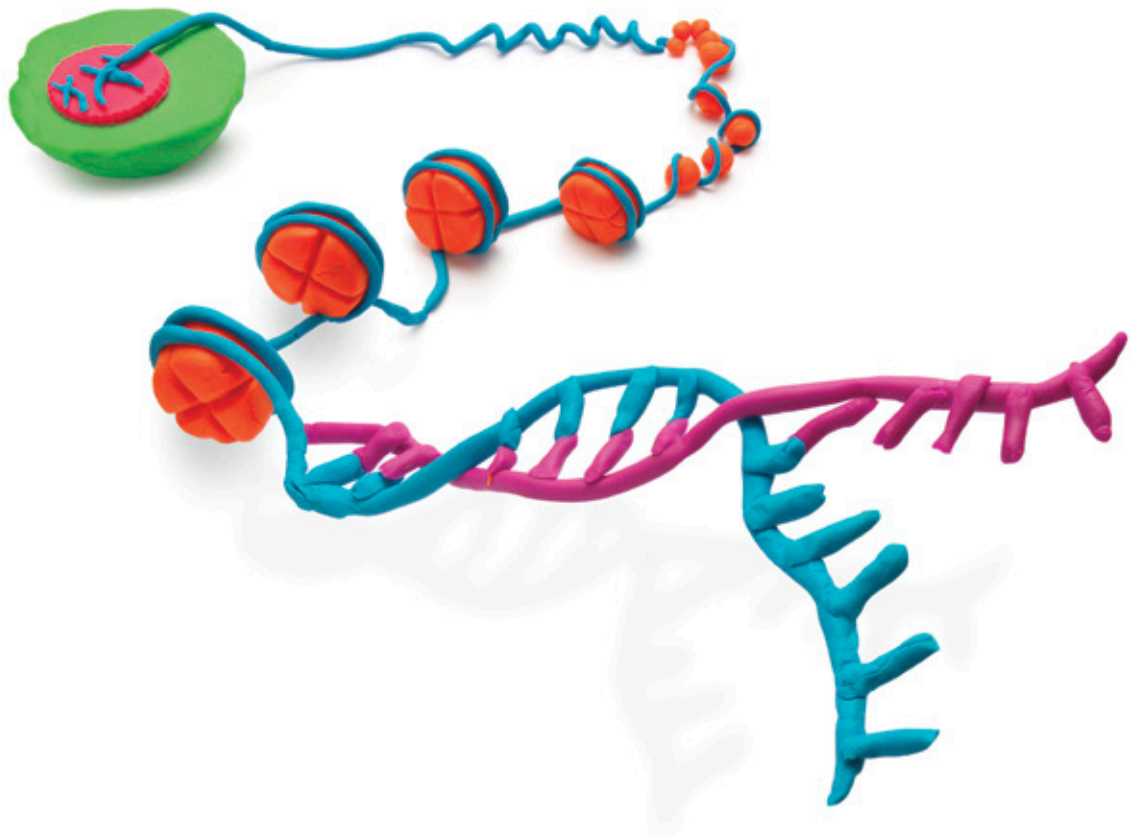
Technical Guide

Guide to Chromatin Immunoprecipitation:
Critical Factors for Success



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Introduction

The eukaryotic nucleus is a dynamic environment of functionally associated nucleic acids, cytoskeletal structure, and maintenance and signaling proteins. The genetic component of the nucleus is packaged as chromatin. Chromatin is a composite of nucleosomal units, each nucleosome formed from the spooling of approximately 147 base pairs of DNA around an octamer of histone subunits (two each of H2A, H2B, H3, and H4). The structure of chromatin varies from tightly compacted heterochromatin, whereby DNA sequences are inaccessible to transcriptional complexes, to an uncoiled euchromatin structure that allows for regulated interaction of proteins with DNA. The structure of chromatin and control of gene expression from its packaged DNA is influenced by epigenetic mechanisms, involving many enzymatic activities that dynamically modify histones and other proteins, in response to cellular cues.

Epigenetics is often described as heritable changes that do not alter the DNA sequence. Examples of these changes include methylation of DNA, modifications to histones, remodeling of chromatin, and signaling via noncoding RNA molecules. While the genome remains relatively static, the complementary epigenome adapts to environmental influences and confers unique, and in some cases heritable, characteristics and phenotypes to different cell types, tissues, and organisms. The epigenome may reflect how complex diseases develop, progress, and transfer from parent to child. It is therefore important to monitor and characterize epigenetic signals in order to fully understand diseases and develop appropriate biomarkers that can serve as diagnostics and targets for therapeutic intervention.

Complex diseases linked to epigenetic mechanisms

Cancer
Cervical carcinoma
Colorectal cancer
Lung cancer
Gastric cancer
Leukemia
Head and neck squamous cell carcinoma
Prostate carcinoma
Hepatocellular carcincoma
Mucoepidermoid carcinoma
Pancreas intraductal papillary mucinous neoplasms
Esophageal squamous cell carcinoma
Oral squamous carcinoma
Beckwith-Wiedmann Syndrome
Prader-Willi syndrome
Angelman Syndrome
Pseudohypoparathyroidism
ICF Syndrome
Rett Syndrome
Systemic Lupus Erythematosus
Atherosclerosis
Endometriosis
Fragile X Syndrome
Alzheimer's disease
Schizophrenia
Depression
Autism
Anxiety

1.1 Chromatin Immunoprecipitation

One of the most powerful tools available for investigating epigenetic mechanisms of disease is chromatin immunoprecipitation (ChIP). ChIP can detect and relatively quantify specific protein-DNA and protein-protein interactions *in vivo* at a single or multiple loci. ChIP involves chemically crosslinking proteins to DNA sequences, which is followed by immunoprecipitation of the crosslinked complexes and analysis of the resultant DNA by endpoint or quantitative polymerase chain reaction (qPCR), microarrays (ChIP-chip), or next-generation sequencing (ChIP-seq).

ChIP can be challenging even for the most experienced researcher. It is a multi-step technique that requires high quality chromatin, robust antibodies, optimized reagents, and protocols to produce reliable and reproducible results. These are typically achieved by either using commercially prepared products and protocols, or undertaking multiple validation experiments. Regardless of which strategy you prefer, this resource book will guide you in tackling some of the problems commonly encountered in ChIP, from sample preparation to DNA analysis.

CHIP TIP

You can use ChIP to discover:

- DNA sequences occupied by specific protein targets
- The binding sites and distribution of a particular protein, such as a transcription factor, throughout the entire genome, under specified cellular conditions
- Gene transcription and polymerase activity
- Complex DNA/protein interactions underlying disease phenotypes
- Modifications to proteins, such as histones, that may influence chromatin structure and gene expression
- Nucleosome architecture and regulation of chromosomal maintenance

Examples of ChIP Data

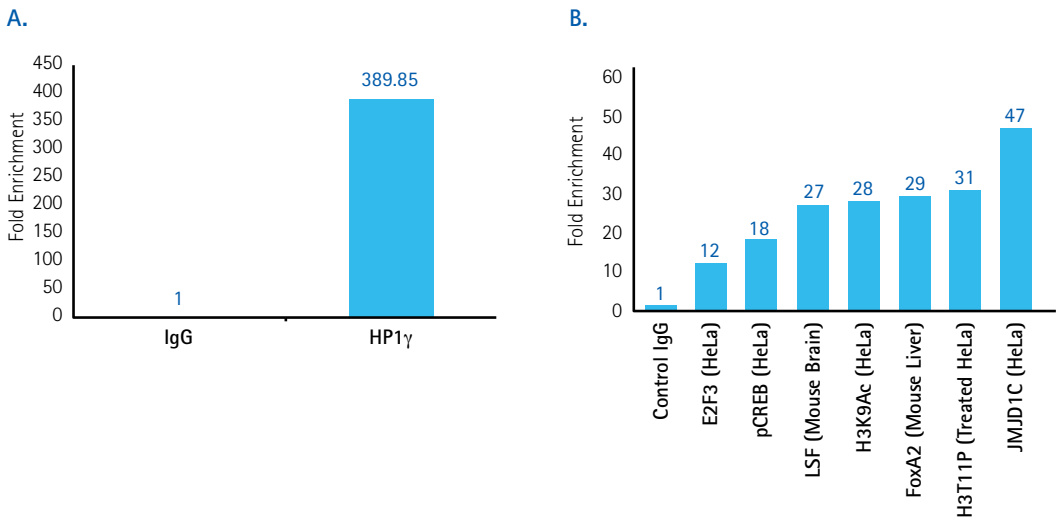

Figure 1. (A) Successful ChIP enrichment of DNA sequences associated with the heterochromatin protein 1dγ (HP1γ), an important marker of gene repression. Data collected using Merck Millipore's ChIPAb+™ Hp1γ antibody/primer set, mouse IgG (nonspecific control) and Merck Millipore's Magna ChIP™ G kit (Catalogue No. 17-10085).

(B) High throughput (96-well plate) ChIP analysis of multiple protein targets to query multiple gene loci under various conditions using Merck Millipore's antibody panels and Magna ChIP™ HT96 Kit (Catalogue No. 17-10077).

ChIP Targets

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ChIP requires a highly epitope-specific antibody that recognizes the protein or modified residues of interest, in their native chromatin states or possible crosslinked conformations. As such, some protein targets or epitopes are more difficult to ChIP than others, due to associations with other proteins *in vivo* or occlusion of the epitope by other means. However, given a range of antibody candidates, many nuclear proteins may be ChIPable. There are a growing number of commercial antibodies that detect many key ChIP targets such as histones and histone modifications, transcription factors, cofactors, nuclear enzymes, and proteins that maintain and repair DNA.

For more information, visit: **www.merckmillipore.com/antibodies**

Before you begin your ChIP experiment, it is critically important to understand the nature of your ChIP target. Consider the following questions:

- Is your ChIP target expressed and under what conditions?
- Is it modified in response to signals?
- Is it located in the nucleus under your conditions of interest?
- Is it a high or low abundance target?
- Does it bind DNA directly or through protein:protein interactions?
- Is it tightly associated with chromatin, such as histones are?
- Will it be analyzed at a single locus or at multiple loci?

2.1 Histones in ChIP

Histones and modified histones are among the most abundant and widely studied ChIP targets, because they undergo a wide variety of post-translational modifications that are associated with biological functions. The family of histones consists of five subtypes: H1, H2 (and its variants), H3, and H4. Histones H2, 3, and 4 form the core structure for nucleosomes, whereas H1 is a linker protein that facilitates the packaging of nucleosomes within chromatin fibers.

Histones undergo a number of post-translational modifications (PTMs): methylation, acetylation, phosphorylation, ubiquitination, sumoylation, citrullination, biotinylation, crotonylation, and ADP ribosylation (See Figure 2).

These PTMs may alter the structure of chromatin, making it less or more accessible to transcriptional complexes. The addition of acetyl groups to histone lysine residues, a process catalyzed by histone acetyltransferases (HATs), reduces electrostatic interactions between histones and DNA molecules, resulting in the destabilization of chromatin structure, greater accessibility of transcriptional complexes to DNA, and potential gene activation. Conversely, removal of this chemical moiety by histone deacetylases (HDACs) often achieves transcriptional repression. Similarly, up to three methyl groups can be added to lysine and arginine residues of H2A, H2B, H3, and H4 by one of several methyltransferase enzymes, depending on the residue being modified.

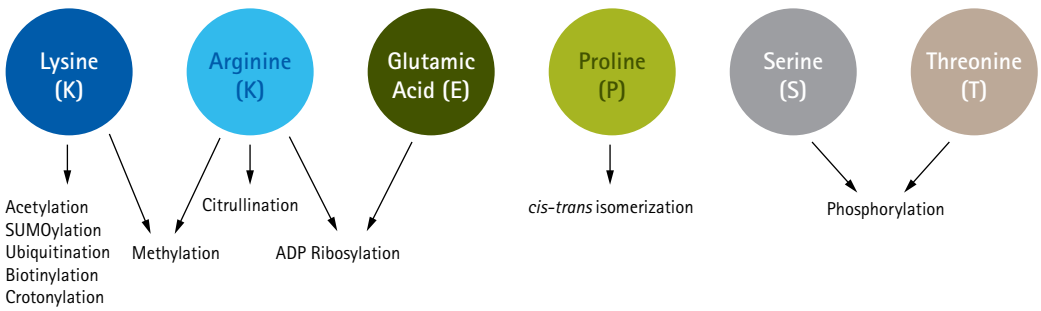

Figure 2. Histone residues and associated post-translation modifications. Adapted from Latham et al, (2007). See http://www.nature.com/nsmb/journal/v14/n11/full/nsmb1307.html

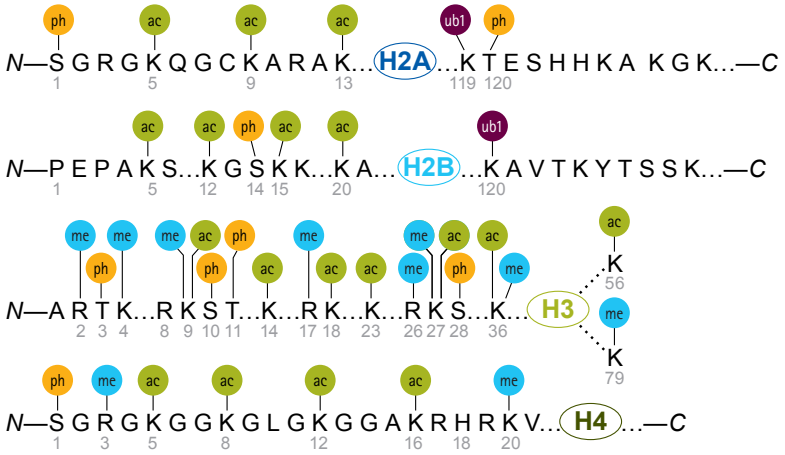

Figure 3: Sequence location of commonly studied histone post-translational modifications:

- ph phosphorylation
- ac acetylation
- ub ubiquitination
- me methylation

Image adapted from Latham et al. , 2007. See http://www.nature.com/nsmb/journal/v14/n11/full/nsmb1307.html

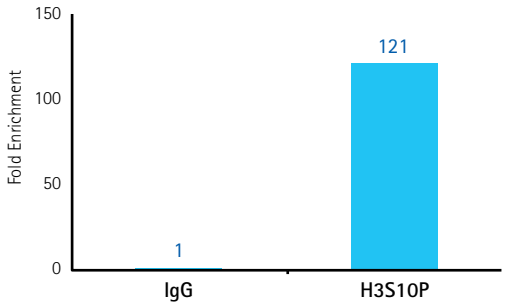

Figure 4: Data collected using Merck Millipore's ChIPAb+™ Phospho-Histone H3 (Ser10) antibody/primer set (Catalogue No. 17-685), mouse IgG (nonspecific control), and Merck Millipore's Magna ChIP™ A/G Kit (Catalogue No. 17-10085).

Methylation of histones can lead to gene expression or repression depending on the pattern of methylated residues. For example, methylation of histone H3 lysines 9 and 27 is a mark of heterochromatin formation and is associated with the activity of the polycomb group of proteins, whereas methylation of H3 lysines 4 and 36 is indicative of euchromatin, gene activation, and trithorax-protein associations.

All histones undergo phosphorylation on multiple serine, threonine and tyrosine residues with varying effects on transcription, chromatin structure, DNA damage pathways, and apoptosis. Site-specific phosphorylation of histone H1, H4 (serine 1), and to a greater extent histone H3 (serine 10 and serine 28), occur during mitosis and contribute to either condensation and segregation, or decondensation of chromosomes. Phospho-histone H3 serine 10, in particular, has been well characterized; it is detectable from G2 to metaphase, and is commonly used as a marker for mitotic cells. In addition, histone phosphorylation may play a role in DNA damage response pathways and apoptosis. The histone variant, H2A.X, is phosphorylated on serine 139 by the ATM/ATK kinases in response to breaks in double-stranded DNA, possibly resulting in the recruitment of DNA repair proteins. These histone modifications may also trigger histone subunit substitution, which involves an array of enzymes and chaperone proteins.

In general, it is the pattern of different histone modifications, the number of modified histone residues, and the spatiotemporal relationship of these modifications—often referred to as the "histone code"—that ultimately determine the effect on chromatin and DNA.

Guarantee Success in ChIP

ChIPAb+™ ChIP–validated histone antibody/primer sets

Description	Catalogue No.
ChIPAb+™ Histone H2A.Z	17-10048
ChIPAb+™ Histone H2B	17-10054
ChIPAb+™ Histone H3 (C-term)	17-10046
ChIPAb+™ Histone H3 (Unmod Lys4)	17-675
ChIPAb+™ Acetyl Histone H3	17-615
ChIPAb+™ Acetyl-Histone H3 (Lys4)	17-10050
ChIPAb+™ Acetyl-Histone H3 (Lys9)	17-658
ChIPAb+™ Acetyl-Histone H3 (Lys14)	17-10051
ChIPAb+™ Monomethyl Histone H3 (Lys27)	17-643
ChIPAb+™ Dimethyl-Histone H3 (Lys4)	17-677
ChIPAb+™ Dimethyl-Histone H3 (Lys9)	17-648
ChIPAb+™ Trimethyl-Histone H3 (Lys4)	17-614
ChIPAb+™ Trimethyl-Histone H3 (Lys4)	17-678
ChIPAb+™ Trimethyl-Histone H3 (Lys9)	17-625
ChIPAb+™ Trimethyl-Histone H3 (Lys27)	17-622
ChIPAb+™ Trimethyl-Histone H3 (Lys36)	17-10032
ChIPAb+™ Trimethyl-Histone H3 (Lys79)	17-10130
ChIPAb+™ Phospho-Histone H3 (Ser10)	17-685
ChIPAb+™ Acetyl Histone H4	17-630
ChIPAb+™ Acetyl-Histone H4 (Lys5)	17-10045

Additional ChIPAb+™ Validated Antibody/Primer Sets are available.

Choose from over 200 additional histone antibodies validated in a range of immunoassays.

For more information, visit: **www.merckmillipore.com/epigenetics**

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The ChIP Experiment

The ChIP experiment can be divided into five main steps:

- Cross link and harvest cells
- Cell Lysis and Chromatin Fragmentation
- Immunoprecipitation
- Wash, Elution, and Crosslink Reversal
- DNA Cleanup and PCR analysis

In a standard ChIP experiment, these five steps are typically accomplished in 2-3 days, but it is possible to complete a full ChIP experiment in one day with alternative protocols. For one-day ChIP experiments, we suggest a kit such as the Magna ChIP™ protein A/G kit (Catalogue No. 17-10085) or the EZ-Magna ChIP™ protein A/G kit (Catalogue No. 17-10086). Rapid protocol kits provide a full set of optimized reagents, and in some cases, control antibodies and PCR primers. The rapid and standard protocols vary primarily in the time required for immunoprecipitation. The rapid protocol is recommended when using ChIP-validated antibodies against abundant targets. Use the standard protocol when using uncharacterized antibodies or less abundant targets. For detailed protocols, download the Magna ChIP™ user guide (Catalogue No. 17-10086). For additional options for kits, antibodies and beads for chromatin IP, you can also visit: **www.merckmillipore.com/epigenetics**

A standard ChIP experiment requires:

- Cultured cells or tissue sample
- Growth medium
- Crosslinking reagent
- Glycine
- Cell lysis reagent
- Antibody specific to the protein target
- Control antibody
- Magnetic or agarose beads conjugated to Protein A and/or Protein G
- Proteinase K
- Wash and elution buffers
- Reagents for agarose electrophoresis
- PCR primers and reagents

Step	Rapid Protocol (1 day)	Standard Protocol (2–3 Days)
Cross link and harvest cells	0.5 hrs	0.5 hrs
Cell lysis/chromatin fragmentation	0.76 hrs	0.76 hrs
Immunoprecipitation	2 hrs	**OVERNIGHT**
Wash, Elution, Crosslink Reversal	3 hrs	3 hrs
DNA Cleanup, PCR	3 hrs	3 hrs

Reliable, Hassle-Free Results

Chromatin Immunoprecipitation Kits:

ChIP kits offer a ready-to-use and reliable approach to ChIP. At Merck Millipore we offer a variety of ChIP kits based on both magnetic beads as well as agarose beads. Magnetic beads utilize a magnetic separation device for processing, and are generally preferred by laboratories performing ChIP due to their ease of use along with better and more reliable recovery of input beads.

Magna ChIP™ Kits Offer

- **Full set of optimized and quality controlled reagents** proven to work in ChIP
- **Detailed protocols** for cells and tissues
- **Time savings:** avoid making reagents and conducting multiple validation and troubleshooting experiments
- **Faster protocols** that enable ChIP in one day using magnetic beads
- **Advanced protocols** that enable up to 96 ChIP reactions at once in a single plate
- **Positive and negative controls; EZ-Magna ChIP™ and EZ-ChIP™ kits** come with IgG controls and well-designed qPCR primers
- **Genome-wide kits** for microarray and ChIP-seq analyses
- **Expert technical support** and troubleshooting

Description	Catalogue No.
Magna ChIP™ A Kit	17-610
Magna ChIP™ G Kit	17-611
EZ-Magna ChIP™ A Kit	17-408
EZ-Magna ChIP™ G Kit	17-409
Magna ChIP™ A/G Kit	17-10085
EZ-Magna ChIP™ A/G Kit	17-10086
Magna ChIP™ HT96 Kit	17-10077
EZ-Magna ChIP™ HT96 Kit	17-10078
Magna ChIP-Seq™ Chromatin Immunoprecipitation and Next Generation Sequencing Library Preparation Kit	17-1010
Magna ChIP²™ Universal Chromatin Immunoprecipitation DNA Microarray Kit	17-1000
Magna ChIP²™ Universal Chromatin Immunoprecipitation DNA Microarray Quad Kit	17-1004
Magna ChIP™ G Tissue Kit	17-20000
Magna ChIP™ Protein A+G Magnetic Beads	16-663
Magna ChIP™ Protein G Magnetic Beads	16-662
Magna ChIP™ Protein A Magnetic Beads	16-661
ChIP Assay Kit (Agarose)	17-295
EZ-ChIP™ Kit (Agarose)	17-371

For additional details on ChIP kits please refer to pages 27-30.

3.1.1. Sample Preparation

▶ **GOAL:** To prepare sufficient amounts of cultured cells or tissue sections/samples appropriate for ChIP

ChIP can be performed on either cultured cells or tissues. In either case, it is important to consider the homogeneity of your sample, as each contributing cell type may have a different chromatin structure. It is also important to determine how many ChIP reactions you will be performing. The number of cells required for each ChIP reaction will depend on the abundance of the protein target. Factors to consider when deciding the "cell equivalents of chromatin" required per ChIP reaction include the quantity of available epitopes in your cells as well as the quality (affinity and specificity) of your antibody. It is possible to achieve successful enrichment with as few as 1 X 10^4 cells per ChIP, if you are using high quality antibodies against abundant epitopes, such as RNA polymerase II and some types of histone modifications. We recommend increasing the amount of cells when the antibody is less optimal or the number of epitopes per cell is low. Tables 1 and 2 provide guidelines for the number of cells required for each type of protein target. You should scale cell numbers according to the performance of your antibody to optimize highest signal-to-noise ratio relative to a nonspecific control (normal IgG or negative-location control).

Tissue samples contain heterogeneous cell populations and can be especially challenging in ChIP. You may use either cryosectioned or fresh tissue microdissected into 1-3 mm^3 sections. The amount of tissue you use for ChIP will also depend on the type of tissue, the relative abundance of your protein target, and the robustness of your ChIP antibody. As a guideline, a pea-size piece of tissue contains approximately 10^7 cells and should be sufficient for 100 ChIP samples. The accuracy of this value, however, depends on the relative cellularity of your tissue and the amount of contaminating extracellular matrix. You should always handle tissue samples carefully and promptly, keeping samples on ice to preserve the sample integrity.

Table 1: Guide to cell numbers for ChIP[1]

Abundance of Protein Target	Molecules per locus	Cells/ChIP	Examples
High	High	10^4	Modified histones, RNA Pol II
Medium	Medium	10^5-10^6	General transcription factors, modifiers (TFIID, PCG)
Low	Low	10^6-10^7	Sequence-specific transcription factors
Low	Indirect binding	10^7 or more	Accessory factors

Table 2: Guide to cell numbers for endpoint analysis

Application	Quantity of ChIP DNA required	Number of Cells
qPCR	Picogram-nanogram	10^5
ChIP-seq	~1-10 nanogram	10^6
ChIP-chip	~10-100 nanogram	10^8

[1] Assumes use of high quality high affinity antibodies

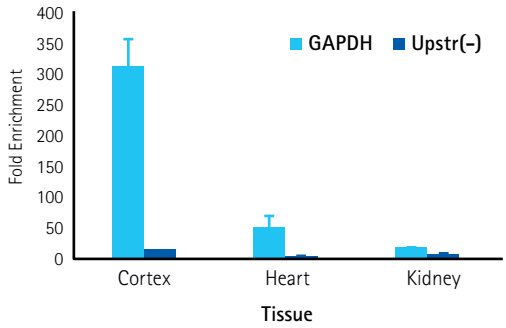


Figure 5: Tissue-specific localization of RNA polymerase II to the GAPDH promoter as revealed using the Magna ChIP™ G Tissue Kit (Catalogue No. 17-20000) and Anti-RNA Polymerase II clone CTD4H8 (Catalogue No. 05-623B). 1 µg of antibody was used to immunoprecipitate chromatin from various mouse tissues. The resulting immunoprecipitated DNA was analyzed by qPCR with primers specific for the mouse GAPDH promoter. qPCR was used to amplify immunoprecipitated chromatin fragments and data were presented as fold relative enrichment to IgG-associated DNA from independent experiments. Fold enrichment was assessed by qPCR with primers upstream of the Dhfr gene (UpStr (-)).

3.1.2. Crosslinking proteins to DNA

▶ **GOAL**: To stabilize *in vivo* associations of protein and DNA

Crosslinking stabilizes the association of your target protein with its interacting DNA sequences. In some cases, target proteins are already tightly associated with DNA and additional chemical crosslinks are not required to preserve the protein/DNA complex during the experimental analysis. This is called native ChIP, or N-ChIP. N-ChIP is suitable for targets such as histones and histone modifications. However, it is important to note that, without additional crosslinking, *in vivo* modifications in nucleosomes may still occur during your analysis.

When targeting proteins that bind weakly to DNA, we highly recommend a crosslinking ChIP (X-ChIP) protocol. X-ChIP may be performed with UV light, formaldehyde, or other chemical crosslinkers. Formaldehyde crosslinking is typically preferred for *in vivo* sample preparations, because this modification is reversible and will allow you to isolate and amplify your ChIP-enriched DNA. Additionally, the crosslinking distance of formaldehyde is only 2 Å (0.2 nm), ensuring that you are crosslinking proteins that are already closely associated with DNA. Formaldehyde will also form crosslinks between DNA-binding proteins and proteins associated with them, facilitating the study of indirect protein/DNA interactions. Formaldehyde crosslinking is able to withstand subsequent experimental manipulations, allowing you to isolate intact protein/DNA complexes. However, formaldehyde may sometimes produce nonspecific crosslinking.

▶ CHIP TIP

- Standard formaldehyde crosslinking conditions are 1% formaldehyde at room temperature for 10 minutes. Crosslinking time can be increased up to 20 minutes to facilitate isolation of weaker or more indirect protein:protein or protein:DNA interactions.
- Use only molecular-biology–grade formaldehyde: Formaldehyde is stablized with methanol, and upon evaporation of the methanol, formaldehyde may form a white precipitate. Do not use formaldehyde that contains this precipitate.
- Long periods of crosslinking may mask epitopes of the target protein and decrease the binding efficiency of your ChIP antibody.
- Inadequate crosslinking may result in loss of your protein/DNA complexes during subsequent steps of the protocol.
- The condition of cells or tissue during treatment with crosslinking reagents may need to be considered (i.e. in presence of culture medium versus phosphate-buffered saline). Culture media contains molecules that can react with formaldehyde and potentially deplete formaldehyde molecules and reduce the expected efficiency of crosslinking.

3.1.3. Cell Lysis

▶ **GOAL**: To disrupt cell and/or nuclear membranes to isolate crosslinked protein/DNA

Cell lysis facilitates the release of the cell nuclei and eliminates components of the cytoplasm that can contribute to background signals. The success of your ChIP assay will depend on the effectiveness of the lysis procedure and the final recovery of chromatin. Some ChIP protocols specify the use of lysis buffers with high concentrations of sodium dodecyl sulfate (SDS), which allows whole cell lysis, whereas others specify the use of buffers for nuclei isolation.

Lysis is achieved by incubation in appropriate concentrations of detergent-based buffers and protease inhibitor cocktail. It is also recommended that you apply mechanical force with a dounce homogenizer, mini-bead beater, vortexer, or glass beads. The method you choose will depend on the type of cells or tissues you are processing. If cell lysis is especially challenging for you, it may be helpful to view the sample before and after lysis under a phase contrast microscope. This will allow you to determine whether the nuclei have been released from your cells, or if using a whole cell lysis buffer, if the entire cell and nuclear membranes have been disrupted.

3.1.4. Fragmentation of chromatin

▶ **GOAL:** To fragment chromatin to lengths (200 to 1000 bp) appropriate for ChIP

Fragmentation ensures that protein/DNA complexes in high–molecular-weight chromatin are soluble and accessible to your ChIP antibody. The method of fragmentation that you choose should depend on whether you performed N-ChIP or X-ChIP (see section 3.1.2). If you are performing N-ChIP, then you should fragment the DNA with appropriate enzymes (such as micrococcal nuclease), because mechanical shearing methods will disrupt native histone/DNA associations. If you are performing X-ChIP, you may choose either mechanical sonication or enzymatic digestion to fragment your DNA. In either case it is important to optimize shearing conditions and use those exact same conditions for each experiment to reduce the potential for variability in the starting chromatin.

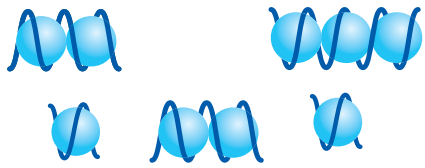


Figure 6: Fragments of chromatin should range from 200 to 1000 bp in length

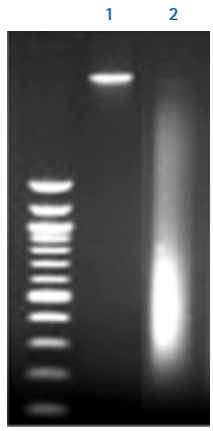


Figure 7. Agarose gel electrophoresis analysis of purified DNA fragments that have undergone sonication (Lane 2) or no sonication (Lane 1), proteinase digestion, crosslink reversal, extraction and precipitation.

We recommend that you analyze the DNA on an agarose gel after each sonication experiment.

▶ CHIP TIP

- It may be challenging to replicate published shearing protocols without optimization. This can be especially true if your instruments differ from those used in those protocols. There are a variety of sonciation instruments, both water bath and probe-based. When using a probe-based sonicator, select a tip that is appropriate for your sample volume. In any event, shearing parameters should be optimized for your sample volume, cell density and cell type.
- Optimization should include the power settings (time on versus time off/rest period) and the number of shearing cycles required to obtain DNA fragments between 200-1000 bp in length. This size is important since downstream analysis assays are typically designed to detect only 100-200 bp amplicons containing a binding site of interest. To perform successful optimization, vary only one parameter for each optimization experiment; for example, keep the power setting constant while varying the number of cycles.
- The efficiency of sonication also varies with each cell type and the number of cell equivalents. You may need to perform separate optimizations for different types of cells or tissues.
- Be careful of time and power settings. Over sonication and too high of a power setting can damage the epitopes you are trying to preserve for the immunoprecipitation step. This will reduce your ChIP signal.
- Always keep lysates ice cold and sonicate at intervals rather than continuously, as sonication produces heat, which can denature chromatin. Avoid foaming during sonication. Foaming can result in surface denaturation of proteins and can result in the loss of chromatin in air bubbles. To avoid this, begin with low power settings and gradually increase the power.
- When optimizing conditions, analyze the length of DNA fragments by agarose gel electrophoresis after each sonication cycle. For optimal sizing, you should perform additional cleanup steps to purify the DNA before electrophoresis. Large, insoluble protein: DNA:RNA complexes resulting from inadequate shearing, may clog the pores of the agarose gel and retard the electrophoresis process. Purify the DNA by digesting the proteins, reversing crosslinks, performing phenol:chloroform extraction and precipitating the clarified DNA.

NOTE that heterochromatin may be resistant to sonication and this may reduce the yield of chromatin.

3.2. Immunoprecipitation

▶ **GOAL:** To use appropriate antibodies to isolate target protein/DNA complexes from chromatin extracts

3.2.1. Monoclonal vs. Polyclonal Antibodies?

Selecting an appropriate ChIP antibody is the one of the most critical steps toward a successful ChIP experiment. Even the highest quality antibodies, which may perform very well in typical Western blot validations, may not be suitable for ChIP. It is best to consider only antibodies that have been validated specifically in ChIP. If your antibodies are not specifically quality controlled and proven to perform in ChIP (e.g. ChIPAb+™ validated antibody primer sets) we suggest you evaluate several potential antibodies before selecting one for your actual ChIP experiments. Below are a few parameters to consider before selecting your antibody.

Either monoclonal or polyclonal antibodies will work for ChIP. A monoclonal antibody recognizes a specific epitope on the target protein. Monoclonals provide the advantage of being highly specific with less of a propensity for nonspecific binding. In addition, monoclonal antibodies perform more consistently from batch to batch due low variability in their clonal nature. However, if the epitope recognized by the monoclonal is masked or altered by previous steps in the protocol, such as crosslinking, then monoclonal antibodies will not be effective in isolating your target protein and its associated DNA sequences. Fortunately, this masking rarely affects monoclonal antibodies.

In contrast, polyclonal antibodies recognize multiple epitopes of a target protein. A polyclonal may therefore be more effective even if a few epitopes are masked by crosslinking. However, because polyclonals recognize multiple epitopes, this can increase the probability that nonspecific binding will occur. In addition, it is important to also consider that the specificity of the polyclonal population may drift over time during immunization, unless the serum from which the antibody is purified is pooled prior to preparation or purification. A related point is that most commercial polyclonal antibodies may differ from batch to batch. The degree of variation will depend upon the manufacturing and quality control practices of the vendor. For example, polyclonal antibodies to modifications have finite amounts of serum available. Antibodies that are in high demand often need to be remade starting with the immunization of a host animal. Consequently, the specificity and affinity of these antibodies can vary from batch to batch. Larger manufacturers of antibodies such as Merck Millipore are able to address this by immunizing multiple animals followed by screening and pooling of materials demonstrating appropriate affinity and specificity. To ensure consistency, the performance of the final antibody can be compared to previous batches. Regardless of your choice of monoclonal or polyclonal, when selecting a commercially prepared antibody, for ChIP, the ideal antibody will have data demonstrating specificity as well data showing reliable performance in ChIP and other key applications.

▶ CHIP TIP

- Whether you select a monoclonal or polyclonal antibody for your ChIP experiment, you must optimize the dilution of your antibody for your specific analysis. If you use excess antibody, you may succeed in immunoprecipitating your target protein, but you may also observe higher nonspecific binding or reduced specific signal. In contrast if use you use too little antibody you will typically observe low recovery of your target see Figure 8.
- For the best results, ChIP antibodies should be well characterized, proven to bind to its target protein, rigorously tested for specificity, and ideally validated in ChIP.
- Just because an antibody works well in a Western blot does not always indicate it will perform well in chromatin Immunoprecipitation. Unlike a Western blot that detects proteins that have been denatured, a ChIP antibody must recognize the target protein in its native state.

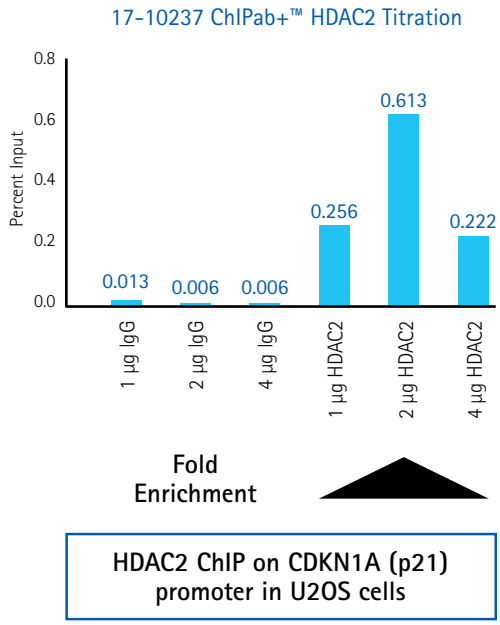


Figure 8. Titration of Input Antibody to Maximize ChIP Enrichment.

3.2.2. Control Antibodies for ChIP

Chromatin is a complex mixture of proteins bound to nucleic acid. To ensure valid results, ChIP experiments should include controls for nonspecific binding of chromatin to your antibody, immunoprecipitation beads, and the resultant ChIP'd DNAs to be analyzed. To control for specificity of your ChIP antibody, you should include the following controls in your ChIP experiment:

- **Negative control antibody**: Use a negative control IgG from the same species and format (e.g. purified, naïve serum, or ascites) as your ChIP antibody. For example, if you use a normal, purified mouse monoclonal ChIP antibody, use a normal purified mouse IgG as your negative control. If you are using a purified rabbit polyclonal ChIP antibody, then use a normal, purified rabbit IgG as a negative control antibody. Alternatively, if an appropriate negative control IgG is not available, you may opt to use the "no antibody" or bead-only approach. Although the matched IgG is typically a better negative control, either the negative control IgG or a bead only control can be used for fold enrichment calculations.
- **Positive control antibody**: Use an antibody of the same species that recognizes an abundant protein target, such as RNA polymerase II or histone subunits (typically H3 or H4 N-term). The positive control antibody helps to validate aspects of the experiment not affected by selection of your ChIP antibody and can be helpful for troubleshooting experiments.

3.2.3. Specificity Testing

Before you select an antibody, it is important to understand the specificity of your antibody. This is particularly true when working with antibodies directed against post-translationally modified proteins such as modified histones. It is important to confirm that the antibodies used detect only epitopes containing your specific modification site.

There are multiple approaches to evaluate the specificity of an antibody, including: dot blots, peptide microarrays; peptide microarrays or peptide inhibition assays. Some labs will perform a number of validation methods, especially for modified histone peptides, to properly evaluate cross-reactivity to similar epitopes, for example between dimethyl and trimethyl adducts on the same histone subunit. For a reliable method of testing specificity for histone antibodies, we recommend AbSurance™ Histone Antibody Specificity Arrays (Catalogue No's. 16-665, 16-668, and 16-667). These arrays provide a total of 89 high quality peptides of histones H2, H3, and H4 on PVDF membranes. The assay is a simple Western blot-like procedure that works with standard chemiluminescent detection systems and in contrast to microarray based approaches, requires no additional software or expensive equipment for detection. For additional information on AbSurance arrays please see page 31.

An example of rigorous specificity testing, including the AbSurance™ Histone Antibody Specificity Array, is shown in Figures 9 and 10 for anti-trimethyl histone H3 (Lys4). Evaluation of specificity by peptide inhibition assay shows weak cross-reactivity with dimethyl histone H3 (Lys4). The histone peptide array, which is a much simpler approach than peptide inhibition assays, shows a similar pattern of cross-reactivity to the dimethyl adduct of histone H3 (Lys4) (See Figure 10).

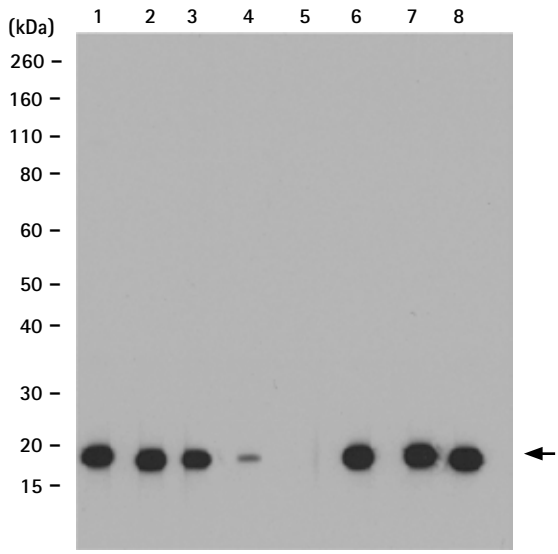
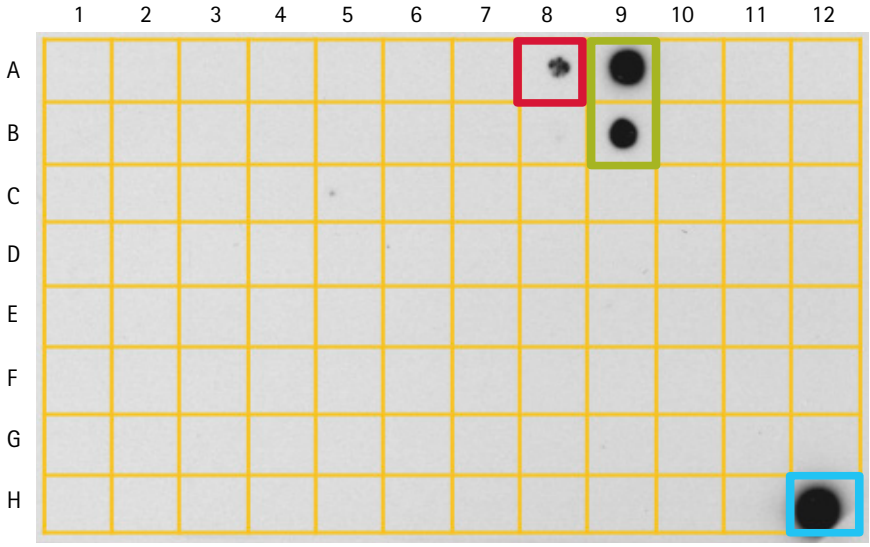


Figure 9: Peptide Inhibition Specificity Assay: Trimethyl Histone H3 (Lys4) (Lane 5) completely blocked the activity of this antibody while dimethyl histone H3 (Lys4) partially inhibited the antibody, indicating a weak cross-reactivity with this adduct. Lane designation partially inhibited the antibody (Lane 4).

Lane 1: no peptide
Lane 2: Unmodified H3 (Lys4)
Lane 3: Monomethyl H3 (Lys4)
Lane 4: Dimethyl H3 (Lys4)
Lane 5: Trimethyl H3 (Lys4)
Lane 6: Trimethyl H3 (Lys9)
Lane 7: Trimethyl H3 (Lys27)
Lane 8: Trimethyl H4 (Lys20)

Arrow indicates Di/Trimethyl Histone H3 (Lys4) (~17 kDa)

Trimethyl Histone H3 (Lys4)



Layout of the AbSurance Histone H3 Antibody Specificity Array

	1	2	3	4	5	6	7	8	9	10	11	12	
A	H3 1-19 unmod	H3 1-19 R2me1	H3 1-19 R2me2a	H3 1-19 R2me2s	H3 1-19 T3P	H3 1-19 K4ac	H3 1-19 K4me1	**H3 1-19 K4me2**	**H3 1-19 K4me3**	H3 1-19 R8me1	H3 1-19 R8me2a	H3 1-19 R8me2s	100 ng
B	H3 1-19 unmod	H3 1-19 R2me1	H3 1-19 R2me2a	H3 1-19 R2me2s	H3 1-19 T3P	H3 1-19 K4ac	H3 1-19 K4me1	H3 1-19 K4me2	**H3 1-19 K4me3**	H3 1-19 R8me1	H3 1-19 R8me2a	H3 1-19 R8me2s	10 ng
C	H3 1-19 K9ac	H3 1-19 K9me1	H3 1-19 K9me2	H3 1-19 K9me3	H3 1-19 S10P	H3 1-19 T11P	H3 7-26 unmod	H3 7-26 K14ac	H3 7-26 R17me1	H3 7-26 R17me2a	H3 7-26 R17me2s	H3 7-26 K18ac	100 ng
D	H3 1-19 K9ac	H3 1-19 K9me1	H3 1-19 K9me2	H3 1-19 K9me3	H3 1-19 S10P	H3 1-19 T11P	H3 7-26 unmod	H3 7-26 K14ac	H3 7-26 R17me1	H3 7-26 R17me2a	H3 7-26 R17me2s	H3 7-26 K18ac	10 ng
E	H3 16-34 unmod	H3 16-34 K23ac	H3 16-34 K27ac	H3 16-34 K27me1	H3 16-34 K27me2	H3 16-34 K27me3	H3 16-34 R26me1	H3 16-34 R26me2a	H3 16-34 R26me2s	H3 16-34 S28P	H3 26-44 unmod	H3 26-44 K36ac	100 ng
F	H3 16-34 unmod	H3 16-34 K23ac	H3 16-34 K27ac	H3 16-34 K27me1	H3 16-34 K27me2	H3 16-34 K27me3	H3 16-34 R26me1	H3 16-34 R26me2a	H3 16-34 R26me2s	H3 16-34 S28P	H3 26-44 unmod	H3 26-44 K36ac	10 ng
G	H3 26-44 K36me1	H3 26-44 K36me2	H3 26-44 K36me3	H3 26-44 Y41P	H3 47-65 unmod	H3 47-65 K56ac	H3 71-89 unmod	H3 71-89 K79me1	H3 71-89 K79me2	H3 71-89 K79me3	Rat IgG **10 ng**	Sheep IgG **10 ng**	100 ng
H	H3 26-44 K36me1	H3 26-44 K36me2	H3 26-44 K36me3	H3 26-44 Y41P	H3 47-65 unmod	H3 47-65 K56ac	H3 71-89 unmod	H3 71-89 K79me1	H3 71-89 K79me2	H3 71-89 K79me3	Mouse IgG **10 ng**	Rabbit IgG **10 ng**	10 ng

Figure 10: Specificity screening with Merck Millipore's AbSurance™ Histone H3 Antibody Specificity Array (Catalogue No. 16-667).
This antibody detected trimethyl histone H3 (Lys4) in green, but also confirmed previous peptide inhibition data showing low affinity and slight cross-reactivity with dimethyl histone H3 (Lys4) in red. (Positive control IgG is shown at position 12H in blue.)

3.2.4. Validation Testing

Ideally an antibody will be validated in ChIP. However, the lack of ChIP data is not always an indication that an antibody will not work for ChIP. When selecting an antibody for ChIP studies, consider all application data available for that antibody since typically the greater the variety of applications for which there is data, the higher the likelihood of an antibody performing in ChIP. For example, an antibody validated in immunoprecipitation (IP), immunofluorescence (IF), or immunohistochemistry (IHC) is more likely to produce positive ChIP results than an antibody validated for only Western blot. However, validation in these applications does not guarantee the success of the antibody in ChIP, as successful ChIP antibodies must recognize accessible epitopes that are not affected by the crosslinking methods often used in ChIP.

CHIP TIP

- Select candidate ChIP antibodies that perform in immunocytochemistry, Western blot, and/or immunoprecipitation. Satisfactory performance in these other immunoassays increases the chances of performance in ChIP. Although this does not guarantee performance in ChIP. this can help prioritize candidate antibodies and screen out less desirable ones.

EXAMPLE

Antibody Validation for ChIP: Anti-JMJD6 (ChIPAb+™ Catalogue No. 17-10263)

Immunofluorescence and Western blot data were collected using Merck Millipore's Anti-JMJD6 (Catalogue No. 09-812). ChIP data was collected using the ChIPAb+™ JMJD6 antibody/primer set (Catalogue No. 17-10263), in conjunction with the Magna ChIP™ A kit (Catalogue. No. 17-408).

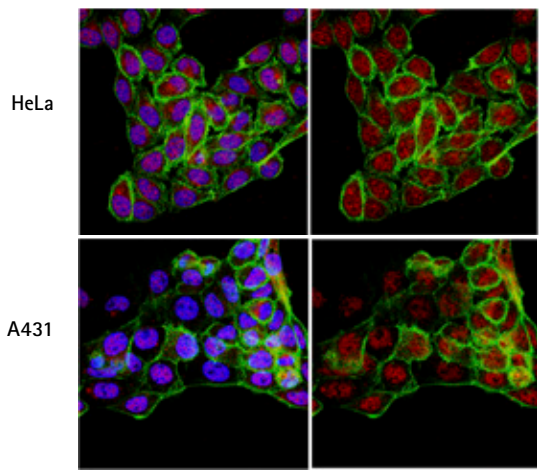


Figure 11: Immunofluorescence analysis showed that Anti-JMJD6 recognizes epitopes in the native conformation inside cells.

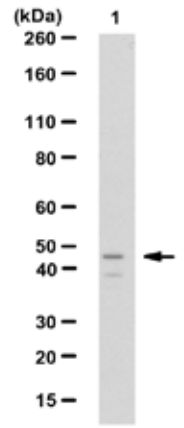


Figure 12: Western blot analysis verified that Anti-JMJD6 immunoprecipitates the JMJD6 protein.

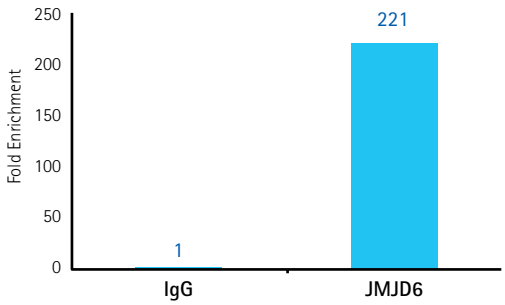


Figure 13: Subsequent testing of JMJD6 in ChIP shows significant fold enrichment of JMJD6-associated DNA.

3.2.5. Application of ChIP Antibody

The ChIP antibody may be conjugated directly to agarose or magnetic beads or it may be immobilized on beads conjugated to protein A and/or protein G. Original ChIP protocols used agarose beads, but many long time ChIP users have moved to magnetic beads. Magnetic beads enable rapid isolation of protein/DNA complexes from the crude chromatin mixture using a magnetic separation device, such as the Magna GrIP™ Rack (Catalogue No. 20-400), PureProteome™ Magnetic Stand (Catalogue No. LSKMAGS08 or LSKMAGS15) or the Magna GrIP™ HT96 rack (Catalogue No. 17-10071).

Magnetic Beads

Advantages
Low nonspecific binding
Blocking and preclearing not required
Easy to handle during washes
Beads are visible in tube
Reproducible results
Disadvantages
Slightly higher cost
Magnetic rack required
Non-porous (binding capacity dependent on bead surface area)

Agarose Beads

Advantages
High capacity binding (porous), which may also increase nonspecific binding
Simple equipment required (centrifugation or filtration)
Slightly lower cost
Disadvantages
Blocking required
Preclearing required
Not visible in tubes
High probability of bead loss during handling

Agarose beads perform well in the hands of many researchers and offer a less expensive, but more time consuming option. These beads require centrifugation for separation and may exhibit nonspecific binding, which may require blocking and lysate preclearing.

Merck Millipore offers both agarose and magnetic beads as well as ChIP kits (see page 7). Our kits and beads are offered in a variety of formats including protein A, protein G, as well as a blend of protein A and G conjugates (protein A/G). The choice of bead type is often influenced by the type of antibody one plans to use for their experiment. Protein A beads exhibit the highest affinity for rabbit polyclonal antibodies, whereas the protein G beads bind a wider range of antibodies including most, but not all, classes of mouse monoclonal IgGs. The protein A/G blend provides the most flexibility in terms of the type of antibody that you can use, because it combines the binding characteristics of both protein A and protein G. Our research and development team has found that the protein A/G blend typically produces lower backgrounds than protein A or G alone, for a wide range of antibodies without compromising the efficiency of pull down (see Figure 14) .

▸ EXAMPLE

Protein A/G Beads: 17-646 ChIPAb+™ Hp1g (CBX3) Protein G Purified Mouse Monoclonal

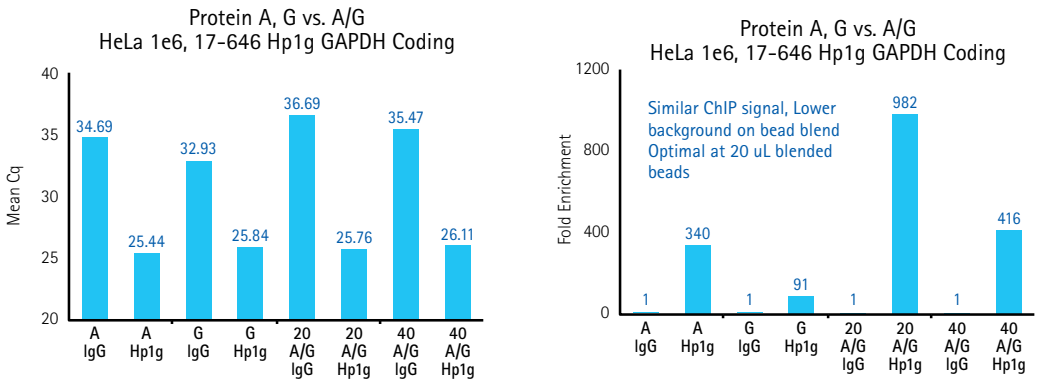


Figure 14: Comparison of ChIP using Protein A or G alone vs. A/G Blends. Consistent with lower background signal, IgG signals are detected later (higher Cq) using protein A/G bead blends as compared to protein A or G beads alone (left graph). In addition similar Cq are detected using protein A/G bead blends as compared to protein A or G alone suggesting no loss of signal. Lower backgrounds with similar recovery of signal result in higher fold enrichments with A/G blends as shown in graph on the right.

Regardless of your choice of beads, the order in which you apply magnetic or agarose beads to your ChIP reaction may affect your ChIP signal. One method is to incubate the beads with the capture antibody (few hours at room temperature, or overnight at 4 °C) followed by addition of chromatin and further incubation (1 hour to overnight with rotation, at 4 °C). Increasing the time of incubation may increase both the background and the ChIP signal; however, antibodies with low affinities for their targets generally do not produce significant ChIP signals without longer (overnight) incubations. Alternatively some protocols incubate the antibody and chromatin and then add beads, or add all three components at the same time. Adding all three components often works, and reduces the time required to perform the overall reaction.

Magnetic Beads

Protein A/G Beads

Description	Catalogue No.
Magna ChIP™ Protein A+G Magnetic Beads	16-663
Magna ChIP™ Protein G Magnetic Beads	16-662
Magna ChIP™ Protein A Magnetic Beads	16-661

For a complete list of ChIP accessories, visit:
www.merckmilipore.com/epigenetics

Magnetic Racks

PureProteome™ Magnetic Stand (Catalogue No. LSKMAGS08)

Effective bead capture: Strong trapezoid-shaped magnet fits tube contours to capture up to 300 µL of beads

Efficient agitation: Removable magnet and unique vortex interface enables thorough mixing

Easy to handle: Ergonomically designed magnetic stand securely holds both 1.5 mL and 2.0 mL tubes

Magna GrIP™ Rack (Catalogue No. 20-400)

Effective bead capture: Polyethylene rack contains 4 neodymium magnets

Versatile: Rack may also be used with either 15 mL or 0.5 mL tubes

Easy to handle: Ergonomically designed magnetic stand has 8 holes suitable for 1.5-2.0 mL tubes or spin columns

3.2.6. Washing the Immunoprecipitate

After immunoprecipitation, the antibodies, beads, and protein A or G often have biomolecules associated with their surfaces that are not related to antigen recognition. It is therefore necessary to perform a series of wash steps with ChIP-specific buffers to remove nonspecific chromatin, protein, and nucleic acids from your immunoprecipitate, because these nonspecific components can significantly increase background signals, produce high variability, and contribute to failure of the ChIP assay. In some cases, multiple buffers (for example, high salt, low salt, lithium chloride, and stringent TE wash), or buffers of increasing stringency, are used to reduce binding of nonspecific molecules. Other protocols involve simpler buffer systems. Regardless of the wash methods you use, you should use consistent wash conditions: maintain consistent buffer temperatures, wash incubation times, and rotation speeds of any apparatus used for washing. In certain cases, background signals may be reduced by increasing the number of washes, although significant improvement of ChIP signals is ultimately determined by the quality of your ChIP antibody and the nature of your ChIP target.

3.3. Elution and Crosslink Reversal

The elution and crosslink reversal steps are necessary to dissociate your chromatin complex from the antibody and beads and to isolate your ChIP'd DNA from the protein portion of the chromatin complex. If you used magnetic beads, elution can be easily done with a magnetic rack and appropriate elution buffers (e.g. sodium carbonate buffers). It is also possible to elute using a peptide competition assay, in which the antibody/protein/DNA complex is incubated with peptides exhibiting greater affinity for the antibody than your target protein, resulting in displacement of your target protein from the antibody complex. This approach may significantly reduce background signals but can be expensive due to the costs of the synthetic peptides.

After elution with agents such as sodium carbonate, and before qPCR analysis, it is critical that you reverse the formaldehyde crosslinks between lysine residues and DNA. Typically, crosslinks are reversed by incubation with proteinase K and heat. You may need to further purify the DNA sample by extraction with a combination of

organic solvents, such as phenol:chloroform extraction (see supplementary protocols). Alternatively, purify the DNA from the digested protein/nucleic acid mixture by silica-based chromatography (i.e. spin columns), by magnetic DNA purification particles, or by chelating agents such as Chelex®. We recommend that you perform an RNAse digestion step prior to proteinase K digestion to remove contaminating RNAs from the ChIP reactions.

3.4. Quantitative Real-Time PCR (qPCR)

After you have completed ChIP and purified your DNA sample, you may choose to perform endpoint or quantitative real-time PCR (qPCR) to quantify the DNA in your samples. In qPCR, your DNA samples are incubated with primers, polymerases, oligonucleotides, and detection fluorophores such as TaqMan® (fluorescent donor:quencher hybridization) specific probes or SYBR® Green intercalating dye (no specific probe required). The DNA sample undergoes cycles of amplification via DNA polymerase, in which products from the previous cycle become templates for the next cycle, thus doubling the amplified DNA in each cycle, in the most optimal reactions. The qPCR assay allows you to quantify initial DNA concentrations from multiple samples in real time by analyzing fluorescent signal intensities that are proportional to the amount of amplicon. For successful ChIP analysis, ensure that your primers amplify the intended sequence with efficiency over 95%, and that they do not form dimers that may diminish the specific signal from qPCR based on SYBR® Green technology.

CHIP TIP

- Before you begin qPCR you must reverse the crosslinks in both your input and test samples with proteinase K and heat (see previous sections). In some cases you may need to purify your DNA by performing a solvent (phenol:chloroform) extraction.

Best practices for conducting qPCR with maximum reproducibility and signal-to-noise ratios can be found at **http://bitesizebio.com/articles/10-tips-for-consistent-real-time-pcr-rtpcr/**

Two commonly used detection methods for qPCR are SYBR® Green technology and TaqMan® probe technology. SYBR® Green is a DNA-binding dye that drastically increases in fluorescence when bound to double-stranded DNA. The fluorescent signal, therefore, increases as the number of copies of double-stranded DNA increases. Detection can also be achieved with TaqMan® probes. In this method, a reporter fluorophore and a quencher fluorophore are incorporated into a single, sequence-specific probe that recognizes your PCR amplicon. In the free probe, the signal from the reporter is quenched by proximity to the quencher fluorophore. Upon hybridization to the amplicon, the 5′→3′ exonuclease activity of the DNA polymerase will degrade the probe, releasing free fluorophore in proportion to the amount of template molecules available for hybridization. In both of the above qPCR techniques, fluorescent signal follows a linear phase, followed by a plateau phase when one component of the reaction becomes rate-limiting.

Comparison of SYBR® Green and TaqMan® Detection Methods

	SYBR® Green	TaqMan®
Detection	Detects double-stranded DNA	Detects amplicon via hybridization and degradation of probe
Throughput	Single target	Can multiplex
Technical Challenge	Low; simple experimental protocol	Moderately high; Requires well designed primers and probes and optimization of each primer/probe set
Cost	+	++
Specificity	+ May detect nonspecific signals such as primer dimers or other nucleic acid molecules not of interest	++ Low nonspecific binding

Fluorescent signals indicate the cycle threshold (Ct)* for each sample. The Ct is the point of the linear phase at which the fluorescent signal exceeds the background. The Ct depends on the amount of DNA in your sample. If your sample contains relatively high amounts of DNA target, fewer cycles will be required to exceed background and the Ct will be low. Conversely, if the quantity of your DNA target is low, more cycles will occur before Ct is achieved. ChIP analysis by qPCR works best when starting with more dilute DNA samples (as opposed to highly concentrated templates which can inhibit Taq polymerase when present in high concentrations). Therefore, follow available protocols describing typical volumes of ChIP'd DNA to analyze by qPCR, such as 2 µL out of 50 µL ChIP sample, to avoid introducing PCR inhibitors into the reaction.

*NOTE: Current guidelines from the "Minimum Information for Publication of Quantitative Real-Time PCR Experiments" (MIQE) advise that "quantification cycle" (Cq) should be used in place of "cycle threshold" (Ct) when reporting qPCR data. For more information on this topic as well as recommendations on appropriate controls to run, standards for data analysis, and error reporting, visit http://www.rdml.org/miqe.php.

3.4.1. Efficiency of qPCR reactions

In order to extract meaningful information from your qPCR analysis, it is important that you measure certain parameters to ensure that the assay itself is working properly. First, test the efficiency (E) of your qPCR reaction. The efficiency of the qPCR reaction is typically expressed as a percentage value, and indicates the percentage of the template that is being amplified in each cycle. The best way to test the efficiency of your reaction is to generate a standard curve by running five-point serial dilutions of a sample of known concentration, and plotting the corresponding Ct values to generate a standard curve.

For the DNA standard used for your qPCR optimization experiments, you can use fragmented, purified genomic DNA, or more conveniently, DNA isolated from your chromatin as your Input sample. This Input sample can be a small percentage (2 to 5%) of your total chromatin sample. The chromatin sample should be proteinase K-digested, crosslink-reversed, and purified to provide a suitable control material for assay development or efficiency calculation. In qPCR instrumentation software, the efficiency is often automatically calculated and reported if you select your sample type as "Standard".

The efficiency of the reaction can be calculated by the formula:

$$\text{Efficiency (E)} = 10^{-1/\text{slope of standard curve}}$$

$$\%\ \text{Efficiency} = (E-1) \times 100$$

In a well-optimized reaction, the efficiency should be between 95 to 105%. If your efficiency falls outside of this range, you may need to explore potential sources of error in your experiment:

1. Run at least three replicates of each dilution, and possibly adjust your dilution
2. Use optimized buffers
   a. Use a commercial mastermix if possible to enable more consistent results.
   b. Run a sample containing no DNA to test your buffers for contaminants
3. Use well-designed primers — ideally, Ct values should be between 18 and 30.
4. If your efficiency is still suboptimal, consider the following possible causes of inefficiency:
   a. Amplicon is too big (keep it between 65 and 150 bases long)
   b. Poor primer integrity (use fresh primers)
   c. Primers too concentrated (change primer dilution to avoid dimers)
   d. Contamination from phenol, salts, or ethanol in the template DNA
   e. Inappropriate instrument baseline or threshold settings
   f. Contamination in no-template control (use dedicated pipettes, UV-irradiated equipment, fresh Milli-Q® water, etc., and setup in separate room)

3.4.2. Data Analysis

There are two approaches to analyzing qPCR data: absolute quantification and relative quantification.

Absolute quantification

Absolute quantification allows the determination of how much DNA is in a given quantity of sample, without performing comparative analyses with other samples. For this analysis you should do the following:

1. Prepare serial dilutions of a sample of known concentration (quantitated, purified Input DNA)
2. Include at least three replicates for each dilution
3. Run these standards alongside your test sample(s)
4. Construct a standard curve of the log quantities of sample versus the Ct values obtained from the qPCR analysis
5. Perform a regression analysis to determine the equation of the standard curve and use this equation to calculate the quantity of DNA in your unknown sample (s).

The quantity of target DNA in the test sample should fall within the linear dynamic range of the qPCR assay. If this is not the case, adjust the dilutions of the standard sample and repeat the experiment.
The fitted value derived from the extrapolated Ct measurement can be reported as "nanograms DNA recovered," "percent of input," "copy number," "mean quantity," as well as other variations that describe number of molecules. Most commonly, "percent of input" is utilized when using Input DNA as the standard in relative standard curve method.

Relative quantification

In relative quantification analysis, the test sample is expressed as a fold change relative to a control sample (immunoprecipitated using normal purified IgG or mock IP). The test sample may also be expressed as a percentage of a reference gene that is known to maintain constant expression levels under the conditions of the experiment, similar to the use of "housekeeping" protein in Western blot analyses, or housekeeping gene cDNAs for qRT-PCR expression analysis. DNA loci known to be unoccupied by the immunoprecipitated protein (negative locus) can be used in this manner as a reference gene compared to known, occupied, positive control DNA loci.

Relative quantification uses the delta Ct (ΔCt) or delta delta Ct (ΔΔCt) methods. These methods assume that the amplification efficiency of your PCR reaction is 100% (e.g. amplicons double at each cycle during the linear phase of the reaction, and E=2), or at least, that all assays in the test have identical efficiency.

The ΔCt Method

The delta Ct method can be used when equal numbers of cells are used to obtain the control and test DNA samples.

Where E=2:

$$\text{Ratio of ChIP DNA/control DNA} = 2^{\text{Ct(control DNA)-Ct(ChIP DNA)}}$$

The 2-ΔΔCt Method

This method is widely used to normalize ChIP DNA to input DNA, and the analysis includes primers to both a positive and a negative locus, with equal amplification efficiency. The input refers to the chromatin sample obtained before immunoprecipitation. The ChIP DNA and dilutions of the input DNA sample are analyzed concurrently to generate their respective Ct values. To calculate the fold enrichment of the ChIP DNA relative to the input sample, complete the following steps:

1. Normalize the Ct values of the ChIP DNA to the Ct value of the input DNA (ΔCt) for each primer set by subtracting the Ct value obtained for the input DNA from the Ct value for ChIP DNA:

   $$\Delta Ct = Ct_{\text{ChIP DNA}} - (Ct_{\text{input}} - \text{Log2 [Input dilution factor]})$$

2. Calculate the percent of input for each ChIP:

   $$\%\text{Input} = 2^{(-\Delta Ct\ [\text{normalized ChIP}])}$$

3. Normalize the positive locus ΔCt values to negative locus (ΔΔCt) by subtracting the ΔCt value obtained for the positive locus from the ΔCt value for negative locus:

   $$(\Delta\Delta Ct = \Delta Ct_{\text{positive}} - \Delta Ct_{\text{negative}})$$

4. Calculate the fold enrichment of the positive locus sequence in ChIP DNA over the negative locus:

   $$\text{Fold enrichment} = 2^{\Delta\Delta Ct}.$$

4

Troubleshooting & Frequenty Asked Questions

Problem/Question	Suggested Steps/Answer
High background in negative control (IgG or mock IP) samples	**Excessive antibody resulting in binding to non-targets:** Optimize the concentration of the antibody.
	Nonspecific binding to beads: Include a pre-clearing step to exclude these non-targets or add a blocking agent to the beads.
	Incomplete fragmentation of chromatin: Optimize the fragmentation process to acquire chromatin lengths between 200-1000 bp. Separately optimize fragmentation for each cell or tissue type. Use siliconized or low retention tubes.
	Contaminated reagents: Ensure that all reagents are freshly prepared and free of contaminants. Increase the number of washes.
	Run a "no DNA" PCR reaction to determine if your sample is contaminated with nucleic acids.
"No DNA" PCR reaction is showing signal	Use pipettes dedicated to PCR, and UV-irradiate pipettes prior to setting up PCR.
	Perform ChIP, DNA purification, and PCR reaction setup in three separate rooms/areas using dedicated pipettes, or set up reactions in a hood.
	Avoid using bottled or otherwise prepackaged water. Use freshly delivered Milli-Q® water from a system containing a UV light source.
	Use aerosol-resistant pipette tips.
	Do not open tubes containing amplified PCR products anywhere near the location of future ChIP or qPCR experiments.
Low recovery of DNA	**Ineffective or low affinity ChIP antibody:** Ensure that you are using an antibody that has been validated in ChIP For a complete selection of ChIP antibodies, see **www.merckmillipore.com/epigenetics.** If you are using a ChIP antibody, increase the incubation time of the antibody.
	Insufficient ChIP antibody: Generally 1-10 µg of ChIP antibody is sufficient. However, the amount of antibody required may depend on the relative abundance of your target protein and the affinity of the antibody for the target.
	Insufficient starting sample: Before crosslinking, prepare a separate plate to determine cell number. Re-evaluate your cell number per reaction. Increase your cell number especially if you are attempting to detect a low abundance target.
	Incomplete cell lysis and ineffective fragmentation: Optimize these steps by varying parameters (see section 5.3) and assessing their effects on chromatin recovery. Use mechanical force such as a dounce homogenizer or glass beads to improve cell lysis. Optimize the fragmentation steps and avoid foaming.
	Over crosslinking: Long incubation in formaldehyde may mask epitopes required for recognition by ChIP antibody. This can be especially problematic if you are using a monoclonal antibody. Over crosslinking may also result in the formation of complexes that are resistant to sonication. Optimize crosslinking steps: the final concentration of formaldehyde should be 1%, and you should determine the most effective crosslinking time before proceeding with the experiment.
	Under crosslinking: Insufficient crosslinking may result in dissociation of target proteins from DNA during subsequent steps of the protocol. Unless you are studying histones and histone modifications, you should use an X-ChIP protocol to stabilize the associations of your target protein with DNA. Increase crosslinking time.
	Low affinity or low quality beads: Protein G magnetic beads bind a wider range of antibodies, including mouse monoclonals. For the most flexibility with antibody choice, we recommend the protein A/G blend (Catalogue No. 16-663).
	PCR primers: Test the efficiency of the primers. Include appropriate controls and redesign primers if necessary.
Should I use a monoclonal or polyclonal antibody?	Either monoclonal or polyclonal antibodies can work for ChIP. Monoclonals are frequently highly specific, but monoclonals can be sensitive to crosslinking conditions. Over crosslinking may mask the target epitope and careful optimization of crosslinking might be required. Polyclonals are less sensitive to over crosslinking conditions, and may produce better enrichment than comparable monoclonals, but polyclonals are more likely to bind to nonspecific targets.
How can I increase the chances of the selected antibody working in ChIP?	Test specificity/crossreactivity to identify the epitopes recognized by your antibody. You will also need to test the antibody in multiple immunoassays such as Western blot, immunocytochemistry, and immunoprecipitation, then make sure the antibody produces good fold enrichment of your target DNA in ChIP.
How much antibody should I use?	We recommend using 2-10 µg of your ChIP antibody depending on the abundance of your protein target and the affinity of your antibody for the target. More antibody does not always equal stronger signal. It is suggested that to get the best ChIP signal the amount of antibody be titrated.
How should I choose a commercial ChIP antibody?	Choose an antibody that has passed multiple specificity/crossreactivity tests, and validated in ChIP and multiple immunoassays. For screening of your histone antibodies, we recommend the AbSurance™ histone antibody specificity Arrays (Catalogue Nos. 16-665, 16-667, and 16-668).

Problem/Question	Suggested Steps/Answer
Do you recommend using tags if I cannot find a suitable ChIP antibody for my study?	Using a tagged antibody in ChIP is a way to get around antibody unavailability, variability and epitope masking in crosslinked chromatin. It is possible that a tag will interfere with transcription factor function. Tags should be evaluated on a case-by-case basis. Switching tags between N and C termini may be good controls.
What factors should I consider if I want to overexpress a protein with a fusion tag?	Any system where overexpression is occurring may introduce false-positive interactions. Ideally, if you are analyzing stably expressing fusion-tagged cell lines, we recommend using high and low expressing cell lines, and performing mock IPs from untransfected cell chromatin, for control comparisons.
What is a good control antibody?	We recommend using normal IgG from the same species as your ChIP antibody, so if you are using a mouse monoclonal, we recommend normal mouse IgG.
What is the advantage of using protein A/G bead blend?	Many antibodies bind to both protein A and G with varying affinity and specificity. Blending protein A and G beads eliminates the need to choose one over the other and to evaluate binding to both types for optimization. In most cases we have seen better fold enrichment and reduced background activity using a protein A/G bead blend compared to using similar quantities of pure protein A or protein G beads.
Will subjecting my chromatin to freeze-thaw cycles affect my ChIP results?	We don't recommend freeze-thaw cycles when handling chromatin samples, but in some cases, this has not affected the ChIP result. This may depend on the buffer in which the chromatin is stored. In some protocols, glycerol is included in the buffer to stabilize epitopes through freeze-thaw cycles.
Why are high volumes of ChIP dilution buffer required before IP? Are different buffers preferred for different beads?	Ten-fold dilution of chromatin is necessary when the lysis buffer used in chromatin fragmentation contains high concentrations of SDS (that is 1% in some cases). Diluting the chromatin ensures that the IP beads and antibody won't be denatured or affected by the presence of the detergent. We use similar buffers for both agarose beads and magnetic beads in our ChIP kits.
How can you determine if you have over crosslinked your sample?	If you have not previously studied this target in ChIP, we recommend treating cultured cells with 1% formaldehyde for 10 minutes at RT. If you can't get enrichment (and expect the protein is present at the location you are looking), then increase crosslinking time to 15 or 20 minutes. If you have overcrosslinked, in general, you will see the same signal in a location-independent manner. Location-independence means that you observe the same signal at a known binding site versus a known negative locus (such as a 4 kb-distant site).
Why can't I crosslink my target to DNA?	You may not be allowing adequate time for crosslinking to occur. Increase the crosslinking time.
Is it necessary to quench the crosslinking step?	Quenching is routinely done with addition of glycine. However, some protocols suggest that washing out the formaldehyde with PBS (when fixing cultured cells) is sufficient to stop crosslinking. This will depend on whether serum is present in the growth media when you are fixing your cells. Glycine quenching is more important if serum proteins are present in the growth media.
Is it necessary to reverse crosslinks and do a solvent extraction of ChIP DNA before agarose gel electrophoresis?	Crosslink reversal is highly recommended before performing this step. Large complexes may clog the pores of the gel, retard electrophoresis, and produce high background. DNA purification may be optional depending on your sample. Crude DNA can be successfully amplified and stained following proteinase K digestion and crosslink reversal.
Should I consider using non-formaldehyde crosslinkers?	It may depend on your particular sample. In some cases dual crosslinkers are recommended. UV crosslinking is also done in some cases; however, UV crosslinks are irreversible and will prevent further analysis of your ChIP DNA.
Is a nuclei isolation step required?	Isolating nuclei prior to extraction of chromatin can reduce background by eliminating cytoplasmic proteins.
How can I tell if I have isolated nuclei from my cells?	You can view the samples on a phase contrast microscope to determine if the nuclei have been released from cells.
How many cells should be used for targets such as accessory factors that are indirectly associated with DNA?	Ten million cells is recommended. See section on "Chromatin Preparation" for further guidelines.
How should I perform ChIP with tissues?	In general, chromatin can be prepared from fresh or frozen tissue, and fixed in 1% formaldehyde-containing solutions for 15 minutes, but there are many variations on how this can be done and how tissues are disaggregated or homogenized prior to chromatin isolation. You may also try the Magna ChIP G Tissue Kit (Catalogue No. 17-20000) for an easier approach to processing your tissue samples for ChIP. There are also protocols available for tissue ChIP from laboratories such as the Farnham lab at UC Davis, as well as several published methods for brain tissue.

Problem/Question	Suggested Steps/Answer
Should I perform enzymatic digestion or sonication to shear my chromatin?	Enzyme digestion may not be as efficient as sonication, and incubating the samples at 37 °C for digestion may result in degradation of epitopes, but digestion may require less optimization than sonication to achieve mono-, di- or trinucleosomes.
What is the best equipment for sonication?	It depends on your particular sample. It is possible to use any type of sonication equipment, but the sonication process must be optimized for each type of sample
If using a probe sonicator, where should the probe tip be placed?	The probe tip should be submerged but not touching the wall of your tube. To prepare chromatin in batch, we recommend using 15 mL conical tubes, with a minimum volume of 0.6 mL (1.2 mL is more consistent), and with a clamp setup to make sure the probe is in the tube near the bottom but not touching the wall. The tube itself should be submerged in an ice bath on an adjustable platform during the sonication process.
How many ChIP reactions can I perform at once?	Commercial methods now exist to enable you to perform up to 96 ChIP reactions at once in a 96-well plate. For high throughput studies, we suggest Merck Millipore's Magna ChIP™ HT96 ChIP Kit (Catalogue No. 17-10077) or the EZ-Magna ChIP™ HT96 Kit (Catalogue No. 17-10078) with included controls.
Should I perform gel analysis or quantitative real-time PCR (qPCR)?	We recommend qPCR because it eliminates the need to optimize cycle number to ensure you are in the logarithmic amplification phase of your assay. Enrichment may be lower with transcription factors, but qPCR allows you to be more quantitative rather than qualitative.
How do you measure percent enrichment?	One approach is to run concurrent PCR reactions of replicate serial dilutions of the input DNA alongside your ChIP DNA and antibody control samples. The Ct values obtained can be used to plot a standard curve and the test samples can be fit to this curve to determine percent enrichment. (see section on ChIP analysis by qPCR).
How do I report error (intra-assay or inter-assay variation) for qPCR results?	Conduct all qPCR in replicates, and calculate the mean Ct and standard error. Then, propagate that standard error throughout your calculations of enrichment using an error propagation calculator, such as http://laffers.net/tools/error-propagation-calculator/
Is it advisable to normalize the test sample to just IgG in the relative Ct method?	The standard practice for comparative analyses is to use mock IP, ChIP IP, and input on both a target amplicon and a reference amplicon. Using just mock IP (IgG) Ct values as a normalizer without the additional samples required is not advisable.
What is an acceptable % input range for the normal IgG control antibody?	The IgG pulldown can be quite variable and qPCR-assay dependent. The same mock IgG sample can have different percent input results in one location of the genome vs. another region based on sequence composition of the assay design. The signal may be the result of nonspecific binding of nucleic acid to tube, to beads, to antibodies. ChIP is relative so it is best not to attempt to conform to a specific percent of input value, but ideally, the IgG value should have Ct values that are nearest to the most dilute sample in your standard curve. Again, ChIP is relative so if your ChIP signal is higher than your IgG signal (within limits of variation in the assay), you have a positive ChIP result.
What is considered a good fold enrichment of my ChIP DNA?	This will depend on your protein target. Abundant targets will likely produce high enrichments relative to low abundance targets. Some labs set a minimum fold enrichment of 5-fold over an IgG control as a minimum threshold for a successful experiment. However, it is best to compare your results to published results rather than conform to a set value.
How can I test my qPCR primers?	You can test your primers by running a qPCR reaction with serial dilutions of a suitable sample of known concentration. If your primers are working optimally, and all other conditions of the reaction have been satisfied, you should observe an approximate doubling of amplicon in each linear phase of the cycle (see section on qPCR).
Should I perform a singleplex or multiplex qPCR assay?	A multiplex experiment may be more suitable when testing multiple samples but will require more complex detection methods such as fluorophore-tagged probes that can easily produce distinct fluorescent signals for each target DNA in your reaction tube. You will therefore need to perform optimizations for each of your primer-probe set and you may incur additional costs. A singleplex experiment is fairly easy to design and multiple samples can be run in parallel with cost-effective DNA-binding dyes such as SYBR® Green and appropriately designed primers (see section on qPCR).
How can I distinguish nucleic acid contaminants from nonspecific binding of SYBR® Green?	You can perform a melt curve analysis in which you manipulate the thermal conditions of the qPCR process to allow your amplicons to melt. You should observe only one peak in the melt curve for your target amplicon. Additional peaks may indicate the presence of primer dimers or contaminants.

Supplementary Protocols

5

5.1. Preparing cells for ChIP

1. If necessary, stimulate or treat adherent mammalian cells at ~80 to 90% confluence in a 150 mm culture dish containing 20 mL of growth media. Include one extra plate of cells to be used solely for estimation of cell number.
2. Prepare 22 mL of 1X PBS (2.2 mL 10X PBS and 19.8 mL water) for each 150 mm culture dish. Store on ice. This will be used for washes and needs to be ice cold.
3. Add 550 µL of 37% formaldehyde (or 1100 µL of 18.5% formaldehyde) directly to 20 mL of growth media to crosslink. Gently swirl dish to mix.
4. Incubate at room temperature for 10 minutes.
5. During the ten minute incubation, prepare 1X protease inhibitor in PBS: Add 2 mL of ice cold 1X PBS to a separate tube for every dish and add 10 µL of the 200X Protease Inhibitor Cocktail III. Store on ice.
6. Add 2 mL of 10X glycine to each dish to quench excess formaldehyde.
7. Swirl to mix and incubate at room temperature for 5 minutes.
8. Place dishes on ice.
9. Aspirate medium, removing as much medium as possible, being careful not to disturb the cells. If you are using suspension cells, spin down cells at 8000 x g for 5 minutes.
10. Add 10 mL of cold 1X PBS to wash cells. Remove 1X PBS.
11. Remove 1X PBS and repeat wash.
12. Add 2 mL of 1X Protease Inhibitor Cocktail III in PBS prepared in Step 6.
13. Scrape cells from each dish into a separate microcentrifuge tube.
14. Spin at 800 x g at 4 °C for 5 minutes to pellet cells.

5.2. Preparing tissues for ChIP

1. Isolate non-fixed fresh tissue as desired. Use a razor blade to cut a pea-size piece of tissue into small pieces (typically 1mm or smaller) to improve crosslink efficiency. Alternatively, a plug of tissue from cryosectioned non-formalin-fixed-paraffin-embedded (FFPE) material can be used to obtain a small sample of interest (please see the Magna ChIP™ G Tissue Kit manual, Catalogue No. 17-20000).
2. Weigh the tissue, and then transfer into a 50 mL tube and wash twice with ice cold 1X PBS.
3. Resuspend tissue in 20 mL ice cold PBS and add 550 µL of 37% formaldehyde (or 1100 µL of 18.5% formaldehyde) to crosslink. Gently swirl dish to mix.
4. Incubate at room temperature for 10 minutes.
5. In the interim, prepare 1X protease inhibitor in PBS: Add 2 mL of ice-cold 1X PBS to a separate tube for every sample and add 10 µL of Protease Inhibitor Cocktail III. Store on ice.
6. Add 2 mL of 10X glycine to quench excess formaldehyde.
7. Homogenize the tissues several times using a Dounce homogenizer (loose pestle).
8. Spin at 800 x g at 4 °C for 5 minutes to pellet cells.

5.3. Optimizing Sonication and Analyzing DNA Fragments

Optimal conditions for shearing crosslinked DNA to 200-1000 base pairs in length depend on the cell type, cell concentration, and the specific sonicator equipment, including the power settings and duration and number of pulses. Approaches for optimizing sonication may include the following:

A. Varying the concentration of cell equivalents per mL of initial buffer with constant sonication parameters.

B. Choosing a fixed concentration of cell equivalents per mL of buffer and varying cycles and/or power settings of sonication.

C. A combination of both approaches.

The protocol below describes optimization following option A and is provided as an example only.

1. Generate a cell lysate by following Section 5.1, but vary your buffer volume per cell amount to generate 3 different microcentrifuge tubes containing several cell equivalent concentrations in the range of 5 x 10^6 per mL to 5 x 10^7 per mL. For HeLa cells, this requires approximately 4 x 10^7 cell equivalents, or approximately four 15 cm plates.
2. Continue with the following Cell Lysis procedure. Each microcentrifuge tube should contain approximately 500 µL of cell lysate.

Volume of Cell Lysis Buffer	Cell Density	Cells Required
500 µL	5 x 10^6/mL	2.5 x 10^6
500 µL	2 x 10^7/mL	1 x 10^7
500 µL	5 x 10^7/mL	2.5 x 10^7

3. Be sure to keep the samples on wet ice at all times. Sonication generates heat which will denature the chromatin.
4. Remove 1 x 10^5 cell equivalents from each condition prior to sonication for analysis of unsheared DNA.
5. For each cell concentration, sonicate each tube for a fixed number of cycles allowing rests between cycles according to the instrument manufacturer's guidelines. For example, using a Misonix 3000 instrument and a No. 419 microtip probe, use six 15 second pulses with 50 second intervals between pulses, with power setting at 6. Keep tubes cool at all times.
6. Remove 1 x 10^5 cell equivalents (20 µL, 5 µL, 2 µL from least to most concentrated sample) of the sonicated chromatin from each condition to a fresh tube.
7. To all samples (unsheared and sheared), add elution buffer to a final volume of 50 µL.
8. Add 1 µL Proteinase K and incubate at 62 °C for 2 hour.
9. Load 10 µL and 20 µL on a 1–2% agarose gel with a 100 bp DNA marker. Loading different amounts helps to avoid under- or overloading
10. Observe which of the shearing conditions gives a smear of DNA in the range of 200–1000 bp.
11. Repeat optimization of the shearing conditions if the results indicate that the resulting DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that you do not alter the cell concentration or volume of lysate per microcentrifuge tube for subsequent chromatin immunoprecipitation experiments.

5.4. DNA Purification

(The following protocol was adopted from Sambrook, et al., 2006.)

1. Centrifuge your samples at full speed for 5 min at RT.
2. Transfer supernatants to fresh microcentrifuge tubes.
3. You can now purify your DNA using spin columns. For solvent extraction continue to Step 4.
4. Transfer the nucleic acid sample to a polypropylene tube and add an equal volume of phenol:chloroform. The nucleic acid will tend to partition into the organic phase if the phenol has not been adequately equilibrated to a pH of 7.8-8.0.
5. Mix the contents of the tube until an emulsion forms.
6. Centrifuge the mixture at 80% of the maximum speed that the tubes can bear for 1 minute at room temperature. If the organic and aqueous phases are not well separated, centrifuge again for a longer time.
7. Normally, the aqueous phase forms the upper phase. However, if the aqueous phase is dense because of salt (> 0.5 M) or sucrose (> 10%), it will form the lower phase. The organic phase is easily identifiable because of the yellow color contributed by the 8-hydroxyquinoline that is added to phenol during equilibration.
8. Use a pipette to transfer the aqueous phase to a fresh tube. For small volumes (< 200 µL), use an automatic pipettor fitted with a disposable tip. Discard the interface and organic phase.
9. Repeat Steps 1–4 until no protein is visible at the interface of the organic and aqueous phases.
10. Add an equal volume of chloroform and repeat Steps 2–4.
11. Recover the nucleic acid by standard precipitation with ethanol.

Kits & Assays

One-Day ChIP Kits

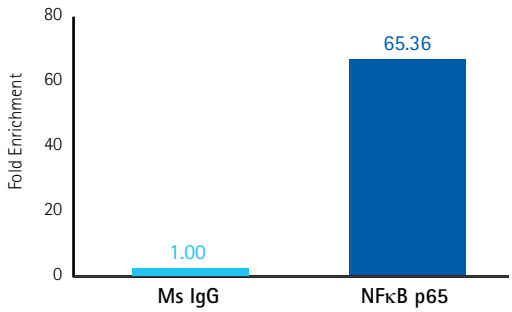
Magna ChIP™ A/G Chromatin Immunoprecipitation Kit

(Catalogue No. 17-10085)

EZ-Magna ChIP™ A/G Chromatin Immunoprecipitation Kit

(Catalogue No. 17-10086)

- Complete ChIP in one day, from cell to PCR results
- Protein A/G magnetic bead blend
- Enrichment of wider range of antibodies
- Suitable for high throughput applications
- Compatible with native ChIP
- EZ-Magna ChIP™ kit with essential positive and negative control antibodies, qPCR primers



Specific localization of NFκB binding via one-day ChIP using the EZ-Magna ChIP™ kit. Sonicated chromatin prepared from serum-starved, TNFα-treated HEK293 cells (~3 x 10^6 cell equivalents per IP) were subjected to chromatin immunoprecipitation using 4 µg of either Normal Mouse IgG, or 4 µg Anti-NFκB p65 (RelA) (components contained in NFκB p65 ChIPAb+™ kit (Catalogue No. 17-10060).

Immunoprecipitation of NFκB p65 (RelA)-associated DNA fragments was verified by qPCR using primers directed against IκBα.

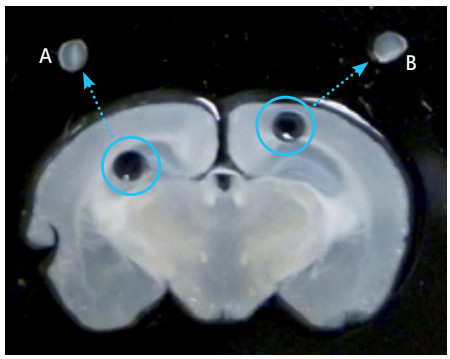
Tissue ChIP

Magna ChIP™ G Tissue Kit

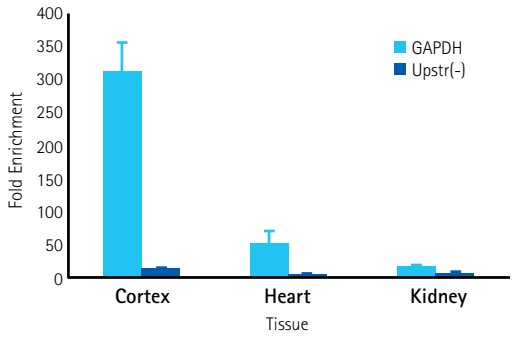
(Catalogue No. 17-20000)

The Magna ChIP™ G Tissue Kit provides the tools necessary to obtain repeatable, reliable, and site-specific tissue biopsies.

- Reliable ChIP from a variety of tissue samples
- Microdissection punch for accurate tissue biopsy
- Complete set of optimized buffers
- Detailed, optimized protocol with guided workflow



Region-Specific Tissue Isolation. A 300 µm coronal mouse brain cryosection was obtained and two microdissections were carried out using the 1 mm microdissection punch provided in the kit. The isolated tissue is shown placed above the dissected region: (A) hippocampus, (B) cortex.



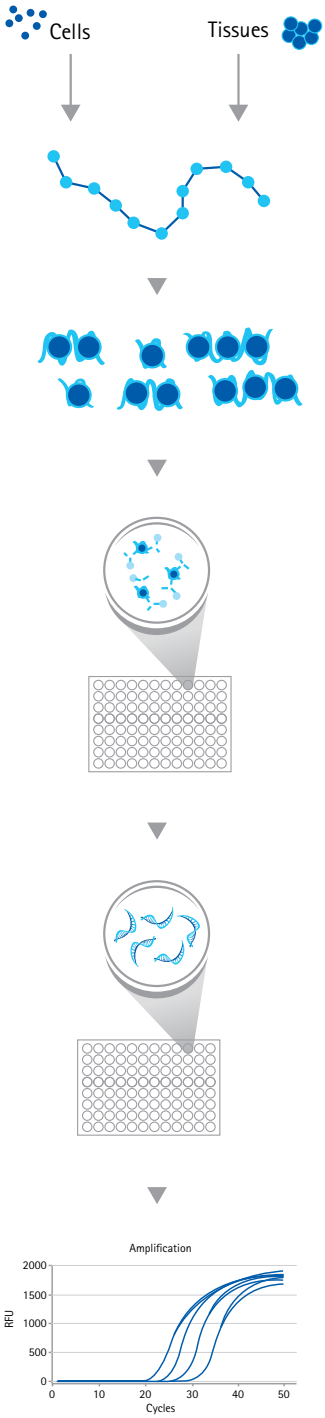
Tissue-specific localization of RNA polymerase II to the GAPDH promoter as revealed using the Magna ChIP™ G Tissue Kit and Anti-RNA Polymerase II clone CTD4H8 (Catalogue No. 05-623B). 1 µg of antibody was used to immunoprecipitate chromatin from various mouse tissues. The resulting immunoprecipitated DNA was analyzed by qPCR with primers specific for the mouse GAPDH promoter. QPCR was used to amplify immunoprecipitated chromatin fragments and data were presented as fold relative enrichment to IgG-associated DNA from independent experiments. For a biological negative control, fold enrichment was assessed by qPCR with primers upstream of the Dhfr gene (UpStr (-)).

High Throughput ChIP Kit

Magna ChIP™ HT96 (Catalogue No. 17-10077)

EZ-Magna ChIP™ HT96 (Catalogue No. 17-10078)

ChIP up to 96 samples in a single experiment. The Magna ChIP™ HT96 system is a simple, effective, 96-well plate-based method for ChIP using as few as 10,000 cells per well. With a streamlined protocol and a proprietary buffer system, the Magna ChIP™ HT96 kit provides excellent sensitivity and lower backgrounds compared to conventional approaches.



In vivo cross-linking
Lysis

Isolation of chromatin cultured cells or tissues

Sonication to shear chromatin

96-well plate immunoprecipitation
- 10,000-100,000 cells per reaction
- Manual or automated processing

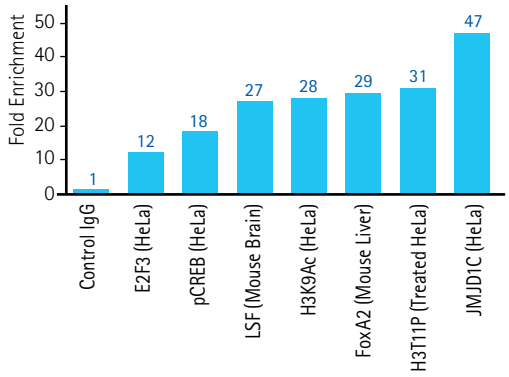
Reversal of cross-links

DNA purification (optional)

Detection
- Quantitative PCR
- Promoter microarray
- Sequencing

Features and Advantages

- Complete set of materials for ChIP of up to 96 samples in a single plate
- Low chromatin requirements: 10,000 to 100,000 cells per reaction
- Magnetic protein A/G bead blend allows the use of a greater variety of antibody subtypes than A or G alone
- Optimized, streamlined protocol allows use of single buffer for sonication, IP, and wash
- Directly analyze resulting DNA without additional clean-up steps
- High fold enrichment using multichannel pipettes or standard automated liquid handling systems
- Protocols for ChIP using cells or tissue
- Available with or without control antibodies and qPCR analysis primers
- Compatible with ChIPAb+™ Antibody/primer sets



High Throughput ChIP with a Variety of Antibodies and Chromatin Types. Chromatin derived from sources indicated was subjected to immunoprecipitation with either specific ChIPAb+™ antibodies (x-axis) or with IgG, using the Magna ChIP™ HT96 multichannel pipette protocol. Assays were performed using conditions described in the respective ChIPAb+™ product user guides.

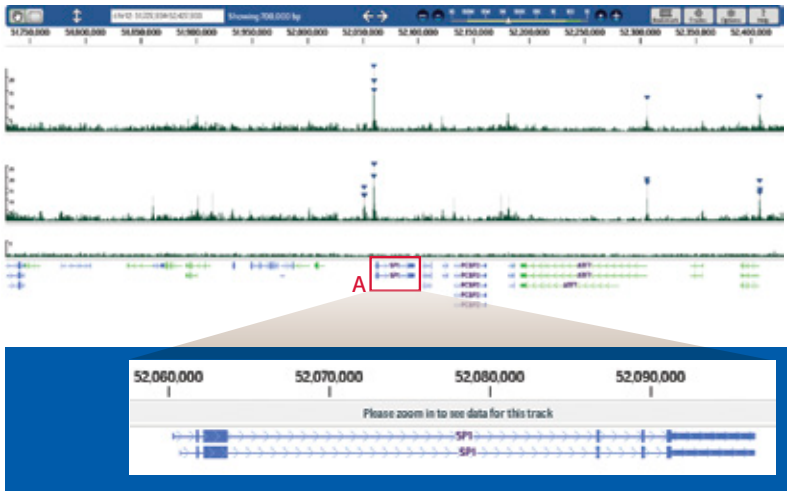
Genome-Wide ChIP Kits – Next Generation Sequencing

Magna ChIP™-Seq Chromatin Immunoprecipitation and Next-Generation Sequencing Library Preparation Kit

(Catalogue No. 17-1010)

- Reliable ChIP-Seq library construction from as little as 1 ng of purified ChIP DNA
- Protein A+G bead blend is compatible with a broader range of antibodies
- Flexible format allows construction of single end, paired end, or barcoded libraries
- Sufficient reagents for up to 10 next generation sequencing library constructions
- Quality-controlled, validated enzymes and buffers in convenient master mix streamline library construction
- Includes validated positive and negative control antibodies and a control primer set
- Proven performance through construction and sequencing of genomic DNA libraries on an Illumina Genome Analyzer II

Next gen sequencing analysis of Sp1-associated DNA library prepared using the Magna ChIP-Seq™ Kit



Effective ChIP and Reliable Next Gen Sequencing Library Construction from Limited Amounts of DNA. Sequencing libraries were constructed using the Magna ChIP-Seq™ Kit (Catalogue No. 17-1010) and the ChIPAb+™ Sp1 antibody/primer set (Catalogue No. 17-601). Libraries were constructed using 1ng, 10ng or an input chromatin sample and sequenced using an Illumina Genome Analyzer. Peak analysis (derived using quantitative enrichment of sequence tags (QuEST)) of the Sp1 locus from confidently mapped reads browsed with DNAnexus™ software shows Sp1 binding (triangles) occurs near expected Sp1 binding sites.

Genome-Wide ChIP Kits—Microarray Analysis

Magna ChIP²™ DNA Microarray Kits

Magna ChIP²™ kits are an easy way to take your ChIP analysis genome-wide. These kits are the first and only complete solution that standardizes and simplifies ChIP-chip analysis by combining all necessary and fully optimized reagents with validated protocols and guidelines. Each Magna ChIP²™ kit is designed to ensure success, sensitivity, and reproducibility, using either Agilent® or user-provided DNA microarrays.

Magna ChIP²™ Universal Kits

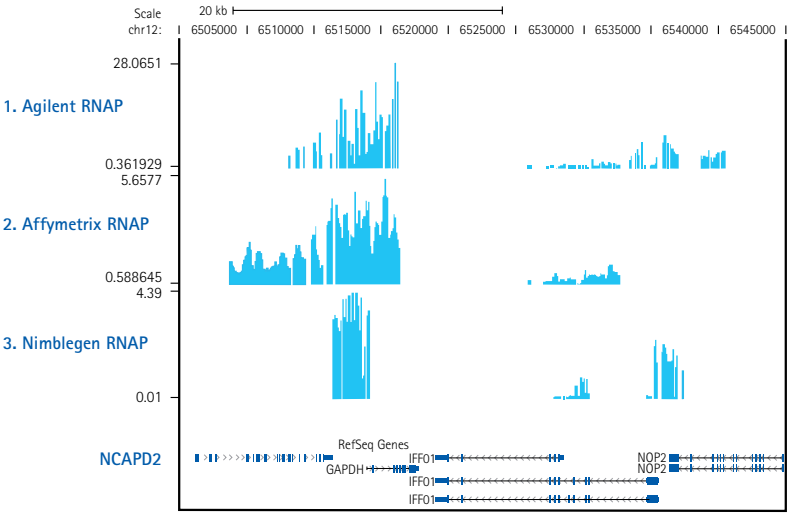
(Catalogue No. 17-1000)

Perform ChIP-chip analysis on virtually any type of microarray with the universal Magna ChIP²™ kits. These kits contain optimized reagents and validated protocols for preparing chromatin that's ready for labeling and hybridization.

Magna ChIP²™ Human and Mouse Promoter Kits (Catalogue No. 17-1001 and 17-1002)

Simplified ChIP-chip analysis with proven reagents and protocols for isolation, amplification, labeling and hybridization, including your choice of either human or mouse Agilent® promoter microarrays.

Comparison of commercially available arrays using Magna ChIP²™ Universal Kit



Magna ChIP²™ kits enable genome-wide ChIP analysis using multiple types of microarrays. Comparative results for the Agilent human 244K promoter array (Top), Affymetrix® human promoter array (Middle) and Nimblegen™ human promoter array (Bottom) using the Merck Millipore Magna ChIP²™ kit.

Histone Arrays

AbSurance™ Histone H2, H3, H4 Antibody Specificity Arrays

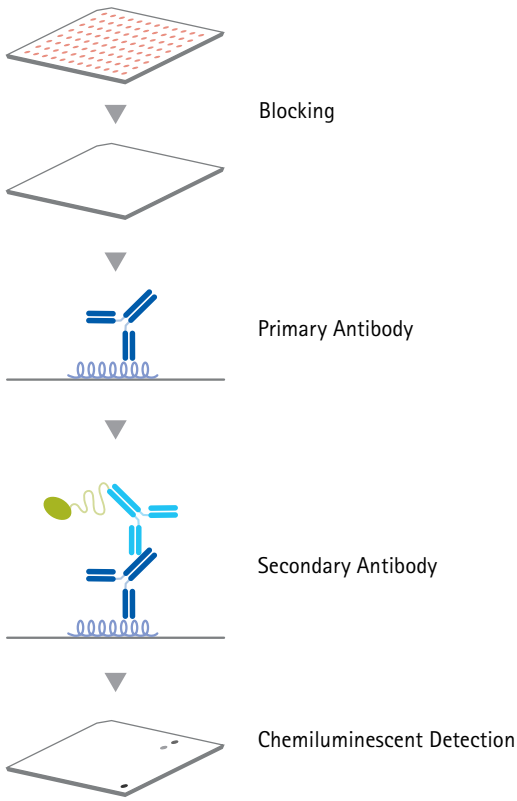
(Catalogue Nos. 16-665, 16-667 and 16-668)

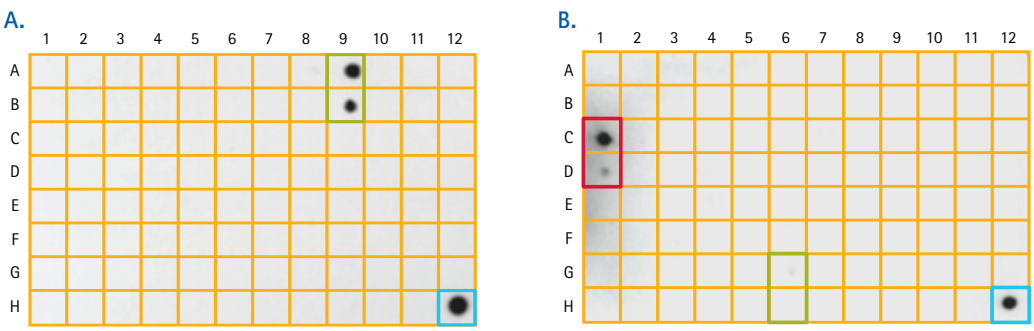
Easily evaluate the specificity and cross-reactivity of your histone antibodies with the same peptide macroarray technology used to screen Merck Millipore's highly characterized and well-published histone antibodies. The AbSurance™ Histone Antibody Specificity Arrays provide an easy and effective Western-blot–like approach to evaluate specificity and cross-reactivity of antibodies for histones H2A, H2B, H3, and H4, without the need for specialized imaging equipment or analysis software.

AbSurance™ Array Features and Benefits:

- High quality purified peptides (>95 % purity)
- A total of 89 peptides (21 acetylated, 43 methylated, 11 phosphorylated, and 14 unmodified)
- Easy-to-handle PVDF membranes
- Consistent and uniform spotting of peptides using a proprietary process
- Sensitive chemiluminescent detection using either film or CCD imagers
- Easy data analysis—no additional software required
- Built-in positive control primary antibodies from rat, mouse, sheep, and rabbit
- Available as an individual H3 or H2A, H2B, H4 Array, or as a complete set

Intuitive, convenient, Western blot–like protocol





AbSurance™ Detects Specific and Non-Specific Interactions.

A. Detection of specific interactions using Merck Millipore's antibody to trimethyl histone H3 lysine 4 (Catalogue No. 05-745R). AbSurance™ array shows expected specificity for H3K4me3 peptide (9A and 9B, green box) without cross-reaction to other peptides. Primary antibody used at 1:2,000 dilution. Positive control rabbit IgG shown at position 12H (blue box).
B. Detection of nonspecific interactions using an antibody to acetyl histone H3 lysine 56 obtained from Supplier E. AbSurance™ array data demonstrate weak recognition of the target H3K56ac peptide (6G and 6H green box) but strong cross-reactivity with H3K9ac (1C and 1D red box). Primary antibody used at 1:2,000 dilution. Positive control rabbit IgG shown at position 12H (blue box).

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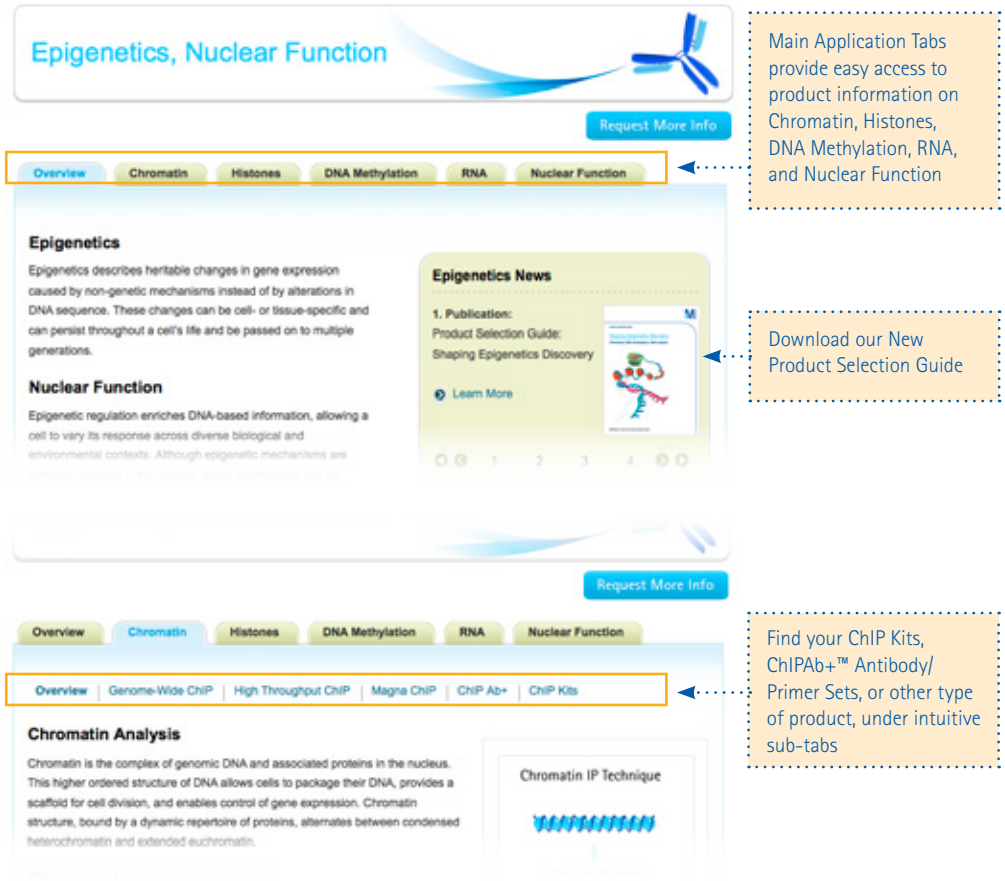

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