A GUIDE TO SUCCESSFUL CHROMATIN IP

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ChIP

Chromatin Immunoprecipitation is used to examine interactions between protein and DNA within the natural chromatin context of the nucleus. This guide highlights critical steps in the Cell Signaling Technology ChIP protocol and demonstrates how specific protocol changes can affect the final outcome of your experiment.

Cell Signaling TECHNOLOGY®

Introduction

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

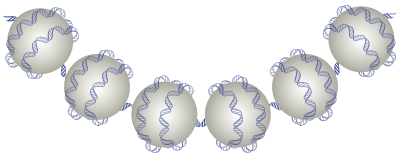
These experiments are sensitive to the integrity of the chromatin, the quality of the protein epitope, and the specificity of the immunoprecipitating antibody. These variables become even more critical when the protein-DNA interaction under investigation is of low abundance and/or low stability.

High abundance, stable interactions, like those between histones and DNA, occur often enough that they may still be detected even if the signal to noise ratio is less than ideal. In contrast, low abundance or less stable interactions, such as the binding of a transcription or co-factor to a specific gene, may fall below the limit of detection if the protocol fails to safeguard the integrity of the protein and the DNA, or if it relies on an antibody that is not highly specific to the target of interest. Therefore, an optimized ChIP protocol will protect the specific signal, limit the background noise, and allow the interaction of interest to shine through.

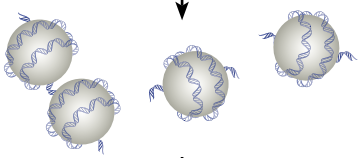
This guide highlights critical steps in an enzymatic digestion based ChIP protocol and demonstrates how protocol changes can affect the final outcome of your experiment.

ChIP Overview

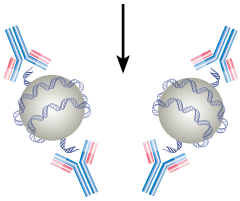
Cells are fixed with formaldehyde to fix proteins (both histone and non-histone) to DNA.



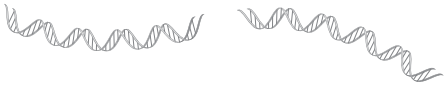
Chromatin is digested with Micrococcal Nuclease into 150-900 bp DNA-protein fragments.



An antibody specific to a protein of interest (histone or non-histone) is added. The complex co-precipitates and is captured by Protein G beads.

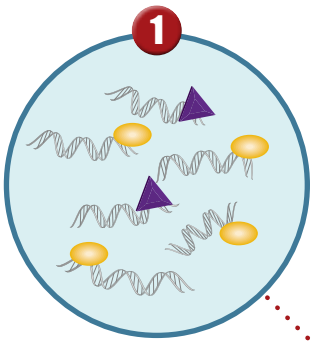


The DNA is purified in preparation for downstream analysis.



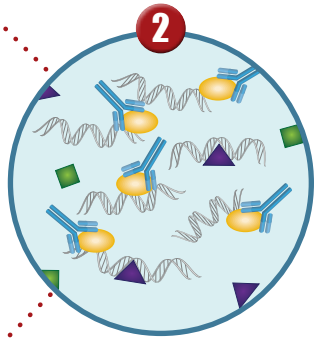
4 Step Protocol to a Better ChIP Experiment

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



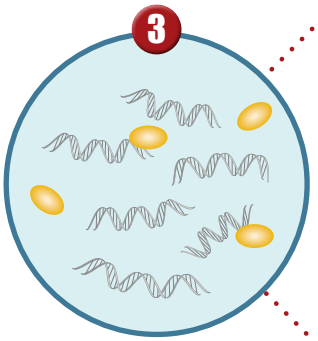
Chromatin Preparation

- Enzyme versus sonication
- Properly digested chromatin



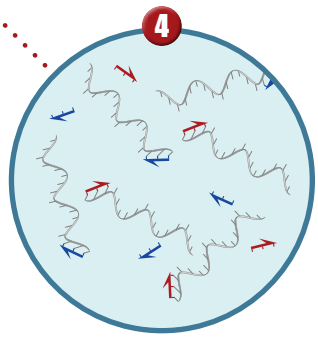
Immunoprecipitation

- Antibody concentration
- Bead selection
  - ChIP grade versus IP grade beads
  - Agarose versus magnetic beads



Cross-Link Reversal and DNA Isolation

- Proteinase K treatment
- DNA purification



Downstream Analysis

- Real-time PCR
- Next-generation Sequencing

The importance of a well-validated antibody.

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

The criteria listed below can be used as a guideline to help ensure your antibody is properly validated for ChIP.

Target Specificity

- Expected expression in positive/negative control cell lines, knockout cells, or siRNA-treated cells.
- Appropriate expression in response to enzyme-specific activators and/or inhibitors.
- Specificity of modification-specific histone antibody verified by peptide array or peptide ELISA.

Signal-to-Background Ratio

- Assessed using isotype controls to ensure acceptably low background signal.
- Enrichment of known target genes at least 10-fold above background, as shown by real-time PCR analysis.

Please visit our website for a full list of ChIP validated antibodies.

www.cellsignal.com/chipabs

Be in Control of Your ChIP Experiment

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

Positive Controls

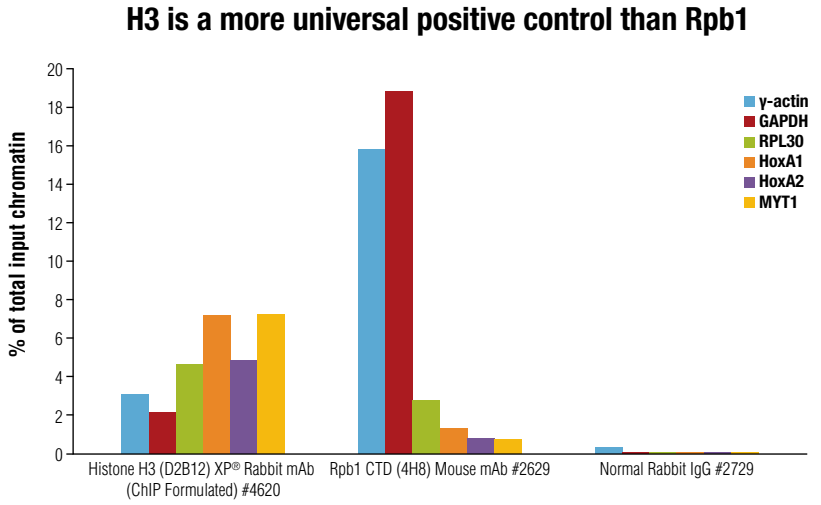
Many commercially available kits are supplied with an antibody to Rpb1 (the largest subunit of RNA polymerase II), for use as a positive control antibody. However, Rpb1 is only highly enriched at sites of active transcription, so if your locus of interest is an inactive site, Rpb1 will not provide a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

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

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

Negative Controls

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



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

Critical steps in the recommended protocol–how critical are they?

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

Chromatin Preparation

Enzymatic Digestion Versus Sonication

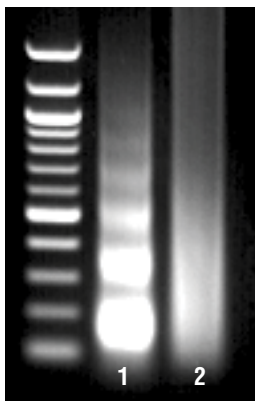
Many labs rely on sonication to prepare their chromatin for immunoprecipitation. While effective, sonication requires exposing the chromatin to harsh, denaturing conditions (i.e., high heat and detergent) that can damage both antibody epitopes and genomic DNA. Further, sonication is inconsistent. The method and quality of the prep will vary depending on the type and brand of sonicator you are using and on the condition of the sonicator probe used. Furthermore, there may only be a few second difference between having chromatin that is under or over-processed. As a result, chromatin preparations of consistent fragment size are difficult to generate with this method.

In contrast, enzymatic digestion uses micrococcal nuclease, which cuts in the linker region between nucleosomes to gently fragment the chromatin. This process does not require high heat or detergent and provides consistent results if the recommended ratio of enzyme to cell number is used. Thus, enzymatic digestion is simple to control, protects chromatin and antibody epitopes from shearing or denaturation, and results in a consistent, high-quality chromatin preparation that is conducive to immunoprecipitation. **(B)**

To demonstrate that enzyme-digested chromatin is more conducive to immunoprecipitation than sonicated chromatin, we performed ChIP using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) and another company's sonication-based ChIP kit. Chromatin was prepared using either enzymatic digestion (according to the SimpleChIP kit instructions) or sonication (according to the other company's instructions) and then subjected to immunoprecipitation using reagents from either the SimpleChIP kit or the other company's kit.

The enzyme digested chromatin showed more robust enrichment of target DNA loci than sonicated chromatin, using either the other company's IP module or the SimpleChIP module. This was especially apparent when less stable interactions, such as the binding of polycomb group proteins (Ezh2 [4] or SUZ12 [5]) to specific genes were assayed. **(C)**

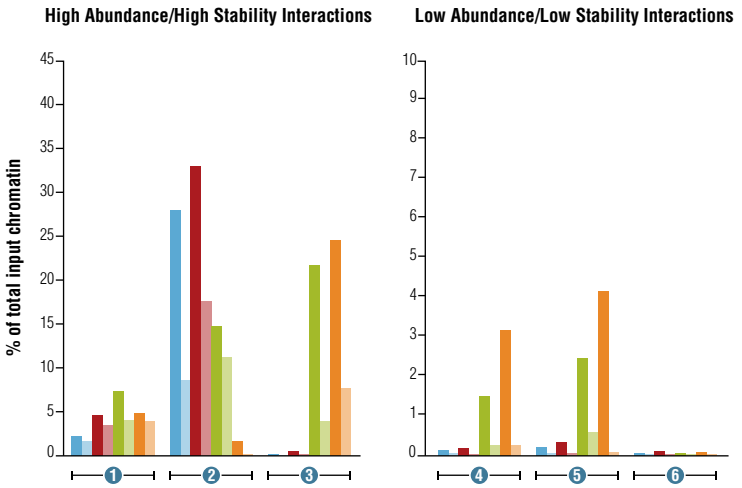
Enzymatic digestion reproducibly fragments chromatin into an array of uniform pieces



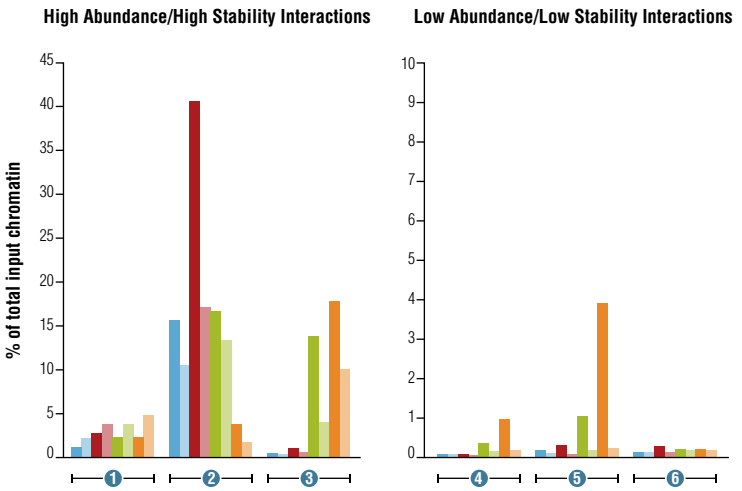
(B) DNA was purified after enzymatic digestion (Lane 1) or sonication (Lane 2) and and observed on a 1% agarose gel.

Target DNA loci are better immunoprecipitated from enzyme digested chromatin than sonicated chromatin

SimpleChIP IP Module



Other Company's IP Module



GAPDH
- Digested Chromatin
- Sonicated Chromatin

RPL30
- Digested Chromatin
- Sonicated Chromatin

HoxA1
- Digested Chromatin
- Sonicated Chromatin

HoxA2
- Digested Chromatin
- Sonicated Chromatin

1. Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620
2. Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751
3. Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb #9733
4. Ezh2 (D2C9) XP® Rabbit mAb #5246
5. SUZ12 (D39F6) XP® Rabbit mAb #3737
6. Normal Rabbit IgG #2729

(C) ChIP was performed using the SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic Beads) #9005 and another company's sonication-based ChIP kit. Chromatin was prepared using either enzymatic digestion (according to the SimpleChIP kit instructions) or sonication (according to the other company's instructions) and subjected to immunoprecipitation with the indicated panel of antibodies. The immunoprecipitated DNA was quantifed by real-time PCR and is presented as a percent of the total input chromatin.

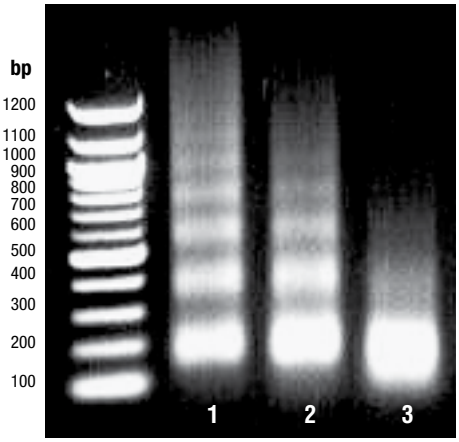
Sonication Post-enzymatic Digestion to Release Chromatin from the Nucleus

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

Properly Digested Chromatin

We recommend observing a sample of your digested chromatin on a 1% agarose gel before moving on to the immunoprecipitation step. Properly digested chromatin (Lane 2) will be sheared into mono-, di-, tri-, tetra- and penta-nucleosomal units, which will migrate as bands from 150 to 900 base pairs (bp) in length. Under-digested chromatin (Lane 1) will include larger (>900 bp) DNA fragments, whereas over-digested chromatin (Lane 3) will migrate as a single band of around 150 bp (mono-nucleosome). **(D)** Observing your chromatin on a gel will ensure optimal enrichment and signal to noise ratio. Chromatin fragments that are too large (> 900 bp) can lead to lower signal resolution and increased background. In contrast, signal resolution may be negatively impacted if the fragments are too small (< 150 bp), as these fragments are too small to be amplified by PCR.

Properly digested chromatin migrates between 150-900 bp



(D) Enzyme-digested chromatin was run on an agarose gel. Lane 1 shows chromatin that is underdigested. Lane 2 shows properly digested chromatin and Lane 3 shows chromatin that is overdigested.

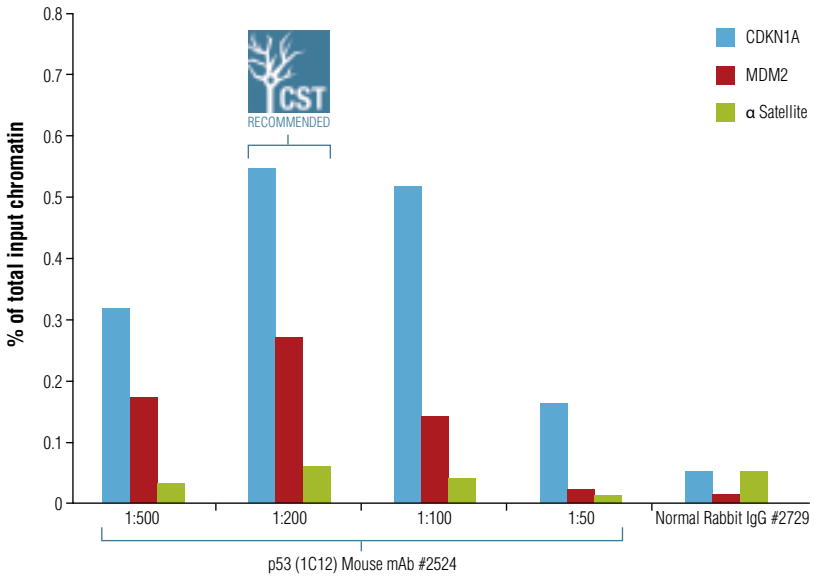
Immunoprecipitation

Antibody Concentration

The concentration of antibody used during the immunoprecipitation step can dramatically affect your ChIP results. If the antibody concentration is too high, relative to the amount of chromatin, it may saturate the assay, leading to lower specific signal and/or increased background noise. Conversely, if the concentration of the antibody is too low it may fail to bind all of the target protein in the IP sample, resulting in less efficient immunoprecipitation.

CST has titrated the p53 (1C12) Mouse mAb #2524 and recommends its use at a 1:200 dilution in the SimpleChIP assay. Adding more or less antibody to the mix by altering the dilution factor actually has a negative effect on target enrichment. **(E)**

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



(E) ChIP was performed with increasing amounts of p53 (1C12) Mouse mAb #2524 using SimpleChIP® Plus Enzymatic Chromatin IP Kit (Magnetic beads) #9005 to determine the optimal antibody dilution. CST recommends using this antibody at a dilution of 1:200.

TROUBLESHOOTING TIP

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

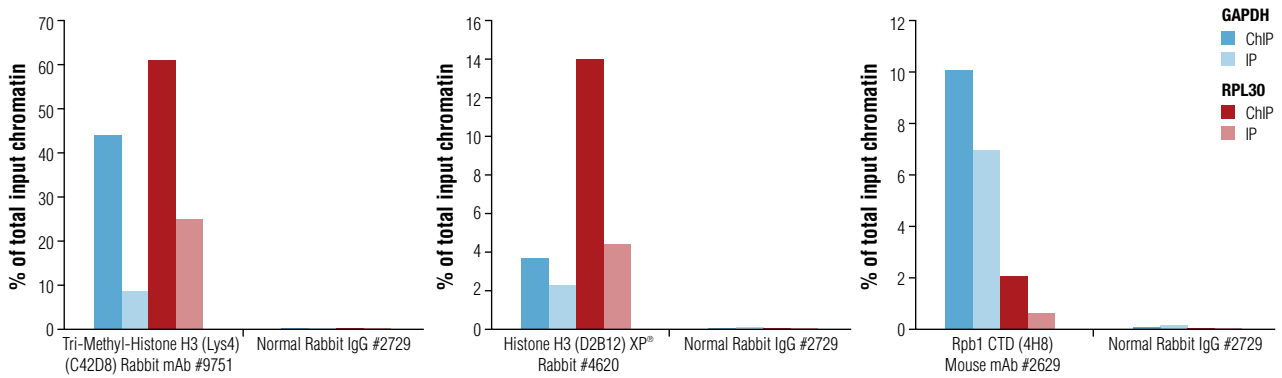
Bead Selection

ChIP-grade Versus IP-grade Beads

Note that not all protein G beads are equal. For example, CST offers both ChIP-grade and IP-grade beads and although they are approved for similar applications they should not be used interchangeably.

CST ChIP-Grade Protein G Beads contain a truncated form of recombinant Protein G that is covalently coupled to nonporous paramagnetic particles (magnetic beads) or agarose beads. Unlike the IP-grade beads, the ChIP-grade beads are supplied in a BSA-containing buffer, which blocks non-specific binding of proteins and DNA, allowing the ChIP-grade beads to provide a cleaner signal than the IP-grade beads. The difference in ChIP quality is apparent from the resulting real-time PCR signal which indicates that ChIP-grade magnetic beads provide higher enrichment and lower background signal when compared to the IP-grade magnetic beads. **(F)**

ChIP-grade Beads perform better than IP-grade Beads



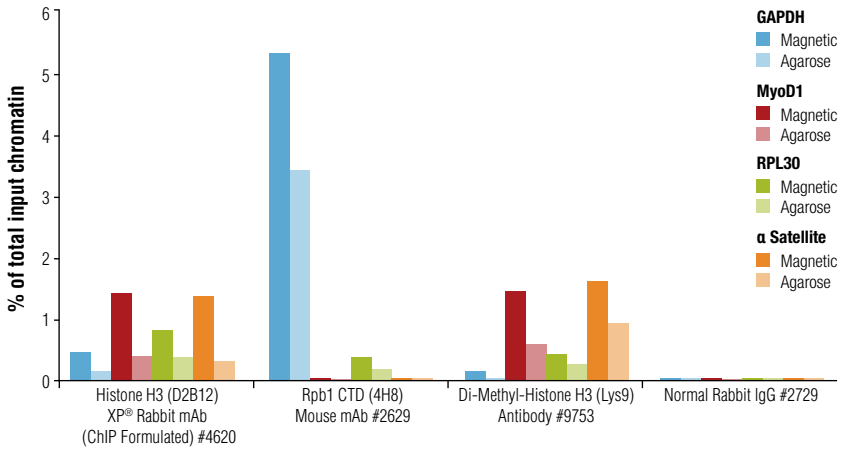
(F) ChIP was performed using digested chromatin from HeLa cells and the indicated antibodies, using either ChIP-Grade Protein G Magnetic Beads #9006, or IP-Grade Protein A Magnetic Beads #8687. Purified DNA was analyzed by quantitative real-time PCR, using SimpleChIP® Human GAPDH Exon 1 Primers #5516 and SimpleChIP® Human RPL30 Exon 3 Primers #7014. The quantified DNA is expressed as a percent of the total input chromatin.

Agarose Versus Magnetic Beads

The ChIP-grade Protein G Magnetic Beads were designed in partnership with New England Biolabs (NEB) for optimal performance in the ChIP assay.

Although the magnetic beads are more expensive and require the purchase of a magnetic rack they do have several advantages over the agarose beads. First, when we perform side-by-side comparisons of agarose and magnetic beads we find the magnetic beads perform better than the agarose beads with regards to higher enrichment and lower background signal **(G)**. Second, the magnetic rack holds the magnetic beads against the side of the IP tube, which makes washes easier to perform and more effective, since wash solutions can be completely removed without losing beads to pipetting. Lastly, although price consideration may make agarose beads seem like the better choice for performing IP, they are not suitable if you intend to perform ChIP-sequencing experiments. Agarose beads rely on sonicated salmon sperm DNA in addition to BSA to block against background signal. The DNA will carry over and be read as part of the sequencing run, skewing the final results.

Magnetic Beads perform better than Agarose Beads



(G) ChIP was performed using digested chromatin from HeLa cells and the indicated antibodies, using either ChIP-Grade Protein G Magnetic Beads #9006, or ChIP-Grade Protein G Agarose Beads #9007. Purified DNA was analyzed by quantitative real-time PCR, using SimpleChIP® Human GAPDH Exon 1 Primers #5516, SimpleChIP® Human MyoD1 Exon 1 Primers #4490, SimpleChIP® Human RPL30 Exon 3 Primers #7014, and SimpleChIP® Human α Satellite Repeat Primers #4486. The quantified DNA is expressed as a percent of the total input chromatin.

Cross-link Reversal and DNA Purification

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

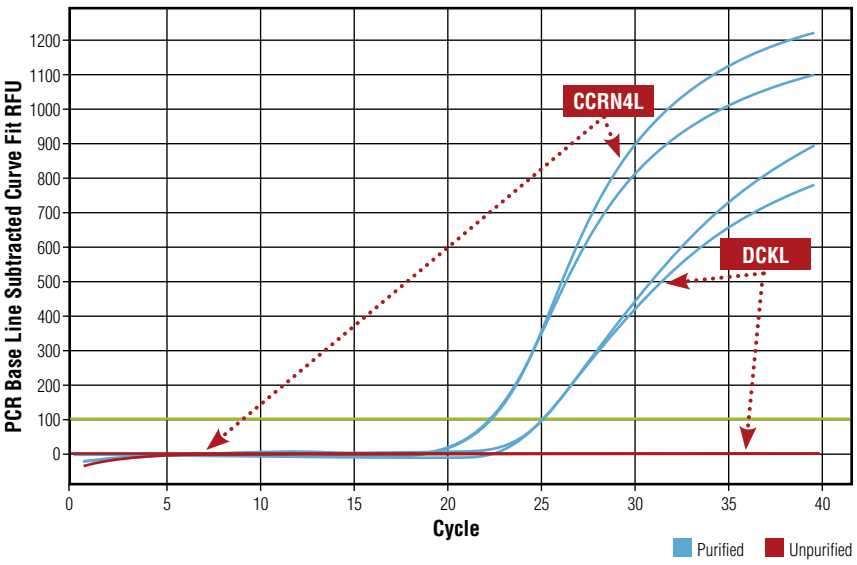
Proteinase K Digestion

The DNA must be separated from the bound protein and this is accomplished by subjecting the immunoprecipitated material to high heat and proteinase K digestion, which reverses the protein-DNA cross-links and digests the protein elements of the chromatin. Proteinase K digests the protein elements of the chromatin as well as any nucleases that may be present in the sample, so this step has the added benefit of protecting the DNA from degradation. Digested protein will negatively affect downstream DNA analysis (i.e., real-time PCR, sequencing, etc), so it will need to be removed using the DNA purification step discussed next.

DNA Purification

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

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



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

Downstream Analysis

There are several approaches available for analyzing the purified DNA that was immunoprecipitated with the protein target of interest. We will describe the two most commonly used methods below.

Real-time PCR

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

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

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

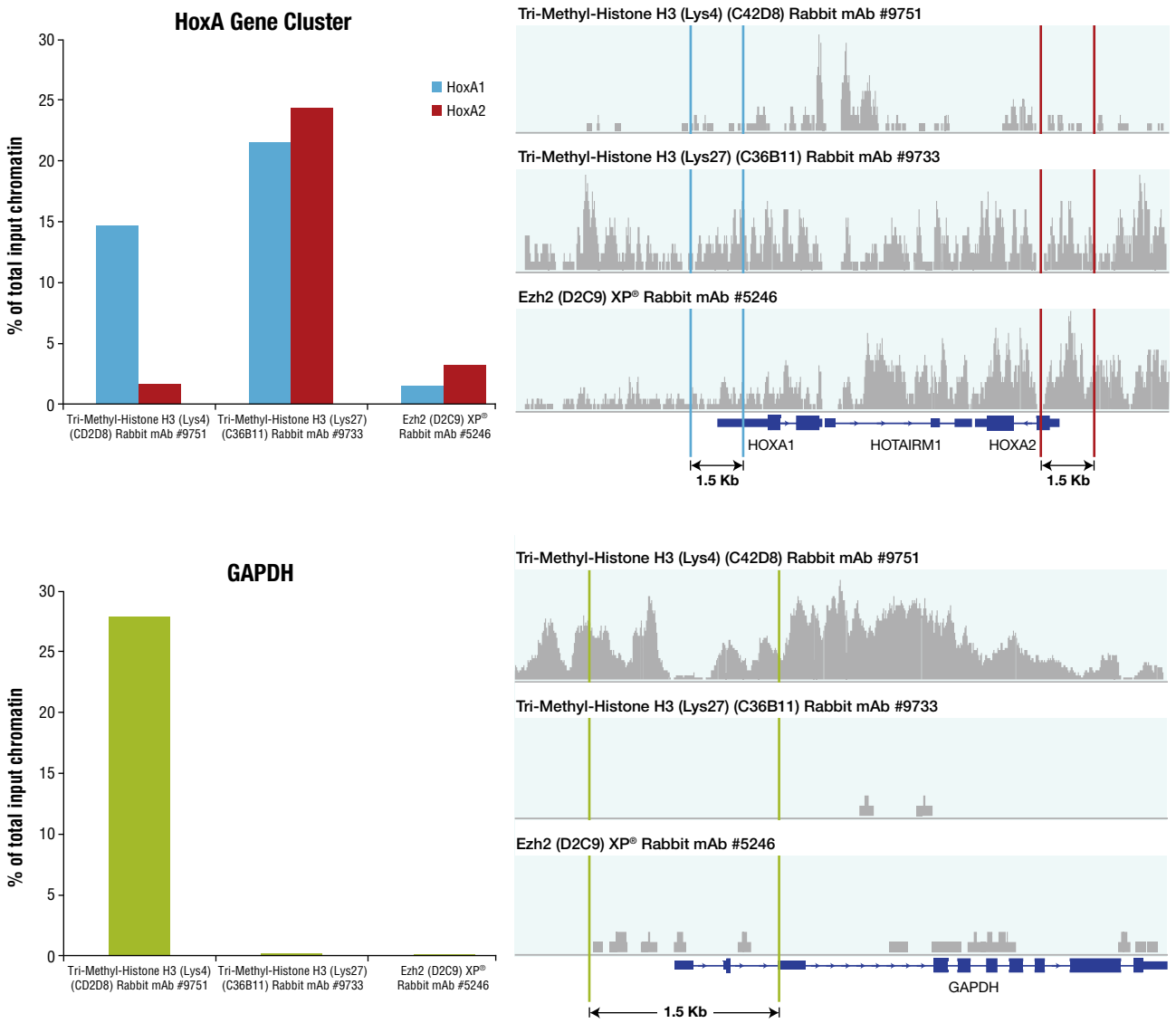
TROUBLESHOOTING TIP

CST recommends using real-time PCR to analyze ChIP results, because it is quantitative and standard PCR is not. In addition, we offer positive and negative control primers, suitable for real-time PCR, for each of our ChIP validated antibodies. Investigators may use these primers to confirm that their chromatin and antibody are conducive to IP.

Next-Generation Sequencing (NGS)

Next generation sequencing (NGS) may be used to analyze immunoprecipitated DNA after ChIP, using a protocol termed ChIP-seq. ChIP-seq provides the user with a high resolution, genome-wide view of protein/DNA interactions that isn't achievable using real-time PCR. For example, in the experiment below IPs were performed with antibodies to the active histone mark H3K4me3, the inactive histone mark H3K27me3, and Ezh2, a polycomb group transcription factor that deposits the H3K27me3 mark. It's known that Ezh2 binds to the HoxA Gene cluster, but not the GAPDH gene and this is shown below by both real-time PCR and next generation sequencing. **(I)**

Importantly, the real-time PCR approach only allows for the examination of preselected genomic loci, while NGS allows the investigator to generate a profile of H3K4me3 and H3K27me3 marks and Ezh2 binding sites across the genome. The wealth of information obtained using this approach makes ChIP-seq a powerful technique for studying the epigenetic changes that occur during either normal development or pathological states.



(I) Chromatin immunoprecipitations were performed with cross-linked chromatin from 4 x 10^6 NCCIT cells and either 10 µl of Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, 10 µl of Tri-Methyl-Histone H3 (Lys27) (C36B11) Rabbit mAb #9733, or 5 µl of Ezh2 (D2C9) XP® Rabbit mAb #5246. The enriched DNA was quantified by real-time PCR using SimpleChIP® Human HoxA1 Intron 1 Primers #7707, SimpleChIP® Human HoxA2 Promoter Primers #5517 (top left), and SimpleChIP® Human GAPDH Exon 1 Primers #5516 (bottom left). The amount of immunoprecipitated DNA in each sample is represented as a percentage of the total input chromatin. DNA sequencing libraries were generated using the NEBNext® ChIP-Seq Library Prep Master Mix Set for Illumina (Cat. #E6240: New England Biolabs, Ipswich, MA) and 10 ng of DNA from each IP or input control. The DNA libraries were sequenced using an Illumina® MiSeq® Sequencer and the obtained sequences were mapped to the UCSC Human Genome Assembly (hg19). The data was visualized using Integrative Genomics Viewer (Helga Thorvaldsdóttir, James T. Robinson, Jill P. Mesirov. Integrative Genomics Viewer (IGV): high-performance genomics data visualization and exploration. Briefings in Bioinformatics 2012) and enrichment of H3K4me3, H3K27me3 and Ezh2 binding at the HoxA1, HoxA2 (top right), and GAPDH (bottom right) genes is shown. Red, blue, and green vertical lines indicate the areas of the genome interrogated with primer sets used for real-time PCR amplification.

ChIP Protocols

An optimized protocol is necessary to achieve consistent, reliable ChIP results. For the best possible results, Cell Signaling Technology (CST) strongly recommends using our protocols, which are the result of extensive in-house validation performed at CST and ensure accurate and reproducible results.
ChIP specific protocols can be found by visiting our web pages.

www.cellsignal.com/chip

Chromatin Immunoprecipitation (ChIP)

Troubleshooting Guide

A. Expected Chromatin Yield

When harvesting cross-linked chromatin from tissue samples, the yield of chromatin can vary significantly between tissue types. The table below provides a range for the expected yield of chromatin from 25 mg of tissue compared to 4 x 10^6 HeLa cells, and the expected DNA concentration, as determined in Section IV of the protocol. For each tissue type, disaggregation using a BD™ Medimachine system (BD Biosciences) or a Dounce homogenizer yielded similar amounts of chromatin. However, chromatin processed from tissues disaggregated using the Medimachine typically gave higher IP efficiencies than chromatin processed from tissues disaggregated using a Dounce homogenizer. A Dounce homogenizer is strongly recommended for disaggregation of brain tissue, as the Medimachine does not adequately disaggregate brain tissue into a single-cell suspension. For optimal ChIP results, we recommend using 5 to 10 µg of digested, cross-linked chromatin per IP; therefore, some tissues may require harvesting more than 25 mg per each IP.

Tissue/Cell	Total Chromatin Yield	Expected DNA Concentration
Spleen	20–30 µg per 25 mg tissue	200–300 µg/ml
Liver	10–15 µg per 25 mg tissue	100–150 µg/ml
Kidney	8–10 µg per 25 mg tissue	80–100 µg/ml
Brain	2–5 µg per 25 mg tissue	20–50 µg/ml
Heart	2–5 µg per 25 mg tissue	20–50 µg/ml
HeLa	10–15 µg per 4 x 10^6 cells	100–150 µg/ml

B. Optimization of Chromatin Digestion

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

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

Problem	Possible Causes	Recommendation
Concentration of the digested chromatin is too low (low chromatin yield).	Not enough tissue or cells were added to the chromatin digestion or cell nuclei were not completely lysed after digestion.	Add additional chromatin to each IP to give at least 5 µg/IP and continue with protocol.
		Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Some tissues may require processing of more than 25 mg per IP. The amount of tissue can be increased to 50 mg per IP, while still maintaining efficient chromatin fragmentation and extraction.
		Increase the number of sonications following chromatin digestion. Visualize cell nuclei under microscope before and after sonication to confirm complete lysis of nuclei.
Chromatin is under-digested and fragments are too large (greater than 900 bp). Large chromatin fragments can lead to increased background and lower resolution.	Too many cells or not enough micrococcal nuclease was added to the chromatin digestion.	Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add less tissue or cells, or more micrococcal nuclease to the chromatin digest. See Section B for optimization of chromatin digestion.
	Tissue or cells may have been over cross-linked. Cross-linking for longer than 10 min may inhibit digestion of chromatin.	Perform a time course at a fixed formaldehyde concentration. Shorten the time of cross-linking to 10 min or less.
Chromatin is over-digested and fragments are too small (exclusively 150 bp mono-nucleosome length). Complete digestion of chromatin to mono-nucleosome length DNA may diminish signal during PCR quantification, especially for amplicons greater than 150 bp in length.	Too few cells or too much micrococcal nuclease added to the chromatin digestion.	Weigh tissue or count a separate plate of cells prior to cross-linking to determine accurate cell number. Add more tissue or cells, or less micrococcal nuclease to the chromatin digest. See Section B for optimization of chromatin digestion.
No product or very little product in the input PCR reactions.	Not enough DNA added to the PCR reaction or conditions are not optimal.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	PCR amplified region may span nucleosome-free region.	Optimize the PCR conditions for experimental primer set using purified DNA from cross-linked and digested chromatin. Design a different primer set and decrease length of amplicon to less than 150 bp (see primer design recommendations in ChIP Protocol Section of our website)
	Not enough chromatin added to the IP or chromatin is over-digested.	For optimal ChIP results, add 5 to 10 µg chromatin per IP.
No product in the positive control histone H3-IP RPL30 PCR reaction.	Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.	Be sure to add 5 to 10 µg of chromatin and 10 µl of antibody to each IP reaction and incubate with antibody overnight and an additional 2 hr after adding Protein G beads.
	Incomplete elution of chromatin from Protein G beads.	Elution of chromatin from Protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
Quantity of product in the negative control Rabbit IgG-IP and positive control histone H3-IP PCR reactions is equivalent (high background signal).	Too much or not enough too little chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.	For optimal ChIP results, add 5 to 10 µg of chromatin and 10 µl of histone H3 antibody to each IP reaction. Reduce the amount of normal rabbit IgG to 1 µl per IP.
	Too much DNA added to the PCR reaction or too many cycles of amplification.	Add less DNA to the PCR reaction or decrease the number of PCR cycles. It is very important that the PCR products are analyzed within the linear amplification phase of PCR. Otherwise, the differences in quantities of starting DNA cannot be accurately measured. Alternatively, quantify immunoprecipitations using real-time quantitative PCR.
No product in the Experimental Antibody-IP PCR reaction.	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough antibody added to the IP reaction.	Typically a range of 1 to 5 µg of antibody are added to the IP reaction; however, the exact amount depends greatly on the individual antibody. Increase the amount of antibody added to the IP.
	Antibody does not work for ChIP.	Find an alternate antibody source.

Recommended Application Solutions

Cell Signaling Technology offers kits and reagents to support your ChIP protocol. These products are used by CST to validate our primary antibodies for ChIP, so you can be confident they will provide consistent, reliable results.

ChIP Products Available from CST

SimpleChIP Kits

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

Beads and Magnetic Rack

ChIP-grade Protein G Magnetic Beads #9006
ChIP-grade Protein G Agarose Beads #9007
6-Tube Magnetic Separation Rack #7017

Individual Kit Components

Micrococcal Nuclease #10011

SimpleChIP kit components listed by protocol step:

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

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

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

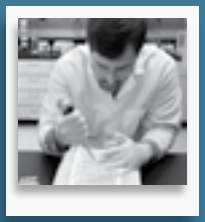
Downstream Analysis
SimpleChIP® Human RPL30 Exon 3 Primers 1
SimpleChIP® Human RPL30 Intron 2 Primers 1

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

www.cellsignal.com/chipabs

Technical Support

At CST, providing exceptional customer service and technical support are top priorities. The same scientists who work at the bench daily to produce and validate our antibodies are available to answer your questions and help troubleshoot your experiments.



FRONT COVER PHOTO:
Chris, Senior Group Leader, Development
has been with CST since 2005.

By Region

UNITED STATES
Orders: 877-616-2355 | orders@cellsignal.com
Support: 877-678-8324 | support@cellsignal.com
www.cellsignal.com

CHINA
Tel: +86-21-58356288
Support (China): 4006-473287/GreatQ | tech@cst-c.com.cn
Support (Asia Pacific): csttechasia@cst-c.com.cn
www.cst-c.com.cn

EUROPE, MIDDLE EAST & AFRICA
Tel: +31 (0)71 720 0200
Support: eusupport@cellsignal.eu
www.cellsignal.com

JAPAN
Tel: 03-3295-1630 | Support: info@cstj.co.jp
www.cstj.co.jp

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