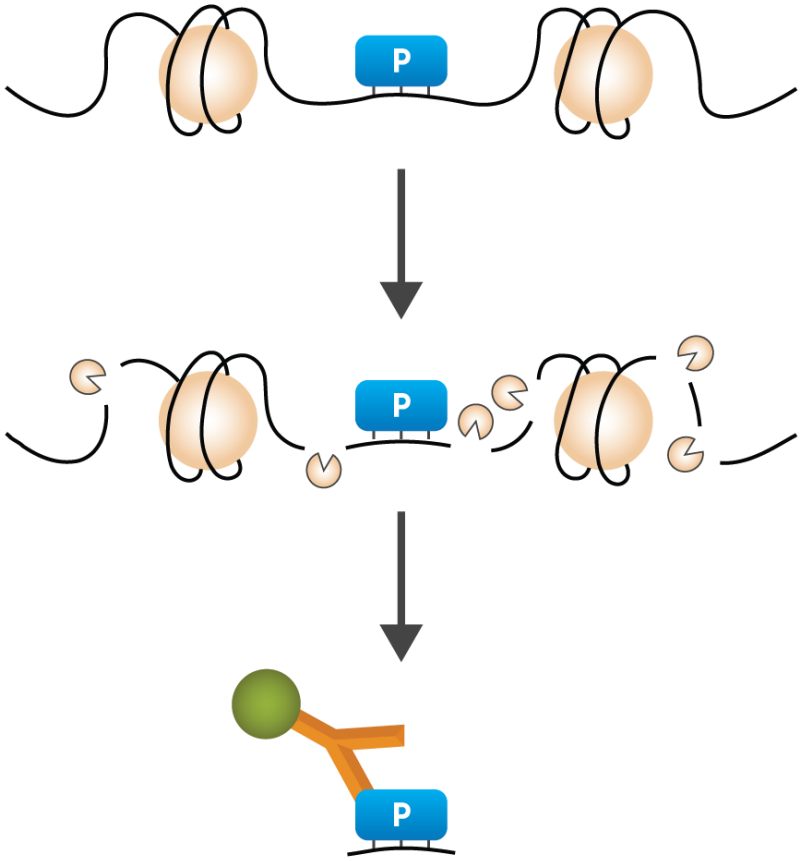
X-ChIP



Crosslinked ChIP or X-ChIP uses covalently and reversibly bonded protein-chromatin complexes for immunoprecipitation. Crosslinking of the target protein and DNA is carried out using reagents such as formaldehyde to prevent their separation during cell lysis and chromatin fragmentation. Formaldehyde works well in crosslinking direct protein-DNA interactions. However, alternative methods of crosslinking are required while investigating proteins that do not bind directly to DNA (protein-protein-DNA complexes). Crosslinking is a time-sensitive step, and excessive reagent can lead to a reduction in protein (antigen) availability for immunoprecipitation.

Following crosslinking, sonication can be performed to generate chromatin fragments approximately 2-3 nucleosomes in length (500-700bp). The cell lysate contains both fragmented chromatin and cell debris, which must be purified to increase the quality of immunoprecipitation. Corresponding antibodies can then be used to capture the chromatin fragments.

N-ChIP



Native ChIP or N-ChIP is used to investigate very strongly bound proteins in chromatin such as histones. In such cases, crosslinking is not required. Instead, native chromatin isolated from cells is directly digested using micrococcal nucleases (O'Neill and Turner, 2003). As a result, N-ChIP cannot be used to investigate non-histone proteins that have weaker affinities to DNA. This is especially true in cases where proteins bind indirectly to the genome. Digestion with nucleases creates chromatin fragments containing monosomes (approx 175bp); therefore, N-ChIP has a greater resolution than X-ChIP. However, certain transcription factors can bind to internucleosomal regions and cannot be detected using N-ChIP. It should be noted that enzyme digestion does not completely generate random fragments like sonication. Rather, micrococcal nucleases have been shown to have a sequence bias, favoring certain areas of the genome sequence. Therefore, certain genomic loci may be overrepresented due to uneven DNA fragmentation. Following chromatin fragmentation, purification, immunoprecipitation, and analysis are conducted in steps similar to X-ChIP.

Advantages and Disadvantages of X-ChIP and N-ChIP

	Advantages	Disadvantages
N-ChIP	• Proteins remain in the native state and antibodies are generally raised against unfixed antigens, leading to better antigen-antibody binding and recognition • High resolution (175bp/mononucleosome) • Due to the abundance of histone proteins, PCR amplification is not necessary • Requires less cellular material, facilitating investigation in rare tissues and biopsies • More predictable antibody binding specificity	• Can only be used for histone proteins • In some cases, the histone-DNA complex may separate • Since nucleosomes are dynamic structures, rearrangement may occur during enzyme digestion • Micrococcal nucleases show sequence preference – resulting in chromatin bias and possibly inaccurate results
X-ChIP	• Can be used to investigate histone and non-histone proteins including DNA-protein, RNA-protein, and protein-protein complexes • Preferred when native chromatin is difficult to prepare (e.g. yeasts) • Since crosslinking is strong, it can be used to detect even transient protein bindings • Possibility of chromatin rearrangements is minimized during preparation and precipitation • DNA-protein complexes are stable and do not separate during lysis	• Crosslinking affects protein-antibody recognition, leading to less efficiency • Due to random fragmentation, the chromatin fragment size varies, which could result in lower resolution • PCR is necessary due to insufficient chromatin recovery • Transient proteins may be fixed due to formaldehyde crosslinking, producing false positives • Over-fixation can cause ineffective sonication and uneven fragmentation

DOWNSTREAM ANALYSIS

Downstream analysis refers to the assays and technologies used to analyze DNA fragments isolated by immunoprecipitation. Chromatin crosslinks are reversed and DNA purification is performed using PCR purification kits or phenol-chloroform extraction with ethanol precipitation. Several approaches are available to analyze purified chromatin fragments. Analysis of specific known genomic loci can be carried out by PCR using specifically designed primers. The advancement of next-generation sequencing (NGS) methods has facilitated genome-wide analysis of protein binding, ChIP-Seq and ChIP-chip are the two most commonly used high-throughput sequencing methods.

ChIP-Quantitative PCR (ChIP-qPCR)

qPCR or real-time polymerase chain reaction quantifies amplified DNA fragments in real- time. ChIP-qPCR is commonly used to detect protein-DNA interactions at known binding sites in the genome. It can also be used to quantify the enrichment of protein in response to external stimuli. qPCR analysis is rapid, time efficient, and cheaper compared to other downstream analysis methods.

However, only a small sequence fragment can be analyzed, unlike high throughput methods. It is also not scalable and cannot be used to detect novel interactions.

ChIP-on-Chip (ChIP-chip)

ChIP-chip uses microarrays chips to analyze protein-interactions and distribution across the genome. Purified DNA fragments obtained after immunoprecipitation are tagged with fluorescent dyes and applied to microarrays. Image analysis of microarray chips provides a high-resolution map of protein dynamics in the genome.

ChIP-chip assays have been used to elucidate protein dynamics in both normal physiological and diseased cells including transcription factor dysfunction, enhancer and repressor binding (Furey, 2012). The assay is rapid and more efficient than qPCR but less efficient than ChIP-Seq.

These assays require a large quantity of starting cellular material and are therefore limited in applicability in rare cells. Microarray sequencing is susceptible to amplification bias. Sequencing also depends on the availability of genome-wide data, so it can be difficult to detect novel sequence interactions. These factors in combination with the high cost of microarrays result in ChIP-Seq being the most preferred downstream analysis method (Pellegrini and Ferrari, 2012)

ChIP-Sequencing (ChIP-Seq)

Combining ChIP with high throughput sequencing methods provides a powerful tool to map protein interactions with nucleic acids across the genome while overcoming most of the limitations of ChIP-chip. The assay also provides a higher resolution per base pair. DNA fragments obtained via immunoprecipitation are amplified and directly sequenced using NGS methods that can sequence nearly a million DNA molecules simultaneously. The sequences are then aligned with whole genome data from the organism to detect protein binding site.

ChIP-Seq has been used to identify transcription factor binding sites, histone modifications, chromatin state in embryonic stem cells and epigenetic regulation in diseased cells. It has also been used to elucidate RNA sequencing and methylation (Furey, 2012).

Unlike ChIP-chip, this assay does not require prior knowledge of genome sequences. This eliminates the inherent bias present from using known probes. The assay is also rapid, cost-effective and highly efficient by requiring very little starting cellular material.

However, the assay is time-consuming and expensive compared to other downstream analysis methods. Additional common limitations include unmappable reads, PCR duplicates, and poor library complexity.

NEW CHIP METHODS

The general ChIP protocol requires up to one million cells and takes 5 days. Recent enhancements and modifications in ChIP have enabled rapid, efficient detection and characterization of DNA-bound proteins across genomes using a reduced number of cells. Complimentary downstream assays have also been developed enabling better resolution and precision. The following section describes some of the latest advances in ChIP.

Bead-Free ChIP

Bead-free ChIP uses protein A or G immobilized beads in spin columns or microplates to elute antibody bound chromatin. The porous beads improve purification of chromatin fragments and reduce non-specific binding. This assay is more rapid, sensitive and requires less starting cellular material (Harmeyer et al., 2015).

Carrier ChIP (CChIP)

The assay uses foreign carrier chromatin from an evolutionarily distant organism such as Drosophila in combination with the target chromatin leading to more efficient precipitation of target chromatin-antibody complex. However, the primers used to sequence target chromatin must be highly specific to overcome background noise due to foreign fragments. The assay has been successfully used to detect histone modifications in stem cells. The primary limitation of this method is that it requires a large quantity of cellular material and is time-consuming (Rodríguez-Ubreva and Ballestar, 2014).

ChIA-PET

Chromatin interaction analysis with paired end tags (CHIA-PET) uses two linker sequences with unique barcodes that are ligated to the end of DNA fragments. These linkers further self-ligate based on proximity leading to the detection of distant DNA – regions that interact with each other or the protein of interest. The assay can detect long-range and short-range interactions between protein/protein complexes and chromatin while efficiently removing background noise. However, this protocol requires a large amount of starting material. Additionally, self-ligation can cause false positives leading to limited sensitivity (Li et al., 2010)

ChIP-exo

ChIP is combined with lambda exonuclease digestion to provide a powerful tool to map specific protein binding sites in the genome. Following immunoprecipitation, a 3'exonuclease is used to lyse the DNA sequences extending from the protein binding site. This step significantly increases the resolution of the assay while reducing background noise (Rhee and Pugh, 2012). This assay has been used to map novel protein interactions. However, this assay cannot be used to investigate complex three-dimensional interactions since simultaneous binding could be read as two binding events rather than as one binding event.

Fast ChIP (qChIP)

As the name implies, fast ChIP significantly reduces time by using an ultrasonic bath that improves immunoprecipitation. Additionally, a resin (Chelex-100) is used to increase the efficiency of crosslink reversal and DNA purification. However, the assay requires a large quantity of starting material, but is scalable to detecting over 1,000 histones and 100 transcription factors simultaneously. It can also be used to examine genomic interactions over several time points (Nelson et al., 2006).

Quick and Quantitative ChIP (Q2ChIP)

Q2ChIP overcomes the need for a large amount of starting material by carrying out crosslinking in the presence of butyrate followed by chromatin purification using tube shift. Further crosslink reversal, protein digestion and increased immobilized antibody beads:chromatin ratio, and DNA elution are simplified into a single step. This assay is significantly more efficient, accurate, and less time-consuming (Dahl and Collas, 2007).

MicroChIP (μCHIP)

MicroChIP uses recent advances in microelectronics for rapid and parallel detection of protein interactions in very small quantities of cells (Dahl and Collas, 2008). μChIP has been used to sequence over 9 modified histone sites from a single batch of 1000 cells. It is therefore applicable to rare tissue samples.

Matrix ChIP

Traditional ChIP assays are performed in test tubes, making it more challenging to observe complex genomic signaling events. As the name implies, microplate-based ChIP, or matrix ChIP, executes the entire assay from immunoprecipitation to DNA purification on the same 96-well microplate, eliminating the need to transfer samples. This simplified assay is high throughput, sensitive, and reproducible. Moreover, the matrix ChIP platform offers the potential for automation (Flanagin et al., 2008).

Pathology Tissue-ChIP (PAT-ChIP)

Detection of protein interactions in patient samples is limited due to the use of formalin-fixed, paraffin-embedded (FFPE) samples that hinder analysis of chromatin structure. PAT-ChIP provides a modified protocol that enables chromatin extraction and immunoprecipitation from FFPE samples that are even several years old. It can thus be utilized as a powerful diagnostic tool to examine epigenetic alterations in cancer and other diseased states (Fanelli et al., 2010).

CHOOSING AND VALIDATING AN ANTIBODY FOR CHIP

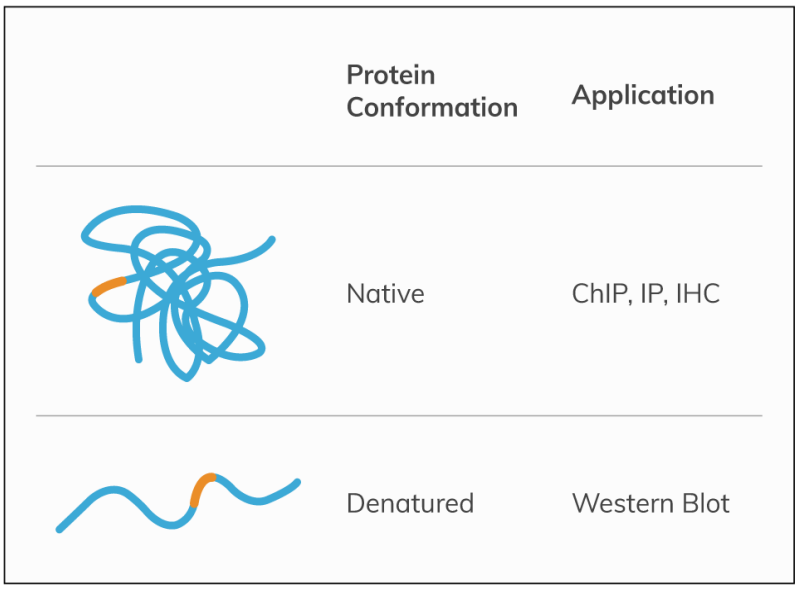
The most critical step of ChIP assay involves using an antibody coupled to beads or other supports to immunoprecipitate the target chromatin-protein complex of interest. Therefore, like other immunoprecipitation based assays, the success of ChIP largely hinges on the specificity and affinity of the chosen antibody. Non-specific antibodies will bind to proteins other than the target, producing background noise that hinders the interpretation of your results. This is especially true when testing rare, low-affinity DNA-protein interactions. For commercial antibodies that have already been ChIP or ChIP-seq validated, previous publications can be used to evaluate its efficacy.

Whether using commercially validated or non-validated antibodies, it is still highly recommended to carefully choose and test the antibody to ensure it indeed recognizes the target protein as advertised. Unfortunately, no standard procedure exists to define antibodies best suited for ChIP. Below, we summarize the most common pitfalls to watch out for when choosing antibodies, and highlight common validation methods that can be used to evaluate the performance of your antibody.

How to Choose an Antibody for ChIP

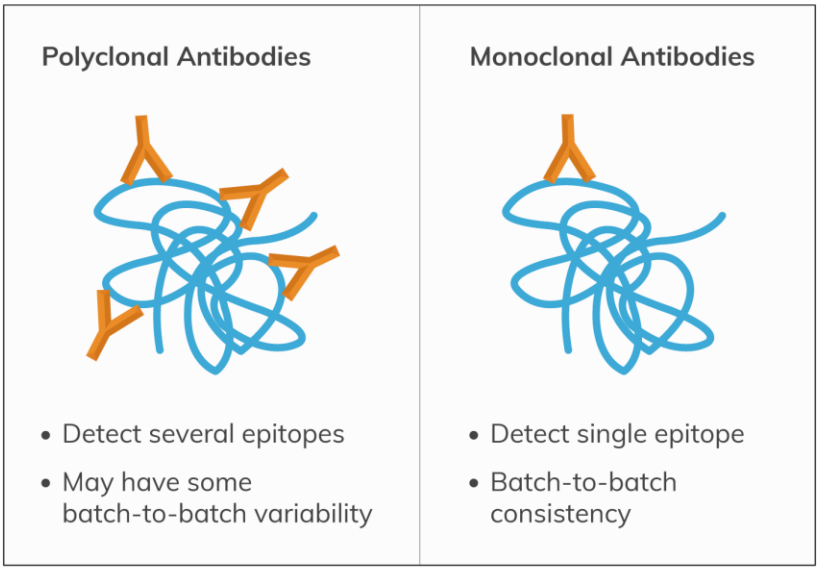
When choosing a ChIP antibody, keep in mind that the antibody must be highly specific and efficiently immunoprecipitate the target protein of choice. Preferably, the chosen antibody must also be affinity-purified. When this is not the case, background noise can be overcome by using sufficiently stringent reagents. While most immunoprecipitation-based experiments require antibodies with high specificities, this is especially critical for ChIP assays. For example, if you wish to evaluate a particular histone modification, the antibody must be specific enough to only recognize histones with the desired group (dimethyl) without pulling down similar modifications (trimethyl, monomethyl). Furthermore, in studies evaluating members of a multigene family, the antibody must target without cross-reactivity to other related family members. In this case, validation of the antibody using mass spectrometry can be carried out. Even low concentrations of non-target proteins can dramatically alter results and lead to misinterpretation of binding affinities. Antibody specificity can generally be tested using peptide inhibition western blots, ELISA, or peptide dot blots.

If a ChIP validated antibody is not available, an antibody that has been successfully used in immunoprecipitation (IP), immunohistochemistry (IHC), or immunocytochemistry (ICC) can be considered. Like ChIP, antibodies in these applications detect a target protein in its native conformation, unlike in western blot where antibodies detect denatured proteins. Therefore, success in these applications is a good predictor of whether the antibody will work for ChIP. Immunofluorescence assays can be used to confirm the antibody recognizes the target protein in its native state, which is crucial for ChIP. Immunoprecipitation studies can help determine optimal concentrations of antibodies to be used. However, it is important to note that not all antibodies that work in standard IP assays will work with ChIP because crosslinking can significantly alter the epitope of target (the portion of the antigen or protein that is recognized and bound by the antibody).



Polyclonal vs Monoclonal Antibodies: Monoclonal, oligoclonal (i.e. pools of monoclonals), and polyclonal antibodies have all been demonstrated to work in ChIP assays. Polyclonal antibodies can recognize multiple epitopes on a target protein. This makes them advantageous for ChIP where crosslinking can mask some epitopes. Yet, this ability also renders polyclonal antibodies to bind to non-specific proteins, resulting in low signal:noise ratios. This can lead to variation between batches, resulting in antibodies of different efficiency and specificity.

Monoclonal antibodies can only recognize a single epitope on the target protein resulting in high specificity, low non-specific binding and background noise. Additionally, due to the low variability in their clonal nature, subsequent batches are consistent to one another. Despite these advantages, if the target protein forms complexes with other proteins, the target epitope will be masked and unrecognizable with a monoclonal antibody. This is also true for X-ChIP where crosslinking with formaldehyde alters most epitopes. Unless a monoclonal antibody has been specifically designed and purified for use in ChIP, it is advisable to choose a polyclonal antibody.



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antibodies have undergone rigorous processes to ensure high specificity, sensitivity, and affinity, so you can work with confidence and generate publishable data. Visit www.bosterbio.com to find the antibody for your protein of interest.

How to Validate an Antibody for ChIP

A recent study tested over 200 commercially available antibodies targeted at histone modifications and detected over 25% to be non-specific. They also found variability between different batches of the same antibody (Wardle and Tan, 2015). Therefore, it is prudent to perform your validation tests even when the commercial antibody is ChIP-grade. Furthermore, antibodies behave differently in different cells, so the antibody must be validated in your specific cells of interest. Most journals also require validation studies as proof of antibody specificity before publication (Baker, 2015). Additionally, projects like the ENCODE and modENCODE consortia have laid down guidelines for validating ChIP reagents and antibodies to establish accuracy, reproducibility, and comparability of data. The most commonly used validation procedures are summarized below.

Primary validation of the antibody is carried out using immunoblot or western blot. Cell or nuclear lysate containing the target protein can be added to the antibody to check the presence of an immunoreactive band at the corresponding molecular weight of the target protein. It is possible that the antibody will recognize proteins other than the target protein, producing multiple bands. If the non-specific binding proteins are non-nuclear or non-DNA binding, it will not interfere with the ChIP assay. Immunoprecipitation can also be performed with the antibody along with mass spectrometry-based sequencing to isolate the protein of interest (Wardle and Tan, 2015).

Validation via immunoblotting may not be possible in all cases. For example, transcription factors are expressed only at low concentrations and will not form a detectable band. In this situation, the target protein can be cloned and overexpressed in cells to obtain concentrations necessary for immunoprecipitation (Morley et al., 2009).

Additionally, the antibody can be validated using immunofluorescence to visualize antibody-bound protein inside the nucleus. Recently, SILAC assays (stable isotope labelling of amino acids in cell culture) have been gaining popularity in validating antibodies (Edfors et al., 2018).

Generally, secondary validation is carried out using a standard ChIP assay. However, some scientists argue it would be more economically prudent to perform a low cost ChIP using cells of interest before immunoprecipitation analysis since antibodies that work in IP do not always work with ChIP (Wardle and Tan, 2015). A standard ChIP assay would include a control locus where the target protein has been known to bind and a negative control locus where the protein does not bind. The eluted antibody-chromatin complex is then run on a gel, blotted and probed using antibody against target protein. This validates the specificity of the antibody and helps detect non-specific binding.

Validation is a strenuous and expensive process with very few antibodies eventually proving to work with ChIP. Consequently, it would be wise to test multiple potential antibodies to target the same protein of interest by recognizing different epitopes. It is more likely one of these antibodies will perform well with ChIP.

For further validation, immunoblot or ChIP assays can be carried out in cells with the target protein knocked out or knocked down. The resulting immunoreactive band must be absent or at least significantly reduced. The ENCODE Consortium guidelines recommend at least 70% reduction of signal in IP and 50% reduction in ChIP-seq. When testing a protein belonging to a protein family, knocking down the related proteins and carrying out ChIP will help ensure the antibody only binds to the target protein.

ChIP and immunoblot assays can also be used to determine the optimal concentration of the antibody that produces the highest signal to noise ratio. In this case, a standard ChIP assay can be carried out using multiple titrated concentrations of the antibody. The generally recommended range is 3–5 µg of antibody for every 25–35 mg of pure monosomes used. Besides the antibody, the protocol used for the assay must also be validated, with the washing conditions optimized for the chosen antibody. Multiple stringencies of washes can be tested to select the optimal range that delivers the highest concentration of antibody-bound chromatin. Generally, NaCl or LiCl washes are used in concentrations ranging between 250-500 mM. For every step of the process, negative control precipitations without the antibody are recommended to evaluate its specificity.

Finally, further validation is required in ChIP-seq assays to confirm the target protein binds to the specific locus. This can be achieved by motif enrichment via qPCR followed by analysis. If the protein does not directly bind to the DNA, the corresponding motif will not be enriched.

A ChIP validated antibody may not be available for targets. This is especially true for rare and less studied species. In such cases, the target protein can be tagged with Myc, His, human influenza hemagglutinin (HA), T7, GST, or V5 peptides epitopes, which can then be easily recognized and precipitated by well characterized commercial antibodies.

However, this method has its disadvantages. The tag might interfere with the protein's ability to bind to the corresponding site on the genome. Some tags are known to cause non-specific interactions with genomic sites. Moreover, the addition of the tag and resulting overexpression can drive non-specific binding. This effect can be somewhat nullified by using endogenous gene regulatory sequences to enhance expression (Poser et al., 2008).

Recently, CRISPR/Cas9 has gained popularity for adding tags to specific loci to produce fusion proteins in a wide range of cells and organisms. This method also overcomes the disadvantage with overexpression and non-specific binding (Mali et al., 2013). Nevertheless, the addition of tags always carries the risk of interfering with endogenous protein function.

CHIP CONTROLS

Controls should be included for every critical step of the ChIP assay in order to validate the antibodies and reagents used. The right controls can make data interpretation accurate and easier. Several controls have been designed and used by different studies to validate various stages of the assay. A few of them are summarized here.

Positive Controls

A sample (5-10%) of input cell lysate that has not been immunoprecipitated can be used as a control to compare with the immunoprecipitated lysate. This allows for data to be interpreted by eliminating nonspecific binding and background noise. Studies investigating histone modifications can simply use purified histone proteins as control to analyze the immunoprecipitated sample.

Antibodies can also be used as controls to test the efficiency of immunoprecipitation and accuracy of the results. A known locus that binds a known protein can be chosen as a positive control. For example, Histone H3 is an excellent choice for a positive control while investigating active genes. Most commercial kits also contain Rpb1 (the largest subunit of RNA polymerase II) for use as a positive control antibody. If you are investigating inactive genes that do not bind to H3 or RNA polymerase II, you will have to choose other antibodies for positive control.

Negative Controls

As a negative control, a locus that does not bind to the target protein of interest should be used. This allows for the determination of background noise. For a negative control, choose an antibody that cannot recognize any chromatin epitope, thus only binding non-specific proteins. Antibodies that only recognize a non-chromatin antigen such as anti-GFP antibody can be used. Antibodies like rabbit IgG (isotype-matched control immunoglobulin) which do not recognize specific epitopes can also detect non-specific interactions. If the concentration of the target protein pulled down using IgG equals the amount pulled down using the experimental antibody, it indicates your antibody isn't working. For ChIP-seq assays, IgG cannot pull down high concentrations or varieties of genomic regions enough to generate a library. Therefore, a quantity of the sample (cell lysate) can be used as a control for both qPCR and sequencing to generate a complex, unbiased library.

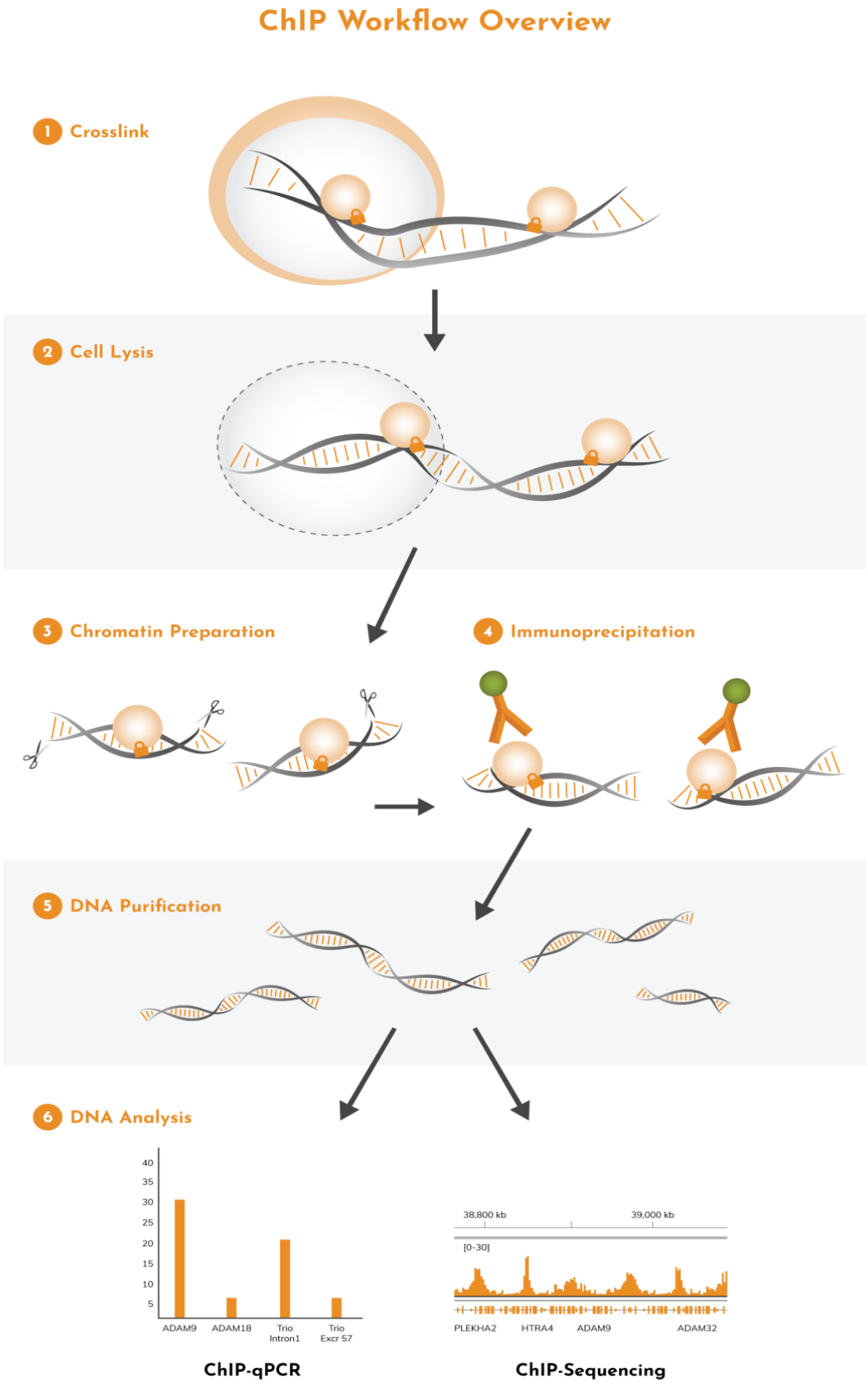
qPCR Analysis

For qPCR analysis, further controls are required to ensure the results are accurate. Certain motifs in the genome can be enriched more than others while some nucleosomes may rearrange during enzyme digestion, both of which can result in bias. It would thus be advantageous to generate PCR primers for several random loci on the genome and compare it with the eluted protein-DNA complex from ChIP.

qPCR also requires positive and negative controls for the target protein of interest. Primers for a locus where the protein of interest is known to bind can be the positive control while primers for one where the target protein is absent represents the negative control. These primers can be subsequently used in RT-PCR to test the specificity of enrichment.

As a general rule, at least 2 positive control loci and 1 known negative loci should be analyzed for immuno-enrichment. The antibody must show a minimum fold enrichment of positive control locus compared to negative control locus. The signal to noise ratio can then be calculated to determine antibody efficiency. Finally, a no-template control (NTC) should always be included in PCR to detect any contamination.

CHIP PROTOCOL



As the name implies, chromatin immunoprecipitation (ChIP) involves the precipitation of a protein-chromatin complex (antigen) using the corresponding antibody. ChIP assays are versatile and can be adapted for a wide range of cells, tissues, and experimental objectives. The general protocol involves the following steps:

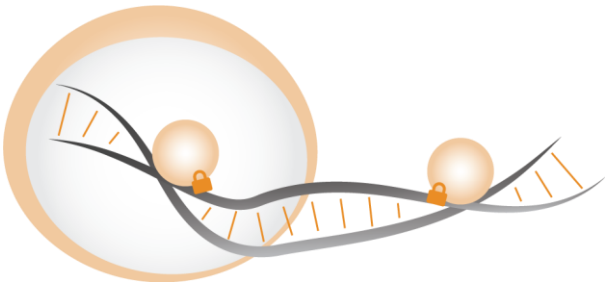
1. Crosslinking of Proteins to DNA (X-ChIP only)
2. Cell Lysis
3. Chromatin Preparation
4. Immunoprecipitation (IP)
5. DNA Preparation
6. DNA Analysis

In this section, we summarize the principles and protocols underlying each step of the ChIP assay. Moreover, we list some optimization tips for researchers that will help you obtain the best possible results.

Before you begin: Considerations for researchers

- **X-ChIP vs N-ChIP:** N-ChIP is the best choice for proteins that bind potently to chromatin. Typically, this includes histone proteins and histone modification proteins. On the other hand, X-ChIP can be used to analyze a wide range of direct or indirectly binding chromatin proteins.
- **Choice of Antibody:** Your choice of antibody will critically impact the efficiency of the assay. Therefore, choose carefully based on the guidelines discussed in previous sections.
- **Controls:** For every step of the assay, it is crucial to include positive and negative controls in order to reliably interpret your results. Guidelines for designing and optimizing controls have been described in previous sections.
- **Single Locus vs Genome-Wide Analysis:** The downstream analysis method should be selected based on the size and number of the target locus. For example, qPCR is applicable for single locus analysis while genome-wide analysis requires ChIP-seq.
- **Cell Count:** The cell count should be optimized for the assay. Generally, a count of 2 x 10^6 cells is recommended for immunoprecipitation. However, the number should be scaled based on the efficiency of your antibody.
- **Cells vs Tissues:** ChIP protocols must be specially adapted for use in tissue samples, particularly with respect to crosslinking and cell lysis.

Step 1: Crosslinking (For X-ChIP Only)



Crosslinking refers to the process of covalently linking two or more molecules together. For target proteins that do not directly bind to DNA and those that bind with low affinity, covalent stabilization of the protein-DNA complex *in vivo* is an essential first step. The reversible crosslinks ensure that the chromatin complex does not separate during cell lysis.

Crosslinking reagents contain two or more reactive ends capable of forming covalent bonds with the functional groups found on proteins. Typically, formaldehyde is recommended for proteins

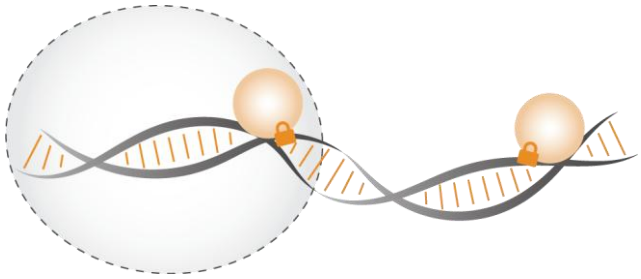
that directly bind to DNA. However, formaldehyde is a zero-length crosslinker (2Å), meaning it cannot form bonds between large protein complexes. For higher order interactions involving large tertiary or quaternary structures, reagents such as EGS (16.1 Å) and DSG (7.7 Å) can be used. Although UV is an effective crosslinker, it is not compatible with ChIP assays as the bonds formed are not reversible.

Compared to cells, tissue samples require longer crosslinking time, so more rapid delivery of reagent is needed to permeate the tissue before the sample starts degrading. Therefore, it is crucial to optimize crosslinking protocol for every cell line and tissue. While inadequate crosslinking can lead to the separation of the protein-DNA complex, over-fixation can prevent efficient chromatin fragmentation. Additionally, bulky crosslinks may mask the target epitopes for the chosen antibody.

Optimization Tips

- **Choosing a crosslinker:** Crosslinkers are chosen based on the functional groups present in the target protein. The molecular span of the crosslinker and its cell permeability should also be considered.
- **Optimizing crosslinking time:** Incubation time should be empirically determined using time course experiments ranging from 2-30 minutes at multiple reagent concentrations. The optimal crosslinking time achieves efficient crosslinking corresponding to the highest antibody-antigen binding.
- **Quality of reagents:** Formaldehyde solutions containing methanol may affect crosslinking, so be sure to use molecular biology grade reagents.
- **Cell culture:** Certain culture mediums can affect the efficiency of formaldehyde crosslinking.
- **Quenching:** Crosslinking should be terminated by incubating with a glycine solution to prevent over-fixation of the sample.

Step 2: Cell Lysis



Lysis of sample cells is carried out to release the DNA-protein complex along with other nuclear components into a solution. Subsequently, the cytoplasmic components are degraded to eliminate background signal (noise). The choice of lysis method and reagent is based on the source of the sample, and the chemical and structural heterogeneity of proteins. For example, tissue samples require more stringent conditions and longer incubation times compared to cell lines.

Lysis buffers can contain a combination of detergents that specifically help lyse and isolate the nuclear fraction of the cell from cytoplasmic components. Crosslinking carried out in the previous step will prevent the detergent from degrading the protein-DNA complex; however, certain detergents can affect the stability of proteins. Therefore, the inclusion of protease and phosphatase inhibitors in the lysis cocktail, such as Boster's Protease Inhibitor Cocktail (Catalog# AR1182) or Phosphatase Inhibitor Cocktail (Catalog# AR1183), will help stabilize the structure and function of target protein.

Cell lysis is a critical step of the ChIP assay, which affects the amount and quality of chromatin recovery. Inefficient removal of cytoplasmic components will also prevent the accurate interpretation of assay results from background noise. In order to optimize cell lysis, it would be beneficial to visualize both lysed and pre-lysed samples under a phase contrast microscope. This would help confirm nuclear lysis.

Boster offers ready-to-use lysis buffers, such as IP Lysis Buffer (Catalog# AR0107), RIPA Lysis Buffer (Catalog# AR0105-100), Cell Lysis Buffer (Catalog# AR0103), which can achieve efficient lysis and protein extraction while avoiding protein degradation. For cells that are especially difficult to lyse, mechanical methods such as sonication may be used.

Optimization Tips

- Visualize cells under a microscope to verify if the nucleus has been released from the cells before proceeding with chromatin preparation.
- If cell lysis is especially hard to achieve, try more stringent lysis buffers and longer incubation times. When no other choice is available, brief sonication or glass dounce homogenizers may be used to achieve mechanical lysis.
- While using mechanical lysis methods, make sure that the cells are incubated on ice throughout the process. Perform time course experiments to determine the optimal time for sonication without causing protein degradation (recommended 30 seconds).

Step 3: Chromatin Preparation

The isolated nuclear fraction contains unbound nuclear proteins, whole chromatin and crosslinked protein-DNA complex. It is essential to fragment high molecular weight chromatin into smaller, workable pieces that render it accessible to antibody binding. The method of chromatin fragmentation is chosen based on the type of ChIP assay. Micrococcal nuclease digestion is essential for N-ChIP while both mechanical and enzymatic fragmentation can be used for X-ChIP assays.

Fragmentation of chromatin is a crucial step that determines the resolution of the ChIP assay, i.e., the strength of accurate determination of the protein-DNA binding site. Generally, fragments of size 200-1000 bp are preferred. However, the efficiency and uniformity of fragmentation will depend on the type of cell line, cell density, presence of crosslinking, and type of method (digestion vs sonication).

Enzymatic Digestion (N-ChIP or X-ChIP): Enzymatic digestion using micrococcal nucleases (Mnases) cleaves double stranded DNA at specific loci, rendering single monosomes (about 175bp). This method of fragmentation achieves uniform sized fragments that can be replicated between multiple experiments. In addition, enzyme digestion avoids the harsh conditions of mechanical fragmentation that can affect the integrity of the target epitope.

However, digested fragments are not completely random because nucleases have affinity toward certain genomic sequences over others. The method is also not ideal for analyzing transcription factors that bind between monosomes since these will be fragmented during digestion.

Optimal incubation time and enzyme concentration should be determined using time course experiments. Agarose gel electrophoresis with a suitable ladder can then be used to determine the conditions that render optimal DNA fragment size.

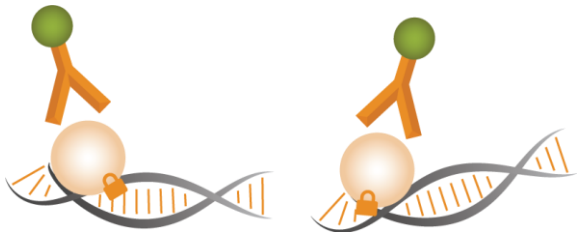
Sonication: Mechanical forces can be used to shear chromatin. These include both traditional probe sonicators or more advanced methods such as water bath sonicators. However, the method is less precise than enzyme digestion, delivering fragments of size ranging between 200-1000 bp. Fragmentation using sonication is difficult to replicate between experiments. Furthermore, heat generated during sonication in combination with stringent reagents can damage the target epitope.

Optimal sonication conditions depend on both cell type and experimental objective. It must be determined empirically using optimization experiments and gel electrophoresis in combination with IP. The optimal sonication time will deliver effective fragments without affecting antigen-antibody binding.

Optimization Tips

- Sonication is one of the most difficult steps to control and requires extensive optimization.
- Certain loci can be overrepresented due to affinity bias of nucleases. Therefore, fragment sizes must be compared using gel electrophoresis.
- For consistent digestion, fresh aliquots of enzyme should be prepared for every experiment.
- If formaldehyde crosslinking prevents access of enzyme to digestion site, switch to sonication.
- Sonication efficiency can be affected by foaming, crosslinking time, cell density and type. To avoid foaming, start at a low power setting before gradually increasing to higher settings.
- Frequent freeze-thaw cycles can damage chromatin integrity.
- If you are performing ChIP assay for the first time, it would be cost-effective to optimize chromatin fragmentation conditions in a small fraction of cells. Conditions that render fragments within 250-700 bp should be chosen.
- Sonication should always be carried out while immersing the sample in an ice bath to prevent overheating.

Step 4: Immunoprecipitation (IP)



Immunoprecipitation is the most critical functional step of the ChIP assay. It involves the affinity purification of a target protein using the corresponding antibody that is immobilized to a solid support such as magnetic or agarose resins. Immunoprecipitation selectively enriches target protein-DNA complex while eliminating other chromatin fragments.

The antibody-target protein-DNA complex is immobilized onto beads or resins in order to enable effective elution. Protein A beads (Catalog# B0001), Protein G beads (Catalog# B0002), and Protein A/G beads are popular bead choices.

High volume of beads can lead to non-specific binding; therefore, the concentration should be optimized for every experiment. While magnetic beads can be easily eluted, agarose beads offer greater binding efficacy due to their porosity. Beads containing immobilized protein A/G are generally preferred due to their combined affinity for a wide range of antibodies.

Choosing a reliable antibody with high affinity and specificity to the target epitope is crucial for effective immunoprecipitation. For mammalian cells, a range of ChIP validated antibodies may already be available. However, in the absence of a ChIP-grade antibody, antibodies must be chosen and validated based on the guidelines described in previous sections.

Antibody concentrations should be optimized for the sample cell type and target protein. Generally, it is recommended to start with concentrations ranging between 0.5-2.0 µg of antibody per 10 µg of chromatin DNA.

The chromatin fragments are incubated with the chosen antibody and beads for time intervals ranging between 2 hours to overnight. The bound antibody-antigen complex is then isolated using centrifugation or magnetic tube racks.

Washing of the beads is essential to remove non-antibody bound chromatin. The choice of washing buffers, their concentrations and washing times depend on the specificity of chosen antibody. This can be empirically determined using optimization experiments.

However, it should be noted that washing will not remove all non-bound chromatin fragments. Thus, it is important to include positive and negative controls to identify and eliminate background noise (described in previous sections). It is also recommended to test the efficiency of IP using qPCR before proceeding with expensive downstream analysis methods.

Optimization Tips

- **Antibody concentration:** Optimal antibody concentration should be calibrated for chosen antibody. Excessive concentrations can contribute to nonspecific binding while low concentrations can result in low enrichment of target protein.
- **Wash buffers:** The stringency of the washing buffer depends on the affinity of the chosen antibody. High stringent reagents can interfere with antibody affinity. Therefore, it is crucial to perform time course experiments with different salt concentrations (e.g. 250-500mM NaCl or LiCl). Optimal stringency is that which reduces background noise without interfering with the ability of the antibody to recognize the target epitope.
- High rpm (>6000rpm) centrifugation of sepharose beads can damage them.
- Antibody selection is the most crucial step of the ChIP assay. Remember to choose an antibody that is able to recognize the target epitope despite crosslinking and chromatin fragmentation.
- A good control antibody is IgG obtained from the same species as the ChIP antibody.
- 5% of sample before adding beads can be used as input control.
- High detergent concentrations will eliminate background noise better. However, it can have detrimental effects on antigen-antibody binding, resulting in low signal. Thus, it is important to achieve the right balance.
- Cell count ranging between 10^6 to 10^7 is recommended for effective immunoprecipitation. Low cell count can result in high background noise, poor signals, and the loss of an effective library generation. However, for rare cell samples, wherein it is harder to obtain large cell numbers, effective immunoprecipitation can still be achieved by using a number of adaptations (Cejas et al., 2016; Dirks et al., 2016; Gilfillan et al., 2012; Xiong et al., 2017).

Step 5: Crosslink Reversal (X-ChIP only) & DNA Purification

Following immunoprecipitation, it is essential to disassociate the chromatin fragment from antibody bound beads to proceed with downstream analysis. If using magnetic beads, elution can be easily carried out using appropriate reagents or magnetic tube racks. If using agarose resins or beads, the antibody-chromatin complex can be eluted using a combination of detergent based reagents (e.g. SDS) and heat. Peptide competition assays using peptides that bind to ChIP antibody with high affinity can also be used for purification, but the method is expensive.

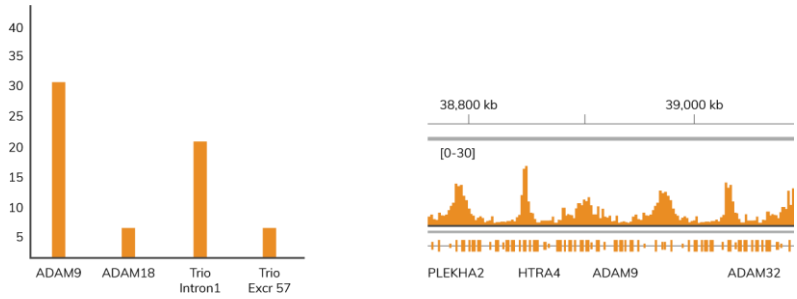
Following elution, crosslinks created between DNA and protein should be removed (X-ChIP only). Typically a combination of reagents including proteinase K (Catalog# AR0056) and heat is used to reverse crosslinks without damaging DNA fragments. Proteinase K cleaves at the carboxyl side of aliphatic, aromatic or hydrophobic residues in proteins. Thus, it can be used to reverse crosslinking while degrading proteins that cause background noise.

Purification of DNA fragments can be carried out using organic solvents such as phenol:chloroform. Column-based purification kits such as spin columns, magnetic DNA purification particles, and chelating agents can also be used. PCR-based purification methods have the most efficiency, but they are expensive.

Optimization Tips

- ChIP can be stopped at this step and the samples stored at -20°C. However, beware that frequent freeze-thaw cycles will damage the integrity of DNA fragments.
- Vortexing the beads while incubating with detergent will keep the beads in suspension and improve elution of DNA fragments.
- It is recommended to perform RNase digestion before adding Proteinase K in order to eliminate RNA from the sample which can cause background noise.

Step 6: DNA Analysis



ChIP-qPCR **ChIP-Sequencing**

After DNA purification, a range of downstream processing assays can be used to analyze protein-DNA interactions. However, regardless of the experimental objective, it is recommended to first quantify the DNA using quantitative PCR (qPCR).

DNA samples are incubated with specially designed primers, polymerase enzymes, oligonucleotides, and detection fluorophores. Since certain areas of the genome will purify better than others (euchromatin>heterochromatin), it is important to include a sample control using a primer designed for multiple random loci of the input genome. Following amplification, fluorescent signal can be used to quantify the sample.

Primers are crucial in determining qPCR efficiency. It is recommended to design primers that achieve at least 95% amplification. Moreover, primer design must eliminate the probability of dimer formation.

Enrichment of DNA fragments in the sample is not directly proportional to the concentration of the target protein bound *in vivo*. This is because immunoprecipitation can be affected by accessibility of epitope, chromatin environment, and experimental conditions. Therefore, the enrichment of target protein is always represented in proportion to input. Consequently, the concentrations of multiple target proteins in the same sample cannot be compared to *in vivo* concentrations.

The purified DNA fragments can be analyzed using a variety of downstream assays. For investigating a single locus, ChIP-PCR or ChIP-qPCR assays prove most cost-effective. However, for genome-wide analysis, ChIP-on-chip using can create a high resolution map of protein binding sites across the genome. ChIP-seq using NGS technologies can also be used to annotate genome-wide protein binding profiles.

Optimization Tips

- **Primer Design:** Primers that have a T_m of 58-60°C (with single peak melting curves) and a GC-content of 30-80% are recommended. In order to prevent primer dimerization, avoid runs of identical nucleotides. In addition, check that the end nucleotides do not contain more than 2 G/C bases.
- Too much input DNA concentration can affect qPCR efficiency**.** Optimal concentration can be empirically determined using serial dilutions.
- qPCR cycles >20 are not recommended.
- **Testing Primers:** While using new primers, primer efficiency should be tested and preferably fall between 90%-105%. Run a qPCR assay with serial dilutions of suitable samples of known concentration. Ideally, amplification should be two-fold in each cycle.
- **Primer Concentration:** Primer matrix experiments should be used to determine optimal primer concentration for both forward and reverse primers.
- **Choice of Detection Platform:** Downstream assays have varying sensitivities with ChIP-Seq producing lower noise than ChIP-on-chip. However, when only small quantities of input samples are available, ChIP-seq can be hindered by poor library complexity and unmappable reads. Recently, many ChIP-seq adaptations have been proposed for rare cell samples (Bolduc et al., 2016; Schmidl et al., 2015).

TROUBLESHOOTING GUIDE

In this section, we provide some troubleshooting tips for some of the most common issues that may happen when performing ChIP. Use the troubleshooting guide below to help identify possible reasons and optimize your experiment.

Issue	Possible reasons	Recommendations
LOW SIGNAL	Over-fragmentation of chromatin can lead to internucleosomal chromatin digestion.	Optimize sonication time to ensure chromatin fragments do not go under 500bp.
	Over-crosslinking can mask epitopes, reducing immunoprecipitation.	Do not crosslink for over 15 minutes. Wash the cells with PBS to remove any leftover reagent.
	Low cell-count.	Optimize starting cell material to isolate at least 25µg of chromatin.
	Low antibody concentration.	Optimize antibody concentration starting with 3-5µg.
	Over-washing may remove antibody-bound chromatin fragments.	Do not use washing buffers containing over 500mM NaCl.
	Ineffective cell lysis can result in low chromatin concentration.	Optimize both buffer and incubation time for efficient lysis. We recommend Boster's IP Lysis Buffer (Catalog# AR0107), RIPA Lysis Buffer (Catalog# AR0105-100), or Cell Lysis Buffer (Catalog# AR0103).
	The chosen antibody may be ineffective.	Include a positive control antibody, such as histone antibody, to verify the protocol.
	Due to weak interaction between protein and DNA, they may have disassociated during lysis.	Choose carefully between X-ChIP and N-ChIP.
	The epitope may be masked due to crosslinking.	Even after crosslinking has been optimized, the target epitope may be masked. Use polyclonal antibodies that can recognize multiple epitopes.
	Ineffective or non-selective binding to agarose beads.	Choose an agarose bead that efficiently binds to the antibody.
	Non-specific antibody binding.	Choose high specificity antibodies.
	Ineffective or non-selective binding to agarose beads.	Choose ChIP-grade beads. Include a pre-binding step where agarose beads are incubated with the sample to remove non-specific binding.

BACKGROUND NOISE	Interference due to reagents used in buffers.	Choose ChIP validated reagents and prepare fresh aliquots for each experiment.
	Ineffective chromatin fragmentation. Large chromatin fragments can cause background noise.	Optimize sonication times. When using enzymes, both concentration and incubation times should be optimized. Fragment size should range between 200-1000bp.
	High antibody concentrations can result in nonspecific binding.	Optimize concentration using control experiments.
	Nucleic acid contamination.	Run a no-DNA PCR reaction as a control to confirm no nucleic acids are present.
	Inadequate washing following immunoprecipitation resulting in contamination due to non-antibody bound chromatin fragments.	Increase the number of washes. You can also increase the salt concentrations in the washing buffers.
	Failure in PCR optimization.	Do not add high concentrations of DNA template to PCR. Additionally, the number of amplification cycles should be optimized.

Issue	Possible reasons	Recommendations
PCR ISSUES		
LOW AMPLIFICATION	Insufficient concentrations.	The concentrations of DNA template, primers and reagents should be optimized Concentration of starting cell material should also be optimized for efficient DNA recovery and amplification.
POSITIVE SIGNAL SEEN IN THE NO TEMPLATE CONTROL	PCR reagents may be contaminated.	Prepare fresh aliquots and repeat the experiment. All the equipment including pipettes should be sterilized.
NO AMPLIFICATION	DNA crosslinks may not have been sufficiently reversed. Over crosslinked cells can also be very hard to lyse. Insufficient crosslinking can result in the separation of the DNA-protein complex.	Optimize crosslinking reagent concentration and incubation times.
	PCR primers may be designed for inter-nucleosomal regions which have been digested during	Perform a positive control experiment and design a new set of primers.

	cell lysis.	
	Failure in immunoprecipitation may result in low recovery of chromatin.	Perform positive control to ensure IP is working.
	Ineffective chromatin fragmentation can result in large fragments.	Optimize fragmentation step.
	PCR reagents may not be working effectively.	Control PCR experiment can identify problems in protocol.
	Incomplete cell lysis.	Perform multiple experiments by varying parameters in order to optimize cell lysis. Mechanical lysis methods can be used in hard to lyse cells.
	Incomplete elution of chromatin from beads.	Carry out elution with consistent mixing so that the beads remain suspended. Never let the beads dry out. Always store them at 4°C.

Issue	Possible reasons	Recommendations
IMMUNOPRECIPITATION ISSUES		
FAILURE IN IMMUNOPRECIPITATION	Over-crosslinking can mask epitopes for antibody recognition.	Perform a time course experiment ranging between 2-30 minutes to optimize crosslinking time.
	Ineffective reversal in crosslinking.	Optimize reversal time and reagent concentration. At least a 15 minute incubation with proteinase K at 95°C is recommended.
	Low chromatin concentration.	Optimize starting cell material. A minimum of 5-10 µg of chromatin is required for efficient IP.
	Damage due to over-lysis of cells.	Optimize both sonication conditions and cycles to deliver optimal size of chromatin fragments without damage to chromatin sequence.
	Over-fragmentation of chromatin.	Over-fragmentation can damage chromatin sequences by masking epitope. Optimize fragmentation conditions.
	Chromatin degradation.	Chromatin may be degraded due to stringent sonication conditions. Ensure the cells are always incubated in ice and keep sonication steps to a minimum.

	Low antibody concentration.	Optimize antibody concentration starting with 1-10 µg of antibody.
	Over-washing can remove antibody-bound chromatin.	Reduce the wash cycles and stringency of wash buffers.
NO BAND IN POSITIVE IP EXPERIMENT	Not enough chromatin or antibody. Antibody incubation time may be too low.	A chromatin concentration of 5-10 µg is recommended for 10 µl antibody. Although a 4 hour incubation may be sufficient for high selectivity antibody, overnight incubation is recommended.

FREQUENTLY ASKED QUESTIONS (FAQs)

1. **Should I crosslink my sample?**

   It is important to choose carefully between N-ChIP and X-ChIP. Generally, histone proteins do not need to be crosslinked. DNA-binding proteins with weaker affinities need to be crosslinked to prevent their separation during cell lysis. However, crosslinking may mask certain epitopes in the chromatin to antibody binding, so we recommend performing a control experiment before making a choice. Crosslinking is also a time control experiment; therefore, be sure to optimize the incubation time.

2. **How can I achieve uniform chromatin fragments?**

   When using enzymes for chromatin fragmentation, use fresh aliquots for every experiment because enzymes may degrade due to storage. It is harder to achieve uniform sized fragments with the mechanical fragmentation method. We recommend including a control at every step to overcome the side effects of varied fragment sizes.

3. **How do I choose an antibody?**

   If no ChIP-grade antibodies are available, antibodies that have worked with other immunoprecipitation experiments will also work. Even when ChIP-grade antibodies are available, it is recommended to characterize their specificity and efficiency using control experiments. Ideally, the chosen antibody should be affinity purified to avoid background noise.

4. **What controls should I use for qPCR?**

   Enzymes used for chromatin fragmentation may show bias towards certain sequences over others. Nucleosomes may also rearrange during enzyme fragmentation. Therefore, it is important to generate a varied library using primers designed towards several loci on the genome. Furthermore, a positive control using cell lysate can help remove background noise.

5. **How do I choose buffers for ChIP assays?**

   It is recommended to use buffers that have previously been validated in ChIP experiments. High stringency buffers can provide cleaner results by reducing background noise and contamination. However, keep in mind that high stringency may damage chromatin structure.

6. **How can I verify my ChIP protocol and reagents are working?**

   Use a positive histone control sample to verify the ChIP reagents are working.

7. **How can I optimize elution from beads?**

   Make sure the agarose beads are not centrifuged at an rpm greater than 6,000 rpm as this can lead to compression of beads and its damage.

8. **How to choose between mechanical fragmentation and enzymatic fragmentation?**

   No matter what method you choose, it is recommended to achieve fragments of size 200-1000 bp. Sonication is the preferred method to shear chromatin as it does not interfere with internucleosomal protein interactions. However, over-sonication can damage chromatin structure and sequence. Furthermore, while investigating proteins that do not strongly bind to DNA, over-sonication can lead to separation of the protein-DNA complex. Therefore, if you choose sonication for your experiment, ensure you optimize the parameters. Enzymatic digestion is gentle and does not affect the structure of chromatin. It is recommended to choose enzymatic digestion while investigating proteins or transcription factors that are rare and bind less stably with DNA. Enzymatic digestion is also reproducible within experiments when compared to sonication which produces completely random fragments. It should be

noted that enzymatic digestion cannot be used while investigating proteins that bind to internucleosomal loci.

9. **Should I use mechanical fragmentation even while carrying out micrococcal nuclease digestion?**

   In some cases, especially while using tissues, enzymatic digestion is not sufficient to carry out chromatin fragmentation. A brief sonication before enzyme digestion can lyse cells and enable enzymes to efficiently permeate them. In other cases, a brief sonication following enzyme digestion can help lyse the nucleus and release the chromatin fragments into the lysate buffer. However, keep in mind that you should not over-sonicate the sample, which may damage the chromatin.

10. **What do I do when no ChIP-grade antibody is available for my chosen protein?**

    Expressing your chosen protein with a fusion tag that can be easily immunoprecipitated using a well-validated antibody is helpful when no efficient antibody is available. However, it should be noted that overexpression of protein can result in false positive interactions. In these cases, it is recommended to carry out a control IP using non-transfected cells for comparison. Sometimes, the tag can interfere with protein interactions, so every tag should be tested on a case-by-case basis.

11. **Should I quench crosslinking?**

    Quenching is carried out by incubating the crosslinked cells with glycine. Some protocols may stop with washing out the crosslinking buffer in order to stop and prevent crosslinking. However, since crosslinking can seriously interfere with the quality of immunoprecipitation, it is recommended to carry out quenching.

12. **How do I choose the right beads for immunoprecipitation?**

    Since protein A and G have a wide range of affinities and specificities, they are recommended for immunoprecipitation. However, each protein has its own specific benefits. We recommend using an appropriate blend of A and G beads in order to utilize the advantages of both beads.

13. **How do I choose the right equipment for sonication?**

    The right sonicator is chosen based on the type of cell or tissue. The size of the probe tip is chosen based on the density and surface volume of the cell medium. For high surface tension liquids, a solid probe is recommended. The depth of the probe should be chosen appropriately to avoid foaming.

14. **Where should the probe be placed during sonication?**

    While the probe tip should be completely submerged in the culture medium, it should not touch the sides or bottom of the tube. The probe tip should be fixed with a clamp to prevent it from moving during sonication.

15. **How do I avoid damage to my cells during sonication?**

    Keep the cells submerged in an ice bath throughout the sonication procedure. Optimize sonication cycles to achieve maximum fragmentation with minimal damage to fragments (you can perform IP to test if the target epitope has been damaged). No matter how many sonication cycles are chosen, each cycle should never exceed 30 seconds.

16. **How do I check if my chromatin has been sufficiently fragmented?**

    Following sonication, it is always recommended to run an agarose gel electrophoresis to test the quality and efficiency of chromatin fragmentation. The sonication protocol that delivers fragments ranging between 200bp to 1Kb with an average of 500bp should be chosen.

17. Is nuclei isolation necessary after cell lysis?

Nuclei lysis is required to release the crosslinked chromatin into the cell lysate. Furthermore, isolating the nuclear lysate from the cytoplasmic component can reduce background noise. We recommend our Cytoplasmic and Nuclear Protein Extraction Kit (Catalog# AR0106) which provides a complete set of extraction reagents that enable the separation of nuclear protein and cytoplasmic fractions from cultured cells and fresh tissues.

18. Why should the chromatin fragments be less than 1000 base pairs?

Uniform sized fragments smaller than 1000bp ensure good resolution for ChIP assays. If the chromatin fragments are larger, your assay could show false positives. For example, although the protein may bind the fragment, its interaction site could be very distant from the target loci being analyzed.

19. How do I verify chromatin fragments have been released from the nucleus?

Use a phase contrast microscope to examine if the nucleus has been lysed. This is the most cost-effective way to confirm cell lysis.

20. What is the optimal concentration of cells for ChIP assays?

Generally, it is recommended to use ten million cells as starting material. For proteins that are rare, 107 cells is recommended. However, the concentration can be chosen based on the amount of DNA required for downstream analysis. While qPCR requires about 105 cells, more complex array assays such as ChIP-chip require over 108 cells.

21. How should the assay protocol be adopted for use in tissues?

ChIP assays have been successfully used for both frozen and fresh tissue samples. Protocol adaptations include increasing formaldehyde incubation time. Tissue homogenization, followed by brief sonication, can help deliver formaldehyde more effectively to the cells. For rare tissue samples, when only a low number of cells are available, several modified protocols have been described (Dahl and Collas, 2008; Rehimi et al., 2017).

22. What is considered a good enrichment in IP?

The pulldown amount typically depends on the abundance of target protein. More abundant proteins will pull down higher concentrations of chromatin complex. However, it is recommended to achieve at least a five-fold enrichment to ensure a successful experiment.

23. How should I quality check my qPCR primers?

qPCR primers can be tested by running quality control experiments with serial dilutions of a sample of known concentration.

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