Applications	Reactivity	Sensitivity	MW (kDa)	Isotype
W IP IHC-P IF-IC F ChIP	H M (Mk) (Pg)	Endogenous	42	Rabbit IgG

Applications Key: W=Western Blotting IP=Immunoprecipitation IHC-P=Immunohistochemistry (Paraffin) IF-IC=Immunofluorescence (Immunocytochemistry) F=Flow Cytometry ChIP=Chromatin IP

Reactivity Key: H=Human M=Mouse Mk=Monkey Pg=Pig

Species cross-reactivity is determined by western blot. Species enclosed in parentheses are predicted to react based on 100% sequence homology.

Protocols

Enzymatic Chromatin IP (Agarose Beads) Protocol

- Appendix A

- Appendix B

Required Reagents

Reagents Included in SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002:

a. Glycine Solution (10X)

b. Buffer A (4X)

c. Buffer B (4X)

d. ChIP Buffer (10X)

e. ChIP Elution Buffer (2X)

f. 5 M NaCl

g. 0.5 M EDTA

h. 1M DTT

i. DNA Binding Reagent A

j. DNA Wash Reagent B

k. DNA Elution Reagent C

l. DNA Spin Columns

m. Protease Inhibitor Cocktail (200X)

n. RNAse A (10 mg/ml)

o. Micrococcal Nuclease (2000 gel units/µl): NEB #M0247S

p. Proteinase K (20 mg/ml): NEB #P8102S

q. SimpleChIP® Human RPL30 Exon 3 Primers #7014

r. SimpleChIP® Mouse RPL30 Intron 2 Primers #7015

s. Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620

t. Normal Rabbit IgG #2729

u. ChIP-Grade Protein G Agarose Beads #9007

Reagents Not included in SimpleChIP® Enzymatic Chromatin IP Kit (Agarose Beads) #9002:

a. PMSF (0.1 M in ethanol)

b. Formaldehyde (37%)

c. Ethanol (96-100%)

d. Isopropanol

e. 1X PBS

f. Nuclease-free water

g. Taq DNA polymerase

h. dNTP Mix

A. *In Vivo* Crosslinking, Nuclei Preparation, and Nuclease S7 Digestion of Chromatin

Before starting:

Stimulate or treat approximately 4 X 10^7 cells for each experiment. This number of cells will generate one chromatin preparation that can be used for up to 10 separate immunoprecipitations.

- For HeLa cells, this is equivalent to 5 x 15 cm culture dishes containing cells that are 90% confluent in 20 ml of growth media.
- Include one extra dish to be used for determination of cell number using a hemocytometer.

Remove and warm:

- 200X Protease Inhibitor Cocktail
- 1 M DTT
- 0.1 M PMSF (not supplied)
- 10X ChIP Buffer and ensure SDS is completely in solution.

Prepare and chill on ice for each set of 4 X 10^7 cells (5 dishes):

- 10 ml 10X glycine
- 200 ml 1X PBS (not supplied)
- 10 ml 1X PBS + 100 µl PMSF
- 10 ml 1X Buffer A (2.5 ml 4X Buffer A + 7.5 ml water) + 5 µl 1 M DTT + 50 µl 200X Protease Inhibitor Cocktail (PIC) + 100 µl PMSF
- 11 ml 1X Buffer B (2.75 ml 4X Buffer B + 8.25 ml water) + 5.5 µl 1 M DTT
- 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer + 900 µl water) + 5 µl 200X Protease Inhibitor Cocktail (PIC) + 10 µl PMSF.

1. To crosslink proteins to DNA, add 540 µl of 37% formaldehyde to each 15 cm culture dish containing 20 ml medium. Swirl briefly to mix and incubate for 10 minutes at room temperature.
   - Final formaldehyde concentration is 1%.
   - Use fresh formaldehyde that is not past the manufacturer's expiration date.
   - Addition of formaldehyde may result in a color change of the medium.
2. Add 2 ml of 10X glycine to each 15 cm dish, swirl briefly to mix, and incubate for 5 minutes 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 1,500 rpm in a bench top centrifuge for 5 minutes at 4 ℃, and wash pellet two times with 20 ml ice-cold 1X PBS. Immediately proceed to step 5.
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. Add 2 ml ice-cold 1X PBS + PMSF to the 15 cm dish of cells. Scrape and resuspend cells into cold buffer. Combine cells from all 5 plates into one 15 ml conical tube. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 minutes at 4 ℃.
5. Remove supernatant and resuspend cells in 10 ml ice-cold Buffer A + DTT + PIC + PMSF. Incubate on ice for 10 minutes. Mix by inverting tube every 3 minutes.
6. Pellet nuclei by centrifugation at 3,000 rpm in a bench top centrifuge for 5 minutes at 4 ℃. Remove supernatant and resuspend pellet in 10 ml ice-cold Buffer B + DTT. Repeat centrifugation, remove supernatant, and resuspend pellet in 1.0 ml Buffer B + DTT. Transfer sample to a 1.5 ml microcentrifuge tube.
7. Add 5 µl of Micrococcal Nuclease, mix by inverting tube several times and incubate for 20 minutes at 37 ℃ with frequent mixing to digest DNA to length of approximately 150-900 bp. Mix by inversion every 3 to 5 minutes.
   - 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 5 µl Micrococcal Nuclease per 4 X 10^7 cells in 1 ml Buffer B + DTT gave the appropriate length DNA fragments.
8. Stop digest by adding 100 µl of 0.5 M EDTA and placing tube on ice.
9. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 minute at 4 ℃ and remove supernatant.
10. Resuspend nuclear pellet in 1 ml of 1X ChIP buffer + PIC + PMSF and split into two tubes of 500 µl. Incubate on ice for 10 minutes.
11. Sonicate each tube of lysate with several pulses to break nuclear membrane. Incubate samples for 30 seconds 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-second 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.
12. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 minutes at 4 ℃.
13. Transfer supernatant to a new tube. This is the cross-linked chromatin preparation, which should be stored at -80 ℃ until further use. Remove 50 µl of the chromatin preparation for Analysis of Chromatin Digestion and Concentration (Section B).

B. Analysis of Chromatin Digestion and Concentration (Recommended Step)

1. To the 50 µl chromatin sample (from Step 13 in Section A), add 100 µl nuclease-free water, 6 µl 5 M NaCl and 2 µl RNAse A. Vortex to mix and incubate samples at 37 ℃ for 30 minutes.

2. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate samples at 65 ℃ for 2 hours.
3. Purify DNA from samples using spin columns as described in Section F.
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).
5. To determine DNA concentration, transfer 2 µl of purified DNA to 98 µl TE to give a 50-fold dilution and read the OD_{260}. The concentration of DNA in µg/ml is OD_{260} 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 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.

C. Chromatin Immunoprecipitation

Before starting:

Remove and warm:

- 200X Protease Inhibitor Cocktail
- 10X ChIP Buffer and ensure SDS is completely in solution.

Remove and place on ice:

- Cross-linked chromatin preparation (from Step 13 in Section A)
- ChIP Grade Protein G Agarose Beads #9007
- Antibodies for IP. This includes the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 and negative control Normal Rabbit IgG #2729.

NOTE: 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.

1. In one tube, prepare enough 1X ChIP Buffer for the desired number of immunoprecipitations. Each precipitation contains 400 µl of 1X ChIP Buffer (40 µl of 10X ChIP Buffer + 360 µl water) and 2 µl Protease Inhibitor Cocktail. When determining the number of immunoprecipitations, the user should include the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 and negative control Normal Rabbit IgG #2729 samples. Place mix on ice.
2. To the prepared ChIP buffer, add the equivalent of 100 µl (5 to 10 µg of chromatin DNA) of the cross-linked chromatin preparation (from Step 13 in Section A) for each 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 Protease Inhibitor Cocktail + 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 ℃ until further use (Step 1 in Section E).
4. For each immunoprecipitation, transfer 500 µl of the diluted chromatin to a microcentrifuge tube and add the immunoprecipitating antibody. The amount of antibody required for IP varies for each antibody and should be determined by the user. For the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620, add 10 µl to the IP

sample. For the negative control Normal Rabbit IgG #2729, add 1 µl (1 µg) to 5 µl (5 µg) to the IP sample. Incubate IP samples 4 hours to overnight at 4 ℃ with rotation.

5. Add 30 µl of ChIP-Grade Protein G Agarose Beads #9007 and incubate for 2 hours at 4 ℃ with rotation.
6. Proceed to Section D.

D. Washing of the Immunoprecipitated Chromatin

Before starting:

Remove and warm:

- 10X ChIP Buffer and ensure SDS is completely in solution.

Prepare and chill on ice for each IP:

- **Low salt wash:** 3 ml 1X ChIP Buffer (300 µl 10X ChIP Buffer + 2.7 ml water)
- **High salt wash:** 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer + 900 µl water) + 70 µl 5 M NaCl

1. Pellet Protein G Agarose Beads in each immunoprecipitation (from Step 5, Section C) by brief 1 minute centrifugation at 6,000 rpm and remove supernatant.
2. Add 1 ml of low salt wash to the beads and incubate at 4 ℃ for 5 minutes with rotation. Repeat steps 1 and 2 two additional times, for a total of 3 low salt washes.
3. Add 1 ml of high salt wash to the beads and incubate at 4 ℃ for 5 minutes with rotation. Immediately proceed to Section E.

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

Before starting:

- Remove and warm 2X ChIP Elution Buffer in a 37 ℃ water bath and ensure SDS is in solution.
- Set a water bath or thermomixer to 65 ℃.
- Prepare 150 µl 1X ChIP Elution Buffer (75 µl 2X ChIP Elution Buffer + 75 µl water) for each washed IP (from Step 3 in Section D) and the 2% input sample (from Step 3 in Section C).

1. Add 150 µl of the 1X ChIP Elution Buffer to the 2% input sample tube and set aside at room temperature until Step 7.
2. Pellet Protein G Agarose Beads by brief 1 minute centrifugation at 6,000 rpm and remove supernatant.
3. Add 150 µl 1X ChIP Elution Buffer to each IP sample.
4. Elute chromatin from the antibody/Protein G beads for 30 minutes at 65 ℃ 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.
5. Pellet Protein G Agarose Beads by brief 1 minute centrifugation at 6,000 rpm.
6. Carefully transfer eluted chromatin supernatant to a new tube.
7. To all tubes, including the 2% input sample from Step 1, add 6 µl 5 M NaCl and 2 µl Proteinase K and incubate 2 hours at 65 ℃.
8. Immediately proceed to Section F. Alternatively, samples can be stored at -20 °. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA Binding Reagent A (Section F, Step 1).

F. DNA Purification Using Spin Columns

Before starting:

- Add 12 ml of isopropanol to DNA Binding Reagent A and 24 ml of ethanol (96-100%) to DNA Wash Reagent B before use. These steps only have to be performed once prior to the first set of DNA purifications.
- Remove one DNA purification spin column and collection tube for each DNA sample from Section E.

1. Add 600 µl DNA Binding Reagent A to each DNA sample and vortex briefly.
   - 4 volumes of DNA Binding Reagent A should be used for every 1 volume of sample.
2. Transfer 375 µl of each sample from Step 1 to a DNA purification spin column in collection tube.
3. Centrifuge at 14,000 rpm 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 375 µl of each sample from Step 1 to the spin column in collection tube. Repeat Steps 3 and 4.
6. Add 700 µl of DNA Wash Reagent B to the spin column in collection tube.
7. Centrifuge at 14,000 rpm 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 14,000 rpm in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA Elution Reagent C to each spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA purification spin column. Eluate is now purified DNA. Samples can be stored at -20 ℃.

G. Quantification of DNA by PCR

Recommendations:

- 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 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 non-specific 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 ℃
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 H_2O	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 ℃	5 min
b.	Denature	95 ℃	30 sec
c.	Anneal	62 ℃	30 sec
d.	Extension	72 ℃	30 sec
e.	Repeat Steps b - d for a total of 34 cycles.		
f.	Final Extension	72 ℃	5 min

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

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.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free H_2O	6 µl
5 µM RPL30 Primers	2 µl
SYBR® Green Reaction Mix	10 µl

4. Start the following PCR reaction program:

a.	Initial Denaturation	95 ℃ 3 min
b.	Denature	95 ℃ 15 sec
c.	Anneal and Extension:	60 ℃ 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 $2^{(C[T]\ 2\%Input\ Sample - C[T]\ IP\ Sample)}$

   C[T] = C_T = Threshold cycle of PCR reaction

Appendix A: Optimization of Chromatin Digestion

Optimal conditions for digestion of cross-linked DNA to 150-900 base pairs in length depend on cell type and cell concentration as well as the concentration of Micrococcal Nuclease. Below is a protocol to determine the optimal digestion conditions for a specific cell type and concentration of cells.

1. Prepare cross-linked nuclei from 4 X 10^7 cells as described in Section A, Steps 1-6.
2. Transfer 200 µl of the nuclei preparation from Step 6 in Section A into 5 individual microcentrifuge tubes and place on ice.
3. Add 5 µl Micrococcal Nuclease stock to 20 µl of 1X Buffer B + DTT (1:5 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 ℃ with frequent mixing.
5. Stop digest by adding 20 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 ℃ and remove supernatant.
7. Resuspend nuclear pellet in 200 µl of 1X ChIP buffer + PIC +PMSF. 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-second 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 ℃.
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 ℃ for 30 min.
12. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate sample at 65 ℃ for 2 hours.
13. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 1 kb DNA marker.
14. Observe which of the digestion conditions produces DNA in the desired range of 150-900 base pairs (1 to 5 nucleosomes). The volume of diluted Micrococcal Nuclease that produces the desired size of DNA fragments using this optimization protocol is equivalent to the volume of Micrococcal Nuclease stock that will need to be added to 4 X 10^7 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 5 µl of stock Micrococcal Nuclease should be added to 4 X 10^7 cells during the digestion of chromatin in Step 7 of Section A.
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.

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 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.	Shorten the time of cross-linking to 10 min.
	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 G).
	Not enough chromatin added to the IP or chromatin is over-digested.	For optimal ChIP results, add 5 to 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 to 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 ℃ 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 chromatin or 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	Not enough DNA added to the PCR reaction.	Add more DNA to the PCR reaction or increase the number of amplification cycles.

reaction.	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 alternative antibody source.

Enzymatic Chromatin IP (Magnetic Beads) Protocol

- Appendix A

- Appendix B

Required Reagents

Reagents Included in SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003:

a. Glycine Solution (10X)

b. Buffer A (4X)

c. Buffer B (4X)

d. ChIP Buffer (10X)

e. ChIP Elution Buffer (2X)

f. 5 M NaCl

g. 0.5 M EDTA

h. 1M DTT

i. DNA Binding Reagent A

j. DNA Wash Reagent B

k. DNA Elution Reagent C

l. DNA Spin Columns

m. Protease Inhibitor Cocktail (200X)

n. RNAse A (10 mg/ml)

o. Micrococcal Nuclease (2000 gel units/µl): NEB #M0247S

p. Proteinase K (20 mg/ml): NEB #P8102S

q. SimpleChIP® Human RPL30 Exon 3 Primers #7014

r. SimpleChIP® Mouse RPL30 Intron 2 Primers #7015

s. Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620

t. Normal Rabbit IgG #2729

u. ChIP-Grade Protein G Magnetic Beads #9006

Reagents Not included in SimpleChIP® Enzymatic Chromatin IP Kit (Magnetic Beads) #9003:

a. 6-Tube Magnetic Separation Rack #7017
b. PMSF (0.1 M in ethanol)
c. Formaldehyde (37%)
d. Ethanol (96-100%)
e. Isopropanol
f. 1X PBS
g. Nuclease-free water
h. Taq DNA polymerase
i. dNTP Mix

A. *In Vivo* Crosslinking, Nuclei Preparation, and Nuclease S7 Digestion of Chromatin

Before starting:

Stimulate or treat approximately 4 X 10^7 cells for each experiment. This number of cells will generate one chromatin preparation that can be used for up to 10 separate immunoprecipitations.

- For HeLa cells, this is equivalent to 5 x 15 cm culture dishes containing cells that are 90% confluent in 20 ml of growth media.
- Include one extra dish to be used for determination of cell number using a hemocytometer.

Remove and warm:

- 200X Protease Inhibitor Cocktail
- 1 M DTT
- 0.1 M PMSF (not supplied)
- 10X ChIP Buffer and ensure SDS is completely in solution.

Prepare and chill on ice for each set of 4 X 10^7 cells (5 dishes):

- 10 ml 10X glycine
- 200 ml 1X PBS (not supplied)
- 10 ml 1X PBS + 100 µl PMSF
- 10 ml 1X Buffer A (2.5 ml 4X Buffer A + 7.5 ml water) + 5 µl 1 M DTT + 50 µl 200X Protease Inhibitor Cocktail (PIC) + 100 µl PMSF
- 11 ml 1X Buffer B (2.75 ml 4X Buffer B + 8.25 ml water) + 5.5 µl 1 M DTT
- 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer + 900 µl water) + 5 µl 200X Protease Inhibitor Cocktail (PIC) + 10 µl PMSF.

1. To crosslink proteins to DNA, add 540 µl of 37% formaldehyde to each 15 cm culture dish containing 20 ml medium. Swirl briefly to mix and incubate for 10 minutes at room temperature.
   - Final formaldehyde concentration is 1%.
   - Use fresh formaldehyde that is not past the manufacturer's expiration date.
   - Addition of formaldehyde may result in a color change of the medium.

2. Add 2 ml of 10X glycine to each 15 cm dish, swirl briefly to mix, and incubate for 5 minutes 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 1,500 rpm in a bench top centrifuge for 5 minutes at 4 ℃, and wash pellet two times with 20 ml ice-cold 1X PBS. Immediately proceed to step 5.
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. Add 2 ml ice-cold 1X PBS + PMSF to the 15 cm dish of cells. Scrape and resuspend cells into cold buffer. Combine cells from all 5 plates into one 15 ml conical tube. Centrifuge cells at 1,500 rpm in a bench top centrifuge for 5 minutes at 4 ℃.
5. Remove supernatant and resuspend cells in 10 ml ice-cold Buffer A + DTT + PIC + PMSF. Incubate on ice for 10 minutes. Mix by inverting tube every 3 minutes.
6. Pellet nuclei by centrifugation at 3,000 rpm in a bench top centrifuge for 5 minutes at 4 ℃. Remove supernatant and resuspend pellet in 10 ml ice-cold Buffer B + DTT. Repeat centrifugation, remove supernatant, and resuspend pellet in 1.0 ml Buffer B + DTT. Transfer sample to a 1.5 ml microcentrifuge tube.
7. Add 5 µl of Micrococcal Nuclease, mix by inverting tube several times and incubate for 20 minutes at 37 ℃ with frequent mixing to digest DNA to length of approximately 150-900 bp. Mix by inversion every 3 to 5 minutes.
   - 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 5 µl Micrococcal Nuclease per 4 X 10^7 cells in 1 ml Buffer B + DTT gave the appropriate length DNA fragments.
8. Stop digest by adding 100 µl of 0.5 M EDTA and placing tube on ice.
9. Pellet nuclei by centrifugation at 13,000 rpm in a microcentrifuge for 1 minute at 4 ℃ and remove supernatant.
10. Resuspend nuclear pellet in 1 ml of 1X ChIP buffer + PIC + PMSF and split into two tubes of 500 µl. Incubate on ice for 10 minutes.
11. Sonicate each tube of lysate with several pulses to break nuclear membrane. Incubate samples for 30 seconds 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-second 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.
12. Clarify lysates by centrifugation at 10,000 rpm in a microcentrifuge for 10 minutes at 4 ℃.
13. Transfer supernatant to a new tube. This is the cross-linked chromatin preparation, which should be stored at -80 ℃ until further use. Remove 50 µl of the chromatin preparation for Analysis of Chromatin Digestion and Concentration (Section B).

B. Analysis of Chromatin Digestion and Concentration (Recommended Step)

1. To the 50 µl chromatin sample (from Step 13 in Section A), add 100 µl nuclease-free water, 6 µl 5 M NaCl and 2 µl RNAse A. Vortex to mix and incubate samples at 37 ℃ for 30 minutes.
2. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate samples at 65 ℃ for 2 hours.
3. Purify DNA from samples using spin columns as described in Section F.
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).
5. To determine DNA concentration, transfer 2 µl of purified DNA to 98 µl TE to give a 50-fold dilution and read the OD_{260}. The concentration of DNA in µg/ml is OD_{260} 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 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.

C. Chromatin Immunoprecipitation

Before starting:

Remove and warm:

- 200X Protease Inhibitor Cocktail
- 10X ChIP Buffer and ensure SDS is completely in solution.

Remove and place on ice:

- Cross-linked chromatin preparation (from Step 13 in Section A)
- ChIP Grade Protein G Magnetic Beads #9006
- Antibodies for IP. This includes the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 and negative control Normal Rabbit IgG #2729.

  NOTE: 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.

1. In one tube, prepare enough 1X ChIP Buffer for the desired number of immunoprecipitations. Each precipitation contains 400 µl of 1X ChIP Buffer (40 µl of 10X ChIP Buffer + 360 µl water) and 2 µl Protease Inhibitor Cocktail. When determining the number of immunoprecipitations, the user should include the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620 and negative control Normal Rabbit IgG #2729 samples. Place mix on ice.
2. To the prepared ChIP buffer, add the equivalent of 100 µl (5 to 10 µg of chromatin DNA) of the cross-linked chromatin preparation (from Step 13 in Section A) for each 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 Protease Inhibitor Cocktail + 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 ℃ until further use (Step 1 in Section E).

4. For each immunoprecipitation, transfer 500 µl of the diluted chromatin to a microcentrifuge tube and add the immunoprecipitating antibody. The amount of antibody required for IP varies for each antibody and should be determined by the user. For the positive control Histone H3 (D2B12) XP® Rabbit mAb (ChIP Formulated) #4620, add 10 µl to the IP sample. For the negative control Normal Rabbit IgG #2729, add 1 µl (1 µg) to 5 µl (5 µg) to the IP sample. Incubate IP samples 4 hours to overnight at 4 ℃ with rotation.
5. Add 30 µl of ChIP Grade Protein G Magnetic Beads #9006 and incubate for 2 hours at 4 ℃ with rotation.
6. Proceed to Section D.

D. Washing of the Immunoprecipitated Chromatin

Before starting:

Remove and warm:

- 10X ChIP Buffer and ensure SDS is completely in solution.

Prepare and chill on ice for each IP:

- **Low salt wash:** 3 ml 1X ChIP Buffer (300 µl 10X ChIP Buffer + 2.7 ml water)
- **High salt wash:** 1 ml 1X ChIP Buffer (100 µl 10X ChIP Buffer + 900 µl water) + 70 µl 5 M NaCl

1. Pellet Protein G Magnetic Beads (from step 5, Section C) by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 minutes for solution to clear and then carefully remove the supernatant.
2. Add 1 ml of low salt wash to the beads and incubate at 4 ℃ for 5 minutes with rotation. Repeat steps 1 and 2 two additional times, for a total of 3 low salt washes.
3. Add 1 ml of high salt wash to the beads and incubate at 4 ℃ for 5 minutes with rotation. Immediately proceed to Section E.

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

Before starting:

- Remove and warm 2X ChIP Elution Buffer in a 37 ℃ water bath and ensure SDS is in solution.
- Set a water bath or thermomixer to 65 ℃.
- Prepare 150 µl 1X ChIP Elution Buffer (75 µl 2X ChIP Elution Buffer + 75 µl water) for each washed IP (from Step 3 in Section D) and the 2% input sample (from Step 3 in Section C).

1. Add 150 µl of the 1X ChIP Elution Buffer to the 2% input sample tube and set aside at room temperature until Step 7.
2. Pellet Protein G Magnetic Beads by placing the tubes in a Magnetic Separation Rack. Wait 1 to 2 minutes for solution to clear and then carefully remove the supernatant.
3. Add 150 µl 1X ChIP Elution Buffer to each IP sample.
4. Elute chromatin from the antibody/Protein G beads for 30 minutes at 65 ℃ 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.
5. Pellet Protein G Magnetic bead by placing the tubes in a Magnetic Separation Rack and wait 1 to 2 minutes for solution to clear.
6. Carefully transfer eluted chromatin supernatant to a new tube.

7. To all tubes, including the 2% input sample from Step 1, add 6 µl 5 M NaCl and 2 µl Proteinase K and incubate 2 hours at 65 ℃.
8. Immediately proceed to Section F. Alternatively, samples can be stored at -20 °. However, to avoid formation of a precipitate, be sure to warm samples to room temperature before adding DNA Binding Reagent A (Section F, Step 1).

F. DNA Purification Using Spin Columns

Before starting:

- Add 12 ml of isopropanol to DNA Binding Reagent A and 24 ml of ethanol (96-100%) to DNA Wash Reagent B before use. These steps only have to be performed once prior to the first set of DNA purifications.
- Remove one DNA purification spin column and collection tube for each DNA sample from Section E.

1. Add 600 µl DNA Binding Reagent A to each DNA sample and vortex briefly.
   - 4 volumes of DNA Binding Reagent A should be used for every 1 volume of sample.
2. Transfer 375 µl of each sample from Step 1 to a DNA purification spin column in collection tube.
3. Centrifuge at 14,000 rpm 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 375 µl of each sample from Step 1 to the spin column in collection tube. Repeat Steps 3 and 4.
6. Add 700 µl of DNA Wash Reagent B to the spin column in collection tube.
7. Centrifuge at 14,000 rpm 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 14,000 rpm in a microcentrifuge for 30 sec.
10. Discard collection tube and liquid. Retain spin column.
11. Add 50 µl of DNA Elution Reagent C to each spin column and place into a clean 1.5 ml microcentrifuge tube.
12. Centrifuge at 14,000 rpm in a microcentrifuge for 30 sec to elute DNA.
13. Remove and discard DNA purification spin column. Eluate is now purified DNA. Samples can be stored at -20 ℃.

G. Quantification of DNA by PCR

Recommendations:

- 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 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 non-specific 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 ℃

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 H_2O	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 ℃	5 min
b.	Denature	95 ℃	30 sec
c.	Anneal	62 ℃	30 sec
d.	Extension	72 ℃	30 sec
e.	Repeat Steps b - d for a total of 34 cycles.		
f.	Final Extension	72 ℃	5 min

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

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.

Reagent	Volume for 1 PCR Reaction (18 µl)
Nuclease-free H_2O	6 µl
5 µM RPL30 Primers	2 µl
SYBR® Green Reaction Mix	10 µl

4. Start the following PCR reaction program:

a.	Initial Denaturation	95 ℃ 3 min
b.	Denature	95 ℃ 15 sec
c.	Anneal and Extension:	60 ℃ 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 $2^{(C[T]\ 2\%Input\ Sample - C[T]\ IP\ Sample)}$

   C[T] = C_T = Threshold cycle of PCR reaction

Appendix A: Optimization of Chromatin Digestion

Optimal conditions for digestion of cross-linked DNA to 150-900 base pairs in length depend on cell type and cell concentration as well as the concentration of Micrococcal Nuclease. Below is a protocol to determine the optimal digestion conditions for a specific cell type and concentration of cells.

1. Prepare cross-linked nuclei from 4 X 10^7 cells as described in Section A, Steps 1-6.
2. Transfer 200 µl of the nuclei preparation from Step 6 in Section A into 5 individual microcentrifuge tubes and place on ice.
3. Add 5 µl Micrococcal Nuclease stock to 20 µl of 1X Buffer B + DTT (1:5 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 ℃ with frequent mixing.
5. Stop digest by adding 20 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 ℃ and remove supernatant.
7. Resuspend nuclear pellet in 200 µl of 1X ChIP buffer + PIC +PMSF. 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-second 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 ℃.
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 ℃ for 30 min.
12. To each RNAse A-digested sample, add 2 µl Proteinase K. Vortex to mix and incubate sample at 65 ℃ for 2 hours.
13. Remove 20 µl of each sample and determine DNA fragment size by electrophoresis on a 1% agarose gel with a 1 kb DNA marker.
14. Observe which of the digestion conditions produces DNA in the desired range of 150-900 base pairs (1 to 5 nucleosomes). The volume of diluted Micrococcal Nuclease that produces the desired size of DNA fragments using this optimization protocol is equivalent to the volume of Micrococcal Nuclease stock that will need to be added to 4 X 10^7 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 5 µl of stock Micrococcal Nuclease should be added to 4 X 10^7 cells during the digestion of chromatin in Step 7 of Section A.
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.

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 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.	Shorten the time of cross-linking to 10 min.
	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	Not enough DNA added to the PCR	Add more DNA to the PCR reaction

little product in the input PCR reactions.	reaction or conditions are not optimal.	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 G).
	Not enough chromatin added to the IP or chromatin is over-digested.	For optimal ChIP results, add 5 to 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 to 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 ℃ 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 chromatin or 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 alternative antibody source.

Flow Cytometry Protocol

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **1X Phosphate Buffered Saline (PBS):** Dissolve 8 g NaCl, 0.2 g KCl, 1.44 g Na_2HPO_4 and 0.24 g KH_2PO_4in 800 ml dH_2O. Adjust the pH to 7.4 with HCl and the volume to 1 L. Store at room temperature.
2. **Formaldehyde** (methanol free).
3. **100% Methanol**
4. **Incubation Buffer:** Dissolve 0.5 g bovine serum albumin (BSA) in 100 ml 1X PBS. Store at 4 ℃.

B. Fixation

1. Collect cells by centrifugation and aspirate supernatant.
2. Resuspend cells briefly in 0.5–1 ml PBS. Add formaldehyde to a final concentration of 2–4% formaldehyde.
3. Fix for 10 min at 37 ℃.
4. Chill tubes on ice for 1 min.
5. For extracellular staining with antibodies that do not require permeabilization, proceed to Section D, Step 1 or store cells in PBS with 0.1% sodium azide at 4 ℃; for intracellular staining, proceed to permeabilization (Section C, Step 1).

C. Permeabilization

1. Permeabilize cells by adding ice-cold 100% methanol slowly to pre-chilled cells, while gently vortexing, to a final concentration of 90% methanol. Alternatively, to remove fix prior to permeabilization, pellet cells by centrifugation and resuspend in 90% methanol.
2. Incubate 30 min on ice.

3. Proceed with immunostaining (Section D, Step 1) or store cells at –20 ℃ in 90% methanol.

D. Immunostaining

NOTE: Account for isotype matched controls for monoclonal antibodies or species matched IgG for polyclonal antibodies. Count cells using a hemocytometer or alternative method.

1. Aliquot 0.5–1x10^6 cells into each assay tube (by volume).
2. Add 2–3 ml Incubation Buffer to each tube and rinse by centrifugation. Repeat.
3. Resuspend cells in 100 µl Incubation Buffer per assay tube.
4. Block in Incubation Buffer for 10 min at room temperature.
5. Add the unconjugated, biotinylated, or fluorochrome-conjugated primary antibody at the appropriate dilution to the assay tubes (see individual antibody datasheet for the appropriate dilution).
6. Incubate for 1 hr at room temperature.
7. Rinse as before in Incubation Buffer by centrifugation.
8. If using a fluorochrome-conjugated primary antibody, resuspend cells in 0.5 ml PBS and analyze on flow cytometer; for unconjugated or biotinylated primary antibodies, proceed to immunostaining (Section D, Step 9).
9. Resuspend cells in fluorochrome-conjugated secondary antibody or fluorochrome-conjugated avidin, diluted in Incubation Buffer at the recommended dilution.
10. Incubate for 30 min at room temperature.
11. Rinse as before in Incubation Buffer by centrifugation.
12. Resuspend cells in 0.5 ml PBS and analyze on flow cytometer; alternatively, for DNA staining, proceed to optional DNA stain (Section E, Step 1).

E. Optional DNA Stain

1. Resuspend cells in 0.5 ml of DNA dye (e.g. Propidium Iodide (PI)/RNase Staining Solution #4087).
2. Incubate for at least 5 min at room temperature.
3. Analyze cells in DNA stain on flow cytometer.

Immunohistochemistry Protocol (Paraffin)

***IMPORTANT:** See product data sheet for the appropriate antibody diluent and antigen unmasking procedure.**IHC Protocol:** Unmasking buffer/antibody diluent.

A. Solutions and Reagents

1. Xylene
2. Ethanol, anhydrous denatured, histological grade (100% and 95%)
3. Deionized water (dH_2O)
4. Hematoxylin (optional)
5. **Wash Buffer:**

   1X TBS/0.1% Tween-20 (1X TBST): To prepare 1 L add 100 ml 10X TBS to 900 ml dH_2O. Add 1 ml Tween-20 and mix.

10X Tris Buffered Saline (TBS): To prepare 1 L add 24.2 g Trizma® base ($C_4H_{11}NO_3$) and 80 g sodium chloride (NaCl) to 1 L dH_2O. Adjust pH to 7.6 with concentrated HCl.

6. ***Antibody Diluent:**

a. **SignalStain® Antibody Diluent** #8112

b. **TBST/5% normal goat serum (#5425):** To 5 ml 1X TBST add 250 µl normal goat serum.

c. **PBST/5% normal goat serum (#5425):** To 5 ml 1X PBST add 250 µl normal goat serum.

1X PBS/0.1% Tween-20 (1X PBST): To prepare 1 L add 100 ml 10X PBS to 900 ml dH_2O. Add 1 ml Tween-20 and mix.

10X Phosphate Buffered Saline (PBS): To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phophate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 7.4.

7. ***Antigen Unmasking:**

a. **Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7•2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.

b. **EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2•2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

c. **TE:** 10 mM Tris/1 mM EDTA, pH 9.0: To prepare 1L add 1.21 g Trizma® base ($C_4H_{11}NO_3$) and 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2•2H_2O$) to 950 ml dH_2O. Adjust pH to 9.0, then adjust final volume to 1000 ml with dH_2O.

d. **Pepsin:** 1 mg/ml in Tris-HCl pH 2.0.

8. **3% Hydrogen Peroxide:** To prepare, add 10 ml 30% H_2O_2 to 90 ml dH_2O.

9. **Blocking Solution:** TBST/5% normal goat serum (#5425): to 5 ml 1X TBST add 250 µl normal goat serum.

10. Biotinylated secondary antibody.

11. **ABC Reagent:** (Vectastain ABC Kit, Vector Laboratories, Inc., Burlingame, CA) Prepare according to manufacturer's instructions 30 minutes before use.

12. **DAB Reagent or suitable substrate:** Prepare according to manufacturer's recommendations.

B. Deparaffinization/Rehydration

NOTE: Do not allow slides to dry at any time during this procedure.

1. **Deparaffinize/hydrate sections:**

a. Incubate sections in three washes of xylene for 5 minutes each.

b. Incubate sections in two washes of 100% ethanol for 10 minutes each.

c. Incubate sections in two washes of 95% ethanol for 10 minutes each.

2. Wash sections twice in dH_2O for 5 minutes each.

C. *Antigen Unmasking

NOTE: Consult product data sheet for specific recommendation for the unmasking solution.

1. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0 then maintain at a sub-boiling temperature for 10 minutes. Cool slides on bench top for 30 minutes.

2. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 minutes at a sub-boiling temperature. No cooling is necessary.

3. **For TE:** Bring slides to a boil in 10 mM TE/1 mM EDTA, pH 9.0 then maintain at a sub-boiling temperature for 18 minutes. Cool on the bench for 30 minutes.
4. **For Pepsin:** Digest for 10 minutes at 37 ℃.

D. Staining

1. Wash sections in dH_2O three times for 5 minutes each.
2. Incubate sections in 3% hydrogen peroxide for 10 minutes.
3. Wash sections in dH_2O twice for 5 minutes each.

   NOTE: Consult product data sheet for recommended antibody diluent.
4. Wash sections in wash buffer for 5 minutes.
5. Block each section with 100-400 µl blocking solution for 1 hour at room temperature.
6. Remove blocking solution and add 100-400 µl primary antibody diluted in recommended antibody diluent to each section. Incubate overnight at 4 ℃.
7. Remove antibody solution and wash sections in wash buffer three times for 5 minutes each.
8. Add 100-400 µl biotinylated secondary antibody, diluted in TBST per manufacturer's recommendation, to each section. Incubate 30 minutes at room temperature.
9. If using ABC avidin/biotin method, prepare ABC reagent according to the manufacturer's instructions and incubate solution for 30 minutes at room temperature.
10. Remove secondary antibody solution and wash sections three times with wash buffer for 5 minutes each.
11. Add 100-400 µl ABC reagent to each section and incubate for 30 minutes at room temperature.
12. Remove ABC reagent and wash sections three times in wash buffer for 5 minutes each.
13. Add 100-400 µl DAB or suitable substrate to each section and monitor staining closely.
14. As soon as the sections develop, immerse slides in dH_2O.
15. If desired, counterstain sections in hematoxylin per manufacturer's instructions.
16. Wash sections in dH_2O two times for 5 minutes each.
17. Dehydrate sections:

a. Incubate sections in 95% ethanol two times for 10 seconds each.

b. Repeat in 100% ethanol, incubating sections two times for 10 seconds each.

c. Repeat in xylene, incubating sections two times for 10 seconds each.

18. Mount coverslips.

Immunofluorescence General Protocol

IMPORTANT: Please refer to the APPLICATIONS section on the front page of product datasheet to determine if this product is validated and approved for use on cultured cell lines (IF-IC), paraffin-embedded samples (IF-P), or frozen tissue sections (IF-F). Please see product datasheet for appropriate antibody dilution and unmasking solution.

A. Solutions and Reagents

NOTE: Prepare solutions with purified water.

1. **10X Phosphate Buffered Saline (PBS):** To prepare 1 L add 80 g sodium chloride (NaCl), 2 g potassium chloride (KCl), 14.4 g sodium phosphate, dibasic (Na_2HPO_4) and 2.4 g potassium phosphate, monobasic (KH_2PO_4) to 1 L dH_2O. Adjust pH to 8.0.
2. **Formaldehyde:** 16%, methanol free, Polysciences, Inc. (cat# 18814), use fresh, store opened vials at 4 ℃ in dark, dilute in PBS for use.
3. **Blocking Buffer:** (1X PBS / 5% normal goat serum (#5425) / 0.3% Triton™ X-100): To prepare 25 ml, add 2.5 ml 10X PBS, 1.25 ml normal serum from the same species as the secondary antibody (e.g., normal goat serum, normal donkey serum) and 21.25 ml dH_2O and mix well. While stirring, add 75 µl Triton™ X-100.
4. **Antibody Dilution Buffer:** (1X PBS / 1% BSA / 0.3% Triton™ X-100): To prepare 40 ml, add 4 ml 10X PBS and 120 µl Triton™ X-100 to 0.4 g BSA. Bring to final volume of 40 ml with dH_2O and mix well.
5. **Fluorochrome-conjugated secondary antibody NOTE:** When using any primary or fluorochrome-conjugated secondary antibody for the first time, titrate the antibody to determine which dilution allows for the strongest specific signal with the least background for your sample.
6. **Prolong® Gold Anti-Fade Reagent** (#9071), with DAPI (#8961).

Reagents specific to IF-P application:

1. **Xylene**
2. **Ethanol**, anhydrous denatured, histological grade, 100% and 95%.
3. **Antigen Unmasking:**

a. **For Citrate:** 10 mM Sodium Citrate Buffer: To prepare 1 L add 2.94 g sodium citrate trisodium salt dihydrate ($C_6H_5Na_3O_7•2H_2O$) to 1 L dH_2O. Adjust pH to 6.0.

b. **For EDTA:** 1 mM EDTA: To prepare 1 L add 0.372 g EDTA ($C_{10}H_{14}N_2O_8Na_2•2H_2O$) to 1 L dH_2O. Adjust pH to 8.0.

B. Specimen Preparation

I. Cultured Cell Lines (IF-IC)

NOTE: Cells should be grown, treated, fixed and stained directly in multi-well plates, chamber slides or on coverslips.

1. Aspirate liquid, then cover cells to a depth of 2–3 mm with 4% formaldehyde in PBS. **NOTE:** Formaldehyde is toxic, use only in fume hood.
2. Allow cells to fix for 15 min at room temperature.
3. Aspirate fixative, rinse three times in PBS for 5 min each.
4. Proceed with Immunostaining (Section C).

II. Paraffin Sections (IF-P)

NOTE: Do not allow slides to dry at any time during this process.

1. **Deparaffinization/Rehydration:**

a. Incubate sections in three washes of xylene for 5 min each.

b. Incubate sections in two washes of 100% ethanol for 10 min each.

c. Incubate sections in two washes of 95% ethanol for 10 min each.

d. Rinse sections twice in dH_2O for 5 min each.

2. **Antigen Unmasking:**

   NOTE: Consult product datasheet for specific recommendation for the unmasking solution.

2.

a. **For Citrate:** Bring slides to a boil in 10 mM sodium citrate buffer pH 6.0, then maintain at a sub-boiling temperature for 10 min. Cool slides on bench top for 30 min.

b. **For EDTA:** Bring slides to a boil in 1 mM EDTA pH 8.0 followed by 15 min at a sub-boiling temperature. No cooling is necessary.

3. Proceed with Immunostaining (Section C).

III. Frozen/Cryostat Sections (IF-F)

1. For fixed frozen tissue proceed with Immunostaining (Section C).
2. For fresh, unfixed frozen tissue, please fix immediately, as follows:

a. Cover sections with 4% formaldehyde in PBS.

b. Allow sections to fix for 15 min at room temperature.

c. Rinse slides three times in PBS for 5 min each.

d. Proceed with Immunostaining (Section C).

C. Immunostaining

NOTE: All subsequent incubations should be carried out at room temperature unless otherwise noted in a humid light-tight box or covered dish/plate to prevent drying and fluorochrome fading.

1. Block specimen in Blocking Buffer for 60 min.
2. While blocking, prepare primary antibody by diluting as indicated on datasheet in Antibody Dilution Buffer.
3. Aspirate blocking solution, apply diluted primary antibody.
4. Incubate overnight at 4 ℃.
5. Rinse three times in PBS for 5 min each.

   NOTE: If using primary antibodies directly conjugated with Alexa Fluor® fluorochromes, then skip to (Section C, Step 8).

6. Incubate specimen in fluorochrome-conjugated secondary antibody diluted in Antibody Dilution Buffer for 1–2 hr at room temperature in dark.
7. Rinse in PBS (Section C, Step 5).
8. Coverslip slides with Prolong® Gold Anti-Fade Reagent (#9071), with DAPI (#8961).
9. For best results, allow mountant to cure overnight at room temperature. For long-term storage, store slides flat at 4 ℃ protected from light.

Immunoprecipitation Protocol / (For Analysis By Western Immunoblotting)

For **shorter assay times** please try our Immunoprecipitation Protocol Utilizing Magnetic Separation / (For Analysis By Western Immunoblotting).

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. 1X Phosphate Buffered Saline (PBS)
2. **1X Cell Lysis Buffer:** (#9803) 20 mM Tris (pH 7.5), 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2.5 mM Sodium pyrophosphate, 1 mM β-glycerophosphate, 1 mM Na_3VO_4, 1 µg/ml Leupeptin

   NOTE: Add 1 mM PMSF immediately prior to use.
3. **Protein A or G Agarose Beads:** (Protein A #9863) Please prepare according to manufacturer's instructions. Use Protein A for rabbit IgG pull down and Protein G for mouse IgG pull down.
4. **3X SDS Sample Buffer:** (#7722) 187.5 mM Tris-HCl (pH 6.8 at 25 ℃), 6% w/v SDS, 30% glycerol, 150 mM DTT, 0.03% w/v bromophenol blue

B. Preparing Cell Lysates

1. Aspirate media. Treat cells by adding fresh media containing regulator for desired time.
2. To harvest cells under nondenaturing conditions, remove media and rinse cells once with ice-cold PBS.
3. Remove PBS and add 0.5 ml ice-cold 1X cell lysis buffer to each plate (10 cm) and incubate the plates on ice for 5 minutes.
4. Scrape cells off the plates and transfer to microcentrifuge tubes. Keep on ice.
5. Sonicate samples on ice three times for 5 seconds each.
6. Microcentrifuge for 10 minutes at 14,000 X g, 4 ℃, and transfer the supernatant to a new tube. If necessary, lysate can be stored at –80 ℃.

C. Immunoprecipitation

Optional: It may be necessary to perform a lysate pre-clearing step to reduce non-specific binding to the Protein A/G agarose beads (See section below).

1. Take 200 µl cell lysate and add primary antibody. Incubate with gentle rocking overnight at 4 ℃.
2. Add either protein A or G agarose beads (20 µl of 50% bead slurry). Incubate with gentle rocking for 1–3 hours at 4 ℃.
3. Microcentrifuge for 30 seconds at 4 ℃. Wash pellet five times with 500 µl of 1X cell lysis buffer. Keep on ice during washes.
4. Resuspend the pellet with 20 µl 3X SDS sample buffer. Vortex, then microcentrifuge for 30 seconds.
5. Heat the sample to 95–100 ℃ for 2–5 minutes and microcentrifuge for 1 minute at 14,000 X g.
6. Load the sample (15–30 µl) on SDS-PAGE gel (12–15%).
7. Analyze sample by Western blotting (see Western Immunoblotting Protocol: Western BSA, Western Milk).

Cell Lysate Pre-Clearing (Optional)

1. Take 200 µl cell lysate and add to either Protein A or G agarose beads (20 µl of 50% bead slurry).
2. Incubate at 4 ℃ for 30 – 60 minutes.
3. Spin for 10 minutes at 4 ℃. Transfer the supernatant to a fresh tube.
4. Proceed to step 1 of Immunoprecipitation.

   NOTE: For proteins with molecular weights of 50 kDa, we recommend using Mouse Anti-Rabbit IgG (Light-Chain Specific) (L57A3) mAb #3677 or Mouse Anti-Rabbit IgG (Conformation Specific) (L27A9) mAb #3678 as a secondary

antibody to minimize masking produced by denatured heavy chains. For proteins with molecular weights of 25 kDa, Mouse Anti-Rabbit IgG (Conformation Specific) (L27A9) mAb #3678 is recommended.

Western Immunoblotting Protocol (Primary Ab Incubation In BSA)

For Western blots, incubate membrane with diluted antibody in 5% w/v BSA, 1X TBS, 0.1% Tween-20 at 4℃ with gentle shaking, overnight.

Products available from Cell Signaling Technology are linked by their respective catalog numbers.

A. Solutions and Reagents

NOTE: Prepare solutions with Milli-Q or equivalently purified water.

1. **1X Phosphate Buffered Saline (PBS).**
2. **1X SDS Sample Buffer:** (#7722, #7723) 62.5 mM Tris-HCl (pH 6.8 at 25 ℃), 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromophenol blue or phenol red.
3. **Transfer Buffer:** 25 mM Tris base, 0.2 M glycine, 20% methanol (pH 8.5).
4. **10X Tris Buffered Saline (TBS):** (#9997) To prepare 1 liter of 10X TBS: 24.2 g Tris base, 80 g NaCl; adjust pH to 7.6 with HCl (use at 1X).
5. **Nonfat Dry Milk:** (#9999) (weight to volume [w/v]).
6. **Blocking Buffer:** 1X TBS, 0.1% Tween-20 with 5% w/v nonfat dry milk; for 150 ml, add 15 ml 10X TBS to 135 ml water, mix. Add 7.5 g nonfat dry milk and mix well. While stirring, add 0.15 ml Tween-20 (100%).
7. **Wash Buffer:** 1X TBS, 0.1% Tween-20 (TBS/T).
8. **Bovine Serum Albumin (BSA):** (#9998).
9. **Primary Antibody Dilution Buffer:** 1X TBS, 0.1% Tween-20 with 5% BSA; for 20 ml, add 2 ml 10X TBS to 18 ml water, mix. Add 1.0 g BSA and mix well. While stirring, add 20 µl Tween-20 (100%).
10. **Phototope®-HRP Western Blot Detection System:** (#7071 anti-rabbit) or (#7072 anti-mouse) Includes biotinylated protein ladder, secondary (#7074 anti-rabbit) or (#7076 anti-mouse) antibody conjugated to horseradish peroxidase (HRP), anti-biotin antibody conjugated to HRP, LumiGLO® chemiluminescent reagent and peroxide.
11. **Prestained Protein Marker, Broad Range (Premixed Format):** (#7720).
12. **Biotinylated Protein Ladder Detection Pack:** (#7727).
13. **Blotting Membrane:** This protocol has been optimized for nitrocellulose membranes, which CST recommends. PVDF membranes may also be used.

B. Protein Blotting

A general protocol for sample preparation is described below.

1. Treat cells by adding fresh media containing regulator for desired time.
2. Aspirate media from cultures; wash cells with 1X PBS; aspirate.
3. Lyse cells by adding 1X SDS sample buffer (100 µl per well of 6-well plate or 500 µl per plate of 10 cm diameter plate). Immediately scrape the cells off the plate and transfer the extract to a microcentrifuge tube. Keep on ice.
4. Sonicate for 10–15 seconds for complete cell lysis and to shear DNA (to reduce sample viscosity).
5. Heat a 20 µl sample to 95–100 ℃ for 5 minutes; cool on ice.

6. Microcentrifuge for 5 minutes.
7. Load 20 µl onto SDS-PAGE gel (10 cm x 10 cm). **NOTE:** CST recommends loading prestained molecular weight markers (#7720, 10 µl/lane) to verify electrotransfer and biotinylated protein ladder (#7727, 10 µl/lane) to determine molecular weights.
8. Electrotransfer to nitrocellulose or PVDF membrane.

C. Membrane Blocking and Antibody Incubations

NOTE: Volumes are for 10 cm x 10 cm (100 cm^2) of membrane; for different sized membranes, adjust volumes accordingly.

1. (Optional) After transfer, wash nitrocellulose membrane with 25 ml TBS for 5 minutes at room temperature.
2. Incubate membrane in 25 ml of blocking buffer for 1 hour at room temperature.
3. Wash three times for 5 minutes each with 15 ml of TBS/T.
4. Incubate membrane and primary antibody (at the appropriate dilution) in 10 ml primary antibody dilution buffer with gentle agitation overnight at 4 ℃.
5. Wash three times for 5 minutes each with 15 ml of TBS/T.

I. For Unconjugated Primary Antibodies

1. Incubate membrane with **appropriate** HRP-conjugated secondary antibody (1:2000) and HRP-conjugated anti-biotin antibody (1:1000) to detect biotinylated protein markers in 10 ml of blocking buffer with gentle agitation for 1 hour at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

II. For HRP Conjugated Primary Antibodies

Skip to Detection of Proteins (Step D).

III. For Biotinylated Primary Antibodies

1. Incubate membrane with HRP-Streptavidin (at the appropriate dilution) in milk for one hour with gentle agitation at room temperature.
2. Wash three times for 5 minutes each with 15 ml of TBS/T.

D. Detection of Proteins

1. Incubate membrane with 10 ml LumiGLO® (0.5 ml 20X LumiGLO®, 0.5 ml 20X Peroxide and 9.0 ml Milli-Q water) with gentle agitation for 1 minute at room temperature. **NOTE:** LumiGLO® substrate can be further diluted if signal response is too fast.
2. Drain membrane of excess developing solution (do not let dry), wrap in plastic wrap and expose to x-ray film. An initial 10-second exposure should indicate the proper exposure time. **NOTE:** Due to the kinetics of the detection reaction, signal is most intense immediately following LumiGLO® incubation and declines over the following 2 hours.

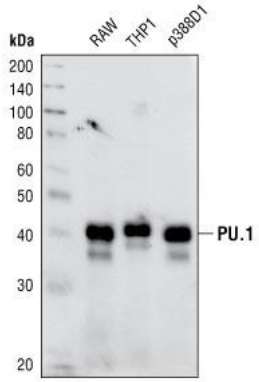
Specificity / Sensitivity

This antibody detects endogenous levels of total PU.1 protein. The antibody does not cross react with other Ets family members.

Source / Purification

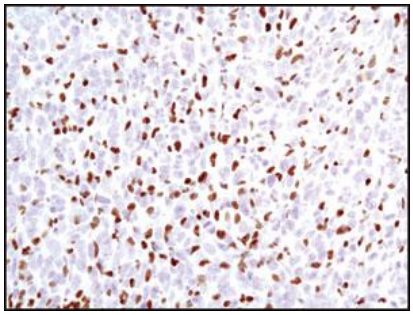
Monoclonal antibody is produced by immunizing animals with a synthetic peptide corresponding to the sequence of human PU.1 protein.

Western Blotting



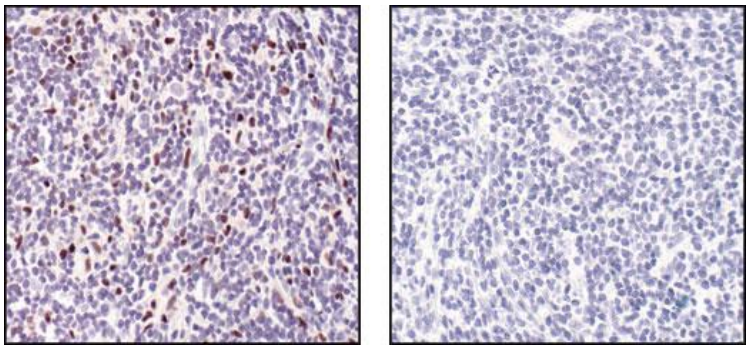
Western blot analysis of extracts from RAW, THP1 and p388D1 cells using PU.1 (9G7) Rabbit mAb.

IHC-P (paraffin)



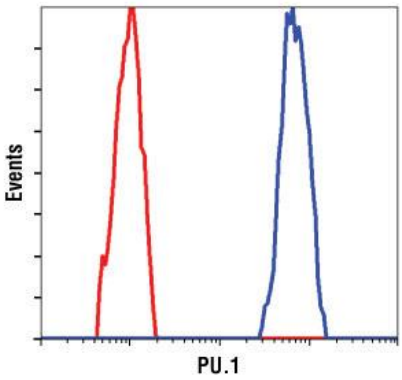
Immunohistochemical analysis of paraffin-embedded 4T1 syngeneic mouse tumor using PU.1 (9G7) Rabbit mAb #2258.

IHC-P (paraffin)



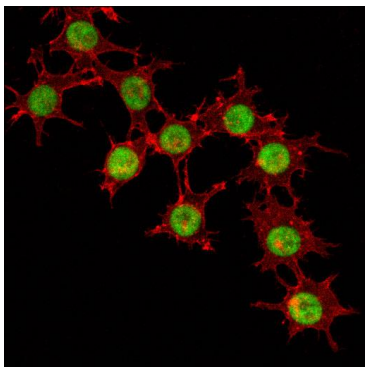
Immunohistochemical analysis of paraffin-embedded Non-Hodgkin's lymphoma using PU.1 (9G7) Rabbit mAb in the presence of control peptide (left) or PU.1 Blocking Peptide #1036 (right).

Flow Cytometry



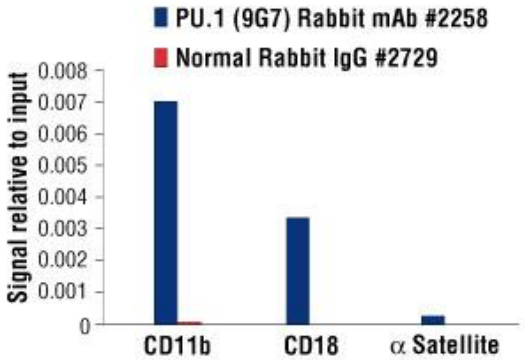
Flow cytometric analysis of untreated THP-1 cells using PU.1 (9G7) Rabbit mAb (blue) compared to a nonspecific negative control antibody (red).

IF-IC



Confocal immunofluorescent analysis of Raw cells using PU.1 (9G7) Rabbit mAb (green). Actin filaments were labeled with DY-554 phalloidin (red).

Chromatin IP



Chromatin immunoprecipitations were performed with cross-linked chromatin from 4 x 10^6 U-937 cells and either 10 µl of PU.1 (9G7) Rabbit mAb or 2 µl of Normal Rabbit IgG #2729 using SimpleChIP®Enzymatic Chromatin IP Kit (Magnetic Beads) #9003. The enriched DNA was quantified by real-time PCR using human CD11b promoter primers, human CD18 intron 1 primers, 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, which is equivalent to one.