Magna ChIP™ G
(Catalog # 17-611)

EZ-Magna ChIP™ G
(Catalog # 17-409)

One-Day Chromatin Immunoprecipitation Kits

FOR RESEARCH USE ONLY.

NOT FOR USE IN DIAGNOSTIC PROCEDURES.

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Version 7.0/ September 2016/ MAGNA0002MAN

Instruction Manual

Each kit contains sufficient reagents for 22 chromatin immunoprecipitation (ChIP) assays.

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Magna ChIP™ and EZ- Magna ChIP™ Kits are available in several formats so you can easily select the optimal kit for your research needs.

I. Magna ChIP™ & EZ-Magna ChIP™ Kit Configurations			
Magna ChIP™ A (Cat. # 17-610)	**Magna ChIP™ G (Cat. # 17-611)**	**EZ-Magna ChIP™ A (Cat. # 17-408)**	**EZ-Magna ChIP™ G (Cat. # 17-409)**
MAGNA0001 (Store at 4°C)	MAGNA0002 (Store at 4°C)	MAGNA0001 (Store at 4°C)	MAGNA0002 (Store at 4°C)
MAGNA0003 (Store at -20°C)	MAGNA0003 (Store at -20°C)	MAGNA0004 (Store at -20°C)	MAGNA0005 (Store at -20°C)

II. Kit Description

Quantity: Two boxes containing all necessary reagents to perform 22 individual chromatin immunoprecipitation (ChIP) reactions. Supplied buffers are sufficient to generate chromatin from up to five 15 cm plates of cultured cells, each plate providing up to 10 chromatin preparations (varies with cell and assay type).

Storage and Stability: Upon receipt, store components at the temperatures indicated on the labels. Kit components are stable for 1 year from date of shipment when stored as directed.

Use: The Magna ChIP™ kit contains reagents optimized for immunoprecipitation of chromatin from mammalian cells. The EZ-Magna ChIP™ kit contains all elements of the Magna ChIP™ kit, but also includes IP and PCR controls to ensure successful optimization of the assay. For Catalog # 17-409, EZ-Magna ChIP™ G, the positive control antibody is a mouse monoclonal antibody to RNA Polymerase II that will detect RNA Polymerase II of human, mouse, rat and yeast origins. The negative control is Normal Mouse IgG which controls for the non-specific immunoselection of chromatin by immunoglobulins. The Control Primer mix is included for detection of a 166 base pair region of the human GAPDH promoter by both end-point and real-time quantitative PCR. Use of these primers with DNA from species other than human is not recommended. Detection of the DNA region, gene or promoter of interest in immunoprecipitated chromatin must be empirically determined by the researcher. PCR using promoter-specific primers is recommended for detection and analysis of enriched DNA.

Related Products:

Catalog # 17-295	Chromatin Immunoprecipitation (ChIP) Kit
Catalog # 17-245	Acetyl-Histone H3 Immunoprecipitation (ChIP) Assay Kit
Catalog # 17-229	Acetyl-Histone H4 Immunoprecipitation (ChIP) Assay Kit
Catalog # 16-157	Protein A agarose/Salmon Sperm DNA
Catalog # 16-201	Protein G agarose/Salmon Sperm DNA
Catalog # 17-371	EZ ChIP™ Kit
Catalog # 17-375	EZ-Zyme Chromatin Preparation Kit
Catalog # 17-610	Magna ChIP™ A Kit
Catalog # 17-408	EZ-Magna ChIP™ A Kit
Catalog # 20-400	Magna Grip Rack (8 Well)
Catalog # 20-153	ChIP Dilution Buffer
Catalog # 20-154	Low Salt Immune Complex Wash Buffer
Catalog # 20-155	High Salt Immune Complex Wash Buffer
Catalog # 20-156	LiCl Immune Complex Wash Buffer
Catalog # 20-157	TE Buffer
Catalog # 20-158	0.5M EDTA
Catalog # 20-159	5M NaCl
Catalog # 20-160	1M Tris-HCl, pH 6.5
Catalog # 20-163	SDS Lysis Buffer

Millipore's complete list of ChIP qualified antibodies can be found at http://www.millipore.com

III. Magna ChIP™ KIT COMPONENTS

Provided Kit Components (Note Storage Temperatures)

MAGNA0002 Contents (17-611 & 17-409) Store at 4°C		
Component	**Catalog #**	**Quantity**
Magnetic Protein G beads	CS200638	450 µL
ChIP Dilution Buffer	CS200624	12.5 mL
Low Salt Wash Buffer	CS200625	12.5 mL
High Salt Wash Buffer	CS200626	12.5 mL
LiCl Wash Buffer	CS200627	12.5 mL
TE Buffer	CS200628	12.5 mL
Cell Lysis Buffer	CS200634	5 mL
Nuclear Lysis Buffer	CS200623	5 mL
ChIP Elution buffer (w/o Proteinase K)	CS200629	5 mL
10X Glycine	20-282	11 mL
10X PBS	20-281	24 mL
Store the Following at Room Temperature Upon Receipt		
Spin Filters	20-290	22 Filters
Collection Tubes	20-291	22 Tubes
Bind Reagent A	20-292	25 mL
Wash Reagent B	20-293	12.5 mL
Elution Reagent C	20-294	1.5 mL

MAGNA0003 Contents (17-611) Store at -20°C		
Protease Inhibitor Cocktail II, 200X	20-283	110 µL ****Contains DMSO**
Proteinase K	20-298	600 µg K in 60 µL

MAGNA0005 Contents (17-409) Store at -20°C		
Protease Inhibitor Cocktail II, 200X	20-283	110 µL ****Contains DMSO**
Proteinase K	20-298	600 µg K in 60 µL
Control Primers	22-004	75 µL of 5 µM of each control primer specific for human GAPDH. FOR: 5'-TACTAGCGGTTT TACGGGCG-3' REV: 5'-TCGAACAGGAG GAGCAGAGAGCGA-3
Anti-RNA Polymerase II, clone CTD4H8	05-623B	25 µg
Normal Mouse IgG	12-371B	25 µg

Required Materials Not Provided

Reagents

- Cells, stimulated or treated as desired
- Antibody of interest for chromatin immunoprecipitation
- 37% Formaldehyde
- *Taq* DNA polymerase for standard PCR
- dNTPs, 2.5 mM each
- SYBR®-Green Master Mix for qPCR
- DNase and RNase-free sterile H_2O

Equipment

- Magnetic Separator (Magna Grip Rack (8 Well), Catalog #20-400)
- Vortex mixer
- Rotating wheel/platform
- Microfuge
- Sonicator
- Thermomixer or Hybridization Oven
- Variable temperature water bath or incubator
- Timer
- Variable volume (5-1000 μL) pipettors + tips
- Cell scraper
- Microfuge tubes, 1.5 mL
- Thermal cycler
- PCR tubes, 0.2 mL

  Filter-tip pipette tips

IV. CHROMATIN IMMUNOPRECIPITATION BACKGROUND

Chromatin Immunoprecipitation (ChIP) is a powerful technique for mapping the *in vivo* distribution of proteins associated with chromosomal DNA. These proteins can be histone subunits and post-translational modifications thereof or other chromatin associated proteins such as transcription factors, chromatin regulators, etc. Additionally, ChIP can be used to identify regions of the genome associated with these proteins, or conversely, to identify proteins associated with a particular region of the genome. Genome-wide maps of protein-DNA interactions or histone modifications using ChIP can provide insight into gene regulatory networks.

ChIP methodology often involves protein-DNA and protein-protein crosslinking, fragmentation of the crosslinked chromatin, and subsequent immunoprecipitation with an antibody specific to a target protein associated with the fragmented chromosomal DNA. The DNA fragments isolated in complex with the target protein can be identified by a variety of methods including PCR, DNA microarray and DNA sequencing. Standard or quantitative PCR can be performed to verify whether a particular DNA sequence (the gene or region of the genome) is associated with the protein of interest. The combination of ChIP and promoter or genomic tiling microarrays (ChIP:CHIP) allows genome-wide identification of DNA-binding sites for chromatin-associated proteins with precise resolution. Recently, direct high-throughput sequencing of immunoprecipitated DNA material or high-throughput sequencing of a plasmid library constructed from immunoprecipitated chromosomal DNA has become a useful alternative to ChIP-CHIP in mapping the protein-DNA interactions across mammalian genomes.

The standard ChIP protocol is laborious and time consuming. With **Magna ChIP™,** the amount of time required to perform the ChIP experiment can be reduced from three days to one. Additionally, the smaller reaction volume of the Magna ChIP™ increases the relative concentration of the antibody enabling the ChIP reaction to be performed with reduced amounts of both antibody and chromatin material. Finally, using magnetic protein A or G beads, **Magna ChIP™** is compatible with automated high throughput platforms that allow a large number of ChIP reactions to be carried out simultaneously. The **Magna ChIP™** kit contains all buffers and reagents required to perform successful ChIP experiments from mammalian cells. **EZ-Magna ChIP™** additionally contains essential controls such as anti-RNA Polymerase II, Normal Mouse IgG and Control Primers to ensure successful protocol optimization and adoption. RNA Polymerase II catalyzes the transcription of protein-encoding genes and is present at the promoter region of actively transcribed genes.

The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene is a housekeeping gene and is constitutively transcribed in most growing mammalian cells. Upon immunoprecipitation of chromatin with anti-RNA Polymerase II, the resulting DNA is enriched for the GAPDH gene (as well as all RNA PolII transcribed genes), whereas immunoprecipitation with Normal Mouse IgG should not result in significant GAPDH enrichment. For DNA purification, the **Magna ChIP™** kit incorporates a unique polypropylene spin column which contains a specially activated silica membrane filter that captures DNA and separates it from contaminating proteins and other cellular debris. In combination with the included binding and wash buffers, the spin column provides rapid purification of chromatin DNA without the need for phenol chloroform extractions or ethanol precipitation. The purified DNA can be directly subjected to a variety of applications including quantitative PCR, amplification/labeling for ChIP-CHIP, ultrahigh-throughput sequencing and ChIP cloning.

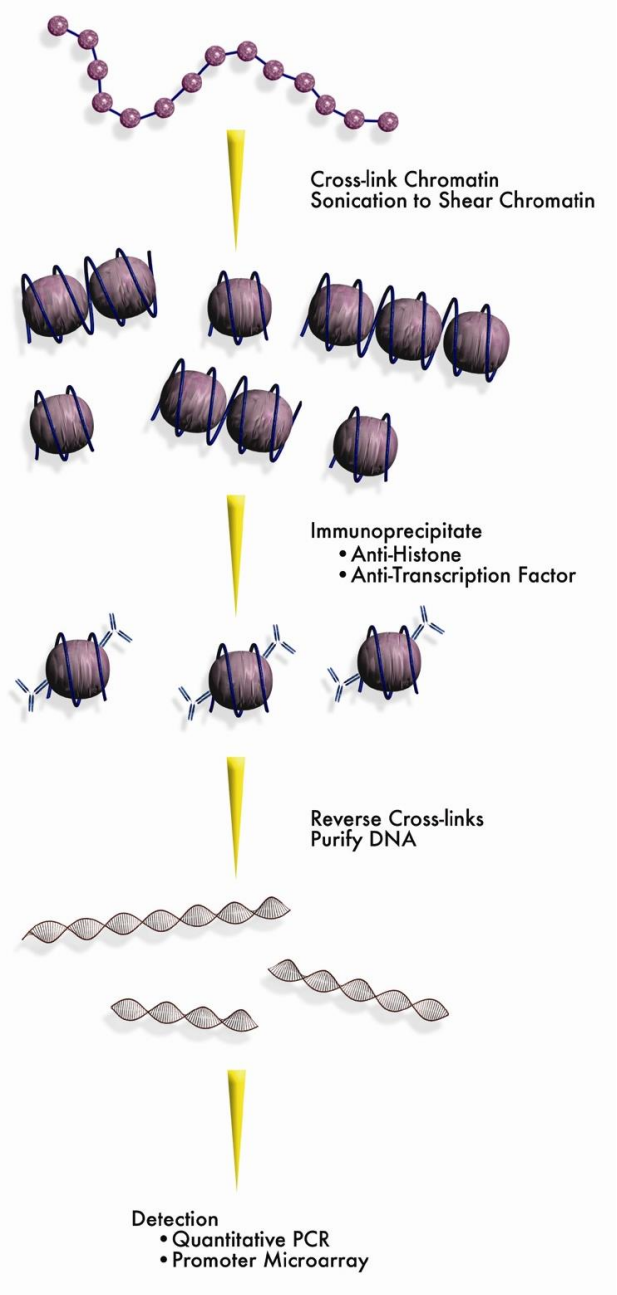
V. CHROMATIN IMMUNOPRECIPITATION (ChIP) ASSAY OVERVIEW

A. Chromatin Sample Prep and Immunoselection

Grow cells and treat with formaldehyde.
Formaldehyde treatment crosslinks proteins to DNA to ensure co-precipitation

Lysis and sonication of the cells. Cells are broken open and sonication is performed to shear the chromatin to a manageable size. Generally, it is recommended to generate fragments of 200-1000 bp of DNA because it is small enough to achieve a high degree of resolution during the detection step. It is critical that average fragment size be confirmed empirically by gel electrophoresis.

Immunoselection. ChIP is very similar to a standard immunoprecipitation using a primary antibody in combination with a directly conjugated or protein A or G-conjugated solid support matrix such as agarose or magnetic beads. The immunoselection step enriches for the specific DNA-protein complex of interest**.**



B. DNA Purification and Detection

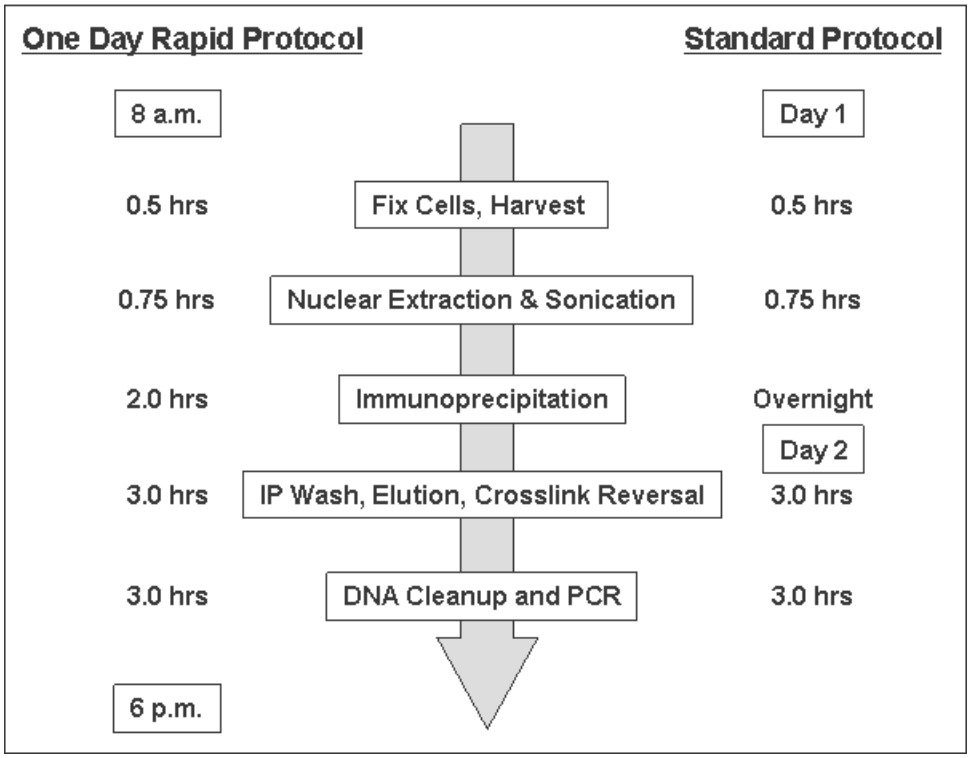
Purification of the DNA. Protein-DNA crosslinks are reversed and DNA is purified to remove the chromatin proteins and to prepare the DNA for the detection step.

Detection. This is the most variable step of the procedure because of the number of detection methods that can be employed. The most meaningful results will be obtained with quantitative PCR for this step. Real Time Quantitative PCR (qPCR) is ideal but this method requires a specialized PCR machine that may not be available. For standard PCR, primer selection is critical and must be designed with close adherence to the following guidelines:

Primer Length:	24 nt
Optimum T_m:	60°C
Optimum GC:	50%
Amplicon size:	100-700 base pairs

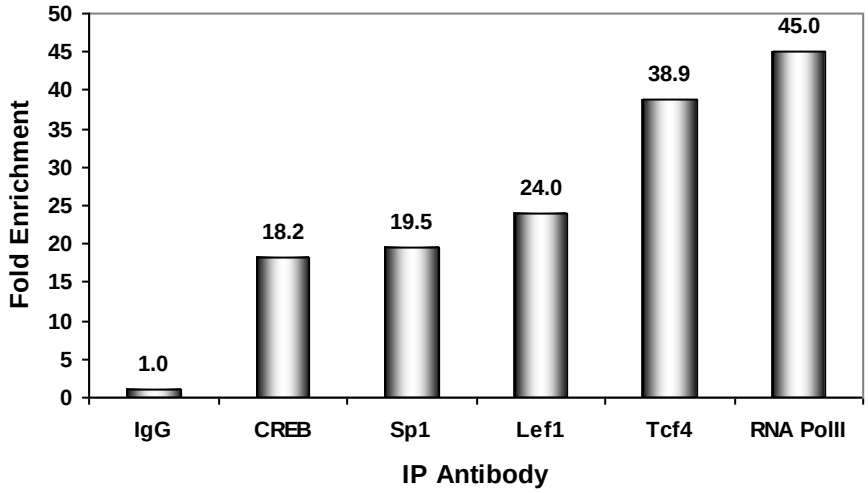
After standard PCR, the fragments are run on agarose or polyacrylamide gels and the gels are stained and imaged.

VI. Comparison of Standard and Rapid Magna ChIP™ Protocols



Comparison of the Rapid Magna ChIP™ and Standard protocols. The protocols vary only in the time required for immunoprecipitation. The example of the Rapid Protocol shown above is only recommended for experiments with a limited number of samples. For additional guidelines on selecting the appropriate protocol, see detailed instructions on page 9.

VII. Performance of various ChIP antibodies using Rapid Magna ChIP™ Protocol



Chromatin from various cell lines was subject to immunoprecipitation with the indicated antibodies using the Rapid ChIP™ protocol (2 hour immunoprecipitation) with **Magna ChIP™ A** (Cat.# 17-610) or **Magna ChIP™ G** (Cat.# 17-611). The IgG used for relative comparison was either Rabbit Purified IgG (Cat.# PP64B) or Mouse Purified IgG (Cat.# 12-371B), depending upon the ChIP antibody. Quantitative PCR data is presented as fold relative enrichment to IgG from independent experiments. Antibodies and chromatin utilized were as follows:

ChIPAb+ CREB (Cat.# 17-600): 4 µg rabbit polyclonal affinity purified antibody immunoprecipitated from **ChIPable Chromatin HEK293** (Cat.# 12-704) and assayed with an NR4A2 promoter PCR assay.

ChIPAb+ Sp1 (Cat.# 17-601): 4 µg rabbit polyclonal affinity purified antibody immunoprecipitated from **ChIPable Chromatin HeLa** (Cat.# 12-687) and assayed with a DHFR promoter PCR assay.

ChIPAb+ Lef1 (Cat.# 17-604): 4 µg mouse monoclonal affinity purified antibody immunoprecipitated from **ChIPable Chromatin HT29** (Cat. #12-705) and assayed with a c-myc promoter PCR assay.

ChIPAb+ Tcf-4 (Cat.# 17-607): 4 µg mouse monoclonal affinity purified antibody immunoprecipitated from **ChIPable Chromatin HT29** (Cat. #12-705) and assayed with a c-myc promoter PCR assay.

A**nti-RNA Polymerase II clone CTD4H8** (Cat.# 05-623B): 1 µg mouse monoclonal affinity purified antibody immunoprecipitated from **ChIPable Chromatin HeLa** (Cat.# 12-687) and assayed with a GAPDH promoter PCR assay.

For a complete listing of Millipore's ChIPAb+ validated antibody/primer sets, visit www.millipore.com and search 'ChIPAb+'.

VIII. DETAILED CHROMATIN IMMUNOPRECIPITATION PROTOCOL

Magna ChIP™ or **EZ-Magna ChIP™** can be performed in a single day, from sample extraction to data analysis, using the Rapid Magna ChIP™ protocol and ChIP-validated antibodies to abundant ChIP targets. Alternatively, a more sensitive protocol is provided for use with antibodies of unknown quality or for less abundant ChIP targets. The primary difference between these two protocols is the length of time required for the immunoprecipitation which can be performed in as little as one hour or as long as overnight. Both time management strategies are diagrammed on page 7. Additionally, for advanced users, an abbreviated protocol card (Cat.# CR200818) is provided and may be used in place of the detailed protocol.

A. *In vivo* Crosslinking and Lysis

Prior to starting this section:

- Stimulate or treat, if necessary, adherent mammalian cells at ~80-90% confluency in a 150mm culture dish containing 20ml of growth media.
    - For HeLa cells, this is approximately 1 x 10^7 cells. This will generate a preparation of chromatin sufficient for up to 10 separate immunoprecipitations.
    - The volume of buffers supplied in the kit are sufficient to generate chromatin from up to five 150 mm plates of cultured cells, each plate providing up to 10 chromatin preparations (varies with cell and assay type). Chromatin from alternate culture vessels can be isolated with slight modifications to the protocol.
    - Include one extra plate of cells to be used solely for estimation of cell number.
    - Cell numbers can be scaled according to the performance of the antibody. For example, EZ-Magna ChIP control antibodies can perform successful ChIP on as few as 1 X 10^5 HeLa cells. The protocol is written for simplicity using 1 X 10^6 cells per ChIP to ensure optimal performance of the control antibodies.
- Obtain ice for incubation of PBS (see Step 3) and for incubating culture dish (see Step 6).
- Prepare 42 ml of 1X PBS (4.2 ml 10X PBS and 37.8 ml water) for each 150 mm culture dish and put on ice. This will be used for washes and needs to be ice cold.
- Warm Nuclear Lysis Buffer to room temperature to ensure SDS is in solution before proceeding with cell lysis.
- Remove Protease Inhibitor Cocktail II and thaw at room temperature for use in Steps 3 and 13. This product contains DMSO and will remain frozen below 18.4°C.

1. Add 550 µl of 37% formaldehyde (or 1100 µl of fresh 18.5% formaldehyde) to 20 ml of growth media to crosslink. Gently swirl dish to mix.
    - Final concentration for formaldehyde is 1%.
    - Use high quality formaldehyde. Do not use if formaldehyde is past the expiration date as suggested by the manufacturer. To make fresh formaldehyde before each experiment, see Appendix B.
2. Incubate at room temperature for 10 minutes.
    - Agitation of cells is not necessary.
3. Meanwhile, remove 2 ml of ice cold 1X PBS to a separate tube for every dish and add 5 µl of Protease Inhibitor Cocktail II to each 1 ml of 1X PBS and put on ice.
4. Add 2 ml of 10X Glycine to each dish to quench unreacted formaldehyde.
5. Swirl to mix and incubate at room temperature for 5 minutes.
6. Place dishes on ice.

7. Aspirate medium, removing as much medium as possible, being careful not to disturb the cells.
8. Add 20 ml of cold 1X PBS to wash cells.
9. Remove PBS and repeat PBS wash, steps 8 and 9.
10. Add 2 ml cold PBS containing 1X Protease Inhibitor Cocktail II to each dish (prepared in Step 3).
11. Scrape cells from each dish into a separate microfuge tube.
12. Spin at 800 x g at 4°C for 5 minutes to pellet cells.
13. During spin, prepare 0.5 ml of cell Lysis Buffer containing 2.5 µl of Protease Inhibitor Cocktail II for each microfuge tube.
14. Remove supernatant. For chromatin preparation from cultured cells, the fixed cell pellet can be snap frozen in liquid nitrogen at this point and stored at -80°C for several months if desired.
15. Resuspend cell pellet in 0.5 ml of cell Lysis Buffer containing 1X Protease Inhibitor Cocktail II.
16. Incubate on ice for 15 minutes, vortex the cell suspension briefly every 5 minutes.
    - (Optional) At the end of the incubation, homogenize the cell suspension 10 times in a Dounce homogenizer to facilitate the release of the nuclei.
17. Spin the cell suspension at 800 x g at 4°C for 5 minutes.
18. During spin, add 2.5 µl of Protease Inhibitor Cocktail II to each 0.5 ml of Nuclear Lysis Buffer required.
19. Remove supernatant and resuspend cell pellet in 0.5 ml of Nuclear Lysis Buffer.
    a. For every 1 x 10^7 HeLa cells, 0.5 ml of Nuclear Lysis Buffer is recommended for this protocol. Adjust accordingly if different cell concentrations are used as the ratio of lysis buffer to cell density is important for reliable cell lysis.
20. If optimal conditions for sonication have already been determined, proceed to Section B. Otherwise, see Appendix A.

B. Sonication to Shear DNA

Prior to starting this section:

Optimal conditions need to be determined to shear crosslinked DNA to ~200-1000 base pairs in length. See Appendix A for a typical protocol. Once shearing conditions have been optimized, proceed with the steps below.

1. If desired, remove 5 µl of cell lysate from Section A, Step 19 for agarose gel analysis of unsheared DNA.
    - If cell lysate from Section A, Step 19 was previously frozen, thaw on ice.
2. Sonicate cell lysate on wet ice.
    - Sonication conditions must be empirically determined using methods described in Appendix A. The efficiency of sonication depends upon cell type, cell equivalents and instrumentation. Where possible, consult your instrument manufacturer's guidelines for instrument operation. An example of sonicated HeLa cell chromatin fractionated suitably for use with **Magna ChIP™** is shown in Figure A (page 14).
    - Keep cell lysate ice-cold. Sonication produces heat, which can denature the chromatin. Allow time between cycles of sonication to prevent sample overheating.
3. Spin at a minimum of 10,000 x g, but not exceeding 15,000 x g, at 4°C for 10 minutes to remove insoluble material.

4. If desired, remove one 5 µl aliquot for agarose gel analysis of the sheared DNA.
    - To prepare an aliquot for agarose gel analysis, follow the protocol in Appendix A, starting at Step VII.
5. Remove supernatant to fresh microfuge tubes in 50 µl aliquots.
    - Each 50 µl aliquot contains 1 x 10^6 cell equivalents of lysate which is enough for one immunoprecipitation.
    - Sheared crosslinked chromatin can be stored at -80°C for up to 3 months.

C. Immunoprecipitation (IP) of Crosslinked Protein/DNA

Prior to starting this section:

- Remove Protease Inhibitor Cocktail II and thaw at room temperature for use in Step 3. This product contains DMSO and will remain frozen below 18.4°C.

1. Prepare enough Dilution Buffer containing protease inhibitors for the number of desired immunoprecipitations and store on ice.
    - Each IP requires the addition of 450 µl of Dilution Buffer and 2.25 µl of Protease Inhibitor Cocktail II.
    - For **EZ-Magna ChIP™** (Cat.# 17-409), samples include the positive control, Anti-RNA Polymerase II, and the negative control, Normal Mouse IgG, and the antibody of interest (user supplied). It is recommended that the user include a negative control IgG of the same species as the antibody of interest.
2. Prepare microfuge tubes each containing 50 µl of sheared crosslinked chromatin (Section B, step 5) for the number of desired immunoprecipitations and put on ice. If chromatin has been previously frozen, thaw on ice.
    - Alternatively, if multiple immunoprecipitations will be performed from the same chromatin preparation, place the entire volume for the number of desired immunoprecipitations in one large tube that will be able to accommodate a volume of 0.5 ml for each IP.
    - Each 50 µl will contain ~1 x 10^6 cell equivalents of chromatin.
3. Add 450 µl of Dilution Buffer containing Protease Inhibitor Cocktail II into each tube containing 50 µl of chromatin.
    - Alternatively, if multiple immunoprecipitations will be performed from the same chromatin preparation, use the appropriate volume of Dilution Buffer containing Protease Inhibitor Cocktail II for the correct number of immunoprecipitations.
4. Remove 5 µl (1%) of the supernatant as “Input” and save at 4°C until Section D, step 1.
    - If different chromatin preparations are being carried together through this protocol, remove 1% of the chromatin as Input from each.
5. Add the immunoprecipitating antibody and 20 µl **fully suspended** protein G magnetic beads.

    ******Make sure the magnetic bead **slurry** is **well mixed** before removing appropriate volume for IP, as magnetic beads will settle on the bottom of the tube over time.
    - For the positive control, anti-RNA Polymerase, add 1.0 µg of antibody per tube.
    - For the negative control, Normal Mouse IgG, add 1.0 µg of antibody per tube.
    - For user-provided antibody and controls, add between 1-10 µg of antibody per tube. The appropriate amount of antibody needs to be empirically determined depending upon antibody titre, purity and specificity.

6. Incubate for **1 hour** to **overnight** at 4°C with rotation.
    - Magna ChIP™ experiments can be performed following either **one day** or **two day** protocols (see pg. 7). It may be possible to reduce the incubation time of the IP from overnight to 1-4 hrs. This depends on many factors (antibody, gene target, cell type, etc.) and will have to be tested empirically.
    - For ChIP validated antibodies or antibodies of known performance characteristics, the Rapid Protocol often provides comparable results versus overnight incubation, and offers significant time savings and convenience.
7. Pellet Protein G magnetic beads with the magnetic separator (Magna Grip Rack (8 Well), Cat.# 20-400, sold separately) and remove the supernatant completely.
8. Wash the Protein G bead-antibody/chromatin complex by resuspending the beads in 0.5 ml each of the cold buffers in the order listed below and incubating for 3-5 minutes on a rotating platform followed by magnetic clearance and careful removal of the supernatant fraction:
    a. Low Salt Immune Complex Wash Buffer (Cat.# 20-154), **one wash**
    b. High Salt Immune Complex Wash Buffer (Cat.# 20-155), **one wash**
    c. LiCl Immune Complex Wash Buffer (Cat.# 20-156), **one wash**
    d. TE Buffer (Cat.# 20-157), **one wash**

D. Elution of Protein/DNA Complexes and Reverse Crosslinks of Protein/DNA Complexes to Free DNA

1. Thaw Proteinase K and warm the ChIP Elution Buffer (w/o Proteinase K) to room temperature to ensure SDS is in solution before proceeding. Prepare the final elution buffer for all IP tubes as well as all Input tubes (see Section C, step 4).
    - For each tube, prepare elution buffer as follows: 100 µl ChIP Elution Buffer (w/o Proteinase K), 1 µl Proteinase K.
2. Incubate at 62°C for 2 hours with shaking.
    - Shaking incubation can be accomplished with equipment such as an Eppendorf Thermomixer, Labnet Shaking incubator, or a standard roller bottle hybridization oven.
3. Incubate at 95 °C for 10 minutes.
4. Cool the samples down to room temperature.
5. Separate beads using a magnet and remove supernatant to a new tube.

E. DNA Purification Using Spin Columns

1. Remove one Spin Filter in Collection Tube and one separate Collection Tube for each sample tube from Section D.
2. Add 0.5 mL of Bind Reagent "A" to each 100 µl DNA sample tube (Immunoprecipitations and Inputs) and mix well.
    - 5 volumes of Bind Reagent "A" should be used for every 1 volume of sample.
    - A precipitate may be observed. This will not interfere with this procedure.
3. Transfer the sample/Bind Reagent "A" mixture to the Spin Filter in Collection Tube.
4. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
5. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
    - If a precipitate formed in Step 2, it may be observed in the bottom of the Collection Tube and this will not interfere with this procedure.
6. Put the Spin Filter back into the same Collection Tube.
7. Add 500 µl of the Wash Reagent "B" to the Spin Filter in Collection Tube.

8. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
9. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
10. Put the Spin Filter back into the same Collection Tube.
11. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
12. Discard the Collection Tube and liquid.
13. Put the Spin Filter into a clean Collection Tube.
14. Add 50 µl of Elution Buffer "C" directly onto the center of the white Spin Filter membrane.
15. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
16. Remove and discard Spin Filter. Eluate is now purified DNA. It can be analyzed immediately or stored frozen at -20°C.

F. PCR of Controls

Standard end-point PCR

Note: Filter-tip pipette tips are recommended for use in this section to minimize risk of contamination.

1. Label the appropriate number of 0.2 mL PCR tubes for the number of samples to be analyzed and place on ice.
    - At a minimum, there will be 4 DNA samples to undergo PCR using the Control Primers included in this kit: positive and negative control antibody immunoprecipitations, Input and a "no DNA" tube as a control for DNA contamination.
    - The Control Primers are specific for the human GAPDH gene. It is recommended that the user design appropriate specific primers (using the guidelines on page 5) for DNA from other species and determine the PCR reaction conditions empirically.
2. Add 2 µl of the appropriate sample to the PCR tube and return to ice.
3. Add the appropriate amount of reagents to each PCR reaction tube on ice, adding the H_2O first and the *Taq* polymerase last, as indicated in Table I.
    - It is recommended that the user employ a Hot-Start™ *Taq* polymerase. If a Hot-Start *Taq* polymerase is not used, *Taq* must be added to each tube after the initial denaturation step.
    - If a master reaction mix is desired, dispense enough reagents for at least one extra tube to account for loss of volume.

Table I. PCR reagent volumes

Reagent	Volume for 1 reaction (µl)
DNA	2.0
H_2O	12.6
10X PCR Buffer (w/o $MgCl_2$)	2.0
$MgCl_2$ (50 mM)	0.6
2.5 mM dNTP	1.6
Control Primers	0.8
Taq (5 U/µL)	0.4

4. Place the PCR reaction tubes in a thermal cycler.
5. Start the following PCR reaction program:

Initial Denaturation	94°C	3 min	
Denature	94°C	20 sec	repeat for a total of 32 times
Anneal	59°C	30 sec	
Extension	72°C	30 sec	
Final Extension	72°C	2 min	

6. Remove the PCR tubes. Reactions can be stored at -20°C.

7. Remove 10 µL of each PCR reaction for analysis by 2% agarose gel electrophoresis with a 100 bp DNA marker. The expected size of the PCR product is 166 base pairs. See Figure B (below) for an example.

Real-time Quantitative PCR

1. Add 2 µl of the sample to the PCR plate suitable for your real time instrument of choice (Performing triplicate of qPCR reactions per ChIP sample is recommended).
2. Prepare a master reaction mix as shown in Table II. Dispense enough reagents for at least one extra tube to account for loss of volume.
3. Add 23 µl of qPCR mix to the 2 µl of the sample.
4. Use caps or an optical tape to seal the plate and start the qPCR reactions.

Table II. qPCR reagent setup and running parameters

qPCR reagent assembly for 1 reaction:	
ddH_2O	9.5 µl
SYBR®-Green Master Mix	12.5 µl
Primer mix	1 µl
Total	23 µl

qPCR parameters:

Initial Denaturation 94°C 10 min

Denature 94°C 20 sec

Anneal and Extension: 60°C 1 min } 50 times

*The polymerase chain reaction (PCR) is covered by one or more of the following U.S. patents: 4,683,202; 4,683,195; and 4,889,818 issue to Cetus Corporation and owned and licensed by Hoffman-LaRoche Molecular Systems, Inc. Purchase of the **Magna ChIP™ Kit** does not convey a license to use the PCR process covered by these patents. Purchasers of this product must obtain a license to use the PCR process before performing PCR.*

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Figure A: DNA Sonication

Sheared chromatin from formaldehyde-crosslinked HeLa cells was prepared by following Section A (all steps), Section B (steps 1-4) and Appendix A (Option 1) of the Magna ChIP™ protocol. 20 µL sheared (lane 2) chromatin was then electrophoresed through a 2% agarose gel and stained with ethidium bromide. Lane 2 shows that the majority of the DNA has been sheared to a length between 200 bp and 1000 bp.

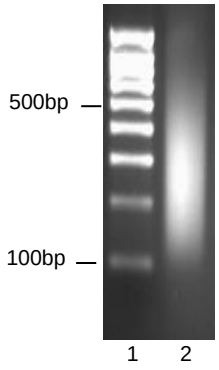


Figure B: PCR Analysis of Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed according to the Magna ChIP™ protocol using chromatin from HeLa cells and either anti-Pol II (Cat.# 05-623B) or Normal mouse IgG (Cat. # 12-371B) as the immunoprecipitating antibody. Purified DNA was then analyzed by PCR using Control Primers specific for the GAPDH promoter. PCR product was observed in the anti-Pol II ChIP (lane 3) and substantially less was detected in the Normal mouse IgG ChIP (lane 2). GAPDH promoter specific DNA was also observed in the 2% Input (lane 4) and not in the “No DNA” PCR control (lane 1).

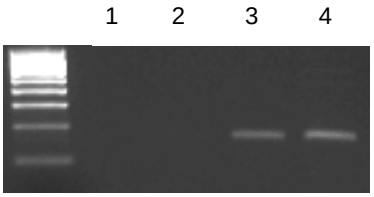
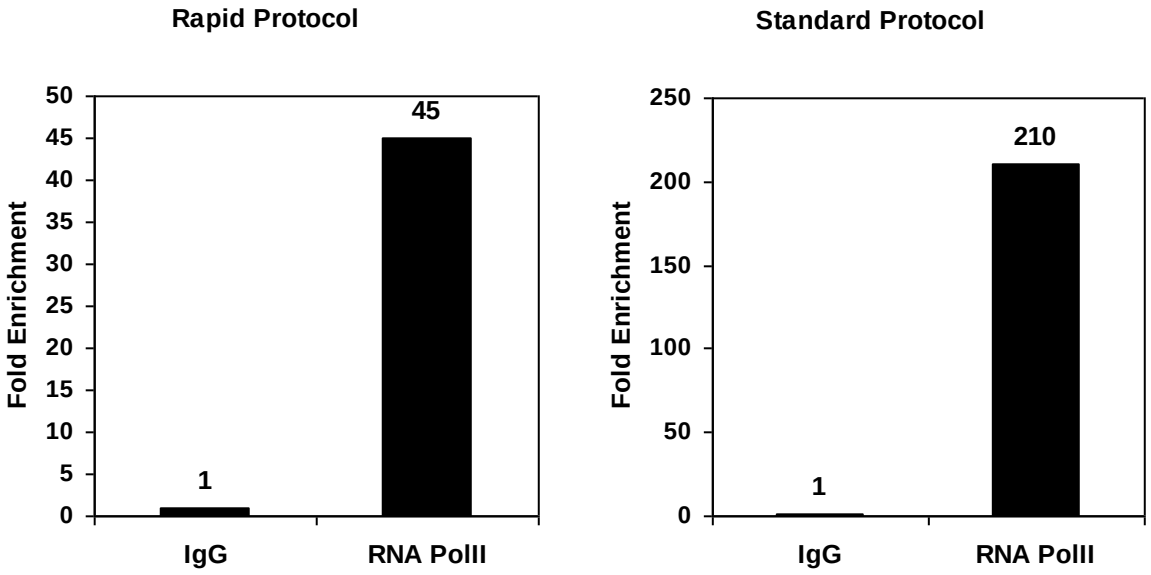


Figure C: PCR Analysis of Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed according to the Magna ChIP™ protocol using chromatin from HeLa cells and either anti-Pol II (Cat.# 05-623B) or Normal mouse IgG (Cat.# 12-371B) as the immunoprecipitating antibody at either a 2 hour immunoprecipitation (Rapid Protocol) or an overnight incubation (Standard Protocol). Purified DNA was then analyzed by qPCR using Control Primers specific for the GAPDH promoter. Fold Enrichment is expressed as the ratio of Pol II signal to IgG signal calculated by extrapolation from a standard curve of Input DNA dilutions.



IX. APPENDIX A: Optimization of DNA Sonication

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

i. Varying the concentration of cell equivalents per mL of initial Cell Lysis buffer with constant sonication parameters,
ii. Choosing a fixed concentration of cell equivalents per mL of Cell Lysis Buffer and varying cycles and/or power settings of sonication
iii. A combination of both approaches

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

I. Generate a cell lysate by following Section A, Steps 1-14, but vary the Cell Lysis Buffer volume per cell amount in Step 15 to generate 3 different microfuge tubes containing several cell equivalent concentrations in the range of 5 x 10^6 per mL to 4-5 x 10^7 per mL. For HeLa cells, this requires approximately 4 x 10^7 cell equivalents, or approximately four 15 cm plates. Continue following the nuclear extraction procedure to Step 19. Each microfuge tube should contain approximately 500 µL of cell lysate.

II. Continue following the nuclear extraction procedure through Step 19.

Volume of Cell Lysis Buffer	Cell Density	Cells required
500 uL	5 x 10^6/mL	2.5 x 10^6
500 uL	2 x 10^7/mL	1 x 10^7
500 uL	4-5 x 10^7/mL	2.5 x 10^7

III. Be sure to keep the samples on wet ice at all times.

- The sonication generates heat which will denature the chromatin.

IV. Remove 1 x 10^5 cell equivalents from each condition prior to sonication for analysis of unsheared DNA.

V. For each cell concentration, sonicate each tube for a fixed number of cycles allowing rests between cycles according to the instrument manufacturer's guidelines. For example, using a Misonix 3000 instrument and a #419 microtip probe, use six 15 sec pulses with 50 second rest in between pulses and power setting at 6. Keep tubes cool at all times. .

VI. Remove 1 x 10^5 cell equivalents (20 uL, 5 uL, 2 uL from least to most concentrated sample) of the sonicated chromatin from each condition to a fresh tube.

VII. To all the samples (unsheared and sheared), add ChIP elution buffer to a final volume of 50 µL .

Option 1:

1. Add 1 µL of RNase A (10 mg/mL, user provided) and incubate for 30 minutes at 37°C.
2. Add 1 µL Proteinase K and incubate at 62°C for 2 hour.
3. Load 10 µL and 20 µLon a 1-2% agarose gel with a 100 bp DNA marker.
    - Loading different amounts helps to avoid under- or over-loading
4. Observe which of the shearing conditions gives a smear of DNA in the range of 200 bp-1000 bp. See Figure A (page 14) for an example.
5. Repeat optimization of the shearing conditions if the results indicate that the resulting DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that the

user does not alter the cell concentration or volume of lysate per microfuge tube for subsequent chromatin immunoprecipitation experiments.

Option 2:

1. Add 1 μL Proteinase K and incubate at 62°C for 2 hour.
2. Add 0.25 mL of Bind Reagent "A" to each 50 μl chromatin sample tube and mix well.
   - 5 volumes of Bind Reagent "A" should be used for every 1 volume of sample.
   - A precipitate may be observed. This will not interfere with this procedure.
3. Transfer the sample/Bind Reagent "A" mixture to the Spin Filter in Collection Tube.
4. Centrifuge for 30 seconds at a minimum of 10,000 x g but not exceeding 15,000 x g.
5. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
   - If a precipitate formed in Step 2, it may be observed in the bottom of the Collection Tube and this will not interfere with this procedure.
6. Put the Spin Filter back into the same Collection Tube.
7. Add 500 μl of the Wash Reagent "B" to the Spin Filter in Collection Tube.
8. Centrifuge for 30 seconds at a minimum of 10,000 x g but not exceeding 15,000 x g.
9. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
10. Put the Spin Filter back into the same Collection Tube.
11. Centrifuge for 30 seconds at a minimum of 10,000 x g but not exceeding 15,000 x g.
12. Discard the Collection Tube and liquid.
13. Put the Spin Filter into a clean Collection Tube.
14. Add 50 μl of Elution Buffer "C" directly onto the center of the white Spin Filter membrane.
15. Centrifuge for 30 seconds at a minimum of 10,000 x g but not exceeding 15,000 x g
16. Load 10 μL and 20 μL on a 1-2% agarose gel with a 100 bp DNA marker.
    - Loading different amounts helps to avoid under- or over-loading
17. Observe which of the shearing conditions gives a smear of DNA in the range of 200 bp-1000 bp. See Figure A (page 14) for an example.
18. Repeat optimization of the shearing conditions if the results indicate that the resulting DNA is not in the desired size range. Once optimal conditions have been determined, it is advised that the user does not alter the cell concentration or volume of lysate per microfuge tube for subsequent chromatin immunoprecipitation experiments.

X. APPENDIX B: Preparation of Fresh 18.5% Formaldehyde

This recipe is for making fresh 18.5% formaldehyde from powdered paraformaldehyde to use immediately in the Magna ChIP™ protocol. Use appropriate safety precautions when performing this procedure.

1. Add 4.8 mL of distilled water to a 50 mL conical plastic tube.
2. Add 0.925 g paraformaldehyde.
3. Add 35 μL of 1N KOH.
4. Cap tube tightly and place in a 400-600 mL glass beaker filled with approximately 200 mL of water.
5. Microwave beaker with tube until water in beaker begins boiling.
6. Remove beaker and vortex tube until paraformaldehyde begins dissolving.
7. Repeat steps 5 & 6 until paraformaldehyde is completely in solution. This step may need to be repeated several times.
8. Store on ice until cool.
9. Use immediately.

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XI. CHROMATIN IP OPTIMIZATION AND TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Crosslinking	Not enough or too much crosslinking	The appropriate amount of formaldehyde and time of crosslinking must be determined empirically. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time. HINT: Histones may not need to be crosslinked since they are tightly associated with DNA.
Cell Lysis	Inefficient disruption of cells	It is important to have enough lysis buffer per cell concentration. Follow the guidelines in this protocol. Also, check the cell lysis by looking at a 10 µL portion of the cell lysate under the microscope for intact cells.
Chromatin Shearing	Not enough/too much sonication	Follow Appendix A to obtain the appropriate sized DNA.
	Denaturation of proteins from overheating sample	Keep the sample on ice during the sonication. Shorten the time of each sonication and increase the number of times the sample is sonicated.
Addition of Primary Antibody	Antibody doesn't recognize protein in fixed chromatin	Choose an antibody directed to a different epitope of the antigen. Decrease the amount or time of formaldehyde fixation.
	Not enough or too much chromatin	Perform IP from a dilution series of antibody with a fixed amount of chromatin or vice versa.
	Insufficient incubation time	• Incubate the antibody of interest with the chromatin at 4°C overnight. • Select a different antibody with higher affinity. • Perform a Western blot of the immunoprecipitated protein to verify the antibody can precipitate the antigen of interest.
Addition of Secondary Reagent – Protein G	Not enough beads	The magnetic beads settle to the bottom of the tube over time. Make sure the Protein G magnetic beads are well mixed prior to removing the appropriate volume for IP.
	Incorrect Antibody Class or Isotype	Check that the subclass and isotype of the antibody can bind Protein G. Protein G is not recommended for IgM or chicken Ig.

Step	Potential Problems	Experimental Suggestions
Washing	Not enough washing time	Increase number of washes for each wash buffer.
	Aspiration of the beads during buffer removal	• Make sure there are no beads in the supernatant prior to removing it.
Elution and Reversal of crosslinking	Incomplete elution	• When performing elution, make sure that the temperature is near 60°C. Proteinase K will be inactivated by prolonged incubation at temperatures above 65°C.
	Excessive Crosslinking	• Too much crosslinking may not be reversible. Conduct a time course at a fixed formaldehyde concentration and/or investigate a range of formaldehyde concentrations for a fixed time.
PCR	Incorrect Annealing Temperature or Amplification Conditions	• Ensure amplification reaction program is correctly set on thermal cycler. • Re-examine primers for correct T_m. • Perform PCR on genomic DNA to confirm amplification conditions and ability of primers to generate a single DNA product of the expected size.
	Bad primers	Follow suggestions for primer design in section "Chromatin IP Assay Overview, section B".
	No PCR product	• Increase amount of DNA added to the PCR reaction. • Increase the number of cycles for the amplification reaction.
	PCR product is a smear	• Decrease amount of DNA added to the PCR reaction. • Use HotStart™ *Taq* polymerase to avoid non-specific annealing of primers.
	No difference in quantity between PCR product from RNA Polymerase II and Normal Mouse IgG IPs	• Ensure correct mass of antibody and the correct cell equivalents of chromatin are used for IP as indicated in protocol. Too much antibody and/or chromatin can result in increased non-specific binding. • Dilute DNA with water to decrease amount of DNA added to the PCR reaction. • Decrease the cycle number at which the DNA is analyzed. It is important that the PCR products are analyzed within the linear amplification phase of PCR, in which differences between quantities of starting DNA can be measured.

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