Magna ChIP^{2} TM
Chromatin Immunoprecipitation DNA Microarray Universal Kit

Catalog # 17-1000: Magna ChIP^{2} TM Universal Kit
(3 Assays)

Catalog # 17-1004: Magna ChIP^{2} TM Universal Quad Kit
(12 Assays)

FOR RESEARCH USE ONLY
Not for use in diagnostic procedures

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Introduction

Chromatin Immunoprecipitation (ChIP) is a powerful technique classically used for mapping the *in vivo* distribution of proteins associated with chromosomal DNA. These proteins can be histone subunits, transcription factors or other regulatory or structural proteins bound either directly or indirectly to DNA.

Using high quality antibodies, protein-interacting regions of chromosomal DNA, as well as their post-translational modifications can be detected. Typically either end-point or quantitative PCR is performed to verify whether a particular DNA sequence (the gene or region of the genome) is associated with the protein of interest. Using this classical approach, laboratories can develop assays for detecting the interactions of the proteins of interest with a limited number of known target genes.

To extend chromatin immunoprecipitation analysis genome-wide, researchers are conducting high content analysis using **ChIP-chip.** This technique utilizes chromatin immunoprecipitation (*"ChIP"*) followed by an assay of the immunoprecipitated chromatin on a microarray. (*"chip"*). This combined approach of ChIP and tiling microarrays allows laboratories to determine the locations of binding sites for virtually any chromatin associated protein that can be cross-linked to chromatin and immunoprecipitated with an antibody. In a single experiment it is possible to map the locations of binding sites for histones, transcription factors, enhancers, repressors, silencing elements, insulators, boundary elements, as well as the sequences controlling DNA replication across a genome. By profiling interactions under a variety of biological conditions, changes in the constellation of protein-DNA interactions across the genome can be used to gain a better understanding of gene regulatory networks, cell proliferation, and disease progression.

A typical ChIP-chip assay involves several steps. These steps include protein-DNA and protein-protein crosslinking, fragmentation of the crosslinked chromatin, followed by immunoprecipitation with an antibody targeting a protein associated with the fragmented chromosomal DNA. Next, the DNA fragments (isolated in complex with the target protein) are amplified, labeled and analyzed by promoter or genomic tiling microarrays, thus allowing genome-wide identification of DNA-binding sites for chromatin-associated proteins with precise resolution.

ChIP-chip has become a powerful method to explore chromatin structure and nuclear protein function on a genomic scale. However, ChIP-chip was initially used in only a small number of laboratories due to the complexity, cost and technical challenges of the ChIP-chip protocol. The Magna ChIP^{2} ™ chromatin immunoprecipitation DNA microarray universal kit is designed to facilitate the use of ChIP-chip for beginners, as well as those experienced in the technique for use with Agilent® and other microarray platforms.

Using cultured cells or tissue as starting materials, the Magna ChIP^{2} Universal Kits (cat. # 17-1000 and 17-1004) contain measured buffers and reagents needed to perform three (cat. # 17-1000) or 12 (cat. # 17-1004) chromatin immunoprecipitation comparison experiments. These kits provide optimized reagents for chromatin immunoprecipitation and amplification, in order to yield sufficient starting material for labeling and hybridization using user provided reagents and microarrays of varying designs and feature densities. The kits have been validated using commercially available promoter microarrays including the Agilent® 244K promoter arrays, the Nimblegen® 384K promoter arrays and the Affymetrix® promoter 1.0R arrays.

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Kit Components

Magna ChIP^{2} TM Universal Kit Configurations	
Magna ChIP^{2} TM Universal Kit (3 Assay) (Cat. # 17-1000)	**Magna ChIP^{2} TM Universal Quad Kit (12 Assay) (Cat. # 17-1004)**
Chromatin IP Module I MAGNA0006 (1 EA) (Store at 4°C)	Chromatin IP Module I MAGNA0006 (4 EA) (Store at 4°C)
Chromatin IP Module II MAGNA0007 (1 EA) (Store at -20°C)	Chromatin IP Module II MAGNA0007 (4 EA) (Store at -20°C)
Chromatin Amplification Module MAGNA0012 (1 EA) (Store at -20°C)	Chromatin Amplification Module MAGNA0012 (1 EA) (Store at -20°C)

Chromatin IP Module I MAGNA0006 Store at 4°C		
Component	**Component #**	**Quantity**
Crosslinking buffer[1]	CS203146	60 mL
10X Glycine	CS203147	80 mL
10X PBS	20-281	2 x 24 mL
Lysis Buffer A	CS203148	18 mL
Lysis Buffer B	CS203149	18 mL
Lysis Buffer C	CS203150	10 mL
10% Triton X-100	CS203151	1 mL
Magnetic Protein A/G Beads	CS203152	315 µL
TE	CS200628	12.5 mL
RIPA Buffer	CS203153	26 mL
ChIP Elution Buffer	CS203154	1 mL
10 mM Tris-HCL PH 8.0	CS203155	1 mL
Store the Following at Room Temperature Upon Receipt **Note that some of these components are also used with the Amplification Module**		
Phase Lock Tubes	CS203145	12 EA
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

[1] Requires addition of 37% formaldehyde before use

Chromatin IP Module II MAGNA0007 Store at -20°C		
Blocking Buffer	CS203157	26 mL
200X Protease Inhibitor Cocktail	CS203169	500 µL
RNase A (10 mg/mL)	20-297	60 µL
Proteinase K (10 mg/mL)	20-298	60 µL
5M NaCl	20-159	500 µL
Glycogen (20 mg/mL)	CS202175	40 µL

Chromatin Amplification Module MAGNA0012 Store at -20°C		
10X Blunting Buffer	CS203159	300 µL
100X BSA	CS203160	20 µL
10 mM dNTP Mix	CS203161	70 µL
10 mM dNTP (+dUTP) Mix	CS203205	70 µL
T4 DNA polymerase	CS203162	20 µL
10X Ligase buffer	CS203163	150 µL
15 uM Annealed Linkers	CS203164	200 µL
T4 DNA ligase	CS203165	20 µL
10X PCR Buffer	CS203166	150 µL
40 uM Amplification Primer	CS203167	40 µL
Taq DNA Polymerase	CS203168	30 µL

Components of this kit (with the exception of 20-159) are not available for individual sale.

Materials Required But Not Supplied

Reagents

- Cells, stimulated or treated as desired
- ChIP-qualified antibody of interest for chromatin immunoprecipitation (visit www.millipore.com/antibodies and search under "chromatin immunoprecipitation" for a list of available targets)
- 37% Formaldehyde
- Phenol (e.g. Sigma-Aldrich® P4557)
- Phenol:Chloroform:Isoamyl alcohol (e.g. Sigma-Aldrich® P3803)
- Chloroform:Isoamyl Alcohol (e.g. Sigma-Aldrich® C0549)
- DNase and RNase-free sterile H_2O
- 100% Ethanol
- 70% Ethanol
- 3M NaOAc, pH 5.2 (e.g. Sigma-Aldrich® S7899)
- Liquid Nitrogen

Equipment

- Magnetic Separator (e.g. Millipore Magna GrIP™ Rack (8 Well), Catalog # 20-400)
- Vortex mixer
- Rotating wheel/platform
- Microfuge
- Sonicator
- Thermomixer, heat capable
- 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, aerosol resistant
- NanoDrop® (Thermo-Fisher) or equivalent small volume capable spectrophotometer
- Microarray hybridization equipment, scanner and analysis software
- Dewar flask for liquid nitrogen
- Tissue culture supplies appropriate for your experiment

Hazards:

Protease Inhibitor Cocktail contains DMSO, avoid contact with skin.
This protocol requires use of phenolic extraction reagents, which can cause burns on contact. Use personal protective equipment and caution when extracting samples.
Chromatin preparation may require use of liquid nitrogen. Use PPE when handling liquid N_2 to avoid burns.
Use appropriate fume hoods and venting when working with concentrated formaldehyde solutions. Formaldehyde is toxic by inhalation, skin contact and ingestion so PPE is recommended.

Storage and Stability

MAGNA0006: Store at 4° C, good for 6 months from date of receipt when reagents are stored properly. Please note components to be stored at room temperature upon receipt.

MAGNA0007: Store at -20° C, good for 6 months from date of receipt when reagents are stored properly.

MAGNA0012: Store at -20° C, good for 6 months from date of receipt when reagents are stored properly.

Tips to Help Ensure a Successful Experiment

IMPORTANT: Please read the entire protocol before starting.

ChIP-chip experiments are composed of multiple steps that are carried out over several days. Because this application represents a significant investment of time and materials, it is **strongly recommended** that you carefully plan the entire experiment and decide how to best to manage your time before you begin.

To help ensure a successful experiment, it is critical to take the time to evaluate the samples being prepared after key steps in the protocol. Below are suggested points where an evaluation of your materials can help prevent a waste of time and materials that may lead to an unsatisfactory ChIP-chip result. ***We strongly recommend that you conduct these evaluations at the indicated steps.**

Critical Steps and Important In-Process Assays

1. **Chromatin Evaluation**
   The size of fragmented chromatin can influence the success of the Millipore Magna $ChIP^2$ protocol. This protocol works best when the chromatin size is between 200-1000 bp. Shearing of the chromatin varies greatly, depending on cell type, growth conditions, quantity, volume, crosslinking, and equipment. It may be necessary to optimize sonication conditions by changing the power settings, cycle number and ratios of time ON and time OFF.

   The quality of the chromatin can be analyzed visually by agarose gel, and the quantity of DNA in the chromatin preparations can be measured following DNA extraction (**Section F,** Step 13) by spectrophotometry. Chromatin of good quality (when visually assessed by agarose gel electrophoresis) typically shows a **size distribution of fragments between 200-1000 bp**. (See Fig. A on page 16 for an example of high quality sheared chromatin)

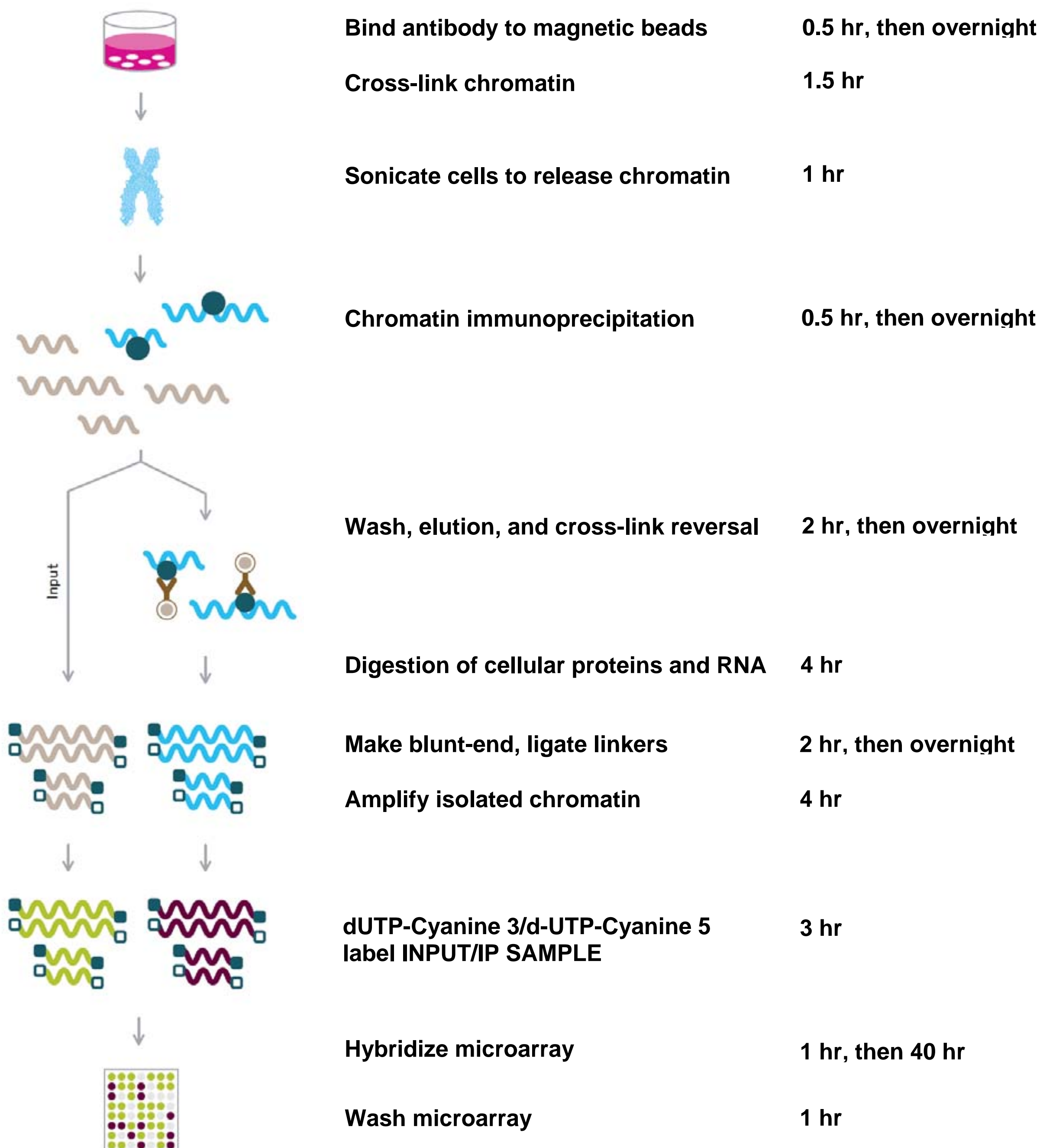
2. **Chromatin Immunoprecipitation Evaluation**
   The success of ChIP-chip is very dependent on how efficiently you can immunoprecipitate your chromatin. To ensure that you are enriching for DNA sequences that are associated with your protein of interest, it is important to evaluate the level of enrichment.

   The success of the chromatin immunoprecipitation can be monitored by a qPCR assay of the relative enrichment of a DNA sequence that is a known target in the ChIP sample, compared to an IgG mock sample. If an IgG control is not performed, a set of negative primers (targeted to a known negative region, such as gene desert) can be used as an internal reference to show that a known target is enriched in the ChIP sample relative to the input. Although some researchers have had success with less than 5 fold enrichment, it is recommended that a minimum of 5 fold enrichment (ΔCt of ~2.3 at 100% efficiency) be achieved before moving forward.

   If you are inexperienced in the methodology of ChIP or unsure of the performance of your antibody in ChIP, you may consider conducting a classical ChIP experiment using products such as Millipore's EZ-Magna ChIP kits (Cat. # 17-408 & 17-409).

3. **Ligation-mediated PCR (LM-PCR) Evaluation**
   The amount of DNA generated by Chromatin IP is not sufficient for microarray analysis. It is necessary to amplify ChIP DNA through ligation mediated PCR (LM-PCR). To verify amplification, it is important to take O.D. measurements of the samples after LM-PCR using a low volume spectrophotometer such as a NanoDrop. For optimal labeling and hybridizing results, LM-PCR amplified samples should have a $\mathbf{A_{260}/A_{280} > 1.7}$ and a $\mathbf{A_{260}/A_{230} > 1.6}$.
   Because there is a PCR bias against DNA of larger size, it is also important to qPCR validate enrichment of the amplified sample before labeling and hybridizing the sample to the array. Refer to **Section I**, step 8 and figure C for an outline of this approach.

ChIP-chip Overview and Time Management



DETAILED ChIP-chip PROTOCOL

The Magna ChIP^2 Universal kit is intended to produce amplified chromatin immunoprecipitated material for use on a customer supplied microarray. The reagents provided allow for a fixed number of chromatin preparations, immunoprecipitations using user supplied antibodies, and subsequent ligation-mediated PCR reactions according to the following product specific quantities:

Cat. # 17-1000: Three independent chromatin preparations from approximately 10^8 cells from culture or tissue. Three independent chromatin immunoprecipitations and up to twenty-four LM-PCR reactions when utilizing the one step amplification protocol (See Section I). When using two step amplification, the reagents supplied are sufficient to provide twelve LM-PCR reactions.

Cat. # 17-1004: Twelve independent chromatin preparations from approximately 10^8 cells from culture or tissue. Twelve independent chromatin immunoprecipitations and up to twenty-four LM-PCR reactions when utilizing the one step amplification protocol (See Section I). When using two step amplification, the reagents supplied are sufficient to provide twelve LM-PCR reactions.

The detailed protocol contained below describes how to prepare samples (Input and IP) for a single chromatin preparation, immunoprecipitation and ligation mediated amplification reaction to allow subsequent labeling and microarray comparisons of a single total sample versus an immunoprecipitated sample using one user supplied antibody. Success of the experiment is largely dependent on an *a priori* knowledge of the performance of that antibody in ChIP and awareness of at least one genomic location where that protein is expected to occupy in a given chromatin sample. The quantity of LM-PCR amplified ChIP sample required will depend on the microarray platform being employed (i.e. number of arrays and feature density) as well as the labeling method being used. Users are encouraged to follow recommendations wherever possible for the downstream workflow in the ChIP-chip experiment when using the Millipore Magna ChIP^2 Universal DNA Microarray Kit with respect to 3rd party provided labeling products and microarrays.
Note: All references to reagents being "chilled" refers to temperatures at approximately 2 to 8°C.

A. Magnetic Bead Preparation

Note: Separation times listed below are dependent on the strength of the magnet used. This protocol was developed using the Millipore Magna GrIP™ Rack (Cat # 20-400). If other magnetic separations stands are used separation times may need to be determined empirically and adjusted.

Important: Perform all steps for binding the antibody to the magnetic beads in a cold room or on ice.

1. Fully re-suspend the magnetic protein A/G beads by inversion or gentle vortexing.

   Note: Protein A/G beads are particulate and will settle over time. It is important to re-suspend the beads to ensure a homogenous solution.

2. Add 100 µL of magnetic protein A/G beads to a microfuge tube, put the tube on a magnetic device and let sit for 1 minute.
3. Carefully aspirate the supernatant with a pipette without disrupting beads, then wash beads by adding 1 mL of Blocking Solution.

4. Remove tubes from magnetic separation device, and shake or agitate the tube gently to re-suspend beads. Return tubes to the magnetic separation device. Allow 1 minute for beads to collect to the side of the tube. Carefully remove supernatant.
5. Repeat wash (steps 3 & 4) two times, using 1 mL of Blocking Solution for each wash.
6. Re-suspend beads in 1 mL of Blocking Solution and add ~10 µg of purified antibody.**
7. Incubate the bead mixture on a rotating platform 45-60 rpm at 4°C for 12-15 hours. Store at 4°C. These beads will be used in **Section D** – Chromatin Immunoprecipitation.

Note: If your antibody of interest is not purified but has been shown to perform specifically in ChIP with smaller amounts of chromatin, the quantity required for ChIP-chip is generally 2-10 fold higher, but may need to be empirically determined.

B. *In vivo* Crosslinking

Prior to starting this section:

- Make fresh 11% cross linking buffer. It is important that this buffer be made fresh.
- Make sufficient buffer for the amount of media used to grow your cells or the volume of tissue you are processing. Each plate processed will require the addition of a 1/10 volume of 11% crosslinking buffer per volume of growth media.
- Note that the 11% refers to the final concentration of formaldehyde in this buffer. When a 1/10 volume of this buffer is added to your cells, the final formaldehyde concentration for fixation is 1%.
- To make the 11% Formaldehyde Crosslinking buffer, mix 37% formaldehyde stock with the crosslinking buffer provided in the kit to achieve a final 11% stock solution. It is suggested that your prepare 20% more than required for the number of plates to be processed. To make it simple to determine how much 11% Crosslinking Buffer to make and what volumes of 37% formaldehyde and Crosslinking buffer to combine, a calculation worksheet is provided below. A blank row is provided at the bottom to enter numbers for your experiment. To use the sheet simply enter or calculate the numbers in each column A-E then use in the formulas shown above the blank row to determine the amount of material to mix, in order to make sufficient 11% Crosslinking Buffer for your experiment.

Worksheet for Preparation of 11% Formaldehyde Crosslinking Solution for Adherent Cells

A	B	C	D	E		
Media Per Plate (mL)	11% Crosslinking Buffer (mL) Required Per Plate	37% Formaldehyde Stock (mL) per plate	Crosslinking Buffer Stock (mL) per Plate	Total Number of Plates	Total mL 37% Formaldehyde to Mix w/ CrossLinking Buffer x 1.2 for 20% extra	Total mL Crosslinking Buffer to Mix w/ Formaldehyde Stock x 1.2 for 20% extra
Example 20 mL	2	0.6	1.4	5	5 plates x 0.6 mL x 1.2 3.6 mL	5 plates 1.4 mL x 1.2 8.4 mL
User defined	= A x 0.1	= B x 0.293	B - C	User defined	= E x C x 1.2	= E x D x 1.2

- Make sure to grow enough cells to generate sufficient chromatin for the experiment. For each immunoprecipitation and chromatin preparation, 5×10^7 to 1×10^8 cells are recommended. To determine the total number of cells, perform a cell count using a hemocytometer. For suspension cells, count an aliquot of cells from the growth vessel and perform the appropriate calculation to estimate the total number of cells. To determine the number of cells for adherent cells, grow an extra plate of cells to use for your cell count. Trypsinize one plate and count the number of cells, using a hemocytometer. Multiply the cell count from that plate by the total number of plates to be used for the experiment to determine the total number of cells in your plates.

  - For HeLa cells, one plate contains approximately 1-2 x 10^7 cells. 5-10 plates (150 mm plate) are needed for a single ChIP-chip experiment.
  - The volume of buffers supplied in the kit is sufficient to prepare chromatin and perform 3 ChIP-chip experiments. The protocol is written for performing one chromatin isolation at a time.
  - Cell numbers can be scaled up or down based on the performance of the antibody. For example, antibodies against abundant epitopes, such as RNA polymerase II and modified histones, can perform a successful ChIP-chip experiment using significantly fewer cells.

    In general, however, if the source of cellular chromatin is not limiting, 1×10^8 cells is a useful guideline quantity.

- Stimulate or treat, if necessary, adherent mammalian cells at ~80-90% confluence in a 150 mm culture dish containing 20 mL of growth media.
- Prepare an ice bucket or other suitable container for incubating PBS and for incubating the culture dish or tissue sample.
- Prepare sufficient 1X PBS (10X PBS diluted with nuclease free water) and store on ice.

  - Adherent cells: ~25 mLs per 150 mm plate + 10 mLs
  - Suspension cells: ~100 mLs per suspension pellet + 10 mLs
  - Tissue: ~160 mLs per tissue isolation

  This will be used for washes and needs to be chilled.

- Remove Protease Inhibitor Cocktail and thaw at room temperature. Note that the Protease Inhibitor Cocktail contains dimethyl sulfoxide (DMSO) and will remain frozen below 18.4°C.

  Caution: Use caution and wear gloves and handling DMSO as the chemical can penetrate skin and mucous membranes.

Protocol for Adherent Cells:

1. Determine the total number of cells in dishes by counting using a hemocytometer. See suggestions above for total number of cells to use.
2. Add 2 mL of 11% crosslinking buffer to 20 mL of growth media to give 1% final formaldehyde concentration. Gently swirl dish to mix.
    - Use 1/10 volume, if using more or less growth media.
3. Incubate at room temperature for 10 minutes to crosslink proteins.
    - Agitating the cells is not necessary.
    - The crosslinking time can be increased to 15 minutes if desired. Additional exposure of the cells to the crosslinking solution may be necessary to detect protein-protein interactions or to detect epitopes of low abundance.
4. Add 2.2 mL of 10X Glycine to each dish to quench unreacted formaldehyde.
    - Use 1/10 total volume in the dish to yield 1X final concentration.
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 chilled 1X PBS to wash cells.
9. Aspirate PBS.
10. Add 5 mL PBS to each 150 mm dish and harvest cells using a silicone scraper.
11. Pool the cells into a 50 mL conical tube, Spin at 800 x g at 4°C for 5 minutes to pellet cells.
12. Re-suspend pellet in 10 mL of 1X chilled PBS per 10^8 cells. Transfer 5 x 10^7 to 1 x 10^8 cells to 15 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with swinging bucket rotor. Discard the supernatant. Continue protocol at **Section C** – Chromatin Preparation.

    Note: Cell pellet can be flash frozen and stored at -80°C at this step.

Protocol for Suspension Cells:

1. Determine the number of total cells by counting using a hemocytometer. See suggestions above for total number of cells to use.
2. Verify volume of culture media in growth chamber then add 1/10 volume of fresh 11% crosslinking buffer directly to the culture media in the flasks. Swirl flasks briefly to mix.
3. Incubate at room temperature for 15 minutes to crosslink proteins.
4. Calculate total volume in flask (original cell culture volume + crosslinking buffer) and add 1/10 total volume of 10X Glycine to flasks to quench the formaldehyde.
5. Swirl to mix and incubate at room temperature for 5 minutes.
6. Spin down the cells at 1,350 x g for 5 minutes at 4°C.
7. Remove supernatant, being careful not to disturb cell pellet.

8. Wash the pellet twice with 50 mL of chilled 1X PBS.
   - Add chilled 1X PBS.
   - Spin down the cells at 1,350 x g for 5 minutes at 4°C between each wash.
   - Remove 1X PBS being careful not to disturb the cell pellet.
   - Re-suspend pellet in 10 mL of chilled 1X PBS per 10^8 cells. Transfer 5 x 10^7 to 1 x 10^8 cells to 15 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with a swinging bucket rotor. Remove and discard the supernatant, being careful not to disturb the cell pellet. Continue protocol at **Section C** – Chromatin Preparation.

     Note: Cell pellet can be flash frozen and stored at -80°C at this step. Maintain on ice before use.

Protocol for Animal Tissue:

Note: It is recommended that tissue being used for ChIP-chip chromatin preparation be freshly isolated and prepared as quickly as possible to retain *in vivo* profiles of protein occupancy. Preparation of chromatin from preserved archival or frozen tissue samples is not recommended. Due to the varied nature of cellularity in tissues of different origin, it is difficult to assess cell number per mass of tissue isolated. The guidelines presented here assume the quantity of tissue processed will yield approximately 10^8 cells but optimization of quantity used per tissue may need to be determined.

1. Dissect tissue of interest and place in chilled 1X PBS in a 50 mL conical tube.
   - Typically 0.1 to 0.3 g of tissue is required for a single ChIP-chip experiment. The exact amount of tissue needed depends upon the abundance of the protein of interest, the affinity of the antibody for the target protein, and the efficiency of crosslinking.
2. Add 10 mL of chilled 1X PBS to a 10 mm tissue culture plate, place animal tissue sample in plate and quickly cut the tissue sample into small pieces (approximately 1 mm cubes) using a new razor blade or scalpel.
3. Transfer diced tissue in chilled 1X PBS to a 50 mL conical tube, and spin down tissues at 1,350 x g for 5 minutes at 4°C. Carefully discard the supernatant, being sure not to disturb the tissue pellet.
4. Re-suspend pellet in 40 mL of room temperature 1X PBS, add 4 mL of 11% crosslinking buffer, and place tube on a rotating platform for 15 minutes at room temperature.
5. Add 4.4 mL of 10X Glycine to each dish to quench unreacted formaldehyde, and place tube on a rotating platform for 5 minutes at room temperature
6. Spin down the tissues at 1,350 x g for 5 minutes at 4°C.
7. Wash the pellets twice with 50 mL of chilled 1X PBS. Remove supernatant.
8. Re-suspend pellet in 2 mL of chilled 1X PBS, transfer to a dounce homogenizer, and homogenize with a type A pestle for 5-10 strokes to break up the tissue until sample is homogenous.

9. Re-suspend pellet in 10 mL of chilled 1X PBS per sample. Transfer cells to 15 mL conical tubes and spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge with a swinging bucket rotor. Remove and discard the supernatant being careful not to disturb the cell pellet. Continue protocol at **Section C** – Chromatin Preparation. Be sure to assess the quality of this chromatin by gel analysis and spectrophotometric determination of the Input chromatin sample at **Section F,** Step 15.

   Note: Cell pellet can be flash frozen in liquid nitrogen and stored at -80°C at this step. Fixed cell pellets can be used after several months of storage.

C. Chromatin Preparation

Prior to starting this section:

IMPORTANT: For the best results, determine optimal conditions need for shearing crosslinked DNA to ~200-1000 base pairs in length. These conditions vary with cell type, length of fixation, quantity of cells, volume, and equipment used. To optimize conditions, a sonication time course experiment followed by agarose gel analysis is suggested. This can be done by sonicating and taking samples after every two cycles of sonication. To analyze these samples on a gel, reverse the crosslinks, purify the nucleic acid and check visually on a gel. Once shearing conditions have been optimized, proceed with the steps below.

IMPORTANT: Add 200X protease inhibitor (final concentration of 1X) to Lysis Buffer A (5 mL per sample), Buffer B (5 mL per sample), and Buffer C (3 mL per sample) before use. Store on ice.

1. Re-suspend each pellet of approximately 10^8 cells in 5 mL of chilled Lysis Buffer A. Rock at 4°C for 10 minutes. Spin at 1,350 x g for 5 minutes at 4°C in a table-top centrifuge. Discard the supernatant.
2. Re-suspend each pellet in 5 mL of Lysis Buffer B. Rock gently at room temperature for 10 minutes. Pellet nuclei in table-top centrifuge by spinning at 1,350 x g for 5 minutes at 4°C. Discard the supernatant.
3. Re-suspend each pellet in 3 mL of Lysis Buffer C.
4. Transfer cells to a 15 mL polypropylene tube.
5. Sonicate the suspension. Samples should be kept in an ice water bath during sonication. **SUGGESTION:** If you use a Misonix 3000, set the power output at 7 and sonicate 8 cycles of 30 seconds ON and 60 seconds OFF to decrease foaming and allow resting of the sample between cycles.
6. Add 300 µL of 10% Triton X-100 to the sonicated lysate and mix by pipetting up and down several times. Split into two 1.5 mL microfuge tubes. Spin at 20,000 x g for 10 minutes at 4°C in a microfuge to pellet debris.
7. Combine supernatants from the two 1.5 mL microfuge tubes into a new 15 mL conical tube for immunoprecipitation.
   - If desired, sample can be snap frozen in liquid nitrogen and stored at -80°C until ready to proceed to immunoprecipitation. It is not recommended to use archived chromatin samples that are more than 3 months old.
8. Save 50 µL of cell lysate from each sample to use as control chromatin (sometimes referred to as Whole Cell Extract or WCE but referred to in future sections as '**Input**'). The input sample is a reference sample used as a comparison to the IP enriched and amplified material you will prepare in the next steps of the protocol. Store at -20°C. The input sample will be used in **Section E,** step 13.

D. Chromatin Immunoprecipitation (ChIP)

1. Remove the antibody conjugated magnetic protein A/G beads prepared in step A from the rotating platform. Remove the unconjugated antibodies by washing 3 times with 1 mL of Block Solution as described below:

   a. Place tubes in magnetic separation device. Allow 1 minute for beads to collect on side of the tube.
   b. Carefully aspirate the supernatant with a pipette without disrupting beads, then wash beads by adding 1 mL of Blocking Solution.
   c. Remove tubes from magnetic separation device and shake or agitate tube gently to re-suspend beads. Return tubes to magnetic separation device. Allow 1 minute for beads to collect to side of the tube. Carefully remove supernatant.

   Note: Separation times are dependent on the strength of the magnet used. This protocol was developed using the Millipore Magna GrIP™ Rack (Cat # 20-400). If other magnetic separations stands are used, separation times may need to be determined empirically and adjusted.

2. Place tubes in magnetic device for 1 minute to collect beads. Remove supernatant.
3. Re-suspend the beads in 100 µL of Block Solution
4. Add 100 µL of antibody/magnetic bead mixture to 3 mL of chromatin, add 15 uL of protease inhibitor if chromatin is not freshly prepared.
5. Gently mix overnight on rotating or rocker platform at 4°C.

E. Immunoprecipitate Washes and Crosslink Reversal

IMPORTANT: Perform the following steps in a 4°C cold room or on ice.

1. Transfer half the volume of the immunoprecipitate to a pre-chilled microfuge tube.
2. Allow tube to sit in magnetic separation rack for 1 minute to collect the beads to the side of the tube. Carefully remove the supernatant without disturbing the beads.
3. Add the remaining immunoprecipitation sample to the tube, allow to sit in magnetic separation rack for 1 minute. Carefully remove supernatant without disturbing beads.
4. Add 1 mL of chilled RIPA Buffer to each tube. Remove tubes from magnetic separation rack and gently shake or agitate tube to re-suspend beads. Replace tubes in magnetic device to collect beads. Remove supernatant.
5. Wash the beads seven additional times with chilled RIPA buffer.
6. Wash once with 1 mL of chilled TE. Allow beads to separate in magnetic rack for 1 minute. Carefully remove supernatant without disturbing beads.
7. Spin the tubes for 3 minutes at 3000 rpm, place tube in the magnetic rack, and remove residual TE with a pipette.
8. Add 210 µL of ChIP Elution Buffer and re-suspend beads.
9. Elute by placing the tubes in a thermomixer at 65°C for 20 minutes with constant shaking.

10. Spin down the beads at maximum speed in a microcentrifuge (~14,000 rpm) for 1 minute at room temperature.
11. Remove 200 µL of supernatant and transfer it to a new 1.5 mL microfuge tube.
12. Reverse the formaldehyde crosslinks by incubating in a water bath at 65°C for 12-15 hrs.
13. Thaw 50 µL of input sample reserved after sonication (see **Section C,** step 8), add 3 volumes (150 µL) of ChIP Elution Buffer and mix. Reverse the crosslinks by incubating in a water bath at 65°C for 12-15 hrs.

F. DNA Extraction and Isolation

1. Add 200 µL of TE to each tube of IP sample and input DNA sample prepared in the previous section (**Section E** – Immunoprecipitate Washes and Cross-link Reversal).
2. Add 8 µL of 10 mg/mL RNase A.
3. Mix and incubate in a water bath for 2 hours at 37°C.
4. Add 8 µL of 10 mg/mL proteinase K to each sample.
5. Mix and incubate in a water bath at 55°C for 30 minutes.
6. Spin down gel in Phase Lock tubes for 30 sec at 14,000 rpm.
7. Put samples in gel phase lock tube. Add 400 µL of buffer saturated phenol and carefully mix the sample by inverting the tube for several times. Spin for 5 min at 14,000 rpm (phenol goes below phase lock gel). Repeat phenol extraction once using the same tube.
8. Add 400 µL of chloroform/isoamyl alcohol and carefully mix by inverting the tube several times. Spin for 5 min @ 14,000 rpm, transfer the top (aqueous) layer to a new tube.
9. Add 16 µL of 5M NaCl, 1.5 µL of 20 µg/µL glycogen and 880 µL of 100% chilled EtOH. Mix well by inverting tube several times.
10. Incubate at -80°C for 30 minutes.
11. Spin down the sample at maximum speed in a microcentrifuge (>14,000 rpm) for 15 minutes at 4°C.
12. Wash the pellets with 500 µL of chilled 70% EtOH. Spin at 14,000 rpm for 5 minutes at 4°C.
13. Air dry pellet for 10 minutes (pellet will turn clear) and re-suspend pellet in 70 µL of 10 mM Tris-HCl, pH 8.0.
14. Save 15 µL of each ChIP sample for analysis.
15. Measure the DNA concentration of input sample with NanoDrop (Thermo-Fisher) or other low volume spectrophotometer and dilute the input sample DNA to 100 ng/µL. Load 0.5 µg of input sample to a 2% agarose gel and checking chromatin size visually.

    - Chromatin of good quality typically shows a smear between 200-1000 bp.
    - If you do not see good quality chromatin, it is strongly suggested that you repeat the chromatin preparation steps to optimize isolation and shearing conditions.

      Figure A shows input sample from a typical chromatin preparation.

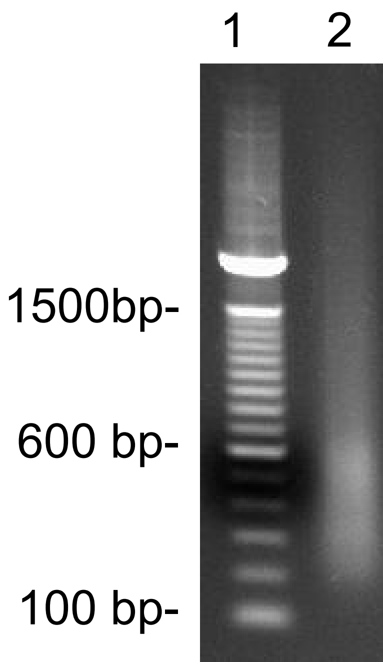


Figure A:
Sheared chromatin from formaldehyde-crosslinked HeLa cells was prepared by following this protocol (Magna ChIP² ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit). Sonication was performed using Misonix 3000 at the following condition: power output at 7 and 8 cycles of 30 seconds ON and 60 seconds OFF. 0.75 µg sheared chromatin (lane 2) was then electrophoresed through a 1.2% 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.

16. Perform qPCR using primers surrounding a known target to validate that the ChIP reaction was successful.
    - Materials for this step are not provided in the Magna ChIP² kits. However, it is strongly recommended that this quality check be done for every ChIP-chip sample. It is essential to validate ChIP quality prior to any further steps. Quality of the chromatin immunoprecipitation should be monitored by qPCR assay to examine the relative enrichment of a known target in the ChIP sample compared to a mock IgG sample or negative reference amplicon (**Figure B**). Preferably, a set of negative primers (targeting a known negative region, such as gene desert) could be used as internal reference to show that a known target is enriched in the ChIP sample relative to the input. Acceptable enrichment is empirical for each ChIP experiment, but in general, the ∆Ct value should be greater than 2.5 or approximately 5 fold enrichment for qPCR primer pairs of high efficiency.

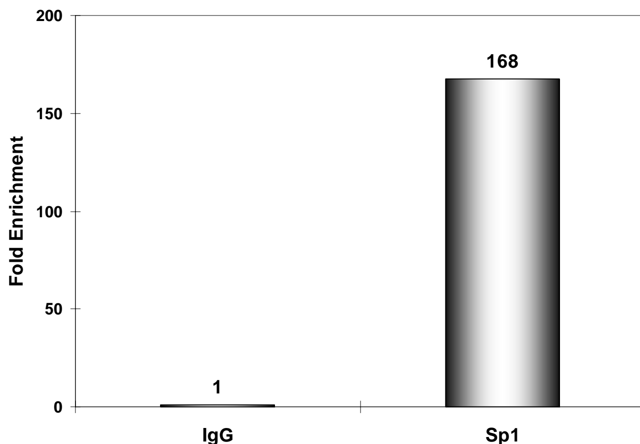


Figure B: Sp1 ChIP pre-amplification fold enrichment. Chromatin immunoprecipitation was performed according to this protocol (Magna ChIP² ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit) using HeLa S3 chromatin and either anti-Sp1 (Millipore cat. # 17-601) or Normal Rabbit IgG (Millipore cat. # PP64B).

1 µL of Sp1 ChIP sample or IgG ChIP sample was used to perform qPCR with 2 µL of 5 µM primers, targeting human DHFR promoter, in a 20 µL reaction. Fold Enrichment is expressed as the ratio of Sp1 signal to IgG signal calculated by extrapolation from a standard curve of Input DNA dilutions.

G. Blunt the DNA ends

IMPORTANT: Perform the following steps with samples on ice.

1. Put 1 µL (100 ng) of input sample DNA into a LPCR tube and add 54 µL ddH_2O. Set up one input sample reaction for each IP sample to be processed.
2. Put 55 µL of each IP sample into separate PCR tubes on ice.
3. Make blunting mix on ice (55 µL of mix per reaction).

Table 1 : Blunting Mix

Stock	1X Mix	Final Concentration[2]
10X Blunting Buffer	11.0 µL	1x
100X BSA	0.5 µL	50 ng/µL
10 mM each dNTP	1.1 µL	100 µM
T4 DNA polymerase	0.2 µL	6 U/mL
ddH_2O	42.2 µL	
Total	55 µL	

4. Add 55 µL of blunting mix to all samples.
5. Incubate for 20 minutes at 12°C in a thermal cycler.
6. Transfer samples to 1.5 mL microfuge tubes, and place tubes on ice.
7. Add 11.5 µL of cold 3 M sodium acetate and 1 µL of 20 µg/µL glycogen to the sample. Keep on ice.
8. Add an equal volume (120 µL) of chilled phenol:chloroform:isoamyl alcohol to the sample. Vortex.
9. Prepare one Phase Lock Gel tube for each IP and input sample by spinning the tube at 14,000 rpm at room temperature for 30 seconds.
10. Transfer the sample to the Phase Lock Gel tube.
11. Spin in a centrifuge at 14,000 rpm for 5 minutes at room temperature.
12. Transfer the aqueous layer to a 1.5 mL microfuge tube.
13. Add 250 µL of 100% EtOH.
14. Incubate at -80°C for 30 minutes.
15. Spin at 14,000 rpm for 15 minutes at 4°C to pellet the DNA.
16. Wash the pellets with 500 µL of chilled 70% EtOH. Spin at 14,000 rpm for 5 minutes at 4°C
17. Air dry pellet for 10 minutes (pellet will turn clear) and re-suspend each pellet in 25 µL H_2O. Keep on ice.

[2] The Final Concentration is the reagent concentration in the final reaction and *not the master mix*

H. Ligate the Blunt-end DNA

1. Make ligase mix on ice (25 µL of mix per reaction).
2. Add 25 µL of ligase mix to 25 µL of sample.

Table 2: Ligase Mix

Stock	1X Mix	Final Concentration[3]
10x ligase buffer	5.0 µL	1x
15 µM linkers	6.7 µL	2 µM
T4 DNA ligase	0.5 µL	4 U/µL
ddH_2O	12.8 µL	
Total	25.0 µL	

3. Incubate overnight at 16°C.
4. Precipitate ligated DNA. Add 6 µL of 3 M sodium acetate and 130 µL of 100% EtOH.
5. Incubate at -80°C for 30 minutes.
6. Spin at 14,000 rpm for 15 minutes at 4°C to pellet the DNA.
7. Wash the pellets with 500 µL of chilled 70% EtOH. Spin at 14,000 rpm for 5 minutes at 4°C
8. Air dry pellet for 10 minutes (pellet will turn clear) and re-suspend each pellet in 25 µL of H_2O.

I. Amplify the IP and Input Samples

NOTE: PCR amplification is very sensitive to DNA contaminants. This requires that care is taken to prevent contamination of stock solutions and reactions with DNA not contained in your chromatin preparation. Below are a few guidelines to help reduce the potential for cross-contamination.

- Set up all reaction mixtures in an area separate from that used for chromatin isolation or PCR amplified product analysis. Some laboratories have a laminar flow hood with a UV light where the PCR master mix is prepared and taken to the template addition area.
- Use disposable tips containing aerosol filters to minimize the potential cross-contamination via pipetting.
- When pipetting, slowly draw in and expel liquids to avoid the creation of aerosols.
- If possible use a separate set of pipets for the PCR master mix. Having a dedicated set of pipettes at specific benches for specific uses is suggested.

[3] The Final Concentration is the reagent concentration in the final reaction and *not the master mix*

- Use a fresh set of gloves to set up reactions.
- Avoid opening tubes containing amplified material in areas where reaction mixes are set-up.
- If contamination is suspected, run a no template control reaction (buffers, primers, dNTP's and enzymes) to see if there is an amplified product.

There are two different amplification protocols in this section. Which one to use is dependent on how much amplified material is required for labeling and hybridization on the user supplied microarray platform. If you are unsure of how much material is required, Amplification option 2 is recommended.

Amplification Option 1: Used if less than 4 µg of amplified DNA is required for labeling and hybridization. This protocol uses a single round of amplification.

Amplification Option 2: Used if more than 4 µg of amplified DNA is required for labeling and hybridization. This protocol enables large-scale amplification of IP and input samples. After 15 cycles of PCR-based amplification, the reaction is diluted and used as a template for a second round of 25 cycles. Remaining templates can be stored long-term at -20°C.

Protocol for Amplification Option 1:

Less than 4 µg of amplified DNA required for labeling and hybridization.

1. Pipette 25 µL each of IP and input sample DNA into separate PCR tubes (0.2 to 0.5 mL).
2. Using reagents and volumes indicated in **Table 3** make sufficient PCR Mix A for all samples to be amplified. To ensure sufficient material make 10-20% more than required for all reactions. Add 15 µL of PCR Mix A to each tube.

***IMPORTANT**: If using Affymetrix arrays use 10 mM dNTP (+dUTP) Mix instead of 10 mM dNTP mix

Table 3: PCR Mix A

Stock	1X Mix	Final Concentration[4]
10X PCR Buffer	4.00 µL	1x
10 mM dNTP mix*	1.25 µL	250 µM
40 uM Amplification Primer	1.25 µL	1 µM
ddH_2O	8.5 µL	
Total	15.00 µL	

3. Prepare PCR Mix B as outlined in **Table 4**. Set aside for use in step 5.
4. Program a thermocycler as outlined in step 6. Place the PCR tubes in a thermocycler, start the program below. After approximately halfway through step1, pause the program.
5. Add 10 µL of PCR Mix B and mix by gently pipetting up and down.

Table 4: PCR Mix B

Stock	1X Mix	Final Concentration[5]
10X PCR Buffer	1.0 µL	1x
Taq DNA polymerase	1 µL	0.1 U/µL
ddH_2O	8 µL	
Total	10.0 µL	

6. Continue the program:

   Step 1: 55°C for 2 minutes

   Step 2: 72°C for 5 minutes

   Step 3: 95°C for 2 minutes

   Step 4: 95°C for 1 minute

   Step 5: 60°C for 1 minute

   Step 6: 72°C for 1 minute

   Step 7: Go to step 4 for 22 times

   Step 8: 72°C for 5 minutes

   Step 9: 15°C HOLD

7. Use spin columns included with the kit to purify amplified DNA, as follows:
   a. Combine 250 µL of Bind Reagent A to a microfuge tube, add amplified sample and mix well.
   b. Transfer the sample to the DNA Purification Column.
   c. Centrifuge for 30 seconds at 14,000 rpm.
   d. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
   e. Put the Spin Filter back into the same Collection Tube.
   f. Add 500 µL of Wash Reagent B to the Column in Collection Tube.

      Note: Wash Reagent B comes pre-diluted with alcohol so no alcohol addition is necessary.

   g. Centrifuge for 30 seconds at 14,000 rpm.

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[4] This is not the reagent concentration in the master mix. *The 1X value given is the concentration in the final reaction.*

[5] The Final Concentration is the reagent concentration in the final reaction and *not the master mix*

h. Remove the Column from the Collection Tube, save the Collection Tube and discard the liquid.

i. Repeat step f-h for an additional wash.

j. Put the Column back into the same Collection Tube.

k. Centrifuge for 30 seconds at 14,000 rpm.

Note: It is important be sure to remove residual wash buffer at this step since residual buffer can interfere with efficiency of elution and potentially interfere with the labeling reaction.

l. Discard the Collection Tube and liquid.

m. Put the Column into a clean Collection Tube.

n. Add 60 µL of DNA Elution Buffer directly onto the center of the Column's white filter membrane. Wet entire membrane and allow at ~10 seconds for elution buffer to diffuse throughout membrane.

o. Centrifuge for 30 seconds at 14,000 rpm.

p. Remove and discard Column. Eluate contains purified DNA. This material can be analyzed immediately or stored frozen at -20°C.

8. Determine total yield using A_{260} measurements using low volume spectrophotometer, then perform optional qPCR using primers surrounding a known target to confirm that amplification did not negatively influence the original enrichment profile of the ChIP sample.

- In most cases, the yield from one step LM-PCR should be greater than 4 µg.
- The quality of LM-PCR amplified sample is essential for labeling and hybridization. For optimal labeling and hybridizing results, LM-PCR amplified samples should have a $A_{260}/A_{280} > 1.7$ and a $A_{260}/A_{230} > 1.6$.
- Using user provided primers to a DNA sequence known to be bound by protein of interest, qPCR validate the amplified sample is enriched for this target relative to the input sample. Although this step can be considered optional, it is strongly recommended the quality of the material be evaluated before moving on to the labeling and hybridizing steps (**Figure C**).

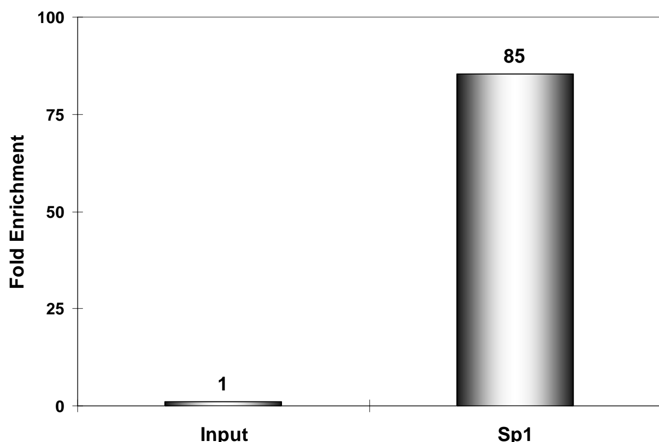

Figure C: Sp1 ChIP post-amplification mass normalized fold enrichment. Chromatin immunoprecipitation was performed according to this protocol (Magna ChIP^2 Chromatin Immunoprecipitation DNA Microarray Universal Kit) using HeLa S3 chromatin and either anti-Sp1 (Millipore cat. # 17-601). 50 ng of LM-PCR amplified Sp1 ChIP DNA or input DNA was used to perform qPCR with 2 µL of 5 µM primers, targeting human DHFR promoter, in a 20 µL reaction. Fold Enrichment is expressed as the ratio of Sp1 signal to input signal calculated by extrapolation from a standard curve of input DNA dilutions.

Protocol for Amplification Option 2:

Use when more than 4 µg of amplified DNA is required for labeling and hybridization.

Note: Perform reaction set-up steps on ice.

1. Pipette 25 µL each of IP and input sample DNA into separate PCR tubes (0.2 to 0.5 mL).
2. Using reagents and volumes indicated in **Table 3**, make sufficient PCR Mix A for all samples to be amplified. To ensure sufficient material, make 10-20% more than required for all reactions. Add 15 µL of PCR Mix A to each sample.

   ***IMPORTANT:** If using Affymetrix arrays use 10 mM dNTP (+dUTP) Mix instead of 10 mM dNTP mix.
3. Prepare PCR Mix B as outlined in **Table 4**. Set aside for use in step 5.
4. Program a thermocycler as outlined in step 6. Place the PCR tubes in a thermocycler, start the program. Approximately halfway through step 1, pause the program.
5. Add 10 µL of PCR Mix B and mix gently by pipetting up and down.
6. Continue the program:

   Step 1: 55°C for 2 minutes

   Step 2: 72°C for 5 minutes

   Step 3: 95°C for 2 minutes

   Step 4: 95°C for 1 minute

   Step 5: 60°C for 1 minute

   Step 6: 72°C for 1 minute

   Step 7: Go to step 4 for 14 times

   Step 8: 72°C for 5 minutes

   Step 9: 15°C HOLD
7. Transfer the material amplified in step 6 to a 1.5 mL microfuge tube and add 475 µL ddH_20 (total volume approximately 525 µL).
8. Set up re-amplification reaction by pipetting 5 µL of diluted PCR product into a PCR tube (0.2 to 0.5 mL).
9. Make a sufficient amount of PCR Mix C using volumes indicated in Table 5. To ensure sufficient material for all tubes make 10-20% more PCR Mix C than required for all reactions.
10. Add 45 µL of PCR mix C to each reaction tube.

Table 5: PCR Mix C

Stock	1X Mix	Final Concentration[6]
10X PCR Buffer	5.00 µL	1x
10 mM dNTP	1.25 µL	250 µM
40 uM Amplification Primer	1.25 µL	1 µM
Taq DNA polymerase	0.5 µL	0.05 U/µL
ddH_2O	37 µL	
Total	45.00 µL	

11. Run the LM-PCR program below in a thermocycler
    - Step 1: 95°C for 2 minutes
    - Step 2: 95°C for 30 seconds
    - Step 3: 60°C for 30 seconds
    - Step 4: 72°C for 1 minute
    - Step 5: Go to Step 2 for 24 times
    - Step 6: 72°C for 5 minutes
    - Step 7: 15°C HOLD
12. Use spin columns included with the kit to purify amplified DNA, as flows:
    a. Combine 250 µL of Bind Reagent A to a microfuge tube add amplified sample and mix well.
    b. Transfer the sample to the DNA Purification Column.
    c. Centrifuge for 30 seconds at 14,000 rpm.
    d. Remove the Spin Filter from the Collection Tube, save the Collection Tube and discard the liquid.
    e. Put the Spin Filter back into the same Collection Tube.
    f. Add 500 µL of Wash Reagent B to the Column in Collection Tube.
    g. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.
    h. Remove the Column from the Collection Tube, save the Collection Tube and discard the liquid.
    i. Put the Column back into the same Collection Tube.
    j. Repeat step f-h for an additional wash.

[6] The Final Concentration is the reagent concentration in the final reaction and *not the master mix*

k. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.

Note: It is important be sure to remove residual wash buffer at this step since residual buffer can interfere with efficiency of elution and potentially interfere with labeling reaction.

l. Discard the Collection Tube and liquid.

m. Put the Column into a clean Collection Tube.

n. Add 60 µL of DNA Elution Buffer directly onto the center of the Column's white filter membrane. Wet entire membrane and allow at ~10 seconds for elution buffer to diffuse throughout membrane.

o. Centrifuge for 30 seconds at a minimum of 10,000 x g, but not exceeding 15,000 x g.

p. Remove and discard Column. Eluate contains purified DNA. This material can be analyzed immediately or stored frozen at -20°C.

13. Determine total yield using A260 measurements using low volume spectrophotometer, then perform optional qPCR using primers surrounding a known target to validate that amplification did not negatively influence the original enrichment profile of the ChIP sample.

- In most cases, the yield from one step LM-PCR should be greater than 4 µg.
- The quality of LM-PCR amplified sample is essential for labeling and hybridization. For optimal labeling and hybridizing results, LM-PCR amplified samples should have a $A_{260}/A_{280} > 1.7$ and a $A_{260}/A_{230} > 1.6$.
- Using user provided primers to a DNA sequence known to be bound by the protein of interest, qPCR validate that the amplified sample is enriched for this target relative to the input sample. Although this step can be considered optional, it is **strongly recommended** the quality of the material be evaluated before moving on to the labeling and hybridizing steps (**Figure C**).

J. Label the Amplified IP and Input Samples, and Hybridize to Microarray

Please refer to protocols and guidelines provided by your array provider for specific details. Reagents for labeling and hybridization are not included in the Magna ChIP^2 Universal Kit. If a kit containing all reagents for the complete ChIP-chip workflow is desired you may want to consider either the Magna ChIP^2 Human Promoter Array Kit (cat. # 17-1001) or the Magna ChIP^2 Mouse Promoter Array Kit (cat. # 17-1002). These kits contain materials for all steps of the ChIP-chip workflow (chromatin IP through hybridization), including three array sets (six slides) for either human or mouse promoters. Please contact www.millipore.com, your local Millipore representative or local office for additional details.

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Examples of Magna ChIP^2 Universal DNA Microarray Array Kit Performance on Three Microarray Platforms

The figures below demonstrate the performance of samples generated using the Magna ChIP^2 Universal kit with various antibodies applied to the Agilent 244K Human Promoter arrays (Agilent P/N G4489A), Affymetrix GeneChip® Human Promoter 1.0R Arrays, and Nimblegen Human RefSeq promoter arrays.

Figure D:

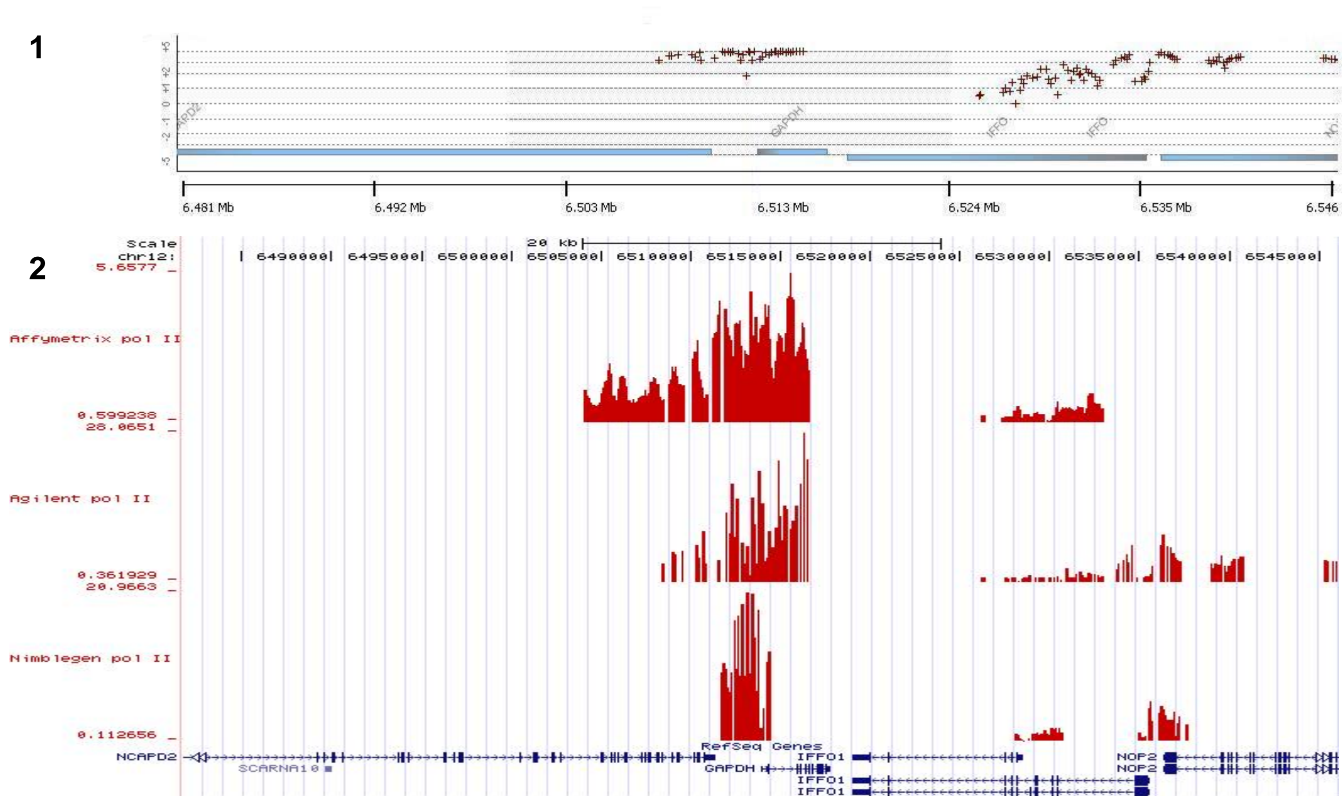

Figure D: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against RNA polymerase II (Millipore cat. # 17-620 and # 05-623) using the Magna ChIP^{2} ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent human 244K promoter array. The array was scanned using the Agilent scanner. The image was extracted using the Agilent Feature Extraction software and analyzed using DNA Analytics 4.0 software (**Figure D1**).
The Agilent dataset was further filtered and visualized using the UCSC genome browser (http://genome.ucsc.edu/) (**Figure D2**: middle panel). Similarly, replicate samples were also labeled and hybridized to the Affymetrix human promoter array (**Figure D2**: top panel) and Nimblegen Human promoter array (**Figure D2**: bottom panel).
Our data showed clear enrichment of RNA polymerase II at the promoter of the GAPDH gene, which is highly expressed and abundantly transcribed in HeLa and many other cell lines and tissues. Our data also showed the enrichment at the GAPDH is independent of the array platform.

Figure E:

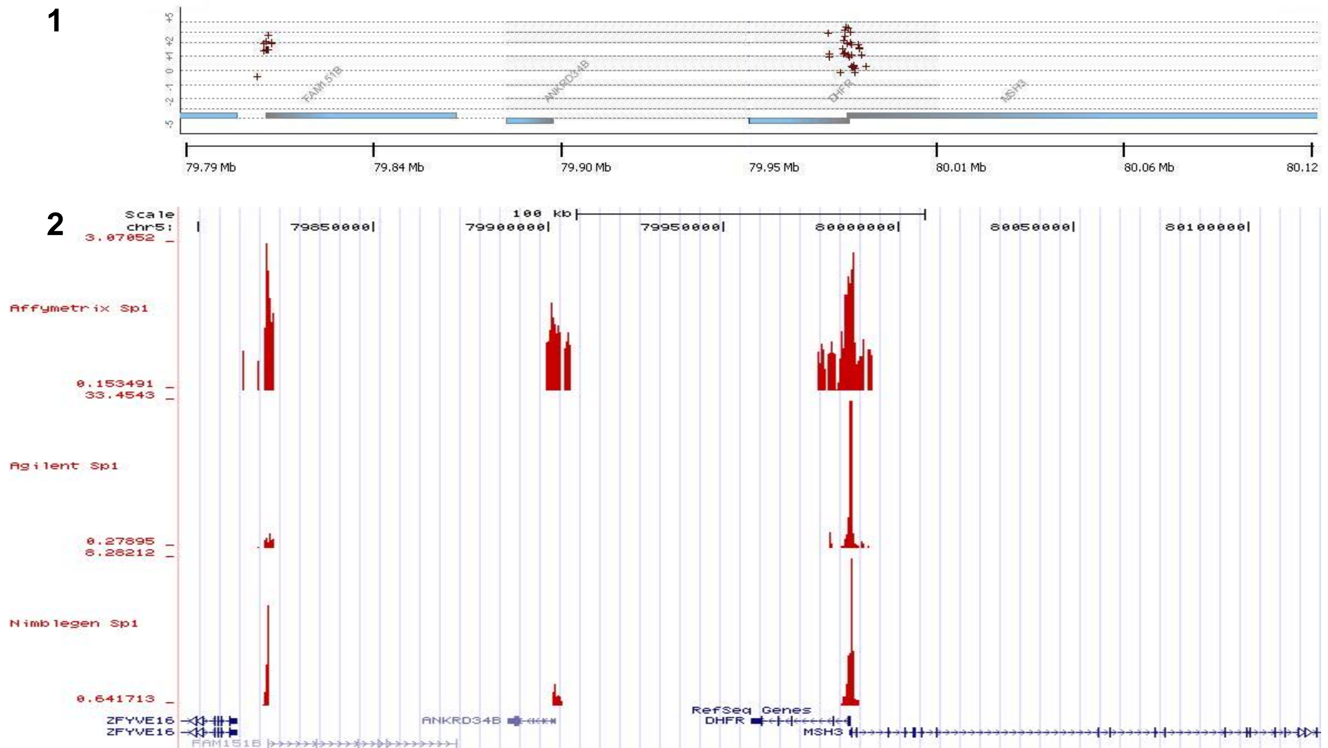

Figure E: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against human transcription factor Sp1 (Millipore Cat. # 17-601) using the Magna ChIP^{2} ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent human 244K promoter array. The array was scanned using the Agilent scanner. The image was extracted using the Agilent Feature Extraction software and analyzed using DNA Analytics software (**Figure E1**).
The data was further filtered and visualized using the UCSC genome browser (http://genome.ucsc.edu/) (**Figure E2**: middle panel). Similarly, replicate samples were also labeled and hybridized to the Affymetrix human promoter array (**Figure E2**: top panel) and Nimblegen human promoter array (**Figure E2**: bottom panel).
Our data showed clear enrichment of Sp1 at the transcription start site of Dihydrofolate Reductase (DHFR), one of the Sp1 target genes. Our data also showed the enrichment at the DHFR is independent of the array platform.

Figure F:

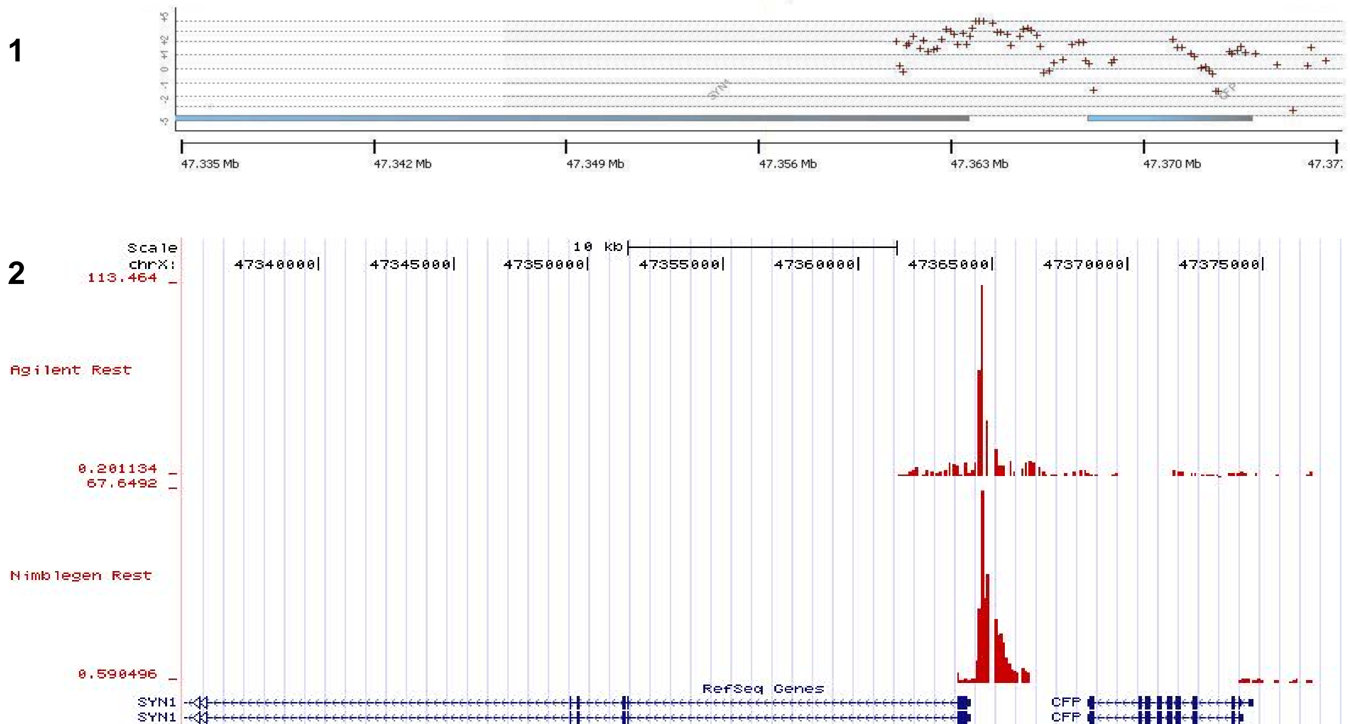

Figure F: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against human transcription factor Rest (Millipore Cat. # 17-601) using the Magna ChIP^{2} ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent human 244K promoter array. The array was scanned using the Agilent scanner. The image was extracted using the Agilent Feature Extraction software and analyzed using DNA Analytics software (**Figure F1**).
The data was further filtered and visualized using the UCSC genome browser (http://genome.ucsc.edu/) (**Figure F2**: Top panel). Similarly, replicate samples were also labeled and hybridized to the Nimblegen human promoter array (**Figure F2**: bottom panel).
Our data showed clear enrichment of Rest at the transcription start site of synapsin I (SYN1), one of the Rest target genes. Our data also showed the enrichment at the SYN1 is independent of the array platform.

CHROMATIN IP OPTIMIZATION AND TROUBLESHOOTING

Experimental Process	Problem	Experimental Suggestions
Crosslinking	Not enough or too much crosslinking	A general guideline for formaldehyde crosslinking of adherent cells is 1% formaldehyde at room temperature for 10 minutes and this condition has proven effective for many published ChIP assays. The time of fixation may be increased to 15 minutes depending upon the sample type (i.e. suspension cells or tissue) and type of molecular interaction being assessed. In order to optimize crosslinking it is useful to perform optimization experiments on small scale chromatin samples to reduce the requirement for higher ChIP-chip cell equivalent requirements. Optimization can be assessed by standard qPCR ChIP experiments to attain highest immunoprecipitation signal and the lowest noise or background signal as assessed by control IgG or reference amplicon signal. Native ChIP (without crosslinking) may also be considered for high affinity proteins such as histones (see reference section).
	How many cells is required for one IP	The amount of cells required for one ChIP experiment is determined by cell type, abundance of protein of interested and antibody quality. We recommend using 0.5-1 X 10^8 cells per ChIP. In some cases, when using high quality antibodies against abundant epitopes such as RNA Polymerase II and histone molecules, significantly fewer cell equivalents of chromatin (i.e 10^6-10^7 cell equivalents) can yield successful results. The success of the experiment will depend on the user defined threshold of difference between immunoprecipitation and input signal.
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	Sonication parameters can be instrument, cell type, and cell number dependent and can also be affected by length of crosslinking time. Sonication for a given cell type can be optimized on small scale with respect to power and cycle number and the volume increased to the guidelines suggested in this manual for higher numbers of cell equivalents required for ChIP-chip. Success of sonication can also be affected by cell density (cell equivalents/volume lysis buffer), and success should be assessed visually using gel electrophoresis of fragmented DNA following protein removal and extraction.
	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.
ChIP	ChIP yield is low	Typically DNA from even large scale ChIP reactions is not detectable using standard NanoDrop or bioanalyzer analysis methods. If ChIP DNA fails to show enrichment in standard qPCR assessment or following LM-PCR amplification, consider using more chromatin or an alternate antibody validated for ChIP (www.millipore.com/antibodies). Use of maximum recovery Eppendof tubes may also increase yield of ChIP DNA.
	Antibody doesn't recognize protein in fixed chromatin	Use ChIP-qualified antibodies when possible. If ChIP-qualified antibodies are not available, antibodies directed against different epitopes of the same protein may be screened for acceptable ChIP activity. Decreasing the time of fixation may also improve ChIP results.
	How much antibody is required per ChIP?	The amount of antibodies for one ChIP experiment is determined by the abundance of the protein of interest and antibody quality and affinity for the target epitope. We recommend using 10 µg of purified antibody per ChIP for transcription factors. It is possible to use less antibody for certain high affinity antibodies and abundant epitopes. If antibodies are supplied in non-purified formats, pre-binding of anti-sera as recommended in this protocol can allow for larger volumes to be used to pre-load the magnetic beads. The capacity of the magnetic beads is approximately 20-30 µg per 100 µL bead slurry.
	Not enough or too much chromatin	Generally speaking too much chromatin is not a concern for ChIP-chip enrichment experiments. Insufficient quantity of input chromatin can be assessed by qPCR of the initial ChIP reaction on a known occupied target vs. reference amplicon.
	Not enough beads	The magnetic beads settle to the bottom of the tube over time. Make sure the magnetic beads are well mixed prior to removing the appropriate volume for IP.
	Antibody Class or Isotype	The immunoprecipitation beads supplied are a mixture of magnetic protein A and magnetic protein G conjugated particles, so isotype specificity of this mixture enables capture of most species of IgG molecules. Capture of IgM and various other Ig isotypes are not recommended with Protein A/G beads. Consult http://www.millipore.com/immunodetection/id3/affinitypurification for additional class and isotype specificity information.

Amplification by LM-PCR	Amplification yield is low	Failure in ligation of adaptors is indicated by failure of material to amplify in the PCR step and inability to maintain enrichment observed following primary ChIP assessment. There are sufficient reagents provided in the Amplification Module to allow multiple LM-PCR reactions. Inefficient ligation of ChIP product could result from inefficient shearing of chromatin resulting in higher than normal molecular weight species following ChIP. If both Input and ChIP samples exhibit yield problems, this may be the case. If only the ChIP sample shows low yield following LM-PCR amplification, more chromatin may be required to achieve adequate adaptor ligation.
	Abnormal A_{260}/A_{280} or A_{260}/A_{230} ratio	Abnormal A_{260}/A_{280} or A_{260}/A_{230} ratios generally indicate contamination of the sample with residual alcohol or other chemicals carrying over from purification of the samples using the DNA purification materials. Ensure that sufficient washes of the columns are employed as instructed, and make certain columns are spun dry following washing and prior to elution of the purified DNA. Abnormal A_{230} peaks in spectrophotometric readings can be caused by residual guanidine HCl or carbohydrates that carry through the purification reaction. Samples that have this contamination can generally be re-purified using additional purification columns in the kit, which are provided in excess.

References

1. **Genome-wide location and function of DNA binding proteins**. Ren B, Robert F, Wyrick JJ, Aparicio O, Jennings EG, Simon I, Zeitlinger J, Schreiber J, Hannett N, Kanin E, Volkert TL, Wilson CJ, Bell SP, Young RA. Science. 2000 Dec 22;290(5500):2306-9.

2. **ChIP-chip for genome-wide analysis of protein binding in mammalian cells.** Kim TH, Barrera LO, Ren B. Curr Protoc Mol Biol. 2007 Jul;Chapter 21:Unit 21.13.

3. **Chromatin immunoprecipitation assay.** Das PM, Ramachandran K, vanWert J, Singal R. Biotechniques. 2004 Dec;37(6):961-9.

4. **Native chromatin immunoprecipitation.** Thorne AW, Myers FA, Hebbes TR. Methods Mol Biol. 2004;287:21-44.

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