A GUIDE TO SUCCESSFUL CHROMATIN IP AND CHROMATIN IP SEQUENCING

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ChIP and ChIP-seq

Chromatin Immunoprecipitation is used to examine interactions between protein and DNA within the natural chromatin context of the nucleus. This guide highlights critical steps of both sonication- and enzymatic-digestion-based ChIP protocols and demonstrates how antibody validation and protocol changes can affect the final outcome of your experiment.

Cell Signaling TECHNOLOGY®

Introduction

Chromatin Immunoprecipitation (ChIP) is used to examine interactions between protein and DNA within the natural chromatin context of the nucleus. ChIP experiments require fixing the cells, which cross-links protein-DNA interactions into place. The chromatin is then broken into fragments and an antibody is used to immunoprecipitate (IP) the protein of interest along with any bound DNA. Finally, the cross-linking is reversed and the precipitated DNA is purified. The purified DNA can be subjected to further analysis, such as standard or quantitative PCR (qPCR, ChIP-qPCR) or next generation sequencing (NGS, ChIP-seq).

These experiments are sensitive to the integrity of the chromatin, the stability of the protein epitope, and the specificity of the immunoprecipitating antibody. These variables become even more significant when the protein-DNA interaction under investigation is of low abundance and/or low stability.

High abundance, stable interactions, like those between histones and DNA, occur often enough that they may still be detected even if the signal-to-noise ratio is less than ideal. In contrast, low abundance or less stable interactions (e.g., transcription factors, cofactors) may fall below the limit of detection if the protocol fails to safeguard the integrity of the protein and the DNA, or if it relies on an antibody with lower affinity to the target of interest. An optimized ChIP protocol will protect the specific signal, limit the background noise, and allow the interaction of interest to shine through.

This guide highlights critical steps for both sonication- and enzymatic-digestion-based ChIP protocols and demonstrates how antibody validation and protocol changes can affect the final outcome of your experiment.

The Importance of a Well-validated Antibody

Antibodies that are not highly specific may bind to unintended targets and increase the background noise, which can make it more difficult to detect less abundant or lower stability interactions.

Here are the steps we take at CST to help ensure your antibody will work as expected in ChIP-qPCR and ChIP-seq. This list can also be used as a general guide to ensure your antibody has been properly validated for use in these applications.

Target Specificity

- Expected detection of protein in positive/negative control cell lines, knockout cells, or siRNA-treated cells
- Appropriate localization in other applications (immunohistochemistry, immunofluorescence) using positive/negative controls
- Appropriate detection of protein in response to specific activators and/or inhibitors
- Histone modification antibody specificity and potential steric hindrance resulting from neighboring modifications determined using histone peptide arrays

Signal-to-Noise Ratio

- Assessed using isotype controls to ensure acceptably low background (noise) signal
- Enrichment of known target genes at least 10-fold above background for histones and 4 to 5-fold for a transcription factor or cofactor, as shown by real-time qPCR analysis

Importantly, good performance in ChIP-qPCR doesn't guarantee an antibody will perform well in ChIP-seq:

- An antibody may not bind well enough to enrich all weaker, less stable sites across the entire genome
- Non-specific antibody interactions generate more background in ChIP-seq; an antibody must show good signal-to-noise ratio across the entire genome
- Antibody epitopes may be masked or partially masked at some gene loci depending on other DNA-bound proteins and the extent of masking can vary across different gene loci

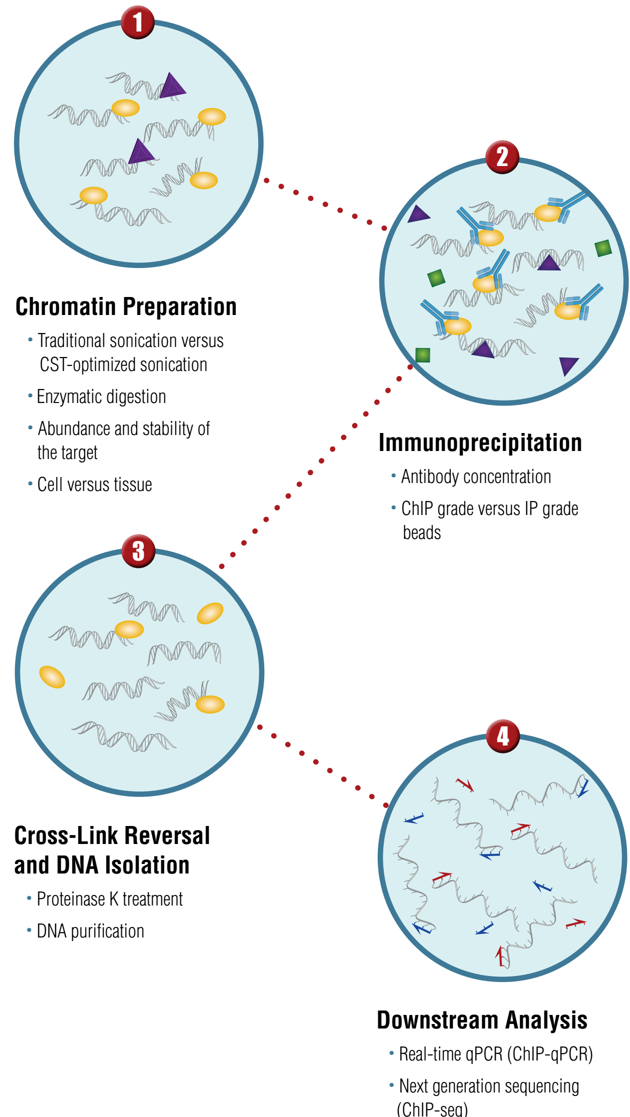
Here are some specific considerations for validating antibodies for ChIP-seq:

- Sufficient number of peaks identified and good signal-to-noise ratio across the genome
- DNA-binding motif analysis for transcription factors
- Comparison to reference data sources like the Encyclopedia of DNA Elements (ENCODE) Project (National Human Genome Research Institute)

Note: Antibodies that have been approved for ChIP-seq by CST are also validated using competitor antibodies against the same target, antibodies to other subunits of a shared protein complex, or antibodies for other targets that bind the same gene loci.

4 Step Protocol to a Better ChIP Experiment

This guide offers insights and explanations for the most critical steps of a ChIP protocol to help you get the most reliable results from your ChIP experiments.



Recombinant Rabbit Monoclonal Antibodies are better than Polyclonal Antibodies for ChIP

According to our internal testing and a study led by researchers at the Broad Institute at MIT, rabbit monoclonal antibodies are a better choice for your ChIP experiments:*

- *Higher specificity than polyclonal antibodies*
- *Renewable source*
- *Better lot-to-lot reproducibility*

**Busby, et al., (2016) Epigenetics & Chromatin 9:49*

Be in Control of Your ChIP Experiment

Adding positive and negative control antibodies to your experimental workflow allows you to have confidence that your assay is working properly and that your results are reliable.

Positive Controls

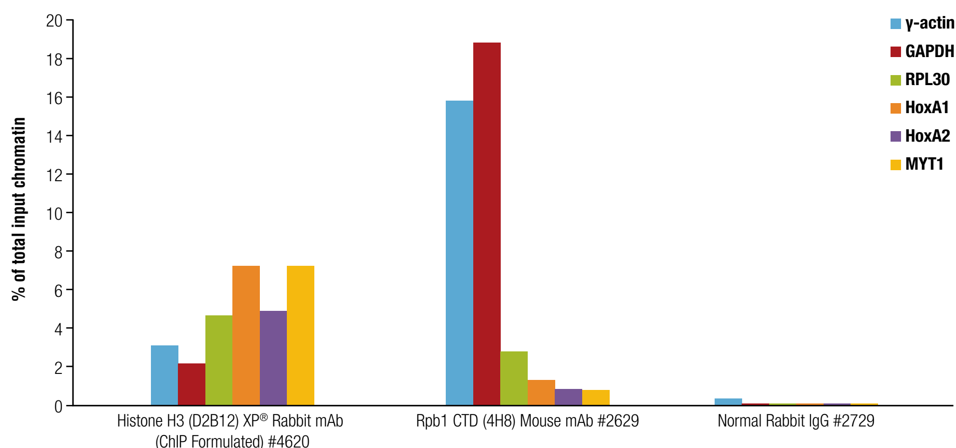
Many commercially available kits are supplied with an antibody to Rpb1 (the largest subunit of RNA polymerase II), for use as a positive control antibody. However, Rpb1 is only highly enriched at sites of active transcription, so if your locus of interest is an inactive site, Rpb1 will not provide a positive control.

CST recommends using Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated). This antibody detects all variants of histone H3 (H3.1, H3.2, H3.3, CENP-A), which are bound to all DNA sequences in the genome. Thus, this antibody provides a universal positive control for your ChIP experiments, independent of the activation status of the locus under examination.

The differences between Rpb1 and Histone H3 are demonstrated below. Rpb1 CTD (4H8) Mouse mAb #2629 enriches transcriptionally active loci like RPL30 and GAPDH, but does not enrich less active loci like HoxA1, HoxA2, or MYT1. In contrast, the Histone H3 antibody enriches all loci tested, indicating it can be used as a universal positive control for your ChIP experiments **(Figure 1)**.

H3 is a more universal positive control than Rpb1

Figure 1: ChIP was performed with 10 µg of cross-linked chromatin and the indicated antibodies. The enriched DNA was quantified by qPCR, using primers to the indicated loci, and is presented as a percent of the total input chromatin.



Negative Controls for ChIP-qPCR

Negative control antibodies, like normal rabbit IgG, do not recognize specific epitopes and are therefore useful for measuring non-specific binding during the IP. For example, if the amount of product in the negative control sample is equal to the amount of product in the target-specific sample, then you can conclude that your target-specific antibody is not enriching the target of interest. This result, in combination with a positive histone H3 signal, indicates that your chromatin is intact and it is your target-specific antibody that is not working in the ChIP experiment.

Input Versus IgG Controls for ChIP-seq

When performing ChIP-seq, the normal rabbit IgG pull-down will often not generate enough DNA for a proper library preparation. Additionally, the limited range of genomic regions pulled down by normal rabbit IgG may bias the library that is produced and this may be exacerbated by qPCR during library construction. Instead, input chromatin (i.e., the chromatin that was used for the IP) should be used to control for inherent qPCR and sequencing bias. It produces a complex sequencing library and is therefore most commonly selected as a control for ChIP-seq.

Critical Steps in the Recommended Protocol…
How Critical Are They?

An optimized protocol is necessary to achieve consistent, reliable ChIP results. We have tested common variations in chromatin preparation, immunoprecipitating antibody concentration, and bead selection to determine the factors that yield optimal outcomes. This guide will highlight the data we use to support our protocol recommendations.

Chromatin Preparation

Abundance and Stability of the Target

Before deciding on a method of chromatin preparation, it is helpful to consider the abundance and the stability of the target protein (e.g., histones, transcription factors, cofactors, etc.) that you are trying to examine.

For example, histones are highly abundant structural proteins that serve to compact DNA into chromatin, making histone-DNA interactions highly stable and easy to cross-link to the DNA. In contrast, transcription factors and cofactors vary in abundance and are transiently bound to the chromatin, making their interactions less stable than histone-DNA interactions and more difficult to cross-link to the DNA.

Another factor to consider is that the target of interest may be part of a larger complex. In such cases, the stability of the complex as a whole may affect the outcome of the experiment. Moreover, the complex may mask the epitope, which can make pulling down the relevant protein-bound DNA more challenging.

Traditional Sonication vs. CST-optimized Sonication and Enzymatic Digestion

Mechanical shearing (or sonication) is a popular choice for preparing chromatin for immunoprecipitation. While effective, most traditional sonication protocols require subjecting the chromatin to harsh, denaturing conditions (i.e., high heat, detergent, and shearing force) that can damage both antibody epitopes and the integrity of the chromatin, making it difficult to use ChIP to study transcription factors and cofactors. Moreover, sonication can be inconsistent from experiment to experiment; under-sonication can lead to incomplete chromatin fragmentation, while over-sonication can result in loss of antibody epitopes due to protein denaturation, degradation, and dissociation of the target protein from the DNA.

The quality of the chromatin preparation varies depending on the type and model of sonicator used, the specific sonication buffers used, the time of sonication, and the volume and density of sample. A few minutes may be all that stands between useable chromatin and under- or over-processed chromatin.

To address some of the difficulties associated with traditional sonication, CST has developed a sonication protocol that uses specially formulated cell and nuclear lysis buffers. This approach protects chromatin integrity and antibody epitopes, resulting in increased immuno-enrichment and making it more compatible for use with transcription factors and cofactors, which are labile and have less stable DNA interactions.

Enzymatic digestion is an alternative method of chromatin fragmentation that is compatible with all target types and highly amenable to the immunoprecipitation of transcription factors and cofactors. This approach uses micrococcal nuclease to cut the linker region between nucleosomes, which gently fragments the chromatin into an array of uniform pieces **(Figure 2)**. Enzymatic digestion is done at low heat in a low-detergent buffer and provides consistent results if the recommended ratio of enzyme to cell number is used. Additionally, because it is simple to control and protects chromatin and antibody epitopes from shearing or denaturation, it is good for users who are new to performing ChIP.

Enzymatic digestion and sonication both generate chromatin fragments between 150 and 900 bp

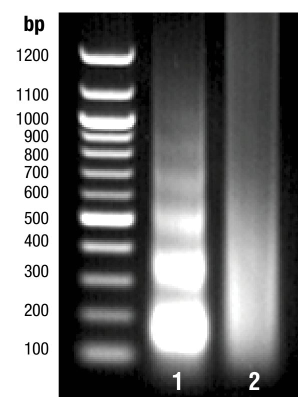


Figure 2: DNA was purified after enzymatic digestion (Lane 1) or sonication (Lane 2) and observed on a 1% agarose gel.

An example of how altering the method of chromatin preparation can affect your final results is shown below **(Figure 3)**. While each of the three fragmentation methods (CST-enzymatic, CST-sonication, the more traditional sonication) generates chromatin that is equally amenable to enrichment of chromatin containing Tri-Methyl Histone H3 (Lys9) (Figure 3, upper panel), the more traditional sonication method fails to generate chromatin that can be enriched for TCF4/TCF7L2 (Figure 3, middle panel) or Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (Figure 3, lower panel).

Traditional Sonication Versus CST-optimized Sonication and Enzymatic Digestion

Figure 3: Chromatin was cross-linked and then fragmented with micrococcal nuclease (CST enzymatic: blue track; CST sonication buffer: red track; traditional sonication buffer: green track) and immunoprecipitated using the indicated antibodies. DNA was prepared for sequencing as outlined in the protocol for the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005.

Preparing Chromatin from Cells

The number of cells needed to generate sufficient quantities of immuno-enriched DNA for a ChIP-qPCR or ChIP-seq reaction will need to be determined empirically. At CST, we have found that 4 x 10^6 cells generally work well for all target types. However, the number of required cells will depend on the abundance of the chromatin-associated target protein, as well as the quality of the antibody used for ChIP. Quite often, fewer cells can be used for more abundant proteins, such as histones, as the target protein is in abundance.

Preparing Chromatin from Tissue

Inherent variability between tissue types and between samples of the same tissue can make it difficult to get sufficient soluble chromatin for your ChIP experiment. The enzymatic and sonication protocols provided by CST work well to efficiently generate chromatin from tissues that is highly amenable to ChIP. We prepared chromatin from mouse liver using both CST sonication and enzymatic methods and performed ChIP-seq using Tri-Methyl-Histone H3 (Lys4), FoxA1/HNF3α, RING1B, and SUZ12 rabbit monoclonal antibodies **(Figure 4)**. Both methods of chromatin fragmentation resulted in equivalent enrichment of Snai2/Slug, a known target gene of both FoxA1 and Tri-Methyl-Histone H3 (Lys4) (Figure 4, upper panels), and equivalent enrichment across the HoxA gene cluster, a known target of both RING1B and SUZ12 (Figure 4, lower panels).

Importantly, we have found that for the sonication method in particular, we can improve both transcription factor and cofactor enrichment from tissue by increasing the cross-linking step during chromatin preparation **(Figure 5)**.

Sonication and enzymatic digestion work equally well to produce chromatin from tissue for ChIP-seq analysis

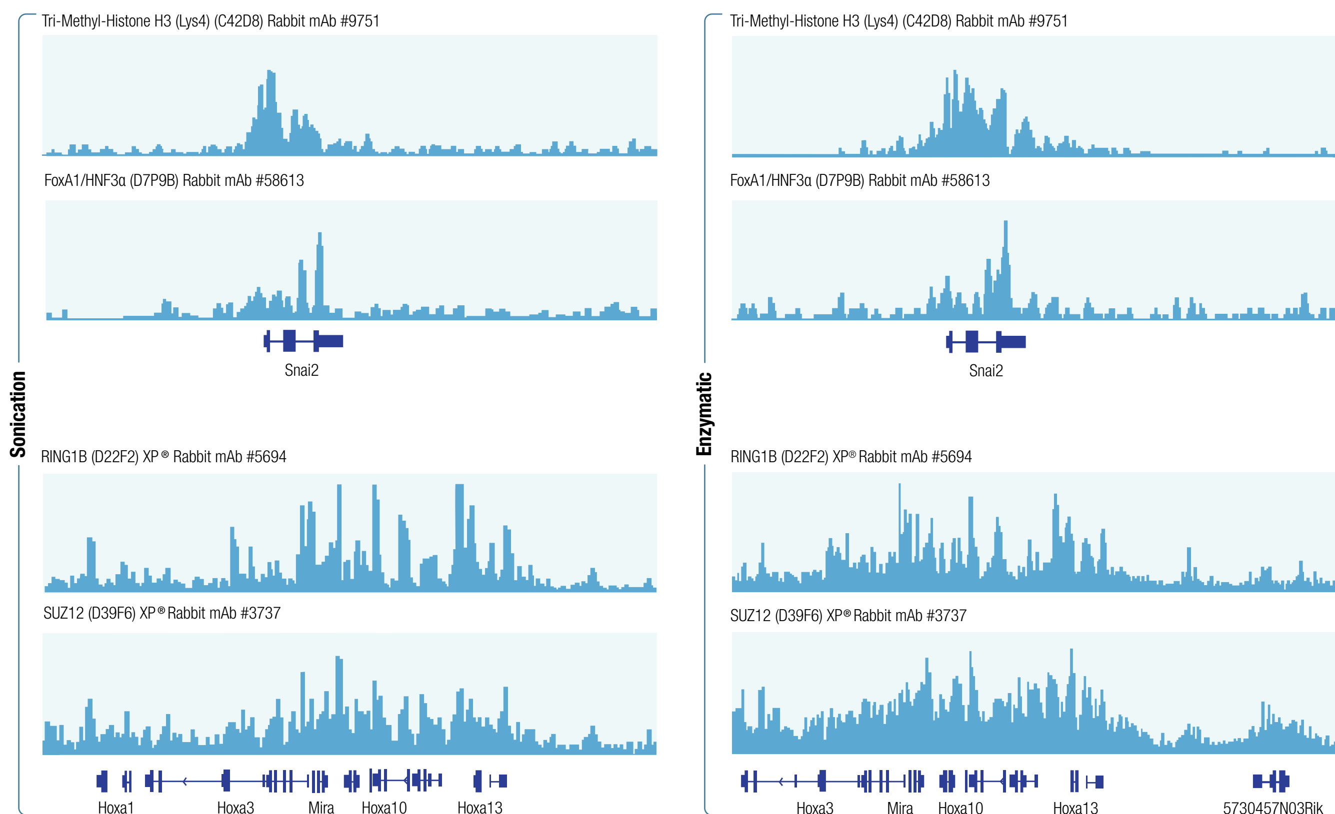


Figure 4: Mouse liver was cross-linked for 10 min (for Tri-methyl-Histone H3 (Lys4) and FoxA1) or 30 min (for RING1B and SUZ12). ChIP was performed using either chromatin sonicated using the CST-optimized protocol or chromatin that was enzymatically digested and immunoprecipitated with the indicated antibodies. DNA was prepared for ChIP-seq according to the SimpleChIP® protocol.

Increasing the cross-linking time to 30 minutes greatly increases the enrichment of chromatin containing RING1B and SUZ12 transcription cofactors from sonicated chromatin

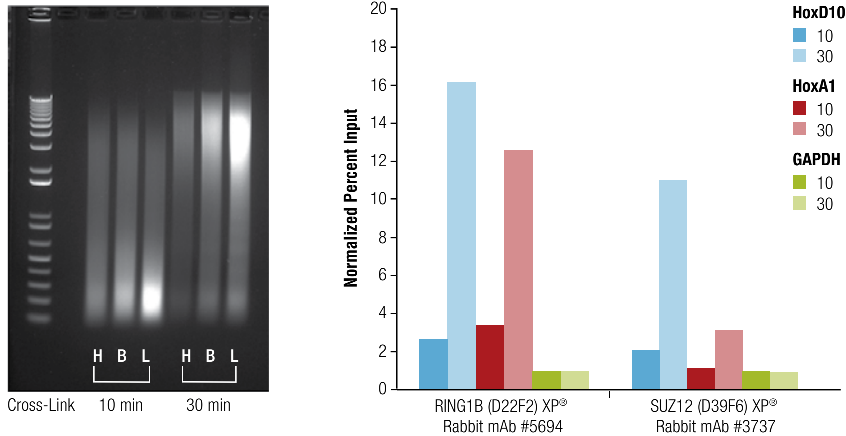


Figure 5: Mouse heart (H), brain (B), and liver (L) were cross-linked for 10 min or 30 min as indicated (left panel). The chromatin was prepared and sonicated for 4 min. ChIP was performed using chromatin prepared from heart tissue with the indicated antibodies using the SimpleChIP® Plus Sonication Chromatin IP Kit #56383 and the enriched DNA was quantified by real-time PCR using primers to the indicated genes (right panel). The amount of immunoprecipitated DNA in each sample is represented as normalized signal to the negative loci GAPDH, which is equal to one.

After Enzymatic Digestion, Light Sonication is Required to Release Chromatin from the Nucleus

The SimpleChIP® Enzymatic Digestion kit protocol recommends sonicating the chromatin preparation, which may seem counterintuitive. We recommend this step because incubation in cell lysis and chromatin digestion buffers A and B does not completely lyse the cell and nuclear membranes of formaldehyde cross-linked cells. Instead, buffers A and B permeabilize the cell, allowing the micrococcal nuclease to enter and digest the chromatin. A short sonication step is required to release the chromatin from the nuclei and into solution. Sonication at this stage does not further fragment the chromatin.

Properly Digested Chromatin

We recommend observing a sample of your chromatin on a 1% agarose gel before moving on to the immunoprecipitation step **(Figure 6)**. For enzymatic ChIP, properly digested chromatin (Figure 6, Lane 2) will be sheared into mono-, di-, tri-, tetra- and penta-nucleosomal units, which will migrate as bands from 150 to 900 base pairs (bp) in length. Under-digested chromatin (Figure 6, Lane 1) will include larger (>900 bp) DNA fragments while over-digested chromatin (Figure 6, Lane 3) will migrate as a single band of around 150 bp (mono-nucleosome). Chromatin fragments that are too large (> 900 bp) can lead to lower signal resolution and increased background. In contrast, signal resolution may be negatively impacted if the fragments are less than 150 bp, as these fragments may be too small to be efficiently amplified by qPCR.

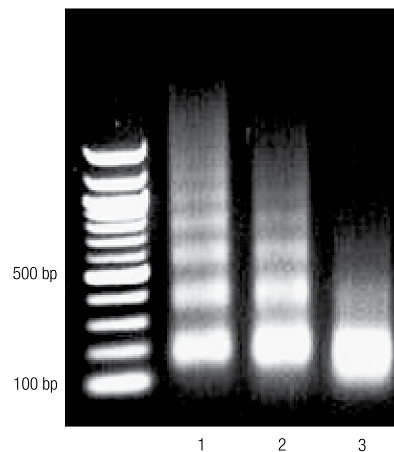


Figure 6. Enzyme-digested chromatin was run on an agarose gel. Lane 1 shows chromatin that is under-digested. Lane 2 shows properly digested chromatin and Lane 3 shows chromatin that is over-digested.

TROUBLESHOOTING TIP

CST provides recommended dilutions on the product sheet for all ChIP validated antibodies. These are provided with each antibody to help you plan successful experiments.

Properly Sonicated Chromatin

When preparing chromatin, using the sonication method, it is important to optimize the number of cycles to ensure that your chromatin fragments are within the appropriate size range (100 to 500 bp). As shown below **(Figure 7)**, increasing cycles of sonication result in reduced size of chromatin fragments (left panel). However, the corresponding ChIP-PCR data (right panel) indicates that over-sonication of the chromatin significantly impairs enrichment of both cofactor ß-Catenin and transcription factor TCF4/TCF7L2-containing chromatin.

Cycles of sonication should be optimized to ensure properly sized chromatin fragments

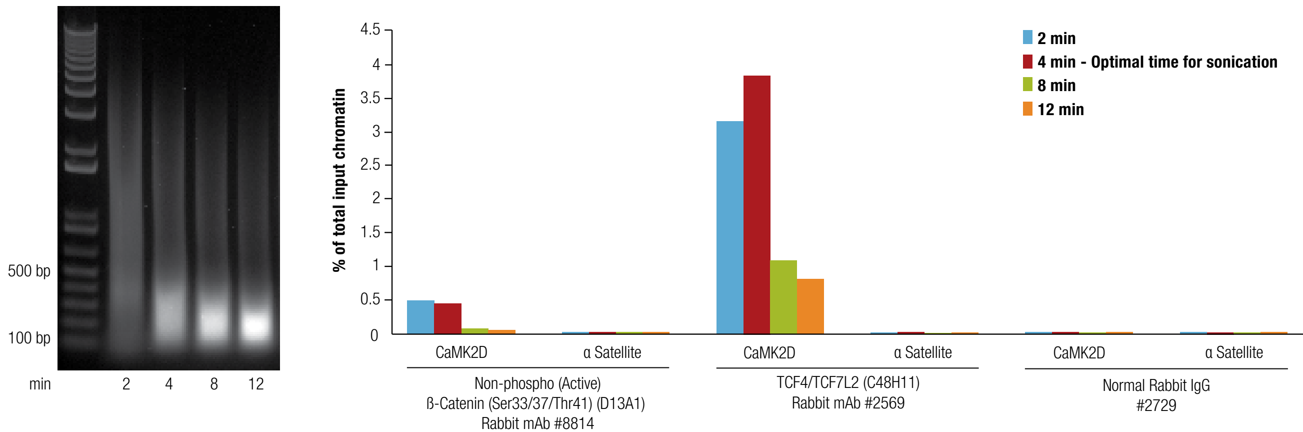


Figure 7: HCT 116 cells were cross-linked for 10 min, sonicated for the time indicated, and DNA was purified and analyzed on a 1% agarose gel, as described in the SimpleChIP® Plus Sonication Chromatin IP Kit #56383 (left panel). ChIP was performed with the indicated antibodies using the SimpleChIP® Plus Sonication Chromatin IP Kit. The enriched DNA was quantified by real-time PCR using primers to the indicated loci and plotted as signal relative to the total amount of input chromatin (equivalent to 100%; right panel).

Immunoprecipitation

Antibody Concentration

The antibody concentration used during the immunoprecipitation step can dramatically affect your ChIP results. If the antibody concentration is too high relative to the amount of chromatin, it may saturate the assay, leading to lower specific signal and/or increased background noise. Conversely, if the concentration of the antibody is too low, it may fail to bind all of the target protein in the IP sample, resulting in less efficient immunoprecipitation. Adding more or less antibody to the IP by altering the dilution factor can have a negative effect on target enrichment **(Figure 8)**.

Use of too much or too little antibody negatively effects ChIP results

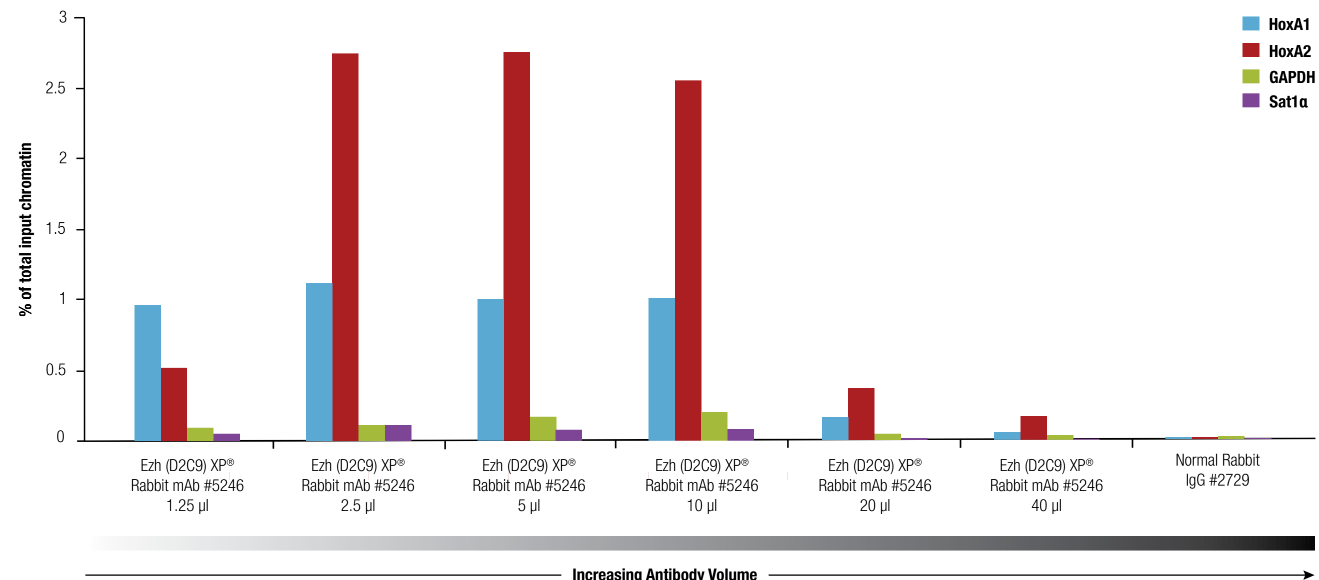


Figure 8: Ezh2 (D2C9) XP® Rabbit mAb #5246 was titrated using the SimpleChIP® Plus Enzymatic Chromatin IP Kit #9005 on cross-linked chromatin prepared from 4 x 10^6 NCCIT cells.

ChIP-grade Versus IP-grade Beads

Not all protein G beads are equal. For example, CST offers both ChIP-grade and IP-grade beads and although they are approved for similar applications, they should not be used interchangeably. CST ChIP-Grade Protein G Beads contain a truncated form of recombinant Protein G that is covalently coupled to nonporous paramagnetic particles (magnetic beads) or agarose beads. Unlike the IP-grade beads, the ChIP-grade beads are supplied in a BSA-containing buffer, which blocks non-specific binding of proteins and DNA, allowing the ChIP-grade beads to provide a cleaner signal than the IP-grade beads. The difference in ChIP quality is apparent from the resulting real-time qPCR signal, which indicates that ChIP-grade magnetic beads provide higher enrichment and lower background signal when compared to the IP-grade magnetic beads **(Figure 9)**.

ChIP Beads / IP-grade Beads

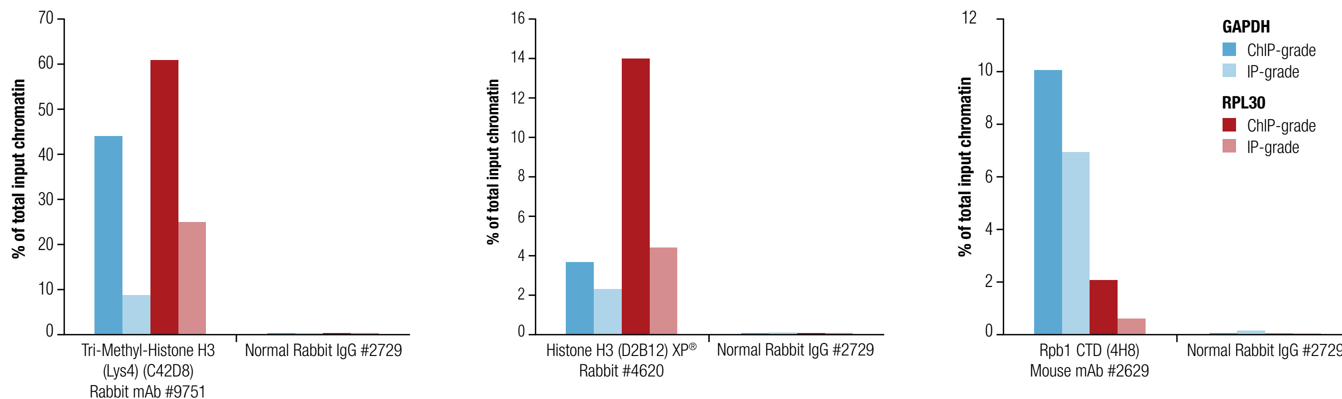


Figure 9: ChIP was performed using digested chromatin from HeLa cells and the indicated antibodies, using either ChIP-Grade Protein G Magnetic Beads #9006, or IP-Grade Protein A Magnetic Beads #8687. Purified DNA was analyzed by quantitative real-time qPCR, using primers to the indicated genes. The quantified DNA is expressed as a percent of the total input chromatin.

Cross-link Reversal and DNA Purification

In the first step of the ChIP protocol the cells are fixed, which cross-links the protein to the DNA and allows the DNA to be immunoprecipitated along with the protein of interest. The cross-linking must be reversed and the DNA must be purified before further downstream analysis can be performed.

Proteinase K Digestion

To reverse crosslinks and separate DNA from bound protein, immunoprecipitated material is subjected to high heat and proteinase K digestion. Proteinase K digests the protein elements of the chromatin as well as any nucleases that may be present in the sample, so this step has the added benefit of protecting the DNA from degradation. Digested protein will need to be removed in the subsequent DNA purification step

DNA Purification

The immunoprecipitated chromatin is eluted, cross-links are reversed, and protein is digested in an SDS-containing buffer that will negatively affect downstream analysis of the DNA. The SDS and digested protein can be removed by phenol-chloroform extraction followed by ethanol precipitation, but we recommend using the DNA purification spin-column and protocol supplied with all SimpleChIP kits. Please note that DNA purification is a necessary step, as unpurified DNA is not suitable for downstream real-time qPCR **(Figure 10)** or NGS analysis.

Purified DNA is amplified by real-time PCR, while unpurified DNA is not amplified

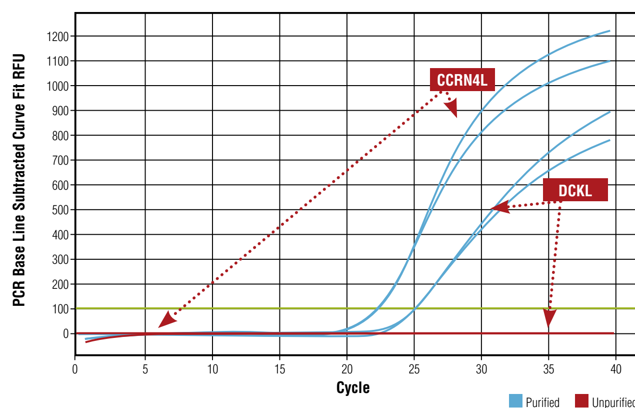


Figure 10: ChIP was performed using Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 and primers against the CCRN4L or DCKL loci. Purified DNA (blue) is amplified while unpurified (red) DNA is not. The green line indicates the threshold level.

> ***Magnetic Beads for ChIP-seq***
>
> *Although price consideration may make agarose beads seem like the better choice for performing IP, they are not suitable if you intend to perform ChIP-sequencing experiments. Agarose beads often rely on sonicated salmon sperm DNA and BSA to block against background signal. The DNA will carry over and be read as part of the sequencing run, skewing the final results.*

Downstream Analysis

There are several approaches available for analyzing the purified DNA that was immunoprecipitated with the protein target of interest. Here we will describe ChIP-qPCR and ChIP-seq.

ChIP-qPCR

The purified DNA may be analyzed using either a standard or quantitative real-time qPCR method. qPCR analysis of the immuno-enriched purified DNA allows the user to analyze a specific protein-gene interaction of interest under different biological conditions. These experiments require primers specific to the genomic region(s) of interest, and thus the enrichment data is limited to the small genomic locus being amplified.

qPCR results can be analyzed using the software provided with the real-time qPCR machine. Alternatively, one can calculate the IP efficiency manually using the equation shown below, which expresses the signal as a percentage of the total input chromatin.

$$\text{Percent Input} = 2\% \times 2^{(C_T\ 2\%\ \text{Input Sample} - C_T\ \text{IP Sample})}$$

Note: C_T = Threshold cycle of PCR reaction

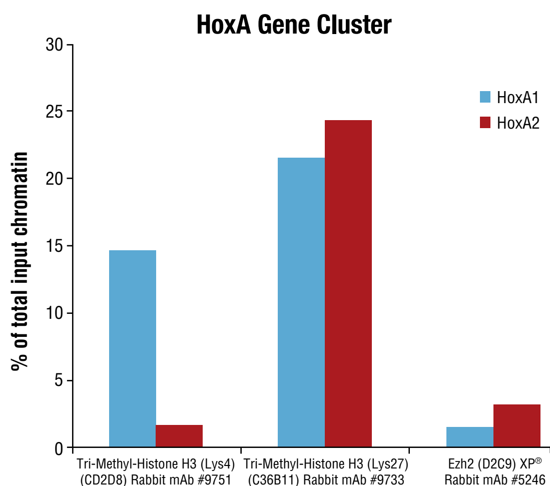
ChIP-seq

NGS may be used to analyze immunoprecipitated DNA after ChIP by a method known as ChIP-seq. ChIP-seq provides the user with a high resolution, genome-wide view of protein-DNA interactions that isn't achievable with ChIP-qPCR.

The power of ChIP-qPCR resides in the fact that it is fast, relatively cheap, and provides a direct answer for whether or not a target is binding to a handful of candidate target genes. However, while it is an effective means of confirming suspected interactions (i.e., it only allows for the examination of preselected genomic loci), it is low-throughput, not scalable, and not conducive to the discovery of novel interactions.

In contrast, ChIP-seq allows the investigator to generate a profile of binding events across the genome **(Figure 11)**. This approach is time-consuming, more expensive, and depends heavily on bioinformatics. That being said, the wealth of information obtained using this approach makes ChIP-seq a powerful technique for studying the epigenetic changes that occur during normal development or pathological progression.

ChIP-q PCR allows for the examination of preselected genomic loci, while ChIP-seq allows for the generation of a genome-wide profile



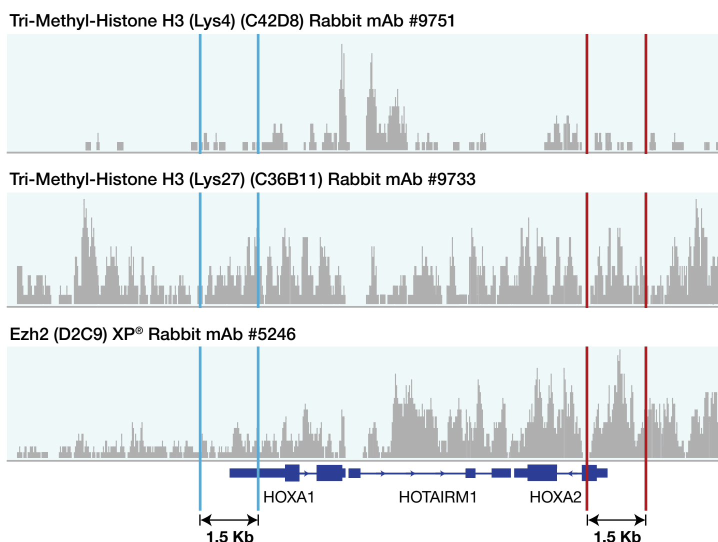


Figure 11: Chromatin immunoprecipitations were performed with cross-linked chromatin from 4 x 10^6 NCCIT cells and either 10 µl of Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, 10 µl of Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb #9733, or 5 µl of Ezh2 (D2C9) XP® Rabbit mAb #5246. The enriched DNA was quantified by real-time PCR using SimpleChIP® Human HoxA1 Intron 1 Primers #7707 and SimpleChIP® Human HoxA2 Promoter Primers #5517. The amount of immunoprecipitated DNA in each sample is represented as a percentage of the total input chromatin. DNA sequencing libraries were generated using the NEBNext® ChIP-Seq Library Prep Master Mix Set for Illumina (Cat. #E6240: New England Biolabs, Ipswich, MA) and 10 ng of DNA from each IP or input control. The DNA libraries were sequenced using an Illumina® MiSeq® Sequencer and the obtained sequences were mapped to the UCSC Human Genome Assembly (hg19). The data was visualized using Integrative Genomics Viewer (Helga Thorvaldsdóttir, James T. Robinson, Jill P. Mesirov. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in Bioinformatics 2012) and enrichment of H3K4me3, H3K27me3 and Ezh2 binding at the HoxA1, HoxA2 genes is shown. Red, and blue vertical lines indicate the areas of the genome interrogated with primer sets used for real-time PCR amplification.

Competitor Comparison

We developed the SimpleChIP® Plus Sonication Chromatin IP Kit to enable successful ChIP-qPCR and ChIP-seq experiments **(Figure 12)**. We compared its performance against two market-leading kits from other companies and found that the CST kit performs equally well for enriching histone proteins (i.e., tri-methyl histone H3 Lys4; Figure 12, top panel). However, when the target is less stable or less abundant (as in the case of either TCF4/TCF7L2-bound or ß-Catenin-bound chromatin) the CST kit provided the strongest signal (Figure 12, middle and lower panel).

CST Sonication kit outperforms other kits for assessing transcription factors and cofactors by ChIP

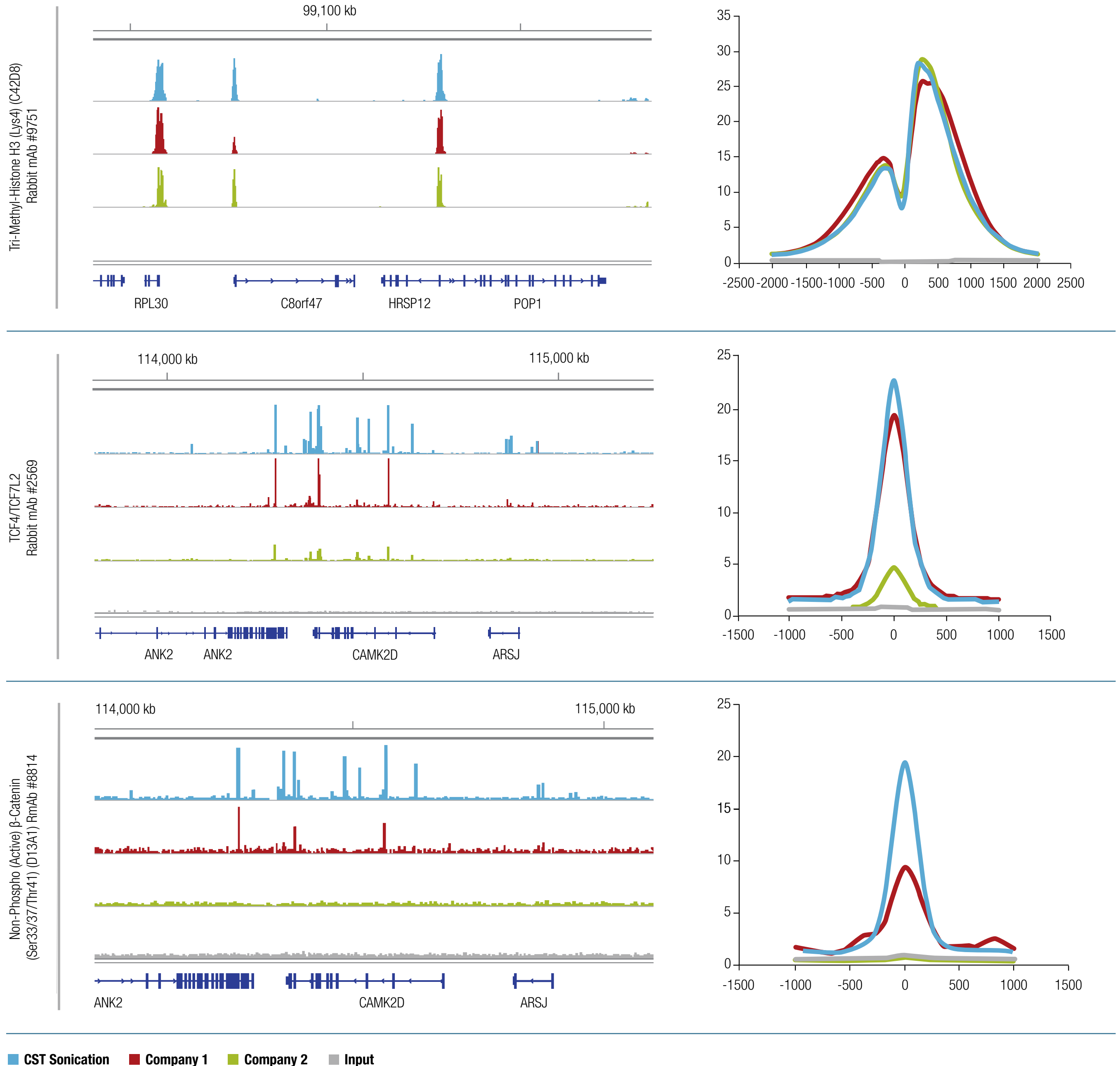


Figure 12: Chromatin was prepared using cross-linked chromatin from 4 x 10^6 HCT 116 cells according to the protocols provided by the kit manufacturers, and immuno-enriched using the indicated antibodies. The ChIP-seq tracks on the left show enrichment of the protein of interest across a localized region of interest. The meta gene analysis on the right depicts the signal-to-noise ratio of all identified peaks found across the whole genome. Input sample was used as a negative control for both ChIP-seq track view and meta gene analysis.

ChIP Products Available from CST

SimpleChIP Kits

- SimpleChIP® Plus Sonication Chromatin IP Kit (Magnetic Beads) #56383
- SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005
- SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003
- SimpleChIP® Plus Enzymatic Chromatin IP Kit (Agarose Beads) #9004
- SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002

ChIP Workflow

- SimpleChIP® Sonication Cell and Nuclear Lysis Buffers #81804
- SimpleChIP® Enzymatic Cell Lysis Buffers A & B #14282
- SimpleChIP® Chromatin IP Buffers #14231
- SimpleChIP® DNA Purification Buffers and Spin Columns #14209
- Micrococcal Nuclease #10011
- Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620
- Rabbit (DA1E) mAb IgG XP® Isotype Control #3900
- Mouse (G3A1) mAb IgG1 Isotype Control #5415
- Normal Rabbit IgG #2729
- ChIP-grade Protein G Magnetic Beads #9006
- ChIP-grade Protein G Agarose Beads #9007
- 6-Tube Magnetic Separation Rack #7017
- 12-Tube Magnetic Separation Rack #14654
- Nuclease-Free Water #12931
- SimpleChIP® Control Primers – *See www.cellsignal.com/chipprimers for a full-list*

Individual Kit Components

SimpleChIP® Plus Sonication Chromatin IP Kits components listed by protocol step:

Chromatin Preparation

- ChIP Sonication Cell Lysis Buffer
- ChIP Sonication Nuclear Lysis Buffer
- Protease Inhibitor Cocktail (200X)
- 1 M DTT
- 10X ChIP Buffer
- Glycine Solution (10X)
- RNase A

Immunoprecipitation

- Protease Inhibitor Cocktail (200X)
- 10X ChIP Buffer (10X)
- Histone H3 (D2B12) XP® Rabbit mAb #4620
- Normal Rabbit IgG (1 µg/µl) #2729
- ChIP-Grade Protein G Beads
- 5 M NaCl

Cross-link Reversal and DNA Isolation

- 5 M NaCl
- ChIP Elution Buffer (2X)
- Proteinase K (20 mg/ml)
- DNA Binding Buffer
- DNA Wash Buffer
- DNA Elution Buffer
- DNA Spin Columns

Downstream Analysis

- SimpleChIP® Human RPL30 Exon 3 Primers 1
- SimpleChIP® Human RPL30 Intron 2 Primers 1

SimpleChIP® Enzymatic Chromatin IP Kit components listed by protocol step:

Chromatin Preparation

- Protease Inhibitor Cocktail (200X)
- 1 M DTT
- 10X ChIP Buffer
- Glycine Solution (10X)
- Buffer A (4X)
- Buffer B (4X)
- Micrococcal Nuclease (2000 gel units/µl)
- 0.5 M EDTA
- RNase A

Immunoprecipitation

- Protease Inhibitor Cocktail (200X)
- 10X ChIP Buffer (10X)
- Histone H3 (D2B12) XP® Rabbit mAb #4620
- Normal Rabbit IgG (1 µg/µl) #2729
- ChIP-Grade Protein G Beads
- 5 M NaCl

Cross-link Reversal and DNA Isolation

- 5 M NaCl
- ChIP Elution Buffer (2X)
- Proteinase K (20 mg/ml)
- DNA Binding Reagent A
- DNA Wash Reagent B
- DNA Elution Reagent C
- DNA Spin Columns

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View a full list of ChIP validated antibodies and SimpleChIP® Control PCR Primers.

www.cellsignal.com/chipabs

ChIP Protocols

An optimized protocol is necessary to achieve consistent, reliable ChIP results. For the best possible results CST absolutely recommends using our protocols, which are the result of extensive in-house validation and will help to ensure accurate and reproducible results. ChIP-specific protocols can be found by visiting our web pages.

www.cellsignal.com/chip

Chromatin Immunoprecipitation (ChIP)

Troubleshooting Guide

A. Expected Chromatin Yield

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. The table below provides ranges for expected total yield of chromatin and expected DNA concentration from 25 mg of tissue or 4 x 10^6 HeLa cells, as determined in Section IV of the protocols.

- In the SimpleChIP® Enzymatic protocol, disaggregation using a BD™ Medimachine system (BD Biosciences) or a Dounce homogenizer yielded similar amounts of chromatin. However, tissue disaggregation using the Mediamachine typically gave higher IP efficiencies compared to disaggregation using a Dounce homogenizer. A Dounce homogenizer is strongly recommended for disaggregation of brain tissue, as the Medimachine does not adequately disaggregate brain tissue into a single-cell suspension.
- In the SimpleChIP® Sonication protocol, a Dounce homogenizer is recommended for all tissue types.

For optimal ChIP results, we recommend using 5 to 10 µg of cross-linked and fragmented chromatin per IP; therefore, some tissues may require harvesting more than 25 mg per each IP.

SimpleChIP® Kit	Enzymatic		Sonication	
Tissue / Cell	Total Chromatin Yield	Expected DNA Concentrations	Total Chromatin Yield	Expected DNA Concentrations
Spleen	20–30 µg per 25 mg tissue	200–300 µg/ml	NT	NT
Liver	10–15 µg per 25 mg tissue	100–150 µg/ml	10–15 µg per 25 mg tissue	100–150 µg/ml
Kidney	8–10 µg per 25 mg tissue	80–100 µg/ml	NT	NT
Brain	2–5 µg per 25 mg tissue	20–50 µg/ml	2–5 µg per 25 mg tissue	20–50 µg/ ml
Heart	2–5 µg per 25 mg tissue	20–50 µg/ml	1.5–2.5 µg per 25 mg tissue	15-25 µg/ml
HeLa	10–15 µg per 4 x 10^6 cells	100–150 µg/ml	10–15 µg per 4 x 10^6 cells	100–150 µg/ml

NT = not tested

B. Optimization of Chromatin Fragmentation

In the SimpleChIP® Enzymatic protocol, optimal conditions for the digestion of cross-linked chromatin DNA to 150–900 bp fragments is highly dependent on the ratio of micrococcal nuclease to the amount of tissue or number of cells used in the digest. Below is a protocol for determination of the optimal digestion conditions for a specific tissue or cell type.

1. Prepare cross-linked nuclei from 125 mg of tissue or 2 x 10^7 cells (equivalent of 5 IP preps), as described in Protocol Sections I, II, and III. Stop after Step 2 of Protocol Section III and proceed as described below.
2. Transfer 100 µl of the nuclei preparation into 5 individual 1.5 ml microcentrifuge tubes and place on ice.
3. Add 3 µl micrococcal nuclease stock to 27 µl of 1X Buffer B + DTT (1:10 dilution of enzyme).
4. To each of the 5 tubes in Step 2, add 0 µl, 2.5 µl, 5 µl, 7.5 µl, or 10 µl of the diluted micrococcal nuclease, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing.
5. Stop each digest by adding 10 µl of 0.5 M EDTA and placing tubes on ice.
6. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 min at 4°C and remove supernatant.
7. Resuspend nuclear pellet in 200 µl of 1X ChIP buffer + PIC. Incubate on ice for 10 min.
8. Sonicate lysate with several pulses to break nuclear membrane. Incubate samples for 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei on a light microscope before and after sonication. HeLa nuclei were completely lysed after 3 sets of 20 sec pulses using a VirTis Virsonic 100 Ultrasonic Homogenizer/Sonicator set at setting 6 with a 1/8-inch probe. Alternatively, nuclei can be lysed by homogenizing the lysate 20 times in a Dounce homogenizer; however, lysis may not be as complete.
9. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 min at 4°C.
10. Transfer 50 µl of each of the sonicated lysates to new microfuge tubes.
11. To each 50 µl sample, add 100 µl nuclease-free water, 6 µl 5 M NaCl and 2 µl RNAse A. Vortex to mix and incubate samples at 37°C for 30 min.
12. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate sample at 65°C for 2 hr.
13. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker.
14. Observe which of the digestion conditions produces DNA in the desired range of 150–900 base pairs (1–6 nucleosomes). The volume of diluted micrococcal nuclease that produces the desired size of DNA fragments using this optimization protocol is equivalent to 10 times the volume of micrococcal nuclease stock that should be added to one IP preparation (25 mg of disaggregated tissue cells or 4 x 10^6 tissue culture cells) to produce the desired size of DNA fragments. For example, if 5 µl of diluted micrococcal nuclease produces DNA fragments of 150–900 bp in this protocol, then 0.5 µl of stock micrococcal nuclease should be added to one IP preparation during the digestion of chromatin in Section III.
15. If results indicate that DNA is not in the desired size range, then repeat optimization protocol, adjusting the amount of micrococcal nuclease in each digest accordingly. Alternatively, the digestion time can be changed to increase or decrease the extent of DNA fragmentation.

In the SimpleChIP® sonication protocol, optimal conditions for the fragmentation of cross-linked chromatin DNA are highly dependent on the number of cells, volume of sample, length of sonication, and sonicator power setting used. For each sonication sample, we recommend using 100–150 mg of tissue or 1 x 10^7 to 2 x 10^7 cells per 1 ml ChIP Sonication Nuclear Lysis Buffer. Below is a protocol for determining the optimal sonication conditions for a specific tissue or cell type.

1. Prepare cross-linked nuclei from 100 to 150 mg of tissue or 1 x 10^7 to 2 x 10^7 cells, as described in Sections I, II, and III. Stop after Step 4 of Section III and proceed as described below.
2. Fragment chromatin by sonication. Optimal sonication conditions can be determined for a given sonicator by varying the number of rounds or duration of sonication at a given power setting (see Step 5 in Section III for optimal power setting using a Branson Digital Sonifier 250 probe sonicator). To determine optimal sonication conditions, set up a sonication time-course experiment and remove 50 µl samples of chromatin after a given round or duration of sonication. For example, take chromatin samples after each 1 to 2 min of sonication.
3. Clarify chromatin samples by centrifugation at 21,000 x g in a microcentrifuge for 10 min at 4°C.
4. Transfer supernatants to new microfuge tubes and add 100 µl nuclease-free water, 6 µl 5 M NaCl #7010, and 2 µl RNAse A #7013. Vortex to mix and incubate samples at 37°C for 30 min.
5. To each RNase A-digested sample, add 2 µl Proteinase K #10012. Vortex to mix and incubate samples at 65°C for 2 hr.
6. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker.
7. Choose the sonication conditions that generate optimal DNA fragment size (see note below) and use for chromatin preparation in Step 5 of Section III. If optimal sonication conditions are not achieved, increase or decrease the power setting of the sonicator and repeat the sonication time course.

NOTE: Optimal sonication conditions can vary with different sample types and fixation times. Use the minimal number of sonication cycles required to generate the desired length of chromatin fragments. Over-sonication, indicated by >80% of total DNA fragments being shorter than 500 bp, can result in excessive damage to the chromatin and lower immunoprecipitation efficiency.

- For sonication of cells fixed for 10 min, optimal sonication conditions will generate a DNA smear with approximately 90% of total DNA fragments less than 1 kb. Increasing the fixation time to 30 min will reduce fragmentation, generating a DNA smear with approximately 60% of total DNA fragments less than 1 kb.
- For sonication of tissues fixed for 10 min, optimal sonication conditions will generate a DNA smear with approximately 60% of total DNA fragments less than 1 kb. Increasing the fixation time to 30 min will reduce fragmentation, generating a DNA smear with approximately 30% of total DNA fragments less than 1 kb.

C. Troubleshooting Table

Problem	Possible Causes	Recommendation
1. Concentration of the fragmented chromatin is too low.	Not enough cells or tissue were used for the chromatin preparation or cell/tissue lysis was incomplete.	If DNA concentration of the chromatin preparation is close to 50 µg/ml, add additional chromatin to each IP to give at least 5 µg/IP and continue with protocol. Count a separate plate of cells before cross-linking to determine an accurate cell number. **Enzymatic:** visualize cell nuclei under microscope before and after sonication to confirm complete lysis of nuclei.
2. Chromatin is under-fragmented and fragments are too large. Large chromatin fragments can lead to increased background and lower resolution.	Cells may have been over-crosslinked and/or too much input material (cells/tissue) were processed.	Shorten the crosslinking time within 10–30 minute range and/or reduce the amount of cell/tissues per sonication. **Enzymatic:** increase the amount of Micrococcal nuclease to the chromatin digestion or perform a time course for enzymatic digestion. **Sonication:** conduct a sonication time course.
3. Chromatin is over-fragmented. Digestion of chromatin to mono-nucleosome length DNA may diminish signal during PCR quantification, especially for amplicons greater than 150 bp in length. Over-sonication of chromatin may disrupt chromatin integrity and denature antibody epitopes.	**Enzymatic:** Not enough cells or too much Micrococcal nuclease added to digestion. **Sonication:** conditions are too harsh.	**Enzymatic:** weigh tissue or count a separate plate of cells prior to cross-kinking to determine accurate cell number. Add more tissue or cells, or less Micrococal nuclease to the chromatin digest. **Sonication:** conduct a sonication time course to find a minimum output/duration to achieve appropriate sonication.
4. No product or very little product in the input PCR reactions.	Not enough DNA added to the PCR reaction or conditions are not optimal.	Add more DNA to the PCR reaction or increase the number of amplification cycles. Optimize the PCR conditions for experimental primer set using purified DNA from cross-linked and fragmented chromatin.
	PCR amplified region may span nucleosome-free region.	Design a different primer set and decrease length of amplicon to less than 150 bp.
	Not enough chromatin added to the IP or chromatin is over-fragmented.	For optimal ChIP results add 5–10 µg chromatin per IP. See recommendations for problems 1 and 3 above.
5. No product in the positive control Histone H3-IP RPL30 PCR reaction.	Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.	Be sure to add 5–10 µg of chromatin and 10 µl of antibody to each IP reaction and incubate with antibody over-night and an additional 2 hr after adding Protein G beads.
	Incomplete elution of chromatin from Protein G beads.	Elution of chromatin from Protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
6. Quantity of product in the negative control Rabbit IgG-IP and positive control Histone H3-IP PCR reactions is equivalent.	Too much or not enough chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.	Add no more than 15 µg of chromatin and 10 µl of histone H3 antibody to each IP reaction. Reduce the amount of normal rabbit IgG to 1 µl per IP.
	Too much DNA added to the PCR reaction or too many cycles of amplification.	Add less DNA to the PCR reaction or decrease the number of PCR cycles. For accurate quantitation, it is critical that PCR products are analyzed within the linear amplification phase of PCR.
7. No product in the Experimental Antibody-IP PCR reaction.	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough antibody added to the IP reaction.	Typically a range of 1 to 5 µg of antibody is added to the IP reaction; however, the exact amount depends greatly on the individual antibody. Increase the amount of antibody added to the IP.
	Antibody does not work for IP.	Choose an alternate, ChIP-validated antibody.

Technical Support

We hope this guide is a helpful resource for performing ChIP in your own lab. Cell Signaling Technology prides itself in providing you with exceptional customer service and support, and we are happy to share our experience with you. Since all of our antibodies are produced in-house, the same scientists who develop and assay these reagents are available as technical resources for our customers. These scientists can be contacted directly and will personally provide technical assistance to you, our customer.

FRONT COVER PHOTO: Fang, ChIP Development Scientist, has been with CST since 2016.

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