Magna ChIP^{2} ™
Chromatin Immunoprecipitation
Promoter Microarray Kit

Catalog # 17-1001: Magna ChIP^{2} ™ Human Promoter 244K Microarray Kit
(6 slides/3 assays)

Catalog # 17-1002: Magna ChIP^{2} ™ Mouse Promoter 244K Microarray Kit
(6 slides/3 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 crosslinked 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 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$^{2\ TM}$ chromatin immunoprecipitation promoter microarray kit is designed to facilitate the use of ChIP-chip for beginners, as well as for those experienced in the technique for use with Agilent® human or mouse promoter microarray platforms.

Using cultured cells or tissue as starting materials, the Magna ChIP^2 Promoter Microarray Kits (Cat. # 17-1001 and 17-1002) contain buffers and reagents needed to perform three chromatin immunoprecipitation comparison experiments. These kits provide optimized reagents for chromatin immunoprecipitation and amplification to yield sufficient starting material for labeling and hybridization using the provided Agilent® 244K human or mouse promoter arrays. The Agilent® 244K promoter arrays provide feature content to interrogate ~17,000 human Refseq promoters (17-1001) or ~17,000 mouse Refseq promoters (17-1002). For ChIP-chip experiments using other types of user-provided arrays, the Magna ChIP^2 Universal DNA Microarray Kits (Cat. # 17-1000 or 17-1004) are recommended.

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Kit Components

Magna ChIP^{2} ™ Promoter Microarray Kit Configurations	
Magna ChIP^{2} ™ Human Promoter Microarray Kit (3 Assays) (Cat. # 17-1001)	**Magna ChIP^{2} ™ Mouse Promoter Microarray Kit (3 Assays) (Cat. # 17-1002)**
Chromatin IP Module I MAGNA0006 (1 EA) (Store at 4°C)	Chromatin IP Module I MAGNA0006 (1 EA) (Store at 4°C)
Chromatin IP Module II MAGNA0007 (1 EA) (Store at -20°C)	Chromatin IP Module II MAGNA0007 (1 EA) (Store at -20°C)
Chromatin Amplification Module MAGNA0008 (1 EA) (Store at -20°C)	Chromatin Amplification Module MAGNA0008 (1 EA) (Store at -20°C)
Labeling Module MAGNA0009 (1 EA) Store at -20°C	Labeling Module MAGNA0009 (1 EA) Store at -20°C
Human Promoter Array Module MAGNA0010 (1 EA) Store at room temperature	Mouse Promoter Array Module MAGNA0011 (1 EA) Store at room temperature

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

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

[1] Requires addition of 37% formaldehyde before use

Amplification Module MAGNA008 Store at -20°C		
10X Blunting Buffer	CS203159	300 µL
100X BSA	CS203160	20 µL
10 mM dNTP Mix	CS203161	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

Labeling Module MAGNA0009 Store at -20°C		
Exo-Klenow	CS203192	20 µL
Labeling Random Primers	CS203191	80 µL
Cyanine 3-dUTP	CS203190	75 µL
Cyanine 5-dUTP	CS203189	75 µL
5X Labeling Reaction Buffer	CS203188	160 µL
10X Labeling dNTP Mix	CS203187	80 µL
Nuclease-free Water	CS203186	400 µL

Human Promoter/Mouse Promoter Array Module MAGNA0010/MAGNA0011 Store at room temperature		
Amicon Ultra-0.5 Filters		15 EA
Amicon Collection Tubes		30 EA
Human Promoter or Mouse Promoter 244K Array Set		3 Sets (2 Slides per Set)
Hybridization Chamber Gaskets		6 gaskets
2X Hybridization Buffer	CS203181	2 vials (1.4 mL)
10X Hybridization Blocking Agent	CS203180	1
Design File for Agilent Arrays		1 CD

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 (with or without 15% methanol)
- 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)
- Human or Mouse Cot-1 DNA (e.g. Life Technologies 15279-011 or 18440-016)
- Oligo aCGH/ChIP-on-chip Wash Buffer 1 & 2 Kit (Agilent 5188-5226)
- Acetonitrile (e.g. Baker 9017-02 or 9017-03)
- Stabilization & Drying Solution (Agilent 5185-5979) or Ozone Barrier slide cover (Agilent G2505-60550)
- 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
- 1.5 mL maximum recovery microfuge tubes (e.g. Axygen MCT-150-L-C or equivalent)
- 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 cultured cell experiment
- Agilent SureHyb Hybridization Chamber (G2534A) or other appropriate hybridization chamber
- Forceps
- Rotisserie hybridization oven
- Sterile 1000 mL bottle
- Glass slide-staining dish
- 1.5 L glass dish
- Magnetic stir plate with heating element
- Magnetic stir bar
- DNA Microarray Scanner (e.g Agilent G2565CA or equivalent microarray scanner)
- Feature Extraction Software (i.e. Agilent's Feature Extraction V 10.7) (http://www.chem.agilent.com/en-US/products/instruments/dnamicroarrays/featureextractionsoftware/)
- ChIP-chip Analysis Software (i.e. Agilent Genomic Workbench V 5.0) (http://www.chem.agilent.com/en-US/products/instruments/dnamicroarrays/dnaanalyticssoftware/)
- Seal-a-Mea® Sealing Device or Nitrogen Purge Box (for microarray slide storage)

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 (PPE) and caution when extracting samples.
- Chromatin preparation may require use of liquid nitrogen. Use personal protective equipment (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.
- Washing Agilent DNA microarrays requires the use of solutions containing acetonitrile. Use appropriate fume hoods and venting when working with acetonitrile. Acetonitrile liquid and vapor can irritate the eyes, nose, throat and lungs. Exposure to acetonitrile can cause fatal cyanide poisoning as it changes to cyanide within the body. Avoid inhalation or exposure to skin by using appropriate PPE.

Storage and Stability

MAGNA0006 Store at 4° C, good for 6 months from date of receipt when reagents are stored properly.
Please note: Some 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.

MAGNA0008 Store at -20° C, good for 6 months from date of receipt when reagents are stored properly.

MAGNA0009 Store at -20° C, good for 6 months from date of receipt when reagents are stored properly.

MAGNA0010/ MAGNA0011 Store at room temperature, 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 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 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**
   Chromatin size is critical to 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 **Figure A** on page 18 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 qPCR of the relative enrichment of a known locus, 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 an **$A_{260}/A_{280} > 1.7$** and an **$A_{260}/A_{230} > 1.6$**.

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.

4. DNA Labeling Evaluation

The amplified DNA is subject to random primed labeling using random primers and Cyanine 3-dUTP and Cyanine 5-dUTP nucleotides. Sufficient fluorophore incorporation and yield must be achieved to allow successful blending of labeled probes and subsequent hybridization. For optimal hybridization results, DNA yield should be great than 5 µg per reaction (i.e. 5 µg each sample per array) , Cyanine 5 specific activity should be greater than 2 pmol/µL and Cyanine 3 specific activity should be greater than 3 pmol/µL as determined using the microarray application on the nanodrop ND-1000 or equivalent.

5. DNA hybridization

The quality of the hybridization can affect the final result greatly. In general, a good hybridization should have low background noise, high signal intensity, high signal to noise ratio and good reproducibility. For example, when an array is scanned using an Agilent scanner, a successful hybridization should have background noise less than 10, signal intensity greater than 50, signal to noise ratio greater than 30 and reproducibility less than 0.2 for both channels (additional details in **Table A and Table B**). See Agilent document G4410-90010 CGH Enzymatic labeling manual V 6.2 for more details (www.agilent.com).

Table A. QC Metric Thresholds for Enzymatic Labeling

Metric	Excellent	Good	Poor
Background Noise	<5	5 to 10	>10
Signal Intensity	>150	50 to 150	<50
Signal to Noise Ratio	>100	30 to 100	<30
Reproducibility	<0.05	0.05 to 0.2	>0.2
DLR Spread	<0.2	0.2 to 0.3	>0.3

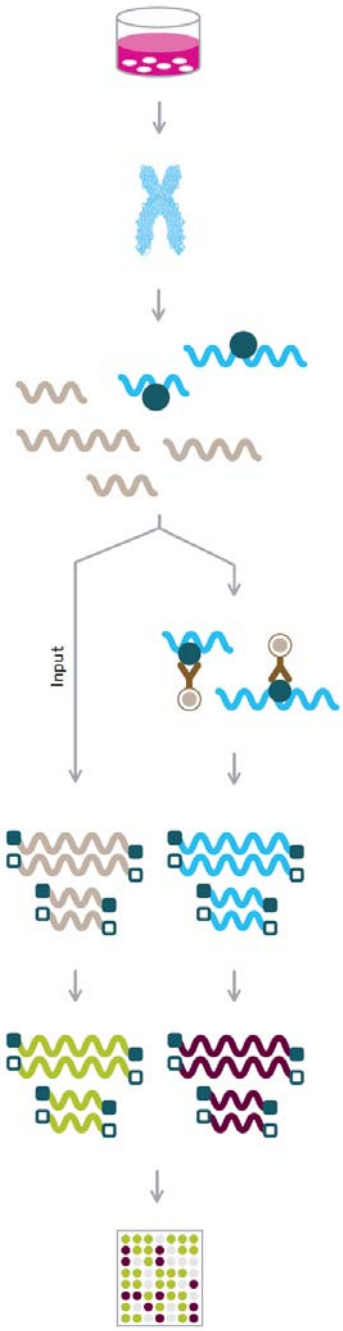
Table A: Quality control criteria for hybridization performance (see G4410-90010 CGH Enzymatic labeling manual V 6.2 for more details).

ChIP_QCM_Dec08 :

Metric Name	Value	UpLim	LowLim
AnyColorPrcntFeatNonUnifOL	0.02	NA	NA
DerivativeLR_Spread	0.71	NA	NA
gRepro	-0.01	NA	NA
g_BGNoise	2.71	NA	NA
g_Signal2Noise	54.87		
g_SignalIntensity	148.51	NA	NA
rRepro	-0.01	NA	NA
r_BGNoise	3.32	NA	NA
r_Signal2Noise	43.23	NA	NA
r_SignalIntensity	143.75	NA	NA

Table B: Example of Metrics for microarray hybridization from an Agilent Feature Extraction QC Report. Background Noise, Signal to Noise, and Signal Intensity values are reported for each dye channel.

ChIP-chip Overview and Time Management



Bind antibody to magnetic beads	**0.5 hr, then overnight**
Cross-link chromatin	**1.5 hr**
Sonicate cells to release chromatin	**1 hr**
Chromatin immunoprecipitation	**0.5 hr, then overnight**
Wash, elution, and cross-link reversal	**2 hr, then overnight**
Digestion of cellular proteins and RNA	**4 hr**
Make blunt-end, ligate linkers	**2 hr, then overnight**
Amplify isolated chromatin	**4 hr**
dUTP-Cyanine 3/d-UTP-Cyanine 5 label INPUT/IP SAMPLE	**3 hr**
Hybridize microarray	**1 hr, then 40 hr**
Wash microarray	**1 hr**

DETAILED ChIP-chip PROTOCOL

The Magna ChIP^2 Promoter Microarray Kits are intended to produce amplified chromatin immunoprecipitated material for use on the provided Agilent® 244K human or mouse promoter arrays. The reagents provided allow for a fixed number of chromatin preparations, immunoprecipitations (using user supplied antibodies), ligation-mediated PCR reactions, labeling reactions, and microarray hybridizations according to the following product specific quantities:

- Three independent chromatin preparations from approximately 10^8 cells from culture or tissue
- Three independent chromatin immunoprecipitations
- Up to twenty-four LM-PCR reactions
- Fifteen labeling reactions
- Six sample hybridization reactions (3 comparisons of 2 microarrays per comparison)

The detailed protocol below describes how to prepare samples (Input and IP) from a single chromatin preparation, immunoprecipitation and ligation mediated amplification reaction for subsequent labeling, hybridization and microarray comparisons of an input 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 any of the Millipore Magna ChIP^2 DNA Microarray Kits.
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 separation 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 or overnight. 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% crosslinking 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 crosslink 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 you 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 x 10^7 to 1 x 10^8 cells are recommended. To determine the total number of cells, perform a cell count using a hemocytometer (such as Millipore's Scepter™ Hand-held Cell Counter). 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. **This 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 x 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.
   - o Use 1/10 volume, if using more or less growth media.
3. Incubate at room temperature for 10 minutes to crosslink proteins.
   - o Agitating the cells is not necessary.
   - o 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.
   - o 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.
   - o Add chilled 1X PBS.
   - o Spin down the cells at 1,350 x g for 5 minutes at 4°C between each wash.
   - o Remove 1X PBS being careful not to disturb the cell pellet.
   - o 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.
   - o 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. Wash the pellets twice with 50 mL of chilled 1X PBS. Remove supernatant.

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 needed for shearing crosslinked DNA to ~200-1000 base pairs in length. These conditions vary with cell type, length of crosslink 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 unbound 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.
    d. Repeat steps a-c (above) two more times for a total of 3 washes.

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. Re-suspend the beads in 100 µL of Block Solution
3. Add 100 µL of antibody/magnetic bead mixture to 3 mL of chromatin, add 15 µL of protease inhibitor if chromatin is not freshly prepared.
4. Gently mix overnight on rotating or rocker platform at 4°C.

E. Immunoprecipate 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 supernatant without disturbing 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** – Immunoprecipate Washes and Crosslink 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.

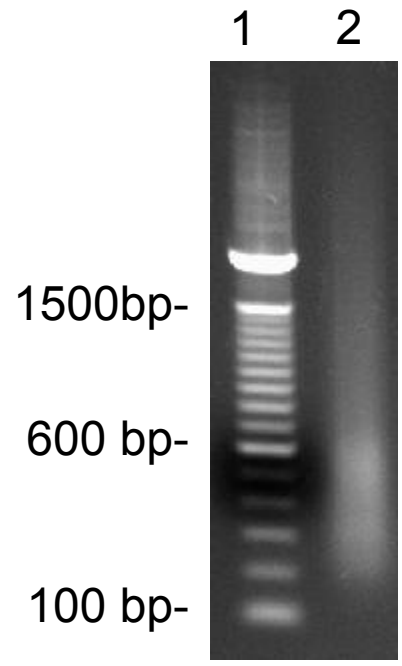


Figure A:
Sheared chromatin from formaldehyde-crosslinked HeLa S3 cells was prepared by following this protocol (Magna ChIP^{2} ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit). Sonication was performed using Misonix 3000 at the following condition: power output at 7 and 8 cycles of 30 seconds 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 ChIP^{2} 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 ΔCt value should be greater than 2.5 or approximately 5 fold enrichment for qPCR primer pairs of high efficiency.

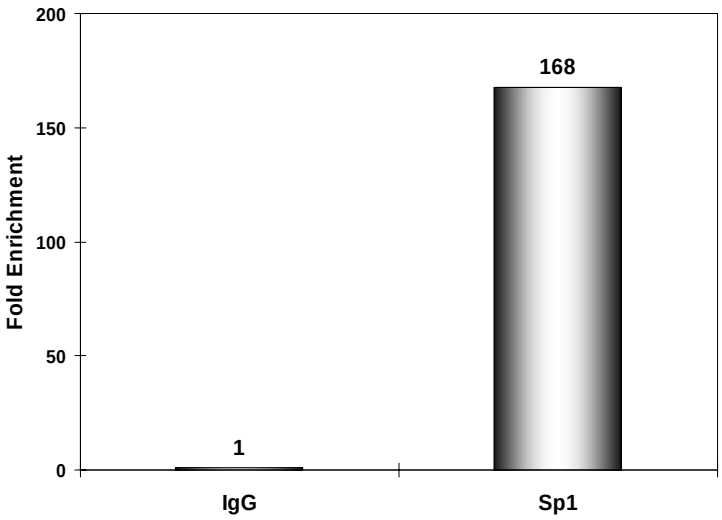


Figure B: Sp1 ChIP pre-amplification fold enrichment. Chromatin immunoprecipitation was performed according to this protocol (Magna ChIP^{2} ™ Chromatin Immunoprecipitation DNA Microarray Universal Kit) using HeLa S3 chromatin and either anti-Sp1 (Millipore Cat. # 17-601) or Normal Rabbit IgG (Millipore Cat. # PP64B).

1 µL of Sp1 ChIP sample or IgG ChIP sample was used to perform qPCR with 2 µL of 5 mM 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]
5x ligase buffer	10.0 µL	1x
15 µM linkers	6.7 µL	2 µM
T4 DNA ligase	0.5 µL	4 U/µL
ddH_2O	7.8 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.

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

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.
- 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.
- 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.

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	

Prepare PCR Mix B as outlined in **Table 4**. Set aside for use in step 5.

3. Program a thermocycler as outlined in step 6. Place the PCR tubes in a thermocycler, start the program below. **After approximately halfway through step 1, pause the program.**
4. 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	

[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*

5. 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

6. 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.
   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 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**).

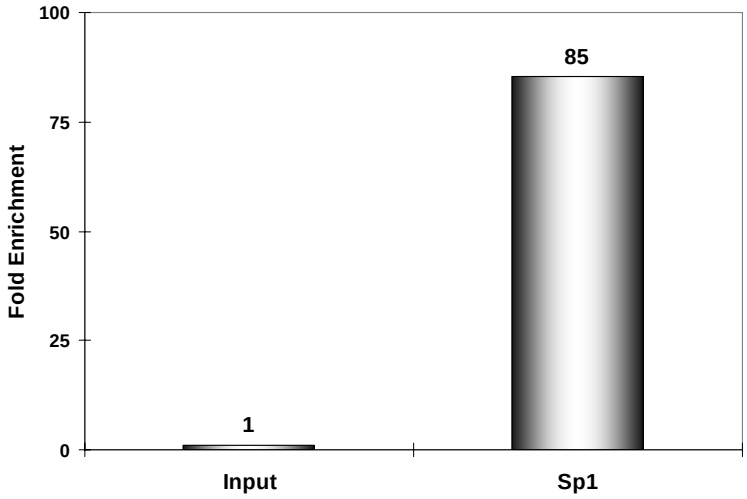


Figure C: Sp1 ChIP post-amplification mass normalized fold enrichment. Chromatin immunoprecipitation was performed according to this protocol (Magna ChIP^2 Chromatin Immunoprecipitation DNA Microarray Universal Kit) using HeLa S3 chromatin and anti-Sp1 (Millipore cat. # 17-601). 50 ng of LM-PCR amplified Sp1 ChIP DNA or input DNA was used to perform qPCR with 2 µL of 5 mM 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** below. 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	

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

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

    Note: It is important 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

This labeling procedure uses random primers and the exo-Klenow fragment to differentially label genomic DNA samples with fluorescent-labeled nucleotides (Cyanine 3-dUTP and Cyanine 5-dUTP). For the ChIP-chip application, the IP sample is labeled with one fluorescent dye while the input sample is labeled with the other. Typically the Input sample is labeled using the Cyanine 3-dUTP and the IP sample is labeled using Cyanine 5-dUTP.
The Magna ChIP^2 ™ Chromatin Immunoprecipitation DNA Microarray Kit contains two microarray slides per 244K promoter set. Each slide requires 5 µg of labeled DNA for each sample to be combined prior to hybridization. For each Input and IP sample prepared in the LM-PCR amplification section, it is necessary to perform duplicate labeling reactions to accommodate the requirement of 5 µg per slide. The protocol below describes labeling of a single sample, but the user should be prepared to label as many samples as are necessary for the number of microarray slides to be hybridized.

IMPORTANT: Cyanine 3-dUTP and Cyanine 5-dUTP are light sensitive. Minimize exposure of the labeling reactions to light throughout the procedure. To prevent degradation of these solutions avoid multiple freeze thaw cycles. Protect Cyanine 3-dUTP and Cyanine 5-dUTP stock solutions from light.

Prior to Starting this section
Equilibrate water baths to 95°C, 37°C and 65°C or prepare a thermocycler for these heating steps.

1. Place 2 µg of amplified input or IP DNA to separate 0.5 mL nuclease-free microfuge tubes labeled for IP (Cyanine 5) or Input (Cyanine 3) labeling. Add nuclease free water to give a total final volume of 26 µL.

   Note: The quantity of DNA used should be equivalent for each sample. Successful amplification can be achieved from as little as 0.5 µg starting DNA. The outcome of the labeling reaction with respect to yield and fluorophore incorporation can be influenced by the quantity and quality of starting material so it is recommended to use as much as is available up to 2 µg/reaction.
2. Add 5 µL of Labeling Random Primers to each reaction tube containing the amplified DNA to give a total final volume of 31 µL. Mix well by gently pipetting up and down.
3. Incubate at 95°C for 3 minutes to denature amplified DNA, then place tubes on ice and incubate for 5 minutes to anneal random primers.

4. While the incubation on ice is taking place, make a sufficient amount of Labeling Master Mix using volumes indicated in **Table 6**. To ensure sufficient material for all tubes, make 10% more Labeling Master Mix than required for all reactions.

Table 6：Labeling Master Mix

Stock	Volume per Reaction	Final Concentration	Amount For Master Mix*
5X Labeling Reaction Buffer	10 µL	1X	
10X Labeling dNTP Mix	5 µL	1X	
1.0 mM Cyanine 5-dUTP or Cyanine 3-dUTP	3 µL	60 µM	
Exo-Klenow	1 µL		
Total	19 µL		

* Multiply volume per reaction by number of reactions then by 1.1 to give 10% more than required

5. Pulse spin tubes containing the DNA to remove any condensation generated upon annealing and hybridization. Add 19 µL of Cyanine 5 Labeling Master Mix to each amplified IP DNA and 19 µL of Cyanine 3 Labeling Master Mix to each amplified input DNA sample to give a total volume of 50 µL. Mix well by gently pipetting up and down several times.
6. Incubate at 37°C for 2 hours.
7. Incubate at 65°C for 10 minutes to inactivate the enzyme, and then place reactions on ice.
8. Reactions can be stored at -20°C in the dark or used immediately.
9. Clean up labeled DNA by removing unincorporated primers and fluorescent nucleotides.
   a. Transfer cyanine 3 and cyanine 5 labeled samples to separate 1.5 mL microfuge tubes and add 430 µL of 1X TE (pH 8.0) to each tube.
   b. For each sample, place an Amicon Ultra-0.5 Filter into an empty 1.5-mL microfuge tube (supplied) and load labeled amplified input or IP sample into the filter. Spin 10 minutes at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
   c. Add 480 µL of 1X TE (pH 8.0) to each filter. Spin for 10 minutes at 8,000 × g at room temperature. Discard the flow-through.
      - There should be small volume of liquid (30 µL-80 µL) remaining in the filter.
   d. Invert the filter into a fresh 1.5-mL microfuge tube (supplied). Spin for 1 minute at 8,000 × g in a microcentrifuge at room temperature to collect purified sample.

      Note: Keep the filter until you are certain that you have collected enough sample for the next step.
   e. Measure and record volume (µL) of each eluate. If sample volume exceeds 80.5 µL return sample to its filter and spin 1 minute at 8,000 × g in a microcentrifuge at room temperature. Discard the flow-through.
   f. Repeat steps d and e (above) until each sample volume is ≤80.5 µL.

g. Bring total sample volume to 80.5 µL with 1X TE (pH 8.0).

10. Take 1.5 µL of each sample to determine the yield and specific activity by using the NanoDrop ND-1000 UV-VIS Spectrophotometer (using the microarray module within the NanoDrop software and select and measure double stranded DNA). For Agilent 244K microarrays, each sample should have the following characteristics:
    DNA yield > 5 µg per reaction
    Cyanine 5 > 2 pmol/µL
    Cyanine 3 > 3 pmol/µL

    If DNA yield is below 5 µg total, you may need to repeat the labeling procedure using more starting material (up to 2 µg of LM-PCR amplified DNA). Increasing the amount of DNA used in labeling may also improve the fluorophore incorporation rate. Excess labeling reactions are provided to mitigate risk of labeling reaction failure for each of these criteria.

    Labeled samples can be stored overnight at -20°C in the dark.

K. Hybridize to microarray

Please Read Before Starting This Section:
The following sections describe the steps required to hybridize and wash Agilent 244K promoter arrays. These steps outline hybridization using the Agilent SureHyb Hybridization Chamber and the hybridization gaskets provided with the kit. Supplementary detailed instructions and helpful hints for the use of the Agilent hybridization chamber and these gaskets can be found in the *Agilent G2534A Microarray Hybridization Chamber User Guide* (G2534-90002). This user guide is provided with the Agilent Microarray Hybridization Chamber Kit (G2534A) and can also be downloaded from the Agilent website (www.agilent.com). If you are not familiar with the use of this hybridization chamber or if some time has passed since you last used it, it is suggested you review these instructions in detail before proceeding. You may also wish to review the Agilent online tutorial available at:
http://www.opengenomics.com/Science/Presentations/Running_a_Microarray_Experiment
to familiarize yourself with hybridization details.

Prior to starting this section:
Equilibrate water baths or heat blocks to 95°C and 37°C to carry out the steps below.

- Prepare 10X Hybridization Blocking Agent by adding 1350 µL nuclease free water.
- Check that the Blocking agent is completely reconstituted before use. You may need to incubate at room temperature for 60 minutes to reconstitute sample before use or storage.
- The 10X Blocking Agent can be prepared in advance and stored at -20°C.

IMPORTANT: Cyanine 3 and cyanine 5 are light sensitive. Use foil and amber tubes to minimize exposure of your samples to light during the hybridization and washing steps.

1. For each DNA microarray, combine the 5 µg of each Cyanine 5-labeled sample and Cyanine 3-labeled sample; adjust the volume to 158 µL with TE.
2. Add 50 µL Human Cot-1 DNA (1.0 mg/mL), 52 µL 10 X Hybridization Blocking Agent and 260 µL 2X Hybridization Buffer to each microfuge tube, Mix contents and quick spin to collect.

3. Heat samples for 3 minutes at 95°C.
4. Immediately transfer the sample tubes to a circulating water bath or heat block at 37°C and incubate for 30 minutes.
5. Spin at 17,900 × g for 1 minute at room temperature to collect the sample.
6. Load a clean gasket slide into the Agilent SureHyb chamber base with the label facing up and aligned with the rectangular section of the chamber base. Ensure that the gasket slide is flush with the chamber base and is not misaligned.
7. Slowly dispense 490 µL hybridization sample onto the gasket well in a "drag and dispense" manner.
8. Place a microarray "active side" down onto the SureHyb gasket slide, so the numeric barcode side is facing up and the "Agilent" barcode is facing down. Verify that the sandwich-pair is properly aligned.
9. Place the SureHyb chamber cover onto the sandwiched slides and slide on the clamp assembly. Hand-tighten the clamp onto the chamber.
10. Vertically rotate the assembled chamber to wet the gasket and assess the mobility of the bubbles. Tap the assembly on a hard surface if necessary to move stationary bubbles.
11. Place assembled slide chamber in rotisserie hybridization oven set to 65°C and hybridize at 20 RPM for 40 hours.

Note: Once the sealed foil pack on microarray slides is opened, unused microarray slides should be either vacuum sealed using a seal a meal type device or otherwise stored in a nitrogen purge box. It is not recommended to store Agilent microarray slides under atmospheric conditions.

L. Wash the microarray slides

- The Agilent Oligo aCGH/ChIP-on-chip Wash Buffer 1 & 2 Kit (Agilent 5188-5226) are available separately and are required for washing Agilent arrays. Agilent's Stabilization & Drying Solution (Agilent 5185-5979) is also available separately and is recommended for to mitigate ozone dependent degradation of Cy5 fluorescent signal. For additional details go to www.agilent.com and search document number 5989-0875en for information about the effects of ozone on Cy5 signal. If preferred, the Ozone Barrier slide cover (Agilent G2505-60550) can be used to mitigate the effects of atmospheric ozone.

Prior to starting this section:

Warm the Oligo aCGH/ChIP-on-chip Wash Buffer 2 to 31°C as follows:

- Dispense 400 mL of Oligo aCGH/ChIP-on-chip Wash Buffer 2 directly into a sterile 1000 mL bottle (for up to 8 slides).
- Tightly cap the 1000 mL bottle and place in a 31°C water bath the night before washing arrays.
- Put a slide-staining dish into a 1.5 L glass dish three-fourths filled with water. Warm to 31°C by storing overnight in an incubator set to 31°C.

1. Fill slide-staining dish #1 with Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature.
2. Place a slide rack into slide-staining dish #2. Add a magnetic stir bar. Fill slide-staining dish #2 with enough Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature to cover the slide rack. Place this dish on a magnetic stir plate.
3. Remove one hybridization chamber from incubator and record time. Record whether bubbles formed during hybridization and if all bubbles are rotating freely.
4. Prepare the hybridization chamber disassembly.
   a. Place the hybridization chamber assembly on a flat surface and loosen the thumbscrew, turning counterclockwise.
   b. Slide off the clamp assembly and remove the chamber cover.
   c. With gloved fingers, remove the array-gasket sandwich from the chamber base by grabbing the slides from their ends. Keep the microarray slide numeric barcode facing up as you quickly transfer the sandwich to slide-staining dish #1.
   d. Without letting go of the slides, submerge the array-gasket sandwich into slide-staining dish #1 containing Oligo aCGH/ChIP-on-chip Wash Buffer 1.
5. With the sandwich completely submerged in Oligo aCGH/ChIP-on-chip Wash Buffer 1, pry the sandwich open from the barcode end only:
   a. Slip one of the blunt ends of the forceps between the slides.
   b. Gently turn the forceps upwards or downwards to separate the slides.
   c. Let the gasket slide drop to the bottom of the staining dish.
   d. Remove the microarray slide and place into slide rack in the slide-staining dish #2 containing Oligo aCGH/ChIP-on-chip Wash Buffer 1 at room temperature. Minimize exposure of the slide to air. **Touch only the barcode portion of the microarray slide on its edges!**
6. Repeat steps step 3 through step 5 for up to seven additional slides in the group. For uniform washing, do up to a maximum of eight disassembly procedures yielding eight microarray slides.
7. When all slides in the group are placed into the slide rack in slide-staining dish #2, stir using setting 4 for 5 minutes.
8. Approximately 1 min before the end of Wash 1, put the prewarmed 1.5 L glass dish filled with water and containing slide-staining dish #3 on a magnetic stir plate with heating element. Fill the slide-staining dish #3 approximately three-fourths full with Oligo aCGH/ChIP-on-chip Wash Buffer 2 (warmed to 31°C). Add a magnetic stir bar. Turn on the heating element and maintain temperature of Oligo aCGH/ChIP-on-chip Wash Buffer 2 at 31°C. Monitor using a thermometer.
9. Take the slide rack out of slide-staining dish #2, place in slide-staining dish #3 and stir using setting 4 for 5 minutes.
10. Slowly remove the slide rack minimizing droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.
11. If you are washing slides in an environment in which the ozone level exceeds 5 ppb, continue at “Stabilization and Drying Solution (optional)” on next section.
12. Scan slides immediately to minimize the impact of environmental oxidants on signal intensities. If necessary, store slides in orange slide boxes in a N_2 purge box, in the dark.

M. Stabilize and Dry slides (optional)

Prior to starting this section

The Agilent Stabilization and Drying Solution contains an ozone scavenging compound dissolved in acetonitrile. The compound in solution is present in saturating amounts and may precipitate from the solution under normal storage conditions. If the solution shows visible precipitation, warming of the solution will be necessary to redissolve the compound. Washing slides using Stabilization and Drying Solution showing visible precipitation will have profound adverse affects on array performance.

PRECAUTIONS AND WARNINGS:
The Agilent Stabilization and Drying Solution is a flammable liquid. Warming the solution will increase the generation of ignitable vapors. Work in a vented fume hood and use gloves and eye/face protection in every step of the warming procedures.
Do not use an open flame or a microwave. **Do not** increase temperature rapidly.
Warm and mix the material away from ignition sources.

- Warm the solution slowly in a water bath set to 37°C to 40°C in a closed container with sufficient head space to allow for expansion. Warm the solution only in a controlled and contained area that meets local fire code requirements. The time needed to completely redissolve the precipitate is dependent on the amount of precipitate present, and may require overnight warming if precipitation is heavy.
- Gently shake the container to obtain a homogenous solution.
- After the precipitate is completely dissolved, allow the solution to equilibrate to room temperature prior to use.

1. In the fume hood, fill slide-staining dish #1 approximately three-fourths full with acetonitrile. Add a magnetic stir bar and place this dish on a magnetic stir plate.
2. In the fume hood, fill slide-staining dish #2 approximately three-fourths full with Stabilization and Drying Solution. Add a magnetic stir bar and place this dish on a magnetic stir plate.
3. Immediately transfer the slide rack to slide-staining dish #1 containing acetonitrile, and stir using setting 4 for 10 seconds.
4. Transfer slide rack to slide-staining dish #2 filled with Stabilization and Drying Solution, and stir using setting 4 for 30 seconds.
5. Slowly remove the slide rack trying to minimize droplets on the slides. It should take 5 to 10 seconds to remove the slide rack.
6. Scan slides immediately to minimize impact of environmental oxidants on signal intensities. If necessary, store slides in original slide boxes in a N2 purge box, in the dark.

   Dispose of acetonitrile and Stabilization and Drying Solution as flammable solvents according to your institutes policy and local regulations.

N. Slide Scanning

Agilent microarrays can be scanned using either an Agilent scanner or other commercially available slide scanners such as the Genepix 4000B scanner. See www.agilent.com for information about other scanners that may be currently compatible with Agilent 244K microarrays.

Suggested Agilent Scanner Settings

1. Assemble the slides into an appropriate slide holder, either version B or A. Place the slides into the slide holder such that the numeric barcode side is visible (*not* the "Agilent"-labeled barcode side).
2. Place assembled slide holders into scanner carousel.
3. Verify scan settings for two-color scans.

Table 7. Scan Settings for 244K Arrays

Scan region	Scan Area (61 x 21.6 mm)
Scan resolution (µm)	5
Dye channel	Red&Green
Green PMT	100%
Red PMT	100%

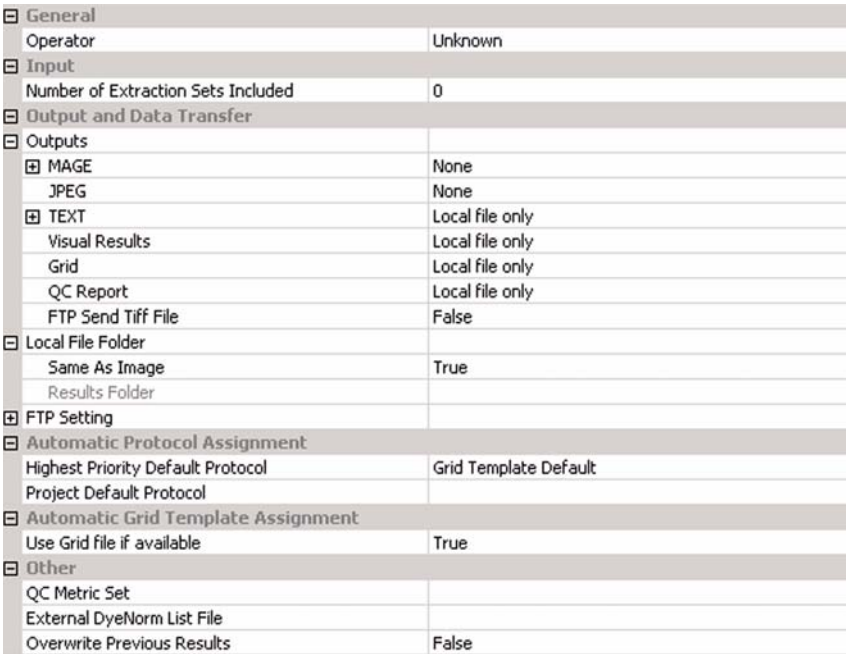
To change any settings, click **Settings > Modify Default Settings**. A window pops up from which you can change the settings.

4. Select settings for the automatic file naming
   - **Prefix 1** is set to **Instrument Serial Number**
   - **Prefix 2** is set to **Array Barcode**
5. Verify that the Scanner status in the main window says **Scanner Ready**.
6. Click **Scan Slot** *m-n* on the Scan Control main window where the letter *m* represents the Start slot where the first slide is located and the letter *n* represents the End slot where the last slide is located.

Gene Pix scanner settings

Only GenePix 4000B scanners are supported for scanning Agilent 244K microarrays.

Refer to the manufacturer's user guide for appropriate scanner settings.



⊟ General	
Operator	Unknown
⊟ Input	
Number of Extraction Sets Included	0
⊟ Output and Data Transfer	
⊟ Outputs	
⊞ MAGE	None
JPEG	None
⊞ TEXT	Local file only
Visual Results	Local file only
Grid	Local file only
QC Report	Local file only
FTP Send Tiff File	False
⊟ Local File Folder	
Same As Image	True
Results Folder	
⊞ FTP Setting	
⊟ Automatic Protocol Assignment	
Highest Priority Default Protocol	Grid Template Default
Project Default Protocol	
⊟ Automatic Grid Template Assignment	
Use Grid file if available	True
⊟ Other	
QC Metric Set	
External DyeNorm List File	
Overwrite Previous Results	False

Figure D. Default settings

4. Check the Extraction Set Configuration.
   a. Select the **Extraction Set Configuration** tab.
   b. Verify that the correct grid template is assigned to each extraction set in the **Grid Name** column. To assign a different grid template to an extraction set, select one from the pull down menu.

      A design file CD is included in the Magna ChIP^2 DNA Microarray Kit that contains the essential files that describe the microarray layout and feature design, including the grid template. This CD can be used to update the Agilent Feature Extraction software.

      If a grid template is not available to select from the pull down menu, you must add it to the Grid Template Browser. To add, right-click inside the Grid Template Browser, select **Add**. Browse for the design file (.xml) and click **Open** to load grid template into the FE database.

      To update to the latest grid templates via Online Update, right-click **Grid Template Browser** and select **Online Update**. You can also download the latest grid templates from Agilent Web site at www.agilent.com/chem/downloaddesignfiles. After downloading, you must add the grid templates to the Grid Template Browser.

      After a new grid template is added to the Grid Template Browser, remember to specify the default protocol for the new grid template if you want the Feature Extraction program to automatically assign a FE protocol to an extraction set.

c. Verify that the protocol **ChIP-v1_95_May07** is assigned to each extraction set in the **Protocol Name** column. To assign a different protocol to an extraction set, select one from the pull down menu.

   The protocols automatically distinguish the formats for processing the data.

   If a protocol is not available to select from the pull down menu, you must import it to the FE Protocol Browser. To import, right-click **FE Protocol Browser**, select **Import**. Browse for the FE protocol (.xml) and click **Open** to load the protocol into the FE database. Visit the Agilent Web site at www.agilent.com/chem/FEprotocols to download the latest protocols.

5. Save the FE Project (.fep) by selecting **File > Save As** and browse for desired location.
6. Verify that the icons for the image files in the FE Project Window no longer have a red X through them. A red X through the icon indicates that an extraction protocol was not selected. If needed, reselect the extraction protocol for that image file.
7. Select **Project > Start Extracting**.
8. After the extraction is completed successfully, view the QC report for each extraction set by double-clicking the QC Report link in the **Summary Report** tab. Determine whether the grid has been properly placed by inspecting Spot Finding at the Four Corners of the Array.

Evaluate the QC report for performance characteristics as indicated in the Agilent G4410-90010 CGH Enzymatic labeling manual V 6.2 and as indicated above in the **Tips to Help Ensure a Successful Experiment** section.

Feature extraction files can be used directly to perform further array analysis using the ChIP-chip module of Agilent Genomic Workbench V 5.0 software, or the ChIP-chip module of DNA Analytics 4.0 software. This software provides further optimization of feature extraction data and visualization tools using the included array design files for the Promoter microarrays. See the software user guide for further details, and check www.agilent.com for further information concerning software upgrades.

For users that are more experienced with ChIP-chip data analysis methodology, feature extraction files can alternately be processed and filtered using third party software tools available for ChIP-chip (or generic microarray) analysis, peak finding tools and other software programs such as Cocas V2.4 software available at http://www.ciml.univ-mrs.fr/software/cocas/index.html.

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 E:

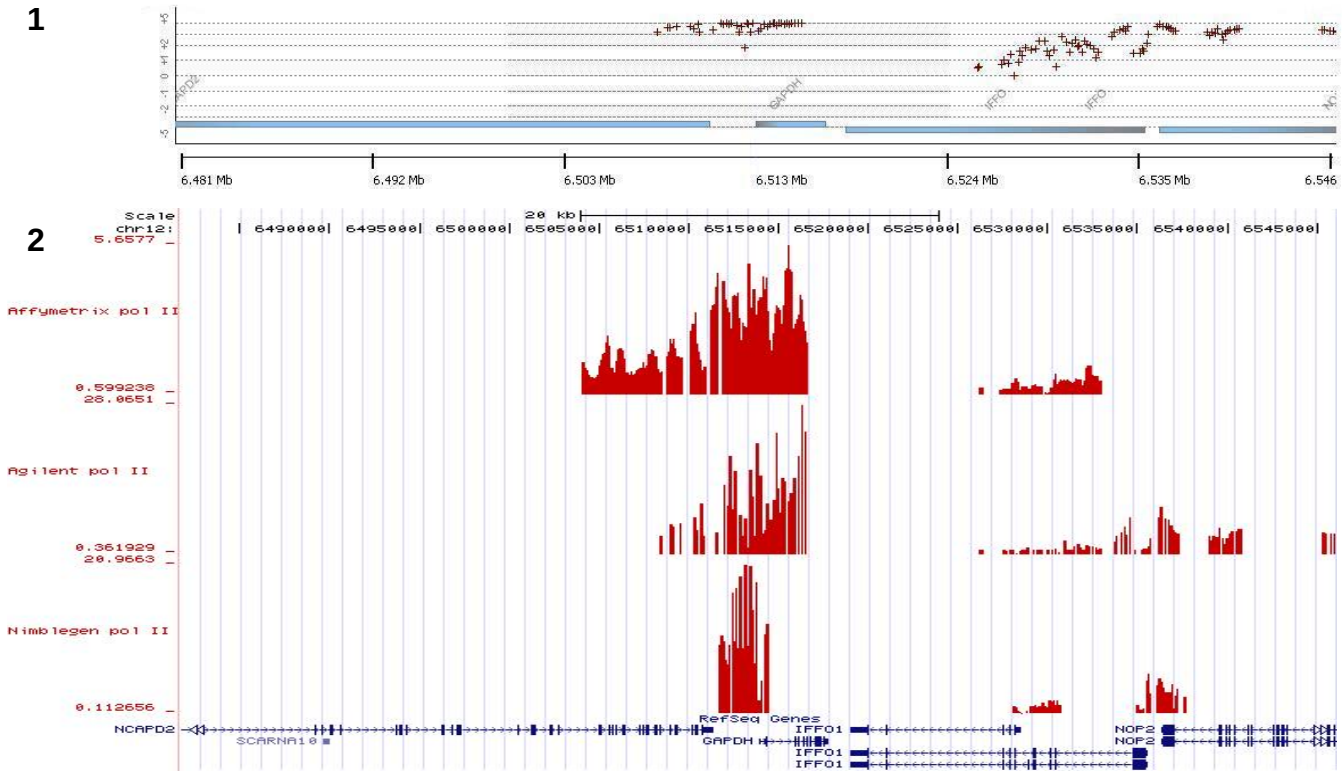


Figure E: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against RNA polymerase II (Millipore Cat. # 17-620 and # 05-623) using the Magna ChIP$^{2\,TM}$ Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent 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 E1**).
The Agilent dataset 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 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 that the enrichment at the GAPDH is independent of the array platform.

Figure F:

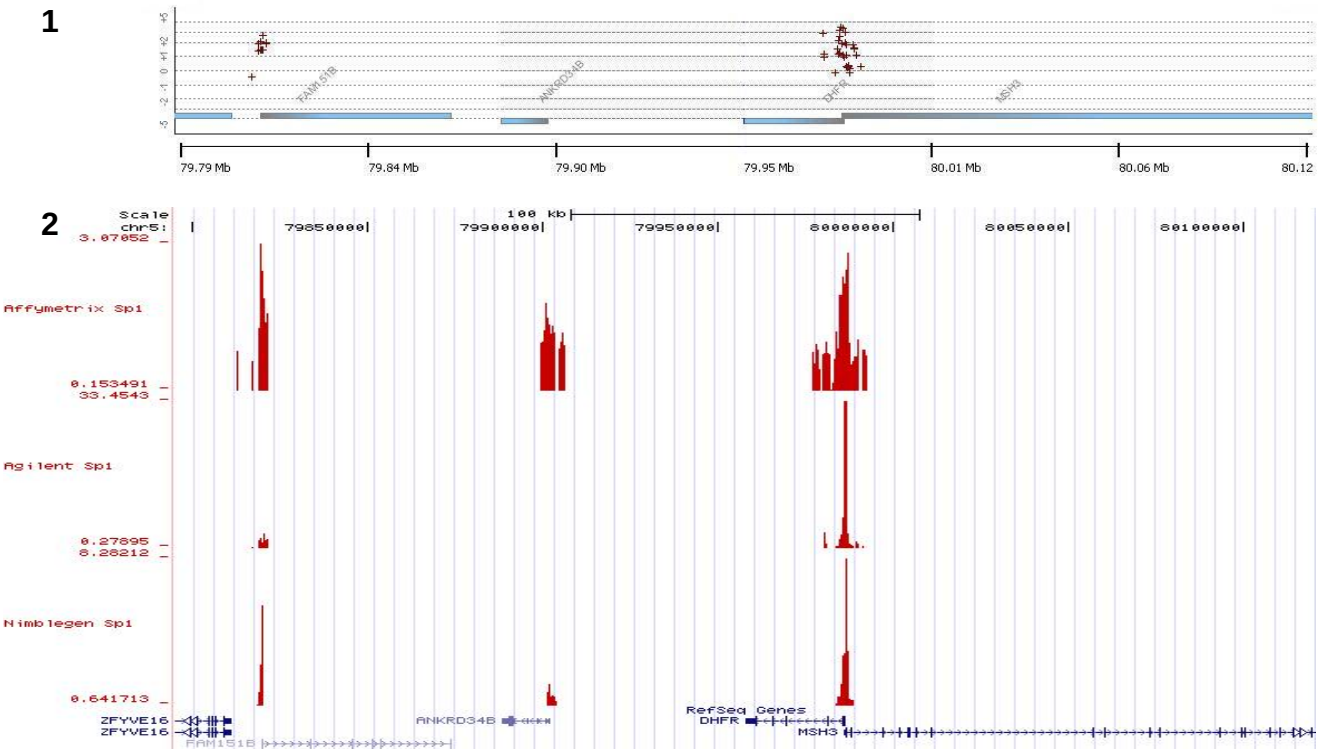
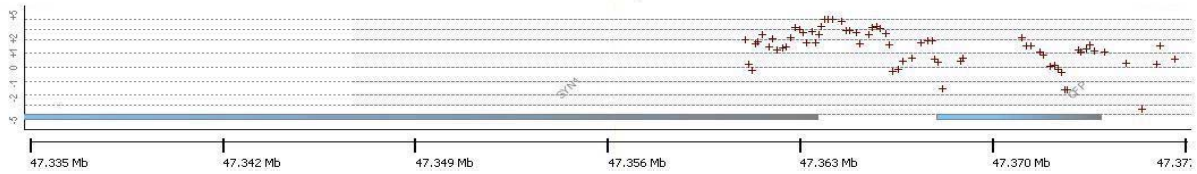


Figure F: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against human transcription factor Sp1 (Millipore Cat. # 17-601) using the Magna ChIP^{2} ^{TM} Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent 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**: middle panel). Similarly, replicate samples were also labeled and hybridized to the Affymetrix human promoter array (**Figure F2**: top panel) and Nimblegen human promoter array (**Figure F2**: 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 G:

1



2

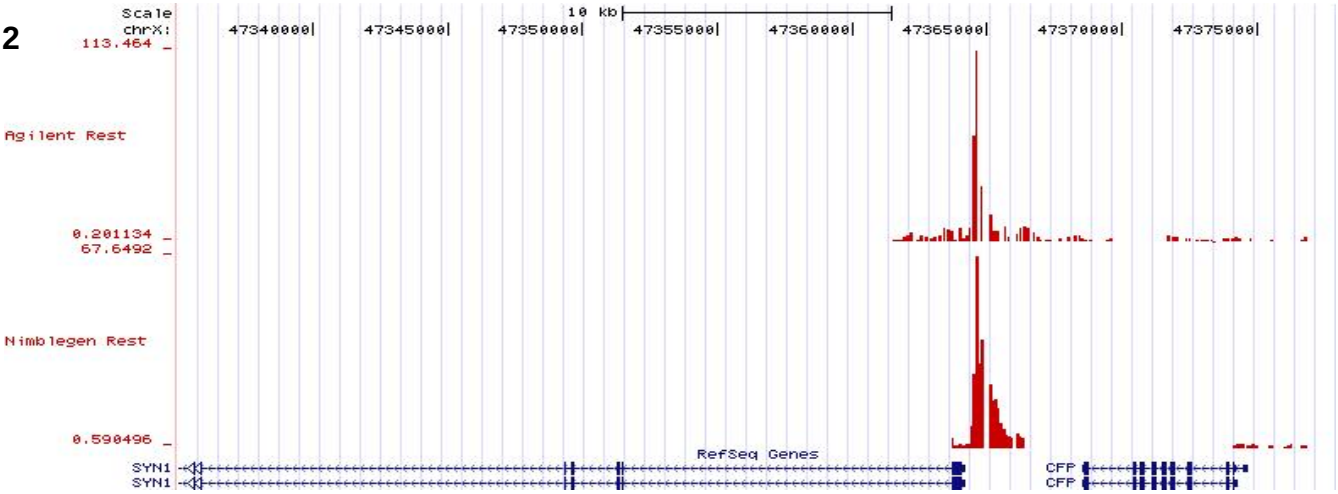


Figure G: Chromatin from HeLa cells was subject to immunoprecipitation with antibody against human transcription factor Rest (Millipore Cat. # 17-601) using the Magna ChIP^{2} ^{TM} Chromatin Immunoprecipitation DNA Microarray Universal Kit. The amplified DNA was labeled and hybridized to the Agilent 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 G1**).
The data was further filtered and visualized using the UCSC genome browser (http://genome.ucsc.edu/) (**Figure G2**: Top panel). Similarly, replicate samples were also labeled and hybridized to the Nimblegen human promoter array (**Figure G2**: 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 crosslink 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 tubes may also increase yield of ChIP DNA.
	Antibody doesn't recognize protein in crosslinked 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 crosslink 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 interested 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.
Labeling	Dye signal is low after a period of time	Cy dyes are sensitive to light. Aliquot Cy3 and Cy5 dUTP dyes upon initial thaw, and avoid exposure to light when stored. Cyanine 5 is additionally sensitive to atmospheric ozone. Ozone barriers may minimize degradation, or use Agilent's Stabilization and Drying Solution to mitigate risk of ozone degradation of the sample on the microarrays.
	Labeling yield and dye incorporation is low	DNA to be labeled contains impurities that affect concentration determination or affect performance of Klenow enzyme in labeling reactions. Make certain A_{260}/A_{280}>1.7 and that A_{260}/A_{230}>1.6. Ensure LM-PCR amplified sample shows a uniform smear on agarose gel electrophoresis. Additional reagents are provided to allow multiple labeling reactions if necessary. If yield of labeled sample is low, increase quantity up to 2 µg used per labeling reaction.
Hybridization	The overall Cy5 intensity is much lower than Cy3 intensity	Cy5 has been shown to be sensitive to ozone degradation, use the Agilent Stabilization and Drying Solution if local ozone level is high (great than 5 ppb).
Data analysis	Data is noisy, false positive rate is high	If ratio of enrichment is used to select binding targets, it may be helpful to filter out the low intensity features using an arbitrary threshold such as 50 FU. Evaluate QC metrics on feature extraction report to evaluate hybridization characteristics.
	no binding target can be identified	It could be that ChIP has failed. Before lableing and hybridization, perform a qPCR with LM-PCR enriched DNA to ensure that ChIP is successful (known target is enriched in amplified ChIP sample).

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