

# A step-by-step guide to successful chromatin immunoprecipitation (ChIP) assays

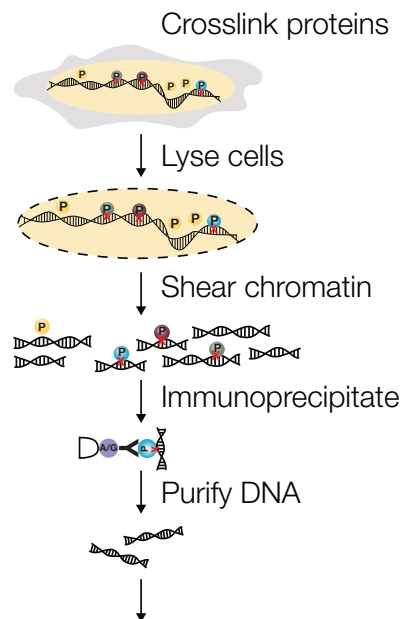
## Introduction

Epigenetics is the study of heritable changes in gene expression that modify DNA, RNA, and protein but do not alter the nucleotide sequence. One common mechanism of regulating epigenetic states is posttranslational modification (PTM). Among the many proteins that are subject to PTM are the histone family of proteins. Histones package genomic DNA into nucleosomes, which enable approximately 2 meters of DNA to fit into a cell's nucleus. The nucleosome contains two subunits, each made of histones H2A, H2B, H3, and H4. Additionally, there is histone H1, which is often called the linker histone. The most prevalent PTMs found on histones are methylation, acetylation, phosphorylation, and ubiquitination.

Chromatin immunoprecipitation (ChIP) is a common technique for studying epigenetics, as it allows the researcher to capture a snapshot of specific protein–DNA interactions. With the advent of next-generation sequencing (NGS), ChIP has become even more powerful—researchers can now get a snapshot of not only specific protein–DNA interactions in a few regions but also genome-wide interactions, by adapting purified ChIP DNA for NGS, called ChIP-sequencing (ChIP-Seq). This snapshot is achieved by crosslinking the DNA and the protein in live cells, then extracting and shearing the chromatin. Finally, the samples are immunoprecipitated with an antibody targeting the protein of interest. The DNA is extracted from the protein and can be evaluated either at specific regions of the genome by quantitative PCR (qPCR) or genome-wide by NGS.

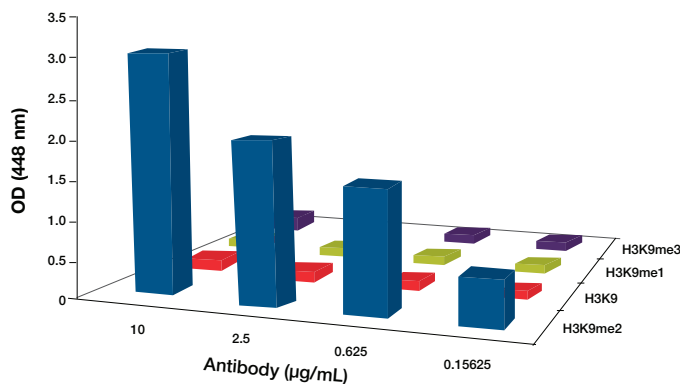
## Choosing an antibody for ChIP

There are two major considerations when choosing an antibody for ChIP—whether it will work in ChIP and whether it is specific. Ideally, the target will have an antibody that has already been shown to work in ChIP or in another immunoprecipitation (IP) application. Monoclonal,



oligoclonal (pools of monoclonals), and polyclonal antibodies all can work in ChIP. The key requirement is that the specific epitope of interest be exposed. One of the advantages of using a monoclonal antibody is that generally it is more specific, but this is associated with a higher likelihood that the one epitope it recognizes is buried. Unless monoclonals are specifically screened or designed for use in ChIP, oligoclonal and polyclonal antibodies are better candidates for recognizing target proteins, as they recognize multiple epitopes of the targets. An alternative approach, if the target of interest does not have an antibody that works for ChIP, is to tag the target with Myc, His, human influenza hemagglutinin (HA), T7, GST, or V5.

The second important consideration in ChIP assays is the specificity of the antibody, because nonspecific antibodies can skew results and your understanding of a target. Although you want your antibody to recognize only your target of interest in all applications, ChIP compounds this issue. For example, if you are interested in knowing where H3K9me2 protein is bound on the genome, you want to ensure that you are pulling down only the dimethyl (me2) mark and not the monomethyl (me1) or trimethyl (me3) mark. H3K9me2 is generally a repressive mark, but H3K9me1 is generally an activating mark. Thus, if the antibody you are using also recognizes H3K9me1 even at a tenth of the stringency that it recognizes H3K9me2 at, the data you are generating will be misleading to your understanding of H3K9me2 (Figure 1). Using an ELISA, we demonstrate that our H3K9me2 antibody (Cat. No. 701783) specifically recognizes the dimethyl mark. This demonstrates the importance of having a very specific antibody for your target. The more specific the antibody, the more robust and accurate your results will be.



**Figure 1. Cross-reactivity in ELISA for Invitrogen™ ABfinity™ anti-H3K9me2 rabbit recombinant monoclonal antibody (Cat. No. 701783).** The assay was performed against H3K9me2, H3K9me1, H3K9, and H3K9me3 by coating the respective antigens at 0.004 µg/mL. The antibody was added at 10, 2.5, 0.625, and 0.156 µg/mL and detected using Invitrogen™ Goat Anti-Rabbit IgG (H+L) Secondary Antibody, HRP conjugate (Cat. No. G21234, 1:5,000 dilution). The plate was developed using Invitrogen™ TMB Stabilized Chromogen (Cat. No. SB02).

Thermo Fisher Scientific offers both agarose (Cat. No. 26156) and magnetic (Cat. No. 26157) ChIP kits that contain most of the reagents necessary for ChIP.

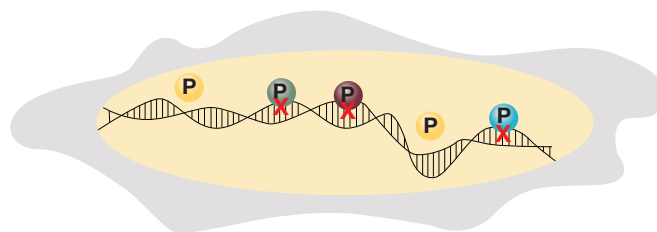
#### A few more considerations before starting ChIP:

- What is the question you are asking? Can it be answered by looking at, for example, ten loci, or do you need genome-wide information? ChIP-Seq is very powerful but also requires significant bioinformatic expertise. If you are unsure, you can start by looking at a handful of loci and later choose to create a ChIP-Seq library if genome-wide information will be useful.

- **Controls are essential for ChIP.** Whether you are comparing the same cell lines or different cell lines and treatments, you need a “no-antibody control” (mock IP) for each IP you are doing. Other controls to consider in order to determine if your ChIP experiment worked:
  - Know a region of DNA that should be enriched in your IP and be amplified by qPCR, to show that your ChIP worked (positive control)
  - Know a region of DNA that you do not expect to be enriched and thus do not expect to be amplified by qPCR, to show that your ChIP is specific (negative control)
- For a standard protocol, you want approximately  $2 \times 10^6$  cells per immunoprecipitation. Recent publications, however, have performed ChIP with multiple orders of magnitude fewer cells.

### The ChIP procedure

#### Step 1: Crosslinking



ChIP assays begin with covalent stabilization of the protein–DNA complexes. Many protein–DNA interactions are transient and involve multiprotein complexes to orchestrate biological functions. As there is constant movement of proteins and DNA, ChIP captures a snapshot of the protein–DNA complexes that exist at a specific time. *In vivo* crosslinking covalently stabilizes protein–DNA complexes.

Researchers can often use a combination of crosslinkers to trap interacting proteins and DNA. These crosslinkers permeate directly into intact cells and effectively lock protein–DNA complexes together, allowing even transient complexes to be trapped and stabilized for analysis. These crosslinkers must be reversible to be used for ChIP.

*In vivo* crosslinking is traditionally and often performed using a formaldehyde solution (Cat. No. 28906), either alone or in combination with other crosslinkers such as Thermo Scientific™ Pierce™ EGS (ethylene glycol bis(succinimidyl succinate), Cat. No. 21565) or DSG (disuccinimidyl glutarate, Cat. No. 20593). Formaldehyde

crosslinking is ideal for two molecules that interact directly. However, formaldehyde is a zero-length crosslinker, limiting its functionality. For higher-order interactions, longer crosslinkers such as EGS (16.1 Å) or DSG (7.7 Å) can trap larger protein complexes with complex quaternary structures. For some histones, native ChIP can be used because the protein–DNA interaction (e.g., H3–DNA and H4–DNA) is inherently very tight, making crosslinking unnecessary.

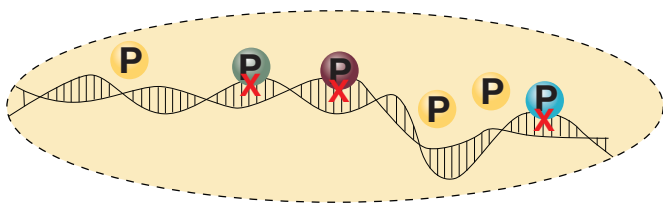
**Note:** This is a point where ChIP can be stopped. After crosslinking, quenching, and washing the cell pellet, it can be stored at –80°C.

#### Tips for crosslinking:

- Determine what type of interactions you are looking for, to decide on what crosslinker to use.
- The duration of the crosslinking step is important. Too little can result in inefficient crosslinking, and too much can result in difficulty lysing and shearing the chromatin to usable sizes. Make sure you quench your crosslinking reaction to ensure that you crosslink only for the desired time.

#### Step 2: Cell lysis

In this step, cell membranes are dissolved with detergent-based lysis solutions to liberate cellular components, and crosslinked protein–DNA complexes are solubilized.



Because protein–DNA interactions occur primarily in the nuclear compartment, removing cytosolic proteins can help reduce background signal and increase sensitivity. The presence of detergents or salts will not affect the protein–DNA complexes, because the covalent crosslinking in step 1 will keep the complexes stable throughout the ChIP procedure. Protease and phosphatase inhibitors (Cat. No. 88668 and 78440) are essential at this stage to maintain intact protein–DNA complexes.

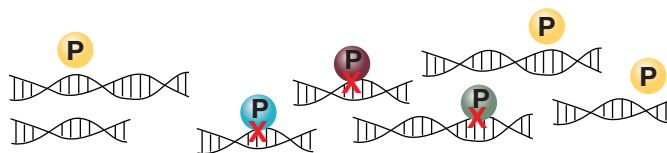
Although mechanical lysis of cells is not recommended, as it can result in inefficient nuclear lysis, some cell types are difficult to lyse by any other method. Reagents such as the Thermo Scientific™ Pierce™ Chromatin Prep Module

(Cat. No. 26158) isolate the nuclear fraction from other cellular components. The module is used to eliminate background signal and enhance sensitivity.

#### Tips for cell lysis:

- Successful cell lysis can be visualized under a microscope. Take a 10 µL sample before and after lysis, and using a hemocytometer, examine the whole cells versus the nuclei.
- The extent of cell lysis can vary depending on the cell type. If the chromatin is not recovered from the nucleus, a more stringent lysis is required. This can be achieved by increasing the incubation time in the lysis buffer, performing a brief sonication in lysis buffer, or using a glass dounce homogenizer.
- If you are using sonication, keep your chromatin on ice at all times and do not pulse for more than 30 seconds at a time to ensure that proteins are not denatured due to excessive heat.

#### Step 3: Chromatin preparation (shearing/digestion)



The extraction step yields all nuclear material, which includes unbound nuclear proteins, full-length chromatin, and the crosslinked protein–DNA complexes. In order to analyze the protein-binding sequences, the extracted genomic DNA must be sheared into smaller, workable pieces. DNA fragmentation is usually achieved either mechanically by sonication or enzymatically by digestion with Thermo Scientific™ micrococcal nuclease (MNase) (Cat. No. 88216).

Ideal chromatin fragment sizes range from 200 to >700 bp; however, DNA shearing is one of the most difficult steps to control. Sonication provides truly randomized fragments, but limitations include:

- The requirement for dedicated machinery, which may need tuning
- Difficulty in maintaining temperature during sonication
- Extended hands-on time
- Extensive optimization steps

Enzymatic digestion with MNase is highly reproducible and more amenable to processing multiple samples. However, enzymatic digestion can lead to variability due to changes in enzyme activity. The enzyme has higher affinity for inter-nucleosome regions and is less random.

**Note:** This is a point where ChIP can be stopped. After shearing/digestion of the chromatin, it can be stored at  $-80^{\circ}\text{C}$ .

#### Tips for shearing or digestion of chromatin:

- Determine shearing or digestion conditions on a fraction of your cells if this is your first time using this cell line. To do this, use lysed cells and run a time course with different numbers of sonication pulses or MNase incubation times. Next, analyze the DNA by treating the chromatin in salt with Invitrogen™ Proteinase K solution (Cat. No. 25530049) at  $65^{\circ}\text{C}$  followed by Invitrogen™ PureLink™ RNase A (Cat. No. 12091021). The DNA can then be cleaned up using either a column or phenol-chloroform extraction, followed by analysis on an agarose gel. Over time you will see reduction in DNA size; choose the time point that has the most DNA in the 250–700 bp range.
- Regardless of the method you choose for shortening your chromatin fragments, confirm that the chromatin length is in the ideal range for every experiment.

#### Step 4: Immunoprecipitation



To isolate a specifically modified histone, transcription factor, or cofactor of interest, ChIP-validated antibodies are used to immunoprecipitate and isolate the target from other nuclear components. This step selectively enriches for the protein–DNA complex of interest and eliminates all other unrelated cellular material.

Selection of the appropriate antibody is critical for successful ChIP assays. Numerous ChIP-validated antibodies are available. For target proteins for which qualified antibodies are unavailable, proteins fused to affinity tags such as HA, Myc, His, T7, V5, or GST can be expressed in the biological samples, and then antibodies against the affinity tags can be used to immunoprecipitate the targets.

The antibody–protein–DNA complex is affinity purified using an antibody-binding resin such as immobilized protein A, protein G, or protein A/G. To reduce background, preclear the lysate by first incubating the lysate with the beads for several hours before adding the antibody. The volume of beads used in each ChIP sample can also influence background, as the increase in bead volume increases nonspecific binding.

After extended incubation, the bead–antibody–protein–DNA complex must be extensively washed and often purified sequentially with low- and high-salt buffers. The protein–DNA complex is then eluted.

#### Bead choice:

- **Magnetic vs. agarose beads**—The advantages of magnetic beads are easy separation and easy visibility of the beads in the tube, leading to less loss of material and highly reproducible results. Agarose beads, however, have a higher capacity for binding due to their porous nature and thus higher surface area. In most cases, bead choice comes down to the preference of the researcher.
- **Protein A vs. protein G vs. protein A/G**—The combination of protein A/G is ideal for most labs. Protein A has the highest affinity for rabbit polyclonal antibodies, and protein G has the best affinity for a range of antibodies. Protein A/G combines the affinities of both protein A and G without reducing the affinity of either for the antibody.

Protein	Type	Cat. No.
Protein A/G	Magnetic	26162
Protein A/G	Agarose	20421
Protein G	Magnetic	88847
Protein G	Agarose	2398
Protein A	Magnetic	88845
Protein A	Agarose	20333

#### Tips for IP:

- Selection of the antibody is very important. Not all antibodies work for ChIP. As your protein of interest is crosslinked to DNA, the epitope that the antibody recognizes needs to be exposed and not buried in the protein–DNA complex. Thus, ideally, you want an antibody that has been shown to work in ChIP, IP, or other application.

- The amount of antibody you use per amount of chromatin also plays a role in ChIP efficiency. Both too little and too much antibody can result in less enrichment of your target of interest.
- Save approximately 5% of your sample before adding beads. This portion will serve as your input control.
- Washing after IP is exceptionally important. Insufficient washing can result in nonspecific signal.

### Step 5: Reversal of crosslinking, and DNA clean-up



Enrichment of DNA bound to the protein of interest is the goal for ChIP. Before the specific DNA products of a ChIP experiment can be quantified, the crosslinks between protein and DNA must be reversed. This is typically done through extensive heat incubations and/or digestion of the protein component with Proteinase K (Cat. No. 25530049).

Proteinase K cleaves at the carboxy site of aliphatic, aromatic, or hydrophobic residues. Because of its broad specificity, Proteinase K is often used to remove proteins from DNA or RNA preparations. Additionally, digestion with Proteinase K eliminates nucleases from the purified DNA, which prevents degradation. Treatment with RNase A (Cat. No. 12091021) is recommended as well to obtain a more pure DNA sample, and is necessary if you are performing ChIP on yeast. A final purification of the DNA from any remaining proteins should be performed using phenol-chloroform extraction or spin columns designed for DNA purification.

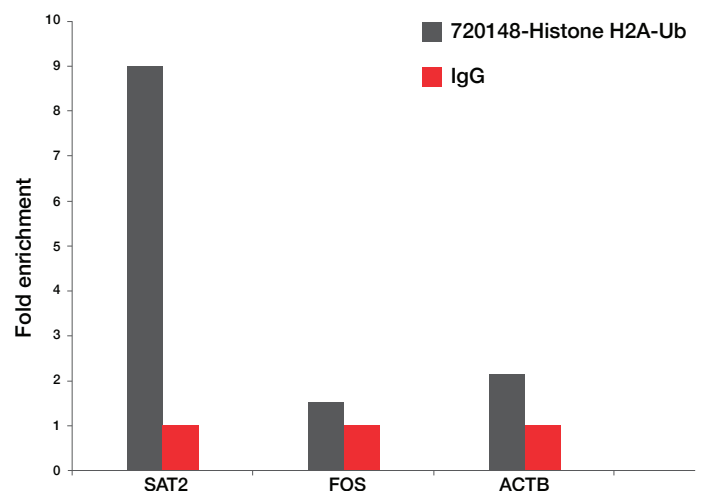
**Note:** This is a point where ChIP can be stopped. After reversal of crosslinks and/or DNA purification, samples can be stored at  $-20^{\circ}\text{C}$ .

### Step 6: DNA quantitation

One of the hallmarks of ChIP is the ability to quantitate the purified DNA products by qPCR; an ideal product for this is Applied Biosystems™ PowerUp™ SYBR™ Green Master Mix (Cat. No. A25742). qPCR enables analysis of target protein–DNA complex levels in different experimental conditions. Figure 2 shows enrichment of histone H2A-Ub by ChIP, where the purified DNA was first analyzed by qPCR for the presence of specific promoter regions before performing ChIP-Seq on the enriched protein. There is a direct correlation between the amounts of immunoprecipitated complex and bound DNA. The purified DNA can be further processed to create an NGS library for ChIP-Seq.

#### Tips for qPCR:

- Design primers to amplify between 100 and 250 bp, with  $T_m$  between  $50^{\circ}\text{C}$  and  $65^{\circ}\text{C}$  and Gs or Cs ideally at the ends of the primers.
- Confirm a single peak on the melting curves.
- Check the efficiency of your primers (if this is the first time using this set of primers). Ideally you want primer efficiency to be between 90% and 105%.
- Even if your experiment is ChIP-Seq, check a few regions of interest by qPCR to confirm that the enrichment worked, before sending your ChIP DNA for sequencing.



**Figure 2. Enrichment of endogenous histone H2A-Ub using anti-histone H2A-Ub rabbit polyclonal antibody.** ChIP was performed using Invitrogen™ anti-H2A-Ub rabbit polyclonal antibody (Cat. No. 720148) on sheared chromatin from  $2 \times 10^6$  HeLa cells using the Applied Biosystems™ MAGnify™ Chromatin Immunoprecipitation System (Cat. No. 49-2024). Normal rabbit IgG was used as a negative IP control. The purified DNA was analyzed on the Applied Biosystems™ 7500 Fast Real-Time PCR System (Cat. No. 4351106) with optimized primers for the region of the inactive SAT2 satellite repeat, used as a positive control target, and for promoters of the active cFOS (FOS) and beta-actin (ACTB) regions, used as negative control targets. Data are presented as fold enrichment of the antibody signal vs. the negative control IgG, calculated using the comparative  $C_t$  method.

## Analyzing your qPCR data to determine if your ChIP worked

- First you need to calculate percent input. The following example assumes the use of a 1% input in your qPCR reaction compared to your ChIP DNA. For the dilution factor (DF) you use the number of cycles that corresponds to the actual dilution factor; for example, for a dilution factor of 100 (based on 1% input), DF is 6.64 cycles ( $\log_2 100$ ).
- Next you need to normalize  $C_t$  to input:  

$$\Delta C_t = C_t [\text{IP}] - (C_t [\text{input}] - \text{DF})$$
- Then calculate the yield or percent input:  

$$\text{Yield (\%)} = (\text{primer efficiency})^{-\Delta C_t} \times 100\%$$
- For fold enrichment, normalize fold change to the no-antibody (IgG) control:  

$$\Delta \Delta C_t = \Delta C_t [\text{IP}] - \Delta C_t [\text{IgG}]$$
- Finally, calculate the fold enrichment:  

$$\text{Fold enrichment} = (\text{primer efficiency})^{-\Delta \Delta C_t}$$

The table below uses the formulas for calculating percent yield and fold enrichment for SAT2 in the H2A-Ub ChIP experiment illustrated in Figure 2. The primer efficiency in this example is 2, and 1% input was used for the qPCR reaction.

SAT2	$C_t$	$\Delta C_t$	Yield (%)	$\Delta \Delta C_t$	Fold enrichment
1% input	24.01	6.64	1.00	NA	NA
IgG	29.63	12.26	0.02	0	1
IP	26.46	9.09	0.18	-3.17	9

## References

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