Magna ChIP™ A/G

(Catalog # 17-10085)

EZ-Magna ChIP™ A/G

(Catalog # 17-10086)

One-Day Chromatin Immunoprecipitation Kits

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures.

USA & Canada Phone: +1(800) 645-5476
In Europe, please contact Customer Service:
France: 0825.045.645;
Spain: 901.516.645 Option 1
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Introduction

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.

ChIP methodology often involves protein-DNA and protein-protein cross-linking, fragmentation of the cross-linked chromatin, and subsequent immunoprecipitation of chromatin with an antibody specific to a target protein. 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. For details on performing ChIP-chip refer to the Magna ChIP^{2}™ user manual (Millipore catalog numbers 17-1000, 17-1001, or 17-1002). Alternatively, high-throughput sequencing of libraries constructed from immunoprecipitated chromosomal DNA (ChIP-Seq) is a powerful alternative to ChIP-chip in mapping the protein-DNA interactions across mammalian genomes. For details on the performance of ChIP-Seq refer to the Magna ChIP-Seq™ kit user manual provided with cat# 17-1010).

Unlike standard ChIP protocols that can be laborious and time consuming, the Magna ChIP kit protocol can reduce the amount of time required to perform a ChIP experiment from three days to one. Additionally, the smaller Magna ChIP reaction volume increases the relative concentration of the antibody enabling the ChIP reaction to be performed with reduced amounts of both antibody and sheared chromatin. Finally because this kit uses a blend of protein A and protein G beads, a wider range of antibody isotypes can be used than A or G alone. This allows a wider variety of antibodies to be used and avoids the need to purchase separate kits for protein A and protein G based immunoprecipitation.

Because Magna ChIP kits use paramagnetic beads they are compatible with automated high throughput platforms, thus allowing a large number of ChIP reactions to be carried out simultaneously.

Overview of Magna ChIP and EZ-Magna ChIP Kits

The Magna ChIP kit contains all buffers and reagents required to perform successful ChIP experiments from mammalian cells. The EZ-Magna ChIP also contains all of the elements of the Magna ChIP kit, plus essential controls such as positive control antibody directed against a housekeeping gene, a negative control IgG and control primers. These standard controls help ensure successful protocol optimization and adoption. In the EZ-Magna ChIP A/G kit, the positive control antibody is a mouse monoclonal antibody with specificity for the CTD region of RNA Polymerase II. This antibody 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 (glyceraldehyde-3-phosphate dehydrogenase) promoter. This housekeeping gene is constitutively transcribed in most growing mammalian cells. Upon immunoprecipitation of chromatin with anti-RNA Polymerase II, the resulting DNA is enriched for GAPDH promoter DNA (as well as all RNA polymerase II transcribed genes), whereas immunoprecipitation with the provided Normal Mouse IgG should not result in significant GAPDH enrichment. The primers provided with the EZ-ChIP kit are appropriate for 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.

For DNA purification after immunoprecipitation and the release of cross links, 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, ultra high-throughput sequencing and ChIP cloning.

Magna ChIP Procedure – Standard vs. One Day Rapid Protocols

The Magna ChIP or EZ-Magna ChIP procedure (sample extraction to data analysis) can be performed in a single day using the Rapid Magna ChIP protocol and ChIP-validated antibodies directed against abundant ChIP targets. Alternatively, a highly 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 below.

Comparison of Standard and Rapid Magna ChIP Protocols

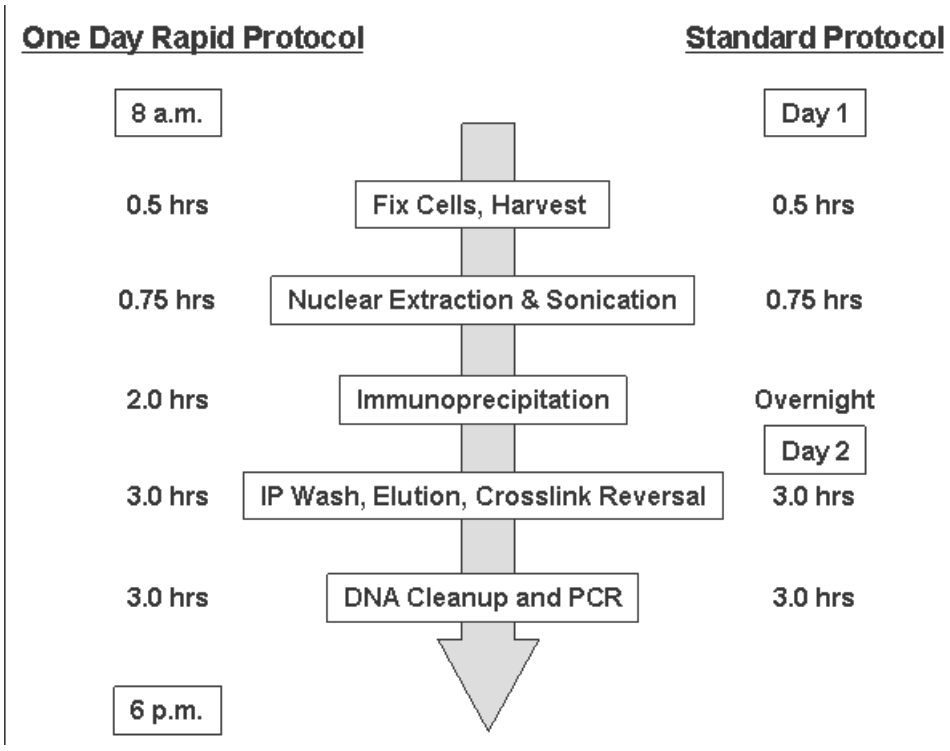


Figure 1. Comparison of the Rapid Magna Chip and Standard protocols. The protocols vary primarily in the time required for immunoprecipitation. The example of the Rapid Protocol shown above is only recommended for experiments with a limited number of samples. For additional guidelines on selecting the appropriate protocol, see detailed protocol on page 10.

Kit Components

This kit provides two boxes containing all necessary reagents to perform 22 individual chromatin immunoprecipitation (ChIP) reactions. Please store at indicated temperature upon receipt. 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).

Magna ChIP & EZ-Magna ChIP Kit Configurations	
Magna ChIP A/G (Catalog No. 17-10085)	**EZ-Magna ChIP A/G (Catalog No. 17-10086)**
MAGNA0017 (Store at 2°C to 8°C)	MAGNA0017 (Store at 2°C to 8°C)
MAGNA0016 (Store at -20°C)	MAGNA0014 (Store at -20°C)

Magna ChIP A/G (2°C to 8°C) MAGNA0017		
Component	**Part #**	**Quantity**
Magnetic Protein A/G Beads	CS204457	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
Magna ChIP A/G (-20°C) MAGNA0014		
Protease Inhibitor Cocktail II, 200X ****Contains DMSO**	20-283	110 µL
Proteinase K (10 mg/mL)	20-298	60 µL
RNAse A (10 mg/mL)	20-297	60 µL
Anti-RNA Polymerase II, clone CTD4H8	05-623B	25 µg
Normal Mouse IgG	12-371B	25 µg
Control Primers	22-004	75 µL
Magna ChIP A/G (-20°C) MAGNA0016		
Protease Inhibitor Cocktail II, 200X ****Contains DMSO**	20-283	110 µL
Proteinase K (10 mg/mL)	20-298	60 µL
RNase A (10 mg/mL)	20-297	60 µL

Materials Required But Not Supplied

Reagents

- Cells, stimulated or treated as desired
- Antibody of interest for chromatin Immunoprecipitation (see page 7)
- 37% Formaldehyde
- *Taq* DNA polymerase (e.g. NovaTaq™ Hot Start DNA Polymerase cat# 71091)
- dNTPs, 2.5 mM each (e.g. Novagen® 10 mM dNTP Mix, Cat.# 71004)
- SYBR® Green Master Mix for qPCR or stock of SYBR Green for blending into qPCR reaction
- DNase and RNase-free sterile H_2O (e.g. Millipore Nuclease-free water cat# 3098)

Equipment

- Magnetic Separator (e.g. Magna GrIP™ Rack catalog #20-400 or PureProteome™ Magnetic Stand, catalog # LSKMAGS08
- 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

Hazards:

- Wear gloves when using this product. Avoid skin contact or ingestion of all reagents and chemicals used in this protocol.
- Protease Inhibitor Cocktail contains DMSO, avoid contact with skin.
- Chromatin preparation may require use of liquid nitrogen. Use personal protective equipment (PPE) when handling liquid N_2 to avoid burns.
- Use PPE, fume hoods and venting when working with concentrated formaldehyde solutions. Formaldehyde is toxic by inhalation, skin contact and ingestion.
- Bind Reagent A (20-292) provided in this kit contains guanidine thiocyanate. This compound is a skin irritant and can form highly reactive compounds when combined with bleach. Do not add bleach or acidic solutions directly to this buffer or solutions containing this buffer.
- Wash Reagent B (20-293) contains ethanol and is flammable. Avoid storing or using this reagent around open flames.

Storage and Stability

MAGNA0017 Store at 2-8° C, good for 6 months from date of receipt when reagents are stored and handled appropriately.

Please note: Some components in this box should be stored at room temperature (18-25° C) upon receipt. Please see “Kit Components” section for details.

MAGNA0014 Store at -20° C, good for 6 months from date of receipt when reagents are stored and handled appropriately.

MAGNA0016 Store at -20° C, good for 6 months from date of receipt when reagents are stored and handled appropriately.

ChIPAb+™ Validated Antibodies and ChIP Qualified Antibodies

For the ChIP application, not all antibodies are capable of effectively precipitating chromatin. Protein conformation, protein interactions (with other proteins or DNA), and the amount of cross-linking can affect whether or not an antibody will work well in ChIP. Consequently, we make a distinction between what we call "ChIP qualified" antibodies, and "ChIP validated" antibodies.

ChIP qualified, or ChIP grade is a term typically used to describe any antibody previously demonstrated to work in ChIP. Although not always directly tested by the supplier, many consider these to be 'validated' for ChIP. For some, this level of validation is sufficient. However, antibody performance in ChIP can vary between different lots. Consequently, in many cases antibodies labeled as ChIP grade fail to perform consistently from lot to lot.

To eliminate this concern, when performing ChIP it is suggested that labs use well characterized antibodies that have been extensively evaluated for specificity, proven to perform in ChIP, and lot validated using ChIP. An example of these types of antibodies are the ChIPAb+ Validated Antibody and Primer Sets. ChIPAb+ antibodies are rigorously validated to ensure specificity and their ability to immunoprecipitate chromatin. In addition, each and every lot of a ChIPAb+ antibody is subject to extensive quality control testing including testing in the ChIP application. ChIPAb+ antibodies are more than just a highly validated antibody. To allow independent verification of performance or for use as a positive control, all ChIPAb+ antibodies include a negative control IgG plus PCR primers directed against a known positive locus. A partial list of ChIPAb+ antibodies is given in the table below.

Catalog Number	Description
17-622	ChIPAb+ Trimethyl-Histone H3 (Lys27)
17-614	ChIPAb+ Trimethyl-Histone H3 (Lys4)
17-658	ChIPAb+ Acetyl-Histone H3 (Lys9)
17-625	ChIPAb+ Trimethyl-Histone H3 (Lys9)
17-648	ChIPAb+ Dimethyl-Histone H3 (Lys9)
17-601	ChIPAb+ Sp1
17-662	ChIPAb+ EZH2, clone AC22
17-615	ChIPAb+ Acetyl Histone H3
17-663	ChIPAb+ EED
17-678	ChIPAb+ Trimethyl-Histone H3 (Lys4)
17-677	ChIPAb+ Dimethyl-Histone H3 (Lys4)
17-10051	ChIPAb+ Acetyl-Histone H3 (Lys14)
17-10050	ChIPAb+ Acetyl-Histone H3 (Lys4)
17-641	ChIPAb+ REST
17-672	ChIPAb+ RNA Pol II
17-608	ChIPAb+ HDAC1
17-10032	ChIPAb+ Trimethyl-Histone H3 (Lys36)
17-661	ChIPAb+ SUZ12
17-10046	ChIPAb+ Histone H3 (C-term)

Catalog Number	Description
17-10044	ChIPAb+ CTCF
17-681	ChIPAb+ Dimethyl-Histone H3 (Lys9)
17-630	ChIPAb+ Acetyl Histone H4
17-613	ChIPAb+ p53
17-603	ChIPAb+ ERα
17-643	ChIPAb+ Monomethyl Histone H3 (Lys27)
17-10048	ChIPAb+ Histone H2A.Z
17-10054	ChIPAb+ Histone H2B
17-10057	ChIPAb+ SMRT
17-10045	ChIPAb+ Acetyl-Histone H4 (Lys5)
17-10098	ChIPAb+ TATA Binding Protein (TBP)
17-10131	ChIPAb+ Phospho-CREB (Ser133)
17-675	ChIPAb+ Histone H3 (Unmod Lys4)
17-10130	ChIPAb+ Trimethyl-Histone H3 (Lys79)
17-600	ChIPAb+ CREB
17-656	ChIPAb+ Sox-2, clone 6F1.2
17-10034	ChIPAb+ EED (Rabbit Poly)
17-685	ChIPAb+ Phospho-Histone H3 (Ser10)
17-620	ChIPAb+ RNA Polymerase II

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

To see all available antibodies visit www.millipore.com/antibodies

Overview of ChIP Workflow

An overview of the key steps in a ChIP protocol is outlined below. A flow chart that outlines the Magna ChIP workflow is presented on page 9. Please see page 10 for a detailed protocol.

A. Chromatin Sample Prep and Immunoselection

Grow cells and treat with formaldehyde.

Formaldehyde treatment cross-links proteins to DNA to ensure co-precipitation

Lysis and sonication of 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 DNA. Protein-DNA cross-links are reversed and DNA is purified to remove the chromatin proteins and to prepare the DNA for the detection step.

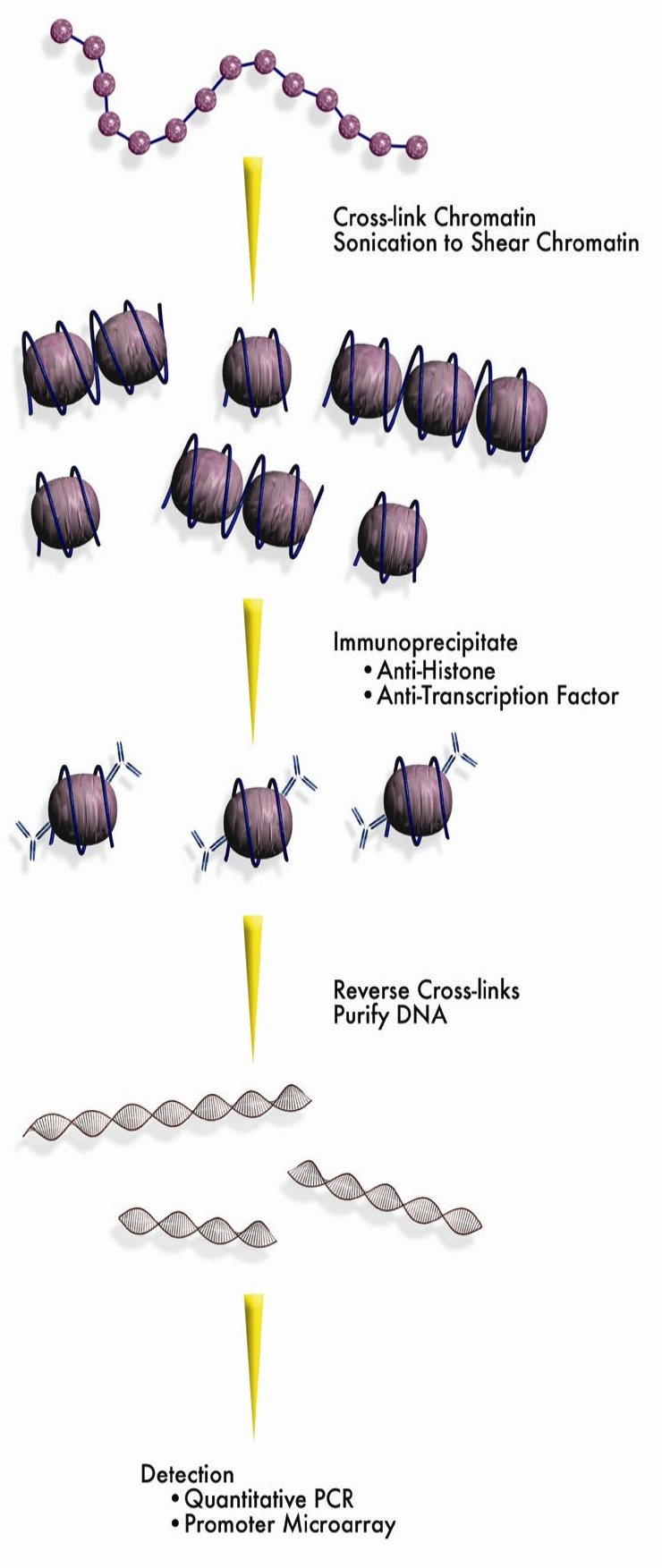
Detection. This is the most variable step of the procedure because of the number of detection methods that can be employed. The most meaningful results will be obtained with quantitative PCR for this step. Real Time Quantitative PCR (qPCR) is ideal, but this method requires a specialized PCR instrument capable of measuring optical changes resulting from the increase in amplified product. 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.

For quantitative PCR careful primer design is also critical. Primer design and amplicon size can vary depending upon the qPCR approach used (i.e. intercalating dye vs. a labeled detection probe). Consult your system manufacturer or reagent provided for specific primer design guidelines.

Figure 2. Workflow



Detailed Protocol

Section I: Chromatin Immunoprecipitation –Please Read Entire Protocol First

A. *In vivo* Cross-linking and Lysis (Cultured Cell Protocol)

Preparation and Important Considerations Before Starting:

- 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 x 10^7 cells. This typically generates 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 based on cell number and culture vessel surface area.
  - 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, Magna ChIP control antibodies can perform successful ChIP on as few as 1 X 10^5 HeLa cells. For simplicity, this protocol is written using 1 X 10^6 cells per ChIP to ensure optimal performance of the control antibodies.
- Obtain ice for incubation of PBS (Step 3) and for culture dish incubation (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 must be ice cold.
- Warm Nuclear Lysis Buffer to room temperature and 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.

Cross Linking and Cell Lysis Protocol

1. Fix cells in 1% formaldehyde by adding 550 µL of 37% formaldehyde (or 1100 µL of fresh 18.5% formaldehyde) to 20 mL of growth media. Gently swirl dish to mix.

   Important: Use high quality formaldehyde. Do not use formaldehyde that is past the expiration date suggested by the manufacturer. For details on how to make fresh formaldehyde before each experiment, see Appendix B on page 20.
2. Incubate at room temperature for 10 minutes. Agitation of the cells is not necessary.
3. During 10 minutes incubation period, remove 2 mL of ice cold 1X PBS and place in a separate tube for every dish to be processed. 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. Use sterile cell scraper to collect cells from each dish and place into a separate microfuge tube.
12. Spin at 800 x g at 4°C for 5 minutes to pellet cells.
13. During spin, for each microfuge tube from step 11, combine 0.5 mL of cell Lysis Buffer with 2.5 µL of Protease Inhibitor Cocktail II.
14. Remove supernatant.

    Tip: For chromatin preparations from cultured cells, fixed cell pellets (without buffer) 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 in a microcentrifuge.
18. During spin, for each microfuge tube to be processed combine 2.5 µL of Protease Inhibitor Cocktail II with 0.5 mL of Nuclear Lysis Buffer.
19. Carefully remove supernatant being careful not to disturb the cell pellet then resuspend cell pellets in 0.5 mL of Nuclear Lysis Buffer.

    Important: 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. The resulting material is sonicated to create appropriately sized chromatin fragments. If optimal conditions for sonication have been determined, proceed to Section B. Otherwise, see page 18, Appendix A Optimization of DNA Sonication.

B. Sonication of Isolated Chromatin to Shear DNA

Prior to starting this section:

- Optimal conditions need to be determined to shear cross-linked DNA to ~200-1000 base pairs in length (Figure 3). See Appendix A for a typical protocol. Once shearing conditions have been optimized, proceed with the steps below.

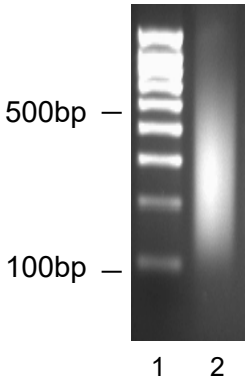


Figure 3: DNA Sonication
Sheared chromatin from formaldehyde-cross-linked HeLa cells was prepared by following all steps of Section A above (*In vivo* Cross-linking and Lysis), and Section B (Sonication Protocol, steps 1-4) and Appendix A (Option 1) of the Magna ChIP protocol. 20 µL sheared (lane 2) chromatin was resolved by electrophoresis 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.

Sonication Protocol

1. If desired, remove 5 µL of cell lysate from Section A (*In vivo* Cross-linking and Lysis), 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.
   - **Note:** Sonication conditions must be empirically determined using methods described in Appendix A (Optimization of DNA Sonication). 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 3 (page 11).
   - **Important:** Keep cell lysates ice-cold. Sonication produces heat, which can denature the chromatin. Allow sufficient time between sonication cycles to prevent sample overheating.
3. Spin at a minimum of 10,000 x g at 4°C for 10 minutes to remove insoluble material. However, do not exceed 15,000 x g to prevent loss of chromatin.
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 cross-linked chromatin can be stored at -80°C for up to 3 months.

C. Immunoprecipitation (IP) of Cross linked Protein/DNA

Prior to starting this section:

- Thaw Protease Inhibitor Cocktail II at room temperature for use in Step 3. Note that this solution contains DMSO and will remain frozen below 18.4°C.
- In advance of step 8, ensure the following buffers are placed on ice
  - Low Salt Wash Buffer
  - High Salt Wash Buffer
  - LiCl Wash Buffer
  - TE Buffer

Immunoprecipitation Protocol

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 A/G** (Cat.# 17-10086), ChIP reaction 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 negative control IgG be from the same species as the antibody of interest.

2. For each immunoprecipitation reaction prepare a microcentrifuge tube containing 50 µL of sheared cross-linked chromatin prepared in Section B (Sonication of Isolated Chromatin to Shear DNA), step 5 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 immunoprecipitation reaction.
   - 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 (Elution of Protein/DNA Complexes and Reverse Cross-links of Protein/DNA Complexes to Free DNA), 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 of **fully resuspended** protein A/G magnetic beads.

   Important: 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 a short period of 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 titer, 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. 4 for additional details). Consequently, 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 A/G magnetic beads with the magnetic separator (e.g. Magna GrIP Rack (8 Well), Cat. # 20-400,) and remove the supernatant completely.

8. Wash the Protein A/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 between each wash followed by magnetic separation and careful removal of the supernatant fraction:

a. Low Salt Wash Buffer (Cat.# CS200625), **one wash**
b. High Salt Wash Buffer (Cat.# CS200626), **one wash**
c. LiCl Wash Buffer (Cat.# CS200627), **one wash**
d. TE Buffer (Cat.# CS200628), **one wash**

D. Elution of Protein/DNA Complexes and Reverse Cross-links of Protein/DNA Complexes to Free DNA

Prior to starting this section:

- Thaw Proteinase K and Warm the ChIP Elution Buffer (w/o Proteinase K) to room temperature to ensure the SDS is in solution before proceeding.

Protocol

1. Prepare the final elution buffer for all IP tubes as well as all Input tubes (prepared in Section C, step 4). For each tube, prepare elution buffer as follows:

ChIP Elution Buffer (w/o Proteinase K)	100□ µL
Proteinase K	1 µL

2. Incubate at 62°C for 2 hours with shaking.
   - Shaking and 62°C incubation can be accomplished with equipment such as an Eppendorf Thermomixer® system, a 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 separation device. Carefully remove and transfer the 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. Do not exceed 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. Do not exceed 15,000 x g.
9. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
10. Place the Spin Filter back into the same Collection Tube.
11. Centrifuge for 30 seconds at a minimum of 10,000 x g. Do not exceed 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. Do not exceed 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 four DNA samples that undergo PCR using the Control Primers included in this kit: positive and negative control immunoprecipitations, Input and a "no DNA" tube as a control for DNA contamination.
   - The Control Primers provided with the EZ-ChIP version of this kit are specific for the human GAPDH gene. If the chromatin used for ChIP is from a different species, it is recommended that the user design species specific primers and determine appropriate PCR reaction conditions. Refer to section B (DNA Purification and Detection on page 8) for primer design guidelines)
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 below.
   - It is recommended that the user employ a Hot-Start *Taq* polymerase (e.g NovaTaq Hot Start DNA Polymerase cat# 71091). 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 used, 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
DNA	2.0 µL
H_2O	12.6 µL
10X PCR Buffer (w/o $MgCl_2$)	2.0 µL
$MgCl_2$ (50 mM)	0.6 µL
2.5 mM dNTP	1.6 µL
Control Primers	0.8µL
Taq (5 U/µL)	0.4µL

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.

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.*

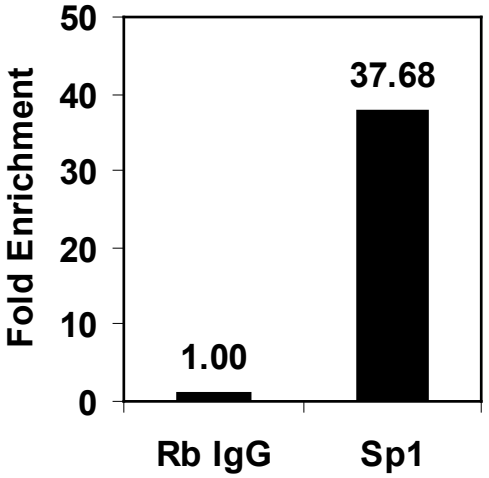


Figure 4: Analysis of Fold Enrichment
Sonicated chromatin prepared from HeLa S3 cells (1 X 10^6 cell equivalents per IP) was subjected to ChIP using 4 µg of either Normal Rabbit IgG, or 4 µg Anti-Sp1 (components contained in Sp1 ChIPAb+ kit cat# 17-601) and the Magna ChIP A+G kit. Immunoprecipitation of Sp1 associated DNA fragments was verified by qPCR using primers directed against DHFR.

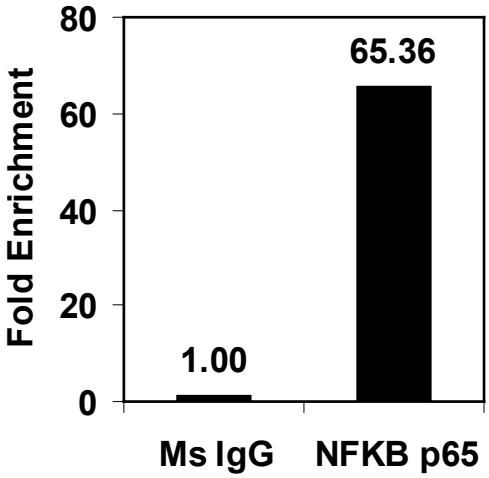


Figure 5: Analysis of Fold Enrichment
Sonicated chromatin prepared from serum starved, TNFα-treated (20ng/mL, 30 min) HEK293 cells (~3 X 10^6 cell equivalents per IP) were subjected to chromatin immunoprecipitation using 4 µg of either Normal Mouse IgG, or 4 µg Anti-NFκB p65 (RelA) (components contained in NFκB p65 ChIPAb+ kit cat# 17-10060). Immunoprecipitation of NFκB p65 (RelA) associated DNA fragments was verified by qPCR using primers directed against IκBα.

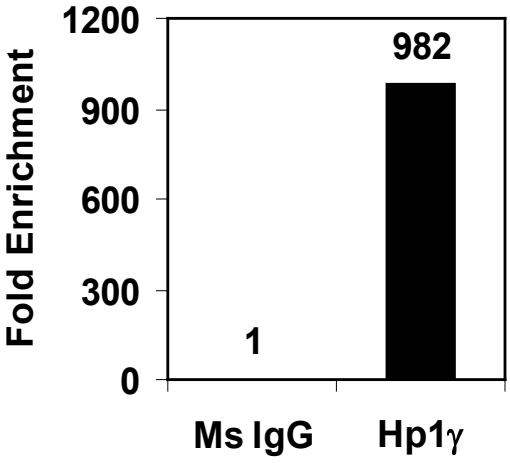


Figure 6: Analysis of Fold Enrichment
Sonicated chromatin prepared from HeLa S3 cells (1 X 10^6 cell equivalents per IP) was subjected to chromatin immunoprecipitation using 5 µg of either Normal Mouse IgG or 5 µg Anti-Hp1γ (components contained in the Hp1γ ChIPAb+ kit cat# 17-646). Immunoprecipitation of Hp1γ associated DNA fragments was verified by qPCR using primers directed against GAPDH.

APPENDIX A: Optimization of DNA Sonication

Optimal conditions for shearing cross-linked DNA to 200-1000 base pairs in length depends on the cell type, cell concentration and the specific sonication 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

Optimization of Sonication

The protocol presented below describes the optimization of sonication following the *In vivo Cross-linking and Lysis* procedure (see page 10). The specific conditions described below are intended as potential starting points for optimization. The specific conditions used for your cell type may vary significantly depending on the sonication system, microtip, and cell type used.

I. Generate a cell lysate by following Section A (In vivo Cross-linking and Lysis procedure page 10), 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 µL	5 x 10^6/mL	2.5 x 10^6
500 µL	2 x 10^7/mL	1 x 10^7
500 µL	4-5 x 10^7/mL	2.5 x 10^7

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

- **Important:** 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 Sonicator® 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 µL, 5 µL, 2 µL from least to most concentrated sample) of the sonicated chromatin from each condition to a fresh tube.

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

Analysis Option 1:

1. Add 1 µL of RNase A (10 mg/mL) and incubate 30 minutes at 37°C.
2. Add 1 µL□ Proteinase K and incubate at 62°C for 2 hours.
3. Load 10 µL and 20 µL on a 1-2% agarose gel with a 100 bp DNA marker and perform electrophoresis.

   Tip: Loading different amounts of DNA sample helps to avoid under- or over-loading of your gel.
4. Observe which of the shearing conditions gives a smear of DNA in the range of 200 bp-1000 bp. See Figure 3 (page 11) for an example.
5. If the results indicate that the resulting DNA is not in the desired size range, repeat the optimization using modified conditions. Once optimal conditions have been determined for a given cell type, it is advised that no alternations be made to the cell concentration or volume of lysate per microfuge tube in subsequent chromatin immunoprecipitation experiments.

Analysis 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.
   - Use 5 volumes of Bind Reagent "A" 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
   - Do not exceed 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. 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
   - Do not exceed 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.
    - Do not exceed 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 Spin Filter membrane.

15. Centrifuge for 30 seconds at a minimum of 10,000 x g.
    - Do not exceed 15,000 x g.
16. Load 10 µL and 20 µL□ on a 1-2% agarose gel with a 100 bp DNA marker.

    Tip: Loading different amounts of DNA sample helps to avoid under- or over-loading of your gel.
17. Observe which of the shearing conditions gives a smear of DNA in the range of 200 bp-1000 bp. See Figure 3 (page 11) for an example.
18. If the data indicates that the resulting DNA is not in the desired size range, repeat the optimization using modified conditions. Once optimal conditions have been determined for a given cell type, it is advised that no alternations be made to the cell concentration or volume of lysate per microfuge tube in subsequent chromatin immunoprecipitation experiments.

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. Formaldehyde and paraformaldehyde can give off vapors that are irritants and both compounds are potential carcinogens. Use appropriate safety precautions and personal protective equipment 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.

CHROMATIN IP OPTIMIZATION AND TROUBLESHOOTING

Step	Potential Problems	Experimental Suggestions
Cross-linking	Not enough or too much cross-linking	The appropriate amount of formaldehyde and time of cross-linking 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 cross-linked 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 A/G	Not enough beads	The magnetic beads settle to the bottom of the tube over time. Make sure the Protein A/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 A/G. Protein A/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 cross-linking	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 Cross-linking	Too much cross-linking 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 Hot Start *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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