#9003

Store at 4°C, RT and –20°C

SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads)

1 Kit
(30 Immunoprecipitations)

Cell Signaling TECHNOLOGY®

Orders ■ 877-616-CELL (2355)
orders@cellsignal.com
Support ■ 877-678-TECH (8324)
info@cellsignal.com
Web ■ www.cellsignal.com

For Research Use Only. Not For Use In Diagnostic Procedures.

Components Ship As: 91820S	Item #	Kit Quantity	Storage Temp
Glycine Solution (10X)	7005	100 ml	4°C
Buffer A (4X)	7006	25 ml	4°C
Buffer B (4X)	7007	25 ml	4°C
ChIP Buffer (10X)	7008	20 ml	4°C
ChIP Elution Buffer (2X)	7009	7 ml	4°C
5 M NaCl	7010	3 ml	4°C
0.5 M EDTA	7011	1 ml	4°C
ChIP-Grade Protein G Magnetic Beads	9006	1 ml	4°C
DNA Binding Buffer	10007	30 ml	Room Temp
DNA Wash Buffer (add 4x volume ethanol before use)	10008	6 ml	Room Temp
DNA Elution Buffer	10009	2 X 1 ml	Room Temp
DNA Purification Columns and Collection Tubes	10010	36 columns	Room Temp

Components Ship As: 45061S	Item #	Kit Quantity	Storage Temp
Protease Inhibitor Cocktail (200X)	7012	750 µl	–20°C
RNAse A (10 mg/ml)	7013	50 µl	–20°C
Micrococcal Nuclease (2000 gel units/µl)	10011	60 µl	–20°C
Proteinase K (20 mg/ml)	10012	100 µl	–20°C
SimpleChIP® Human RPL30 Exon 3 Primers 1	7014	150 µl	–20°C
SimpleChIP® Mouse RPL30 Intron 2 Primers 1	7015	150 µl	–20°C
Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated)	4620	100 µl	–20°C
Normal Rabbit IgG	2729	50 µl	–20°C
DTT (Dithiothreitol)	7016	192.8 mg	4°C

Important: Store DTT at –20°C once in solution.

Storage: All components in this kit are stable for at least 12 months past the reference date indicated on the component label when stored at the recommended temperature and left unused.

Note: Buffer A (4X) #7006, Buffer B (4X) #7007, and ChIP-Grade Protein G Magnetic Beads #9006 contain 0.05% sodium azide.

Reagents not supplied:

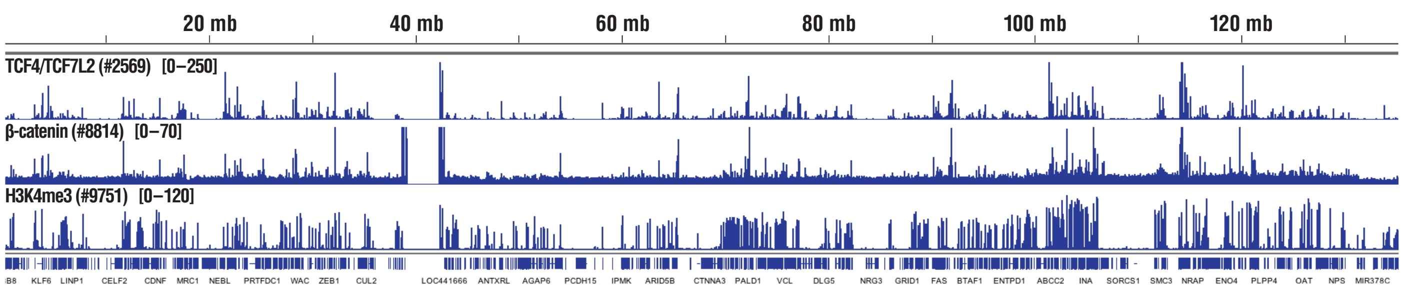
Magnetic Separation Rack #7017/#14654

1. 1X PBS #9872
2. Nuclease Free Water #12931
3. Ethanol (96-100%)
4. Formaldehyde (37% Stock)
5. SimpleChIP® Universal qPCR Master Mix #88989

Please visit www.cellsignal.com/technologies/chip.html for a complete listing of recommended companion products.

Description: The SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003 is designed to conveniently provide reagents needed to perform up to 30 chromatin immunoprecipitations from cells and is optimized for 4 X 10[6] cells per immunoprecipitation. This kit is compatible with ChIP-Seq.

Specificity/Sensitivity: The SimpleChIP® Enzymatic Chromatin IP Kit can be utilized with any ChIP-validated antibody to detect endogenous levels of protein-DNA interactions and histone modifications in mammalian cells (see Figures 1-4). The positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 recognizes many different species of the highly conserved Histone H3 protein, including human, mouse, rat and monkey. Primer sets are included for the human and mouse positive control RPL30 gene loci; however, the use of other species with the kit requires the design of additional control primer sets.



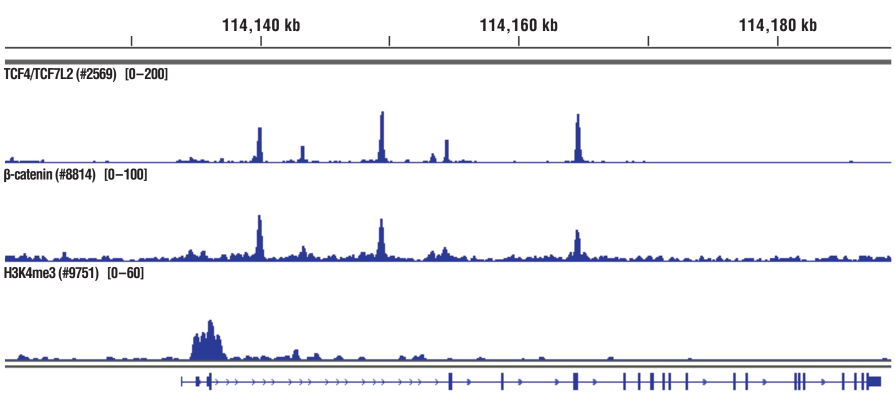


FIGURE 1. Chromatin immunoprecipitations were performed with cross-linked chromatin from HCT 116 cells and either TCF4/TCF7L2 (C48H11) Rabbit mAb #2569, Non-phospho (Active) β-Catenin (Ser33/37/Thr41) (D13A1) Rabbit mAb #8814, or Tri-Methyl-Histone H3 (Lys4) (C42D8) Rabbit mAb #9751, using SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003. DNA Libraries were prepared using DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795. The figure shows binding across chromosome 10 (upper), including ACSL5 (lower), a known target gene of TCF4/TCF7L2, β-Catenin, and H3K4me3.

SimpleChIP® kit co-developed by CST and NEB; Antibodies provided by CST; Enzymes provided by NEB. NEW ENGLAND BioLabs Inc.

© 2022 Cell Signaling Technology, Inc.
SimpleChIP, XP, and Cell Signaling Technology are trademarks of Cell Signaling Technology, Inc.

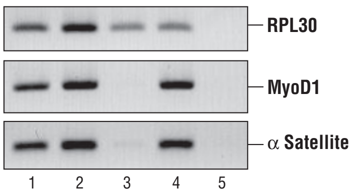


FIGURE 2. Chromatin immunoprecipitations were performed using digested chromatin from HeLa cells and either Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 (lane 2), Rpb1 CTD (4H8) Mouse mAb #2629 (lane 3), Di-Methyl Histone H3 (Lys9) Antibody #9753 (lane 4), or Normal Rabbit IgG #2729 (lane 5). Purified DNA was analyzed by standard PCR methods using SimpleChIP® Human RPL30 Exon 3 Primers #7014, SimpleChIP® Human MyoD1 Exon 1 Primers #4490, and SimpleChIP® Human α Satellite Repeat Primers #4486. PCR products were observed for each primer set in the input sample (lane 1) and various ChIP samples, but not in the Normal Rabbit IgG ChIP sample (lane 5).

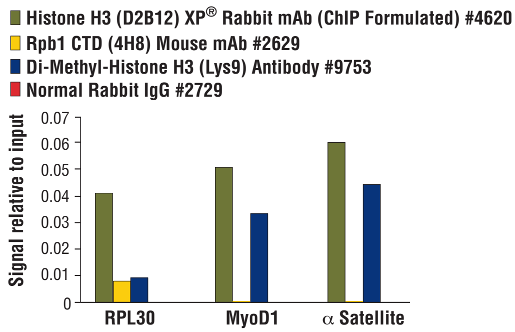


FIGURE 3. Chromatin immunoprecipitations were performed using digested chromatin from HeLa cells and the indicated ChIP-validated antibodies. Purified DNA was analyzed by quantitative real-time PCR, using SimpleChIP® Human RPL30 Exon 3 Primers #7014 (control primer set), SimpleChIP® Human MyoD1 Exon 1 Primers #4490, and SimpleChIP® Human α Satellite Repeat Primers #4486. The amount of immunoprecipitated DNA in each sample is represented as signal relative to the total amount of input chromatin (equivalent to 1).

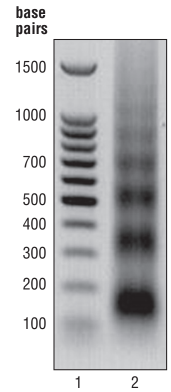


FIGURE 4: HeLa cells were formaldehyde-crosslinked and chromatin was prepared and digested as described in Section A of protocol. DNA was purified as described in Section B and 10 µl were separated by electrophoresis on a 1% agarose gel (lane 2) and stained with ethidium bromide. Lane 2 shows that the majority of chromatin was digested to 1 to 5 nucleosomes in length (150 to 900 bp).

Thank you for your recent purchase. If you would like to provide a review visit cellsignal.com/comments.

www.cellsignal.com

© 2022 Cell Signaling Technology, Inc.

Introduction:

The chromatin immunoprecipitation (ChIP) assay is a powerful and versatile technique used for probing protein-DNA interactions within the natural chromatin context of the cell (1,2). This assay can be used to identify multiple proteins associated with a specific region of the genome, or the opposite, to identify the many regions of the genome associated with a particular protein (3-6). In addition, the ChIP assay can be used to define the spatial and temporal relationship of a particular protein-DNA interaction. For example, the ChIP assay can be used to determine the specific order of recruitment of various protein factors to a gene promoter or to "measure" the relative amount of a particular histone modification across an entire gene locus during gene activation (3,4). In addition to histone proteins, the ChIP assay can also be used to analyze binding of transcription factors, transcription co-factors, DNA replication factors and DNA repair proteins.

When performing the ChIP assay, cells are first fixed with formaldehyde, a reversible protein-DNA cross-linking agent that serves to fix or "preserve" the protein-DNA interactions occurring in the cell (see method overview) (1,2). Cells are then lysed and chromatin is harvested and fragmented using either sonication or enzymatic digestion. The chromatin is then subjected to immunoprecipitation using antibodies specific to a particular protein or histone modification. Any DNA sequences that are associated with the protein or histone modification of interest will co-precipitate as part of the cross-linked chromatin complex and the relative amount of that DNA sequence will be enriched by the immunoselection process. After immunoprecipitation, the protein-DNA cross-links are reversed and the DNA is purified. The enrichment of a particular DNA sequence or sequences can then be detected by a number of different methods.

Standard PCR methods are often employed to identify the DNA sequences or regions of the genome associated with a particular protein or histone modification (1,2). PCR is used to measure the relative abundance of a particular DNA sequence enriched by a protein-specific immunoprecipitation versus an immunoprecipitation with a non-specific antibody control. PCR products are run on an agarose or acrylamide gel to facilitate quantification, and the level of enrichment of the DNA sequence is determined relative to the total amount of input DNA (percent of input). The level of enrichment can also be expressed as fold enrichment above background (enrichment relative to that of the non-specific antibody control). Real-Time PCR provides a more accurate, gel-free system for the quantification of DNA enrichment. Alternatively, the ChIP assay can be combined with genomic tiling micro-array (ChIP on chip) techniques, sequencing, or cloning strategies, which allow for genome-wide analysis of protein-DNA interactions and histone modifications (5-8).

The SimpleChIP® Kit contains buffers and reagents needed to perform the ChIP assay with mammalian cells and works for both histone modifications and non-histone DNA-binding proteins. After cell lysis, the chromatin is fragmented by partial digestion with Micrococcal Nuclease to obtain chromatin fragments of 1 to 5 nucleosomes in size. Enzymatic digestion of chromatin is much milder than sonication and eliminates problems due to variability in sonication power and emulsification of chromatin during sonication, which can result in incomplete fragmentation of chromatin or loss of antibody epitopes due to protein denaturation and degradation. The chromatin immunoprecipitations are performed using antibodies and either ChIP Grade Protein G Agarose or ChIP Grade Protein G Magnetic Beads. After reversal of protein-DNA cross-links, the DNA is purified using DNA purification spin columns provided in the kit. The DNA purification spin columns combine the convenience of spin-column technology with the selective binding properties of a uniquely designed silica membrane that allows for efficient recovery of DNA and removal of protein contaminants without the need for phenol/chloroform extractions and ethanol precipitations. After DNA purification, the enrichment of particular DNA sequences can be analyzed by a variety of methods.

In addition to providing buffers and reagents required to perform the ChIP assay, the SimpleChIP® Kit provides important controls that allow for user determination of a successful ChIP experiment. The kit contains a positive control Histone H3 Rabbit mAb, a negative control Normal Rabbit IgG Antibody and primer sets for PCR detection of the ribosomal protein L30 (RPL30) gene locus (human and mouse primer sets included). Histone H3 is a core component of chromatin in the cell and is bound to most DNA sequences throughout the genome, including the RPL30 locus. Thus, immunoprecipitation of chromatin with the Histone H3 antibody will enrich for the RPL30 gene, while immunoprecipitation with the Normal Rabbit IgG will not result in RPL30 gene enrichment. This enrichment can be quantified using either standard PCR or quantitative real-time PCR methods and the RPL30 primer sets provided in the kit. Importantly, since histone H3 is bound to most DNA sequences throughout the genome, the Histone H3 Rabbit mAb serves as a positive control IP for almost any locus studied, giving the user even more confidence that their ChIP experiment was performed successfully.

The SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003 provides enough reagents to perform up to 30 immunoprecipitations and is optimized for 4 x 10^6 cultulred cells per immunoprecipitation. A ChIP assay can be performed in as little as two days and can easily be scaled up or down for use with more or fewer cells. This kit is compatible with ChIP-Seq.

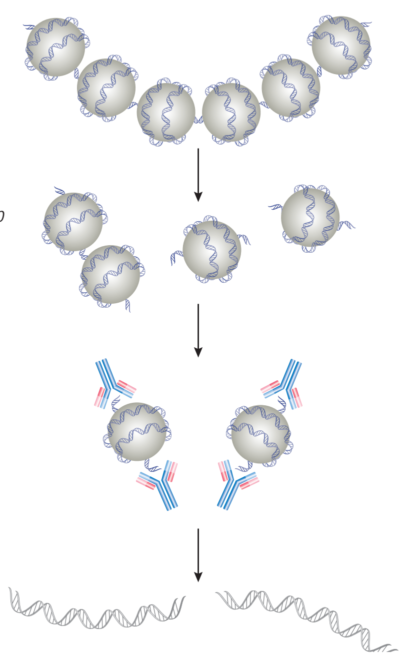
Method Overview

Cells are fixed with formaldehyde to cross-link histone and non-histone proteins to DNA.

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

Antibodies specific to histone or non-histone proteins are added and the complex co-precipitates and is captured by Protein G Agarose or Protein G magnetic beads.

Cross-links are reversed, and DNA is purified and ready for analysis.



Background References:

(1) Orlando, V. (2000) *Trends Biochem Sci* 25, 99–104.

(2) Kuo, M.H. and Allis, C.D. (1999) *Methods* 19, 425–33.

(3) Agalioti, T. et al. (2000) *Cell* 103, 667–78.

(4) Soutoglou, E. and Talianidis, I. (2002) *Science* 295, 1901–4.

(5) Mikkelsen, T.S. et al. (2007) *Nature* 448, 553–60.

(6) Lee, T.I. et al. (2006) *Cell* 125, 301–13.

(7) Weinmann, A.S. and Farnham, P.J. (2002) *Methods* 26, 37–47.

(8) Wells, J. and Farnham, P.J. (2002) *Methods* 26, 48–56.

Chromatin Immunoprecipitation Protocol

!	This ! signifies an important step in the protocol regarding volume changes based on the number of immunoprecipitation preparations (IP preps). One IP prep is defined as 4 x 10^6 tissue cultured cells.
!!	This !! signifies an important step to dilute a buffer before proceeding.
SAFE STOP	This is a safe stopping point in the protocol, if stopping is necessary.

I. Cell Culture Cross-linking and Sample Preparation:

For optimal ChIP results, use approximately 4 X 10^6 cells for each immunoprecipitation to be performed (at least 12 X 10^6 cells are required in order to include positive and negative controls). For HeLa cells, one IP is equivalent to half of a 15 cm culture dish containing cells that are 90% confluent in 20 ml of growth medium. One additional sample should be processed for Analysis of Chromatin Digestion and Concentration (Section III). Since every cell type is different, we recommend including one extra dish of cells in the experiment to be used for determination of cell number using a hemocytometer or cell counter. If desired, five additional chromatin samples should be processed for Optimization of Chromatin Digestion (Appendix A).

Before starting:

(!) All buffer volumes should be increased proportionally based on the number of 15 cm tissue culture dishes (or 1 x 10^7 suspension cells) used in the experiment.

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) #7012 and 10X Glycine Solution #7005. Make sure PIC is completely thawed.
- Prepare 2 ml of Phosphate Buffered Saline (PBS) + 10 µl 200X PIC per 15 cm dish (or 1 x 10^7 suspension cells) to be processed and place on ice.
- Prepare 40 ml of PBS per 15 cm dish (or 1 x 10^7 suspension cells) to be processed and place on ice.
- Prepare 540 µl of 37% formaldehyde per 15 cm dish of cells (or 1 x 10^7 suspension cells) to be processed and keep at room temperature. Use fresh formaldehyde that is not past the manufacturer's expiration date.

1. 1.To crosslink proteins to DNA, add 540 µl of 37% formaldehyde to each 15 cm culture dish containing 20 ml medium. For suspension cells, add 540 µl of 37% formaldehyde to cells suspended in 20 ml medium. Swirl briefly to mix and incubate 10 min at room temperature. Final formaldehyde concentration is 1%. Addition of formaldehyde may result in a color change of the medium.
2. Add 2 ml of 10X glycine to each 15 cm dish containing 20 ml medium, swirl briefly to mix, and incubate 5 min at room temperature. Addition of glycine may result in a color change of the medium.
3. For suspension cells, transfer cells to a 50 ml conical tube, centrifuge at 500 x g in a benchtop centrifuge 5 min at 4°C and wash pellet two times with 20 ml ice-cold PBS. Remove supernatant and immediately continue with Nuclei Preparation and Chromatin Digestion (Section II).
4. For adherent cells, remove media and wash cells two times with 20 ml ice-cold 1X PBS, completely removing wash from culture dish each time.
5. Add 2 ml ice-cold PBS + PIC to each 15 cm dish. Scrape cells into cold buffer. Combine cells from all culture dishes into one 15 ml conical tube.
6. Centrifuge cells at 2,000 x g in a benchtop centrifuge for 5 min at 4°C. Remove supernatant and continue with Nuclei Preparation and Chromatin Digestion (Section II). (**SAFE STOP**) Alternatively samples may be stored at -80°C for up to 3 months.

II Nuclei Preparation and Chromatin Digestion

Before starting:

(!) All buffer volumes should be increased proportionally based on the number of IP preps in the experiment.

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) #7012. Make sure it is completely thawed prior to use.
- Prepare 1 M DTT (192.8 mg DTT #7016 + 1.12ml dH2O). Make sure DTT crystals are completely in solution.

(!!) IMPORTANT: Once in solution, store 1M DTT at -20°C.

- Remove and warm 10X ChIP Buffer #7008 and ensure SDS is completely in solution.
- Prepare 1 ml 1X Buffer A (250 µl 4X Buffer A #7006 + 750 µl water) + 0.5 µl 1M DTT + 5 µl 200X PIC per IP prep and place on ice.
- Prepare 1.1 ml 1X Buffer B (275 µl 4X Buffer B #7007 + 825 µl water) + 0.55 µl 1M DTT per IP prep and place on ice.
- Prepare 100 µl 1X ChIP Buffer (10 µl 10X ChIP Buffer #7008 + 90 µl water) + 0.5 µl 200X PIC per IP prep and place on ice.

1. Resuspend cells in 1 ml ice-cold 1X Buffer A + DTT + PIC per IP prep. Incubate on ice for 10 min. Mix by inverting tube every 3 min.
2. Pellet nuclei by centrifugation at 2,000 x g in a benchtop centrifuge for 5 min at 4°C. Remove supernatant and resuspend pellet in 1 ml ice-cold 1X Buffer B + DTT per IP prep. Repeat centrifugation, remove supernatant, and resuspend pellet in 100 µl 1X Buffer B +DTT per IP prep. Transfer sample to a 1.5 ml microcentrifuge tube, up to 1 ml total per tube.
3. Add 0.5 µl of Micrococcal Nuclease #10011 per IP prep, mix by inverting tube several times and incubate for 20 min at 37°C with frequent mixing to digest DNA to length of approximately 150-900 bp. Mix by inversion every 3 to 5 min. The amount of Micrococcal Nuclease required to digest DNA to the optimal length may need to be determined empirically for individual cell lines (see Appendix A). HeLa nuclei digested with 0.5 µl Micrococcal Nuclease per 4 x 106 cells gave the appropriate length DNA fragments (see Figure 4).
4. Stop digest by adding 10 µl of 0.5 M EDTA #7011 per IP prep and placing tube on ice for 1-2 min.
5. Pellet nuclei by centrifugation at 16,000 x g in a microcentrifuge for 1 min at 4°C and remove supernatant.
6. Resuspend nuclear pellet in 100 µl of 1X ChIP Buffer + PIC per IP prep and incubate on ice for 10 min.
7. Sonicate up to 500 µl of lysate per 1.5 ml microcentrifuge tube 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 under 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 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.
8. Clarify lysates by centrifugation at 9,400 x g in a microcentrifuge for 10 min at 4°C.
9. Transfer supernatant to a new tube. (**SAFE STOP**) This is the cross-linked chromatin preparation, which should be stored at -80°C until further use. Remove 50 µl of the chromatin preparation for Analysis of Chromatin Digestion and Concentration (Section III). This sample may be stored at -20°C overnight.

III Analysis of Chromatin Digestion and Concentration (Recommended Step)

1. To the 50 µl chromatin sample (from Step 9 in Section II), add 100 µl nuclease-free water, 6 µl 5 M NaCl #7010, and 2 µl RNAse A #7013. Vortex to mix and incubate samples at 37°C for 30 min.
2. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate samples at 65°C for 2 h.
3. Purify DNA from samples using DNA purification spin columns as described in Section VI. (**SAFE STOP**) DNA may be stored at -20°C for up to 6 months.
4. After purification of DNA, remove a 10 µl sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 100 bp DNA marker. DNA should be digested to a length of approximately 150-900 bp (1 to 5 nucleosomes; see Figure 4).
5. To determine DNA concentration, transfer 2 µl of purified DNA to 98 µl nuclease-free water to give a 50-fold dilution and read the OD260. The concentration of DNA in µg/ml is OD260 x 2,500. DNA concentration should ideally be between 50 and 200 µg/ml.

NOTE: For optimal ChIP results, it is highly critical that the chromatin is of appropriate size and concentration. Over-digestion of chromatin may diminish signal in the PCR

© 2022 Cell Signaling Technology, Inc.
Cell Signaling Technology is a trademarks of Cell Signaling Technology, Inc. 525_Enzymatic Chromatin Immunoprecipitation Protocol Revision 06/2022

Chromatin Immunoprecipitation Protocol (cont.)

quantification. Under-digestion of chromatin may lead to increased background signal and lower resolution. Adding too little chromatin to the IP may result in diminished signal in the PCR quantification. A protocol for optimization of chromatin digestion can be found in Appendix A.

IV Chromatin Immunoprecipitation

For optimal ChIP results, use approximately 5 to 10 µg of digested, cross-linked chromatin (as determined in Section III) per immunoprecipitation. This should be roughly equivalent to a single 100 µl IP prep from 4 x 10^6 tissue culture cells. Typically, 100 µl of digested chromatin is diluted into 400 µl 1X ChIP Buffer prior to the addition of antibodies. However, if more than 100 µl of chromatin is required per IP the cross-linked chromatin preparation does not need to be diluted as described below. Antibodies can be added directly to the undiluted chromatin preparation for immunoprecipitation of chromatin complexes.

Before starting:

(!) All buffer volumes should be increased proportionally based on the number of immunoprecipitations in the experiment.

- Remove and warm 200X Protease Inhibitor Cocktail (PIC) #7012. Make sure PIC is completely thawed.
- Remove and warm 10X ChIP Buffer #7008 and ensure SDS is completely in solution.
- Thaw digested chromatin preparation (from Step 9 in Section II) and place on ice.
- Prepare low salt wash: 3 ml 1X ChIP Buffer (300 µl 10X ChIP Buffer #7008 + 2.7 ml water) per immunoprecipitation. Store at room temperature until use.
- Prepare high salt wash: 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer #7008 + 900 µl water) + 70 µl 5M NaCl #7010 per immunoprecipitation. Store at room temperature until use.

1. In one tube, prepare enough 1X ChIP Buffer for the dilution of digested chromatin into the desired number of immunoprecipitations: 400 µl of 1X ChIP Buffer (40 µl of 10X ChIP Buffer #7008 + 360 µl water) + 2 µl 200X PIC per immunoprecipitation. When determining the number of immunoprecipitations, remember to include the positive control Histone H3 (D2B12) XP® Rabbit mAb #4620 and negative control Normal Rabbit IgG #2729 samples. Place mix on ice.
2. To the prepared 1X ChIP Buffer, add the equivalent of 100 µl (5 to 10 µg of chromatin) of the digested, cross-linked chromatin preparation (from Step 9 in Section II) per immunoprecipitation. For example, for 10 immunoprecipitations, prepare a tube containing 4 ml 1X ChIP Buffer (400 µl 10X ChIP Buffer + 3.6 ml water) + 20 µl 200X PIC + 1 ml digested chromatin preparation.
3. Remove a 10 µl sample of the diluted chromatin and transfer to a microfuge tube. This is your 2% Input Sample, which can be stored at -20°C until further use (Step 1 in Section V).
4. For each immunoprecipitation, transfer 500 µl of the diluted chromatin to a 1.5 ml microcentrifuge tube and add the immunoprecipitating antibody. The amount of antibody required per IP varies and should be determined by the user. For the positive control Histone H3 (D2B12) XP® Rabbit mAb #4620, add 10 µl to the IP sample. For the negative control Normal Rabbit IgG #2729, add 1 µl (1 µg) to 2 µl (2 µg) to the IP sample. If using antibodies from Cell Signaling Technology, please see recommended dilution listed on the datasheet or product webpage and calculate the amount (µg) of IgG antibody for negative control based on the Cell Signaling Antibody concentration for a fair comparison. Incubate IP samples 4 h to overnight at 4°C with rotation.

NOTE: Most antibodies from Cell Signaling Technology work optimally between 1 and 2 ug per IP sample. In the case where there are multiple samples with varying concentrations, it is best to match the negative control Normal Rabbit IgG #2729 to the highest antibody concentration.

5. Resuspend ChIP-Grade Protein G Magnetic Beads #9006 by gently vortexing. Immediately add 30 µl of Protein G Magnetic Beads to each IP reaction and incubate for 2 h at 4°C with rotation.
6. Pellet protein G magnetic beads in each immunoprecipitation by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 min for solution to clear and then carefully remove supernatant.
7. Wash pellet protein G magnetic beads by adding 1 ml of low salt wash to the beads and incubate at 4°C for 5 min with rotation. Repeat steps 6 and 7 two additional times for a total of 3 low salt washes.
8. Add 1 ml of high salt wash to the beads and incubate at 4°C for 5 min with rotation.
9. Pellet pellet protein G magnetic beads in each immunoprecipitation by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 min for solution to clear and then carefully remove supernatant. Immediately proceed to Section V.

V. Elution of Chromatin from Antibody/Protein G Magnetic Beads and Reversal of Cross-links

Before starting:

(!) All buffer volumes should be increased proportionally based on the number of immunoprecipitations in the experiment.

- Remove and warm 2X ChIP Elution Buffer #7009 in a 37°C water bath and ensure SDS is in solution.
- Set a water bath or thermomixer to 65°C.
- Prepare 150 µl 1X ChIP Elution Buffer (75 µl 2X ChIP Elution Buffer #7009 + 75 µl water) for each immunoprecipitation and the 2% input sample.

1. Add 150 µl of the 1X ChIP Elution Buffer to the 2% input sample tube and set aside at room temperature until Step 6.
2. Add 150 µl 1X ChIP Elution Buffer to each IP sample.
3. Elute chromatin from the antibody/protein G magnetic beads for 30 min at 65°C with gentle vortexing (1,200 rpm). A thermomixer works best for this step. Alternatively, elutions can be performed at room temperature with rotation, but may not be as complete.
4. Pellet protein G magnetic beads by placing the tubes in a Magnetic Separation Rack and wait 1 to 2 min for solution to clear.
5. Carefully transfer eluted chromatin supernatant to a new tube.
6. To all tubes, including the 2% input sample from Step 1, reverse cross-links by adding 6 µl 5M NaCl and 2 µl Proteinase K #10012, and incubate 2 h at 65°C. This incubation can be extended overnight.
7. Immediately proceed to Section VI. (**SAFE STOP**) Alternatively, samples can be stored at -20°C for up to 4 days. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA Binding Buffer #10007 (Section VI, Step 1).

VI DNA Purification Using Spin Columns:

Before starting:

- (!!) Add 24 ml of ethanol (96-100%) to DNA Wash Buffer #10008 before use. This step only has to be performed once prior to the first set of DNA purifications.
- Remove one DNA Purification collection tube #10010 for each DNA sample from Section V.

1. Add 750 µl DNA Binding Buffer #10007 to each DNA sample and vortex briefly.
    - 5 volumes of DNA Binding Buffer should be used for every 1 volume of sample.
2. Transfer 450 µl of each sample from Step 1 to a DNA spin column in collection tube.
3. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
4. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
5. Transfer the remaining 450 µl of each sample from Step 1 to the spin column in collection tube. Repeat Steps 3 and 4.
6. Add 750 µl of DNA Wash Buffer #10008 to the spin column in collection tube.
7. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
8. Remove the spin column from the collection tube and discard the liquid. Replace spin column in the collection tube.
9. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA Elution Buffer #10009 to each spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 18,500 x g in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA purification spin column. Eluate is now purified DNA. (**SAFE STOP**) Samples can be stored at -20°C.

VII Quantification of DNA by PCR:

Recommendations:

© 2022 Cell Signaling Technology, Inc.
Cell Signaling Technology is a trademarks of Cell Signaling Technology, Inc. 525_Enzymatic Chromatin Immunoprecipitation Protocol Revision 06/2022

Chromatin Immunoprecipitation Protocol (cont.)

- Use Filter-tip pipette tips to minimize risk of contamination.
- The control primers included in the kit are specific for the human or mouse RPL30 gene (#7014 + #7015) and can be used for either standard PCR or quantitative real-time PCR. If the user is performing ChIPs from another species, it is recommended that the user design the appropriate specific primers to DNA and determine the optimal PCR conditions.
- A Hot-Start Taq polymerase is recommended to minimize the risk of nonspecific PCR products.
- PCR primer selection is critical. Primers should be designed with close adherence to the following criteria:

Primer length:	24 nucleotides
Optimum Tm:	60°C
Optimum GC:	50%
Amplicon size:	150 to 200 bp (for standard PCR)
	80 to 160 bp (for real-time quantitative PCR)

Standard PCR Method:

1. Label the appropriate number of 0.2 ml PCR tubes for the number of samples to be analyzed. These should include the 2% input sample, the positive control histone H3 sample, the negative control normal rabbit IgG sample, and a tube with no DNA to control for DNA contamination.
2. Add 2 µl of the appropriate DNA sample to each tube.
3. Prepare a master reaction mix as described below, making sure to add enough reagent for two extra tubes to account for loss of volume. Add 18 µl of master mix to each reaction tube.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free H2O	12.5 µl
10X PCR Buffer	2.0 µl
4 mM dNTP Mix	1.0 µl
5 µM RPL30 Primers	2.0 µl
Taq DNA Polymerase	0.5 µl

4. Start the following PCR reaction program:

a.	Initial Denaturation	95°C	5 min
b.	Denature	95°C	30 sec
c.	Anneal	62°C	30 sec
d.	Extension	72°C	30 sec
e.	Repeat Steps b-d for a total of 34 cycles.		
f.	Final Extension	72°C	5 min

5. Remove 10 µl of each PCR product for analysis by 2% agarose gel or 10% polyacrylamide gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product is 161 bp for human RPL30 #7014 and 159 bp for mouse RPL30 #7015.

Real-Time Quantitative PCR Method:

1. Label the appropriate number of PCR tubes or PCR plates compatible with the model of PCR machine to be used. PCR reactions should include the positive control histone H3 sample, the negative control normal rabbit IgG sample, a tube with no DNA to control for contamination, and a serial dilution of the 2% input chromatin DNA (undiluted, 1:5, 1:25, 1:125) to create a standard curve and determine the efficiency of amplification.
2. Add 2 µl of the appropriate DNA sample to each tube or well of the PCR plate.
3. Prepare a master reaction mix as described below. Add enough reagents for two extra reactions to account for loss of volume. Add 18 µl of reaction mix to each PCR reaction tube or well.
(**SAFE STOP**) If necessary cover plate with aluminum foil to avoid light and store at 4°C up to 4 hours or -20°C overnight until machine is ready for use.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free H_2O	6 µl
5 µM RPL30 Primers	2 µl
SimpleChIP® Universal qPCR Master Mix #88989	10 µl

4. Start the following PCR reaction program:

a.	Initial Denaturation	95°C 3 min
b.	Denature	95°C 15 sec
c.	Anneal and Extension:	60°C 60 sec
d.	Repeat steps b and c for a total of 40 cycles.	

5. Analyze quantitative PCR results using the software provided with the real-time PCR machine. Alternatively, one can calculate the IP efficiency manually using the Percent Input Method and the equation shown below. With this method, signals obtained from each immunoprecipitation are expressed as a percent of the total input chromatin.

Percent Input = 2% x $2^{(C[T]\ 2\%\text{Input Sample}\ -\ C[T]\ \text{IP Sample})}$
C[T] = C_T = Threshold cycle of PCR reaction

VIII NG-Sequencing Library Construction

The immuno-enriched DNA samples prepared with this kit are directly compatible with ChIP-seq. For downstream NG-sequencing DNA library construction, use a DNA library preparation protocol or kit compatible with your downstream sequencing platform. For sequencing on For sequencing on Illumina® platforms, we recommend DNA Library Prep Kit for Illumina® (ChIP-seq, CUT&RUN) #56795 and its associated index primers Multiplex Oligos for Illumina® (Single Index Primers) (ChIP-seq, CUT&RUN) #29580 or Multiplex Oligos for Illumina® (Dual Index Primers) (ChIP-seq, CUT&RUN) #47538.

Recommendations:

- For transcription factor or co-factor ChIP-seq, use at least 5 ng of ChIP-enriched DNA and amplification of the adaptor-ligated DNA with 10 cycles of PCR.
- For total histone and histone modifications, or input samples, start with 50 ng of ChIP-enriched DNA and amplification of the adaptor-ligated DNA with 6 cycles of PCR.
- For library construction of ChIP-enriched DNA for all target types, perform cleanup of adaptor-ligated DNA without size selection.
- After DNA library construction, check the DNA library for presence of adaptor dimers (~140 bp) using an Agilent High Sensitivity DNA Kit (Agilent Technologies, Cat# G2938-90322), or by agarose gel electrophoresis with 50-100 ng DNA on a 2% agarose TAE gel. If adaptor dimers are present in the DNA library, repeat cleanup of PCR amplified material.
- The quality of the library can also be confirmed using qPCR and primer sets to known positive and negative target loci. Positive primer pairs should still give the same high signal compared to negative primers as seen in the original qPCR analysis of ChIP-enriched DNA.
- After final cleanup and quality checks, prepare final purified library samples at 2-10 nM for high throughput sequencing.

© 2022 Cell Signaling Technology, Inc.
Cell Signaling Technology is a trademarks of Cell Signaling Technology, Inc. 525_Enzymatic Chromatin Immunoprecipitation Protocol Revision 06/2022

APPENDIX A: Optimization of Chromatin Digestion

Optimal conditions for the digestion of cross-linked chromatin DNA to 150-900 base pairs in length is highly dependent on the ratio of Micrococcal Nuclease to the amount of cells used in the digest. Below is a protocol for determination of the optimal digestion conditions for a specific cell type.

1. Prepare cross-linked nuclei from 2 X 10^7 cells (equivalent of 5 IP preps), as described in Sections I, II, and III. Stop after Step 2 of Section II and proceed as described below.
2. Transfer 100 µl of the nuclei preparation into each of the 5 individual 1.5 ml micro-centrifuge 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 16,000 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 30 sec on wet ice between pulses. Optimal conditions required for complete lysis of nuclei can be determined by observing nuclei under 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 9,400 x g 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 h.
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 to 5 nucleosomes, see Figure 4). 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 immunoprecipitation preparation (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 base pairs in this protocol, then 0.5 µl of stock Micrococcal Nuclease should be added to one IP prep during the digestion of chromatin in Section II.
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.

© 2022 Cell Signaling Technology, Inc.
Cell Signaling Technology is a trademarks of Cell Signaling Technology, Inc. 525_Enzymatic Chromatin Immunoprecipitation Protocol Revision 06/2022

APPENDIX B: Troubleshooting Guide

Problem	Possible Causes	Recommendation
1. Concentration of the digested chromatin is too low.	Not enough cells added to the chromatin digestion or nuclei were not completely lysed after digestion.	If DNA concentration of the chromatin preparation is close to 50 µg/ml, add additional chromatin to each IP to give at least 5 µg/IP and continue with protocol.
		Count a separate plate of cells before cross-linking to determine an accurate cell number and/or visualize nuclei under microscope before and after sonication to confirm complete lysis of nuclei.
2. Chromatin is under-digested and fragments are too large (greater than 900 bp).	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.
	Too many cells or not enough Micrococcal Nuclease was added to the chromatin digestion.	Count a separate plate of cells before cross-linking to determine accurate cell number and see Appendix A for optimization of chromatin digestion.
3. 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.	Not enough cells or too much Micrococcal Nuclease added to the chromatin digestion.	Count a separate plate of cells before cross-linking to determine accurate cell number and see Appendix A for optimization of chromatin digestion.
4. No product or very little product in the input PCR reactions.	Not enough DNA added to the PCR reaction or conditions are not optimal.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	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 Section VII).
	Not enough chromatin added to the IP or chromatin is over-digested.	For optimal ChIP results add 5-10 µg chromatin per IP. See recommendations for problems 1 and 3 above.
5. No product in the positive control Histone H3-IP RPL30 PCR reaction.	Not enough chromatin or antibody added to the IP reaction or IP incubation time is too short.	Be sure to add 5-10 µg of chromatin and 10 µl of antibody to each IP reaction and incubate with antibody over-night and an additional 2 h after adding Protein G beads.
	Incomplete elution of chromatin from Protein G beads.	Elution of chromatin from Protein G beads is optimal at 65°C with frequent mixing to keep beads suspended in solution.
6. Quantity of product in the negative control Rabbit IgG-IP and positive control Histone H3-IP PCR reactions is equivalent.	Too much or not enough chromatin added to the IP reaction. Alternatively, too much antibody added to the IP reaction.	Add no more than 15 µg of chromatin and 10 µl of histone H3 antibody to each IP reaction. Reduce the amount of normal rabbit IgG to 1 µl per IP.
	Too much DNA added to the PCR reaction or too many cycles of amplification.	Add less DNA to the PCR reaction or decrease the number of PCR cycles. 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 can not be accurately measured.
7. No product in the Experimental Antibody-IP PCR reaction.	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.
	Not enough antibody added to the IP reaction.	Typically a range of 1 to 5 µg of antibody 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 IP.	Find an alternate antibody source.

© 2022 Cell Signaling Technology, Inc.
Cell Signaling Technology is a trademarks of Cell Signaling Technology, Inc. 525_Enzymatic Chromatin Immunoprecipitation Protocol Revision 06/2022