EZ-ChIP™

Chromatin Immunoprecipitation Kit

Catalog # 17-371

Instruction Manual

Sufficient reagents for 22 chromatin immunoprecipitation (ChIP) assays per kit.

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I. INTRODUCTION

Chromatin Immunoprecipitation (ChIP) is a widely used method to identify specific proteins associated with a region of the genome, or in reverse, to identify regions of the genome associated with specific proteins. These proteins can be isoforms of histones modified at a particular amino acid or other chromatin-associated proteins. When employed with antibodies that recognize histone modifications, ChIP can be used to “measure” the amount of the modification. An example of this would include measurement of the amount of histone H3 acetylation associated with a specific gene promoter region under various conditions that might alter expression of the gene. Histones are not the only proteins that can be studied using this technique. Much of the recent interest has been in analyzing transcription factor distribution throughout the genome or at specific loci.

When performing ChIP, cells are first fixed with formaldehyde to covalently crosslink proteins to DNA. Then chromatin is harvested from the cells and subjected to an immunoselection process, which requires the use of specific antibodies. Any DNA sequences cross-linked to the protein of interest will co-precipitate as part of the chromatin complex. After the immunoselection of chromatin fragments and purification of associated DNA, the detection of specific DNA sequences is performed. If the DNA which will be detected is associated with the protein or histone modification being examined, the relative representation of that DNA sequence will be increased (or enriched) by the immunoprecipitation process.

Generally, standard PCR is performed to identify the DNA sequence (the gene or region of the genome) associated with the protein of interest. The relative abundance of a specific DNA sequence isolated via the protein-specific immunoselection is compared to DNA obtained when using an unrelated antibody control. DNA fragments are run on gels to facilitate quantitation of the PCR products. A much more accurate alternative to standard PCR is real time quantitative PCR (RT-qPCR). Cloning of sequences from a ChIP experiment is also possible, to create libraries of fragments that are enriched for those that interact with a particular protein. The combination of chromatin IP with microarray applications (ChIP on chip) is a novel technique that is becoming more popular, allowing the generation of genome-wide maps of protein-DNA interactions or histone modifications.

The EZ ChIP™ kit contains the buffers and reagents required to perform a successful ChIP from mammalian cells. Importantly, EZ ChIP™ also contains essential controls (anti-RNA Polymerase II, Normal Mouse IgG and Control Primers) to ensure that the user has successfully performed the ChIP assay. RNA Polymerase II is responsible for the transcription of protein coding genes, and therefore, is present at the promoter region of genes that are actively transcribed. The glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene is considered a housekeeping gene and expected to be undergoing transcription in most growing mammalian cells. Upon immunoprecipitation of chromatin with an antibody to RNA Polymerase II, the resulting DNA is enriched for the GAPDH gene (as well as all transcribed genes), whereas immunoprecipitation with Normal Mouse IgG will not result in GAPDH enrichment. The covalent bonds between the DNA and associated proteins are then severed, and the DNA is purified prior to performing PCR. For DNA purification, the EZ ChIP™ kit incorporates a unique polypropylene spin column manufactured by Mo Bio Laboratories, Inc. Each spin column contains a specially activated silica membrane filter that captures DNA and separates it from contaminating proteins and other cellular debris. After soluble contaminants are spun through the filter, the column is washed and then DNA is eluted in a low-salt buffer. Bind Buffer “A”, Wash Buffer “B”, and Elute Buffer “C” are supplied by Mo Bio Laboratories and are RNase and DNase free. This technology from Mo Bio Laboratories, Inc. provides rapid purification of chromatin DNA without the need for phenol chloroform extractions or ethanol precipitation. The purified DNA is subjected to PCR using the Control Primers which are specific to the promoter region of the GAPDH gene.

Kit Description

Quantity: Two boxes containing the necessary reagents to perform 22 chromatin immunoprecipitation (ChIP) assays. This kit also contains reagents to generate chromatin from five 15-cm plates, each of which provides sufficient chromatin for up to 10 individual precipitations

Storage and Stability: Upon receipt, store components at the temperatures indicated on the labels. Storage temperatures are also indicated on page 5 of this manual. Kit components are stable for 1 year from date of shipment when stored as directed.

Use: The EZ ChIP™ kit contains reagents optimized for immunoprecipitation of chromatin from mammalian cells and includes controls to ensure the successful performance of this assay. The included positive control antibody is a mouse monoclonal antibody to RNA Polymerase II and 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. Control Primers are included for detection of a 166 base pair region of the human GAPDH promoter by PCR. Use of these primers for DNA from other species has not been evaluated. 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.

The EZ ChIP™ kit has all the necessary buffers and reagents to perform successful chromatin immunoprecipitation assays, however, careful attention must be paid to the details of the instructions. Follow all the instructions carefully, especially with regard to incubation times and temperatures.

Related Products:

Catalog # 17-295	Chromatin Immunoprecipitation Kit
Catalog # 17-375	EZ-Zyme Chromatin Preparation 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 # 17-610	Magna ChIP™ A Chromatin Immunoprecipitation Kit
Catalog # 17-611	Magna ChIP™ G Chromatin Immunoprecipitation Kit
Catalog # 17-408	EZ-Magna ChIP™ A Chromatin Immunoprecipitation Kit
Catalog # 17-409	EZ-Magna ChIP™ G Chromatin Immunoprecipitation Kit

For a complete list of Millipore's ChIP qualified antibodies, go to www.millipore.com and search "ChIP".

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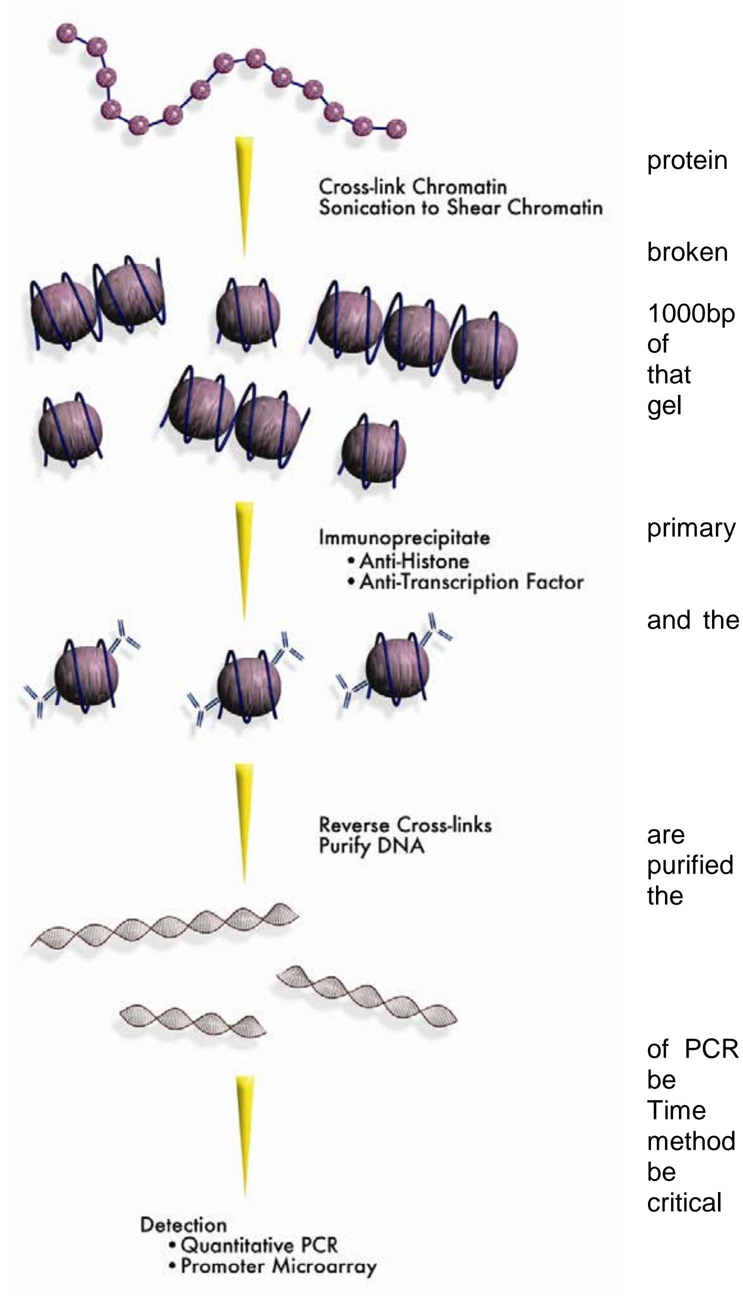
II. CHROMATIN IP ASSAY OVERVIEW

A. Chromatin Sample Prep and Immunoselection



Grow cells and treat with formaldehyde. This treatment crosslinks the proteins to the DNA, ensuring co-precipitation of the DNA with the protein of interest.

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

Immunoselection. This step is very similar to a standard immunoprecipitation, which uses a primary antibody of choice followed by Protein G-conjugated agarose beads as the secondary reagent. This enriches for the protein of interest and the DNA that is specifically complexed with it.

B. DNA Purification and Detection

Purification of the DNA. Protein-DNA crosslinks are reversed during incubation at 65°C 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 and the variability of PCR primer selection. The most meaningful results will be obtained with quantitative PCR for this step. Real Time Quantitative PCR (RT-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 Tm:	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 as appropriate.

III. EZ ChIP™ KIT COMPONENTS

A. Provided Kit Components (Note Storage Temperatures)

Store at 4°C:

ChIP Blocked Protein G Agarose, Catalog # 16-201D. One vial containing **1.5 mL** packed beads with 1.5 mg BSA and approximately 4.5mg recombinant Protein G. Provided as a 50% gel slurry for a final volume of **3 mL** per vial. Suspended in TE buffer, pH 8.0, containing 0.05% sodium azide. Liquid suspension.

ChIP Dilution Buffer, Catalog # 20-153. One vial containing **24 mL**.

Low Salt Immune Complex Wash Buffer, Catalog # 20-154. One vial containing **24 mL**.

High Salt Immune Complex Wash Buffer, Catalog # 20-155. One vial containing **24 mL**.

LiCl Immune Complex Wash Buffer, Catalog # 20-156. One vial containing **24 mL.**

TE Buffer, Catalog # 20-157. Two vials, each containing **24 mL.**

0.5 M EDTA, Catalog # 20-158. One vial containing **250 µL**.

5 M NaCl, Catalog # 20-159. One vial containing **500 µL**.

SDS Lysis Buffer, Catalog # 20-163. One vial containing **10 mL**.

1 M Tris-HCl, pH 6.5, Catalog # 20-160. One vial containing **500 µL.**

10X Glycine, Catalog # 20-282. One vial containing **11 mL**.

10X PBS, Catalog # 20-281. One vial containing **24 mL**.

Store at -20°C:

Protease Inhibitor Cocktail II, Catalog # 20-283. Two vials, each containing **110 µL** of 200X Protease Inhibitor Cocktail II in DMSO.

RNase A, Catalog # 20-297. One vial containing **600 µg** of RNase A in 60 µL sterile water.

Proteinase K, Catalog # 20-298. One vial containing **600 µg** of Proteinase K in 60 µL.

1M $NaHCO_3$, Catalog # 20-296. One vial containing **600 µL**.

Control Primers, Catalog # 22-004. One vial containing **75 µL** of 5 µM of each control primer specific for human GAPDH.

FOR: 5'-TACTAGCGGTTTTACGGGCG-3’
REV: 5'-TCGAACAGGAGGAGCAGAGAGCGA-3'

Anti-RNA Polymerase II, Catalog # 05-623B. One vial containing **25 µg** of Anti-RNA Polymerase II, clone CTD4H8.

Normal Mouse IgG, Catalog # 12-371B. One vial containing **25 µg** of normal mouse IgG.

Store at Room Temperature:

20% SDS, Catalog # 20-280. One vial containing **242 µL** of 20% SDS.

Spin Filters, Catalog # 20-290. One bag containing **22** Spin Filters with Collection Tubes.

Collection Tubes, Catalog # 20-291. One bag containing **22** Collection Tubes.

Bind Reagent A, Catalog # 20-292. One vial containing **25 mL** of Bind Reagent A.

Wash Reagent B, Catalog # 20-293. One vial containing **12.5 mL** of Wash Reagent B.

Elution Reagent C, Catalog # 20-294. One vial containing **1.5 mL** of Elution Reagent C.

B. Required Materials Not Provided

Reagents

- Cells, stimulated or treated as needed for the experimental system
- Antibody of interest for chromatin immunoprecipitation
- 37% Formaldehyde or powdered formaldehyde to prepare a fresh 18.5% solution (see Appendix B)
- *Taq* DNA polymerase
- dNTPs, 2.5 mM each
- DNase and RNase free sterile H_2O

Equipment

- Vortex mixer
- Rotating wheel/platform
- Timer
- Variable volume (5-1000 μL) pipettes + tips
- Microfuge
- Variable temperature water bath
- Cell scraper
- Sonicator
- Microfuge tubes, 1.5 mL
- Thermal cycler
- PCR tubes, 0.2 mL
- Filter-tip pipette tips

IV. CHROMATIN IMMUNOPRECIPITATION 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 150 mm culture dish containing 20 mL of growth media.
  - For HeLa cells, this is approximately 1-2 x 10^7 cells. This will generate a preparation of chromatin that can be used for up to 10 separate immunoprecipitations (varies with cell and assay type).
  - Include one extra dish to be used solely for estimation of cell number.
- 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 SDS 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 Step 3 and 13. This product contains DMSO and will remain frozen below 18.4°C.

1. Add 550 µL of 37% formaldehyde (or 1.15 mL of fresh 18.5% formaldehyde) to 20 mL of growth media to crosslink and gently swirl dish to mix.
   - Final concentration 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, aliquot 2 mL of ice cold 1X PBS into a separate tube for every dish. Add 5 µL of Protease Inhibitor Cocktail II to each 1 mL of 1X PBS and put tubes 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 dish 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 dish (made in Step 3).
11. Scrape cells from each dish into a conical tube.
12. Spin at 700 x g at 4°C for 2-5 minutes to pellet cells.
13. During spin, prepare lysis buffer by adding 5 µL of Protease Inhibitor Cocktail II to each 1 mL of SDS Lysis Buffer required.
    - For every 1 x 10^7 HeLa cells, 1 mL of SDS Lysis Buffer is recommended. Adjust accordingly if using different cell concentrations, as the ratio of lysis buffer to cell density is important for reliable cell lysis.
14. Remove supernatant. (Cell pellets can be frozen at -80°C at this step.)
15. Resuspend each cell pellet in 1 mL of SDS Lysis Buffer containing 1X Protease Inhibitor Cocktail II.
16. Aliquot between 300-400 µL per microfuge tube. (Lysate can be frozen at -80°C at this step.)
17. 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:

The optimal conditions required for shearing crosslinked DNA to ~200-1000 base pairs in length need to be determined. See Appendix A for an example of a protocol. Once optimal shearing conditions have been determined, proceed with the steps below.

1. If desired, remove 5 µL of cell lysate from Section A, Step 16 for agarose gel analysis of unsheared DNA.
   - If cell lysate from Section A, Step 16 was previously frozen, thaw on ice.
2. Sonicate cell lysate on wet ice.
   - HeLa cells in SDS Lysis Buffer at a cell concentration of 1 x 10^7 per mL sheared with 4-5 sets of 10-second pulses on wet ice using a Cole Parmer, High Intensity Ultrasonic Processor/Sonicator, 50-watt model equipped with a 2 mm tip and set to 30% of maximum power gave the appropriate length DNA fragments. See Figure A (page 13).
   - Keep cell lysate ice-cold. Sonication produces heat, which can denature the chromatin.
3. Spin at a minimum of 10,000 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, join the protocol in Appendix A at Step 7.
5. Remove supernatant to fresh microfuge tubes in 100 µL aliquots.
   - Each 100 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 2 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 900 µL of Dilution Buffer and 4.5 µL of Protease Inhibitor Cocktail II.
   - Immunoprecipitations should 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 one microfuge tube containing 100 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 1.1 mL for each IP.
   - Each 100 µL will contain ~1 x 10^6 cell equivalents of chromatin.
3. Add 900 µL of Dilution Buffer containing Protease Inhibitor Cocktail II into each tube containing 100 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. Add 60 µL of Protein G Agarose for each IP.

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   - The Protein G Agarose is a 50% slurry. Gently mix by inversion before pipetting.

- This step serves to "preclear" the chromatin, *i.e.*, to remove proteins or DNA that may bind nonspecifically to the Protein G agarose.
- Alternatively, if multiple immunoprecipitations will be performed from the same chromatin preparation, use the appropriate volume of Protein G Agarose for the correct number of immunoprecipitations.

5. Incubate for 1 hour at 4°C with rotation.
6. Pellet agarose by brief centrifugation (3000-5000 x g for 1 minute).
    - Do not spin Protein G Agarose beads at high speeds. Applying excessive g-force may crush or deform the beads and cause them to pellet inconsistently.
7. Remove 10 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.
8. Collect the remaining supernatant and dispense 1 mL aliquots into fresh microfuge tubes. Discard agarose pellet.
9. Add the immunoprecipitating antibody to the supernatant fraction:
    - 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 determined empirically.
10. Incubate overnight at 4°C with rotation.
    - It may be possible to reduce the incubation time of the IP. This depends on many factors (antibody, gene target, cell type, etc.) and will have to be tested empirically.
11. Add 60 μL of Protein G Agarose to each IP and incubate for 1 hour at 4°C with rotation.
    - This serves to collect the antibody/antigen/DNA complex.
12. Pellet Protein G Agarose by brief centrifugation (3000-5000 x g for 1 minute) and remove the supernatant fraction.
13. Wash the Protein G Agarose-antibody/chromatin complex by resuspending the beads in 1 mL each of the cold buffers in the order listed below and incubating for 3-5 minutes on a rotating platform followed by brief centrifugation (3000-5000 x g for 1 minute) and careful removal of the supernatant fraction:
    a. Low Salt Immune Complex Wash Buffer (Catalog # 20-154), **one wash**
    b. High Salt Immune Complex Wash Buffer (Catalog # 20-155), **one wash**
    c. LiCl Immune Complex Wash Buffer (Catalog # 20-156), **one wash**
    d. TE Buffer (Catalog # 20-157), **two washes**

D. Elution of Protein/DNA Complexes

Prior to starting this section:

- Bring 1 M $NaHCO_3$ to room temperature. A precipitate may be observed but will go into solution once room temperature is achieved. The 1 M $NaHCO_3$ can be vortexed.
- Set water bath to 65°C for use in Section E.

1. Make Elution Buffer for all IP tubes as well as all Input tubes (see Section C, step 7).
    - For each tube, prepare 200 μL of elution buffer as follows: 10 μL 20% SDS, 20 μL 1 M $NaHCO_3$ and 170 μL sterile, distilled water.
2. Alternatively, make a large volume to accommodate all tubes. For example, if there are 10 tubes mix together 105 μL 20% SDS, 210 μL 1M $NaHCO_3$ and 1.785 mL sterile, distilled water.
3. For Input tubes (see Section C, step 7), add 200 μL of Elution Buffer and set aside at room temperature until Section E.
4. Add 100 μL of Elution Buffer to each tube containing the antibody/agarose complex. Mix by flicking tube gently.
5. Incubate at room temperature for 15 minutes.
6. Pellet agarose by brief centrifugation (3000-5000 x g for 1 minute) and collect supernatant into new microfuge tubes.
7. Repeat steps 4-6 and combine eluates (total volume = 200 μL).

E. Reverse Crosslinks of Protein/DNA Complexes to Free DNA

1. To all tubes (IPs and Inputs) add 8 μL 5 M NaCl and incubate at 65°C for 4-5 hours or overnight to reverse the DNA – Protein crosslinks. After this step the sample can be stored at -20°C and the protocol continued the next day.
2. To all tubes, add 1 μL of RNase A and incubate for 30 minutes at 37°C.
3. Add 4 μL 0.5M EDTA, 8 μL 1M Tris-HCl and 1 μL Proteinase K to each tube and incubate at 45°C for 1-2 hours.

F. DNA Purification Using Spin Columns

1. Remove one Spin Filter in Collection Tube and one separate Collection Tube for each sample tube from Section E.
2. Add 1 mL of Bind Reagent "A" to each 200 μL DNA sample tube (IPs 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 600 μL of sample/Bind Reagent "A" mixture to the Spin Filter in Collection Tube.
4. Centrifuge for 30 seconds at a minimum of 10,000 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 it will not interfere with this procedure.
6. Put the Spin Filter back into the ***same*** Collection Tube.
7. Transfer the remaining 600 μL of sample/Bind Reagent "A" mixture from Step 2 into the Spin Filter and repeat steps 4-6.
8. Add 500 μL of the Wash Reagent "B" to the Spin Filter in Collection Tube.
9. Centrifuge for 30 seconds at a minimum of 10,000 but not exceeding 15,000 x g.
10. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.

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

G. PCR of Controls
Option 1: 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 4) for DNA from other species and determine the PCR reaction conditions empirically.
2. Add 2 μL of the appropriate DNA 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 user is not employing a Hot-Start *Taq* polymerase, *Taq* must be added to each tube after the initial denaturation step.
    - If a master reaction mix is desired, make enough reagent for one extra tube to account for loss during dispensing.

Reagent	Volume for 1 reaction (μL)
DNA	2.0
H_2O	13.2
10X PCR Buffer	2.0
2.5 mM dNTP	1.6
Control Primers	0.8
Taq (5 U/μL)	0.4

Table I. PCR reagent volumes

4. Place the PCR reaction tubes in a thermal cycler.

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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 4% agarose gel electrophoresis with a 100 bp DNA size marker. The expected size of the GAPDH Positive Control PCR product is 166 base pairs. See Figure B (below) for an example.

Option 2: 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 issued to Cetus Corporation and owned and licensed by Hoffman-LaRoche Molecular Systems, Inc. Purchase of the EZ 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.

Figure A: DNA Sonication

Unsheared and sheared chromatin from formaldehyde-crosslinked HeLa cells was prepared by following Section A (all steps) Section B (steps 1-4) and Appendix A (steps 7-11) of the EZ ChIP protocol. 20 µL of unsheared (lane 1) and 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.

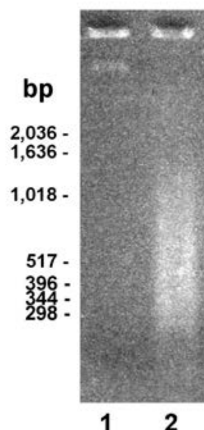
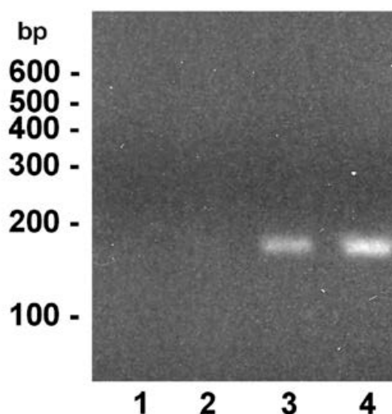


Figure B: PCR Analysis of Chromatin Immunoprecipitation

Chromatin immunoprecipitation was performed using chromatin from HeLa cells and either anti-RNA Polymerase II (Catalog # 05-623) or Normal Mouse IgG (Catalog # 12-371) 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-RNA Polymerase II ChIP (lane 3) and not in the Normal Mouse IgG ChIP (lane 2). GAPDH promoter specific DNA was also observed in the Input (lane 4) and not in the “No DNA” PCR control (lane 1).



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V. 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 (i) and is provided as an example only.

1. Generate a cell lysate by following Section A, Steps 1-16, 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 5×10^6 per mL to 4-5 x 10^7 per mL. For HeLa cells, this requires approximately 4×10^7 cell equivalents, or approximately four 15 cm plates. Continue following the cell lysis procedure through Step 16 adjusting the concentration of each sample according to the table below. Each microfuge tube should contain approximately 500 µL of cell lysate.

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

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

- The sonication generates heat which will denature the chromatin.

3. Remove 1×10^5 cell equivalents from each condition prior to sonication for analysis of unsheared DNA.
4. 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.
5. Remove 1×10^5 cell equivalents (20 µL, 5 µL, 2 µL from least to most concentrated sample) of the sonicated chromatin from each condition to a fresh tube.
6. To all the samples (unsheared and sheared), add ChIP dilution 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 µL on 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.

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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 the empty spin filter 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. Remove and discard the spin filter. The eluate contains the purified DNA.
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 13) for an example.

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.

VI. 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 EZ 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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VII. 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 may need to 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 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. If possible, choose an antibody that has been validated as suitable for ChIP. 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 IP'd protein to make sure the antibody can IP the antigen of interest.
Addition of Secondary Reagent – Protein G agarose	Not enough beads	The Protein G Agarose is a 50% slurry of beads suspended in buffer. The agarose beads settle to the bottom of the tube over time. Make sure the Protein G Agarose is 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. • Spin at the recommended g force.
Elution	Incomplete elution	Check that the pH of 1 M $NaHCO_3$ is ~9 using pH strips. If not, make this solution fresh.
Reversal of crosslinking	Wrong temperature; not enough time	• Protein-DNA crosslinks are reversed at 65°C. A minimum of 4 hrs is required. • 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 in varying amounts the DNA added to the PCR reaction. • Increase the number of cycles for the amplification reaction.
	PCR product is a smear	• Decrease in varying amounts the 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	• 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. • Ensure correct amount 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.

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