Comparison of sample preparation methods for ChIP-chip assays

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A single chromatin immunoprecipitation (ChIP) sample does not provide enough DNA for hybridization to a genomic tiling array. A commonly used technique for amplifying the DNA obtained from ChIP assays is ligation-mediated PCR (LM-PCR). However, using this amplification method, we could not identify Oct4 binding sites on genomic tiling arrays representing 1% of the human genome (ENCODE arrays). In contrast, hybridization of a pool of 10 ChIP samples to the arrays produced reproducible binding patterns and low background signals. However, the pooling method would greatly increase the number of ChIP reactions needed to analyze the entire human genome. Therefore, we have adapted the GenomePlex® whole genome amplification (WGA) method for use in ChIP-chip assays; detailed ChIP and amplification protocols used for these analyses are provided as supplementary material. When applied to ENCODE arrays, the products prepared using this new method resulted in an Oct4 binding pattern similar to that from the pooled Oct4 ChIP samples. Importantly, the signal-to-noise ratio using the GenomePlex WGA method is superior to the LM-PCR amplification method.

INTRODUCTION

The technique of chromatin immunoprecipitation (ChIP) has proven to be a powerful tool, allowing the detection of protein-DNA interactions in living cells. Although this technique was first adapted for use with mammalian cells less than 10 years ago (1,2), it is now the gold standard experiment for the identification of a target gene of a particular transcription factor. Over the last several years, great strides have been made in expanding the use of ChIP from a one gene-at-a-time approach to a global analysis tool through the hybridization of the samples to genomic microarrays (i.e., the ChIP-chip assay). Today, arrays representing promoter regions (3), CpG islands (4–6), or entire genomes (7) are used in combination with ChIP to identify binding sites for transcription factors and components of the transcriptional machinery and to define chromatin structure. However, a single ChIP sample does not provide enough DNA for labeling and hybridization to an array. A commonly used technique for amplifying the DNA obtained from ChIP assays is ligation-mediated PCR (LM-PCR). Unfortunately, we have found that this method often produces very high background when samples are analyzed on genomic tiling arrays. In this study, we have compared three ChIP sample preparation methods that differ in the background noise and reproducibility of binding site identification.

MATERIALS AND METHODS

Cell Culture

Ntera2 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 2 mM glutamine, 100 U/mL of penicillin and streptomycin, and 10% fetal bovine serum (FBS). All cells were incubated at 37°C in a humidified 5% CO₂ incubator.

ChIP-Chip Assays

ChIP assays (1 × 10⁷ cells/assay) were performed following the protocol provided in the supplementary materials (available online at www.BioTechniques.com with updates at genomics.ucdavis.edu/farnham and genomecenter.ucdavis.edu/expression_analysis). The Oct4 antibody used in this study was purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA), and the rabbit anti-goat immunoglobulin G (IgG) was purchased from MP Biomedicals (Solon, OH, USA). For PCR analysis of the ChIP samples prior to product generation, QIAquick®-purified immunoprecipitates (Qiagen, Valencia, CA, USA) were dissolved in 50 μL water. Standard PCRs using 2 μL immunoprecipitated DNA were performed. PCR products were separated by electrophoresis through 1.5% agarose gels and visualized using ethidium bromide.

Three different preparation methods were used to obtain enough ChIP DNA for application to genomic microarrays. ChIP-chip experiments were performed using two independent cultures of cross-linked Ntera2 cells for each method.

Method 1. LM-PCR. For this method, one half of a ChIP sample (from 1 × 10⁷ cells) was used for linker ligation. Amplification of the linker-ligated DNA using LM-PCR is described in detail at genomics.ucdavis.edu/farnham; see also Reference 8.

Method 2. Pooling ChIP samples. For this method, 10 individual Oct4 ChIP assays were performed from each of two sets of 1 × 10⁶ cross-linked cells (1 × 10⁷ cells/ChIP assay). ChIP samples were processed separately following the standard protocol, except that after pre-clearing the chromatin with StaphA cells, all 10 ChIP samples were pooled into one tube for the washing steps. Washes and elution of the pooled ChIPs were then carried out as described in the standard protocol.

Method 3. Whole genome amplification (WGA). An adaptation of the standard protocol for WGA using the GenomePlex® WGA kit (Sigma-Aldrich, St. Louis, MO, USA) was used. Briefly, the initial random fragmentation step was eliminated, and an entire ChIP sample (from 1 × 10⁷ cells) or 10 ng input chromatin were amplified. This usually provides enough sample for one array. However,
if additional product is needed, then a second round of amplification (using 10–20 ng of the first amplification sample) can be performed. A detailed protocol for the WGA method is provided in the supplementary materials.

Biological replicates of LM-PCR products, pooled ChIP samples, and WGA products (a total of six samples) were applied to ENCODE (Encyclopedia of DNA Elements) oligonucleotide arrays (NimbleGen Systems, Madison, WI, USA) containing approximately 380,000 50-mer probes per array, tiled every 38 bp. The regions included on the arrays encompassed the 30 Mb of the repeat masked ENCODE sequences, representing approximately 1% of the human genome. The labeling of DNA samples for ChIP-chip analysis was performed by NimbleGen Systems, Inc. Briefly, each DNA sample (1 μg) was denatured in the presence of 5′Cy3™- or Cy5-labeled random nonamers (TriLink Biotechnologies, San Diego, CA, USA) and incubated with 100 U (exo-) Klenow fragment (New England Biolabs, Ipswich, MA, USA) and dNTP mixture [6 mM each in TE buffer (10 mM Tris, 1 mM EDTA, pH 8.0; Invitrogen, Carlsbad, CA, USA)] for 2 h at 37°C. Reactions were terminated by addition of 0.5 M EDTA, pH 8.0, precipitated with isopropanol, and resuspended in water. Then, 13 μg Cy5-labeled ChIP sample and 13 μg Cy3-labeled total sample were mixed, dried down, and resuspended in 40 μL hybridization buffer (NimbleGen Systems) plus 1.5 μg human COT1 DNA. After denaturation, hybridization was carried out in a MAUI® (MicroArray User Interface) Hybridization System (BioMicro Systems, Salt Lake City, UT, USA) for 18 h at 42°C at the NimbleGen Service Laboratory. The arrays were washed using wash buffer system (NimbleGen Systems), dried by centrifugation, and scanned at 5 μm resolution using the GenePix® 4000B scanner (Axon Instruments, Union City, CA, USA). Fluorescence intensity raw data were obtained from scanned images of the oligonucleotide tiling arrays using NimbleScan™ 2.0 extraction software (NimbleGen Systems). For each spot on the array, log2-ratios of the Cy5-labeled test sample versus the Cy3-labeled reference sample were calculated. Then, the biweight mean of this log2 ratio was subtracted from each point; this procedure is approximately equivalent to mean-normalization of each channel. Sites bound by Oct4 were identified using the peak calling algorithm described in Bieda et al. (9), with minor modifications (available upon request). The peaks called for both biological replicates of the LM-PCR, pooling, and WGA methods are provided as supplementary material. The array data has been deposited into the Gene Expression Omnibus (GEO; series GSE5251).

RESULTS AND DISCUSSION

To identify Oct4 binding sites in the human genome, we first performed a ChIP experiment using an antibody to Oct4 and demonstrated that the Oct4 ChIP sample showed enrichment when primers specific to the NANO1 and EVX1 promoters (known Oct4 binding sites) were used in PCRs, but no enrichment when negative control primers specific for the DHFR gene were used (data not shown). We then used LM-PCR to amplify the Oct4 ChIP samples and hybridized the amplified samples to ENCODE arrays. Using ChIP samples amplified by LM-PCR, we have previously identified binding sites for E2F family members using CpG island (4), promoter (9,10), and genomic tiling (9) arrays. However, using the LM-PCR amplification method, we found that Oct4 binding sites could not be distinguished from the background noise on the arrays (Figure 1, top panel). For example, although the Oct4 binding site in the EVX1 promoter is present on the array used in this study, it could not be identified above background noise. Also, two Oct4 binding sites (confirmed by PCR analysis of ChIP samples) within the EXT1 gene, indicated with arrows in Figure 1, do not show enhanced enrichment as compared with the surrounding DNA. Peak prediction analysis of two biologically independent ChIP-chip assays performed using the LM-PCR method was carried out using a 98th percentile threshold of log2 oligomer ratios and a p value P < 0.0001 (9). Although

![Figure 1. Comparison of three different sample preparation methods for ChIP-chip assays. The hybridization profile of a 300-kb region of chromosome 8 surrounding the EXT1 gene is shown for samples prepared by the LM-PCR (top panel), pooled (middle panel), or WGA (bottom panel) methods. Oct4 binding sites confirmed by PCR are indicated with arrows. ChIP, chromatin immunoprecipitation; LM-PCR, ligation-mediated PCR; WGA, whole genome amplification.](https://www.biotechniques.com)
hundreds of peaks were called for the two arrays using the LM-PCR-derived products, very few peaks were in common on both arrays (Table 1 and supplementary material).

Because known Oct4 binding sites were enriched in the ChIP samples, it was likely that the inability to identify binding sites on the arrays was a result of the amplification method and not inefficient immunoprecipitation. To test this hypothesis, we performed 10 ChIP reactions for each of two biologically independent samples of cross-linked cells. The 10 ChIP samples from a given batch of cells were pooled, and the two pools were applied separately to genomic tiling arrays. We found that the pooling method greatly reduced the background noise on the array and produced reproducible binding patterns (Figure 1, middle panel). In fact, approximately 70% of the peaks identified on one array were identified on the biological replicate array (Table 1 and supplementary material).

Unfortunately, pooling ChIP samples is not always possible (e.g., if using specialized cell types or tumor tissues), and the need to pool 10 ChIP samples for every array would greatly increase the number of ChIP reactions needed to analyze the entire human genome. Therefore, we felt that a different method for amplifying ChIP samples was required. The method of WGA has proven very useful for investigators performing comparative genomic hybridizations (see www.sigmaaldrich.com/sigma/bulletin/wga1bul.pdf). The standard protocol for this technique is to first use a random chemical fragmentation of the genome, producing a series of overlapping short templates averaging 400 bp. Next, the DNA fragments are efficiently primed to generate a library of DNA fragments with defined 3' and 5' termini. This library is then replicated using linear amplification in the initial stages, followed by a limited round of geometric amplifications. Because ChIP samples are obtained using sonicated chromatin that has an average size of 500 bp to 1 kb, we reasoned that the chemical fragmentation step should not be necessary. Therefore, we used an entire ChIP sample (obtained from \(1 \times 10^7\) cells) for the library generation and subsequent amplification. Using this protocol, we found that the predicted Oct4 peaks show a very similar pattern as in the pooled ChIP samples, and the background noise was very low (Figure 1, bottom panel).

Using the WGA method, we found that approximately 63% of the peaks were detected on both arrays (Table 1 and supplementary material). These results are very similar to those obtained by analysis of the arrays hybridized with the pooled samples. One reason why the overlap percentage was not higher than 63%–70% when the pooled and WGA samples were analyzed is due to limitations of the peak-calling program. As shown in Supplementary Figure S1, very similar binding patterns of Oct4 on two arrays can lead to differences in the number and exact positions of called peaks.

The Oct4 binding sites identified using the WGA method were tested by standard PCR analyses using a ChIP sample from a third independent culture of cells (Figure 2). After analyzing 14 predicted Oct4 binding sites, we obtained a 93% confirmation rate, indicating that the WGA amplification method results in an accurate representation of a ChIP sample obtained from a small number of cells.

Conclusions

We have shown that the method of LM-PCR-mediated amplification does not work well for all ChIP samples, perhaps dependent upon the number of binding sites and the abundance of the factor. We have tested a different amplification method, originally developed to provide accurate representation of the genome for studies of copy number changes and SNP analyses in tumor samples. We found that the signal-to-noise ratio obtained from the hybridization of the WGA products to genomic arrays is superior to the LM-PCR method of amplification for ChIP samples, not only for Oct4, but also for a number of other human and mouse transcription factors (data not shown). Based on the low background, reproducibility, and the fact that a single ChIP sample provides sufficient material for several array hybridizations, we recommend the WGA protocol for ChIP-chip analyses.

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COMPETING INTERESTS STATEMENT

R.G. is an employee of NimbleGen Systems Inc. Arrays from this company were used in the ChIP-chip studies. The other authors declare no competing interests.
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Supplementary Material for:
Comparison of sample preparation methods for ChIP-chip assays

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Explanation of the Peak Files

Each of these files can be opened as a table, which lists the called peaks, or imported into Signalmap or the UCSC browser for viewing. The peaks were called using the program described in Bieda et al. (9) using the L1 stringency. The files are labeled by the array number (e.g., 63975), the method of amplification (e.g., Impcr), the stringency (T02P0001), and whether it is the A or B sample. The raw data for each array has been deposited into GEO with the series number GSE5251.

Supplementary Figure S1. Shown are the binding patterns of Oct4 on a region of human chromosome 7 obtained from two biologically independent sets of products prepared using the GenomePlex whole genome amplification method. Also shown are the peaks that are called for the two arrays. Although the binding patterns on the two arrays are very similar throughout the entire region, the precise number and location of the called peaks differs in the two experiments. These differences are due to limitations in the peak-calling program; a broad peak with long shoulders is called as multiple peaks. The precise position of the different peaks, within a single binding region, can differ from array to array.
**Chromatin Immunoprecipitation (ChIP) Assay Protocol**

**Can be prepared in advance and stored at -80°**

**Wash Staph A Cells:**
1. Resuspend 1 gram of lyophilized Staph A cells (Pansorbin®, Calbiochem Cat#507862) in 10 mL of 1X Dialysis Buffer without sarkosyl (DB – srk) (difficult to resuspend, try using a P1000 pipetman w/several mL, then after resuspension add remaining volume OR allow 30 minutes to rehydrate to ease resuspension)
2. Transfer to a 15 mL tube and centrifuge at 6,000 rpm for 5’ at 4°C, pour off supernatant
3. Resuspend pellet in 10 mL 1X DB – srk
4. Centrifuge at 6,000 rpm for 5’ at 4°C, pour off supernatant
5. Resuspend in 3 mL of PBS, 3% SDS, 10% BME (2.25 mL 1X PBS, 450 µL 20% SDS, 300 µL BME) in fume hood
6. Boil for 30’ in fume hood
7. Centrifuge at 6,000 rpm for 5’ at RT, pour off supernatant into chemical waste
8. Wash with 10 mL of 1X DB – srk
9. Centrifuge at 6,000 rpm for 5’ at RT, pour off supernatant
10. Repeat 7, 8 and 9
11. Resuspend in 4 mL of 1X DB – srk
12. Divide into 100 µL aliquots (40x 0.5 mL tubes), snap freeze and store at -80 (or liquid N2) for indefinite time

**Blocking Staph A Cells:**
1. Thaw 1 tube (100 µL each) of Staph A Cells for approximately every 5x10⁷ cells or one tube for four IPs to be used for ChIP analysis.
2. Add 10 µL of 10mg/mL salmon sperm DNA and 10 µL of 10mg/mL BSA for each 100 µL Staph A aliquot and mix by pipette
3. **For best results, incubate on rotating platform overnight at 4°C, or at 4°C for several (≥3 hrs) hours, (or 2 hrs at RT if time constraint)**
4. Transfer to 1.5 mL tube and microfuge at 14,000 rpm for 3’ at 4°C and remove supernatant
5. Wash pellet by resuspending in 1 mL 1X DB – srk
6. Microcentrifuge at 14,000 rpm for 3’ at 4°C and remove supernatant
7. Repeat 5 and 6
8. Resuspend the pellet in 100 µL of 1X DB – srk w/ 1mM PMSF (use 1 µL of 100mM PMSF)
9. Washed and blocked Staph A Cells can be stored at 4°C for up to 2 weeks

**Must be prepared within two weeks of beginning experiment and stored at +4°**
(Prepare 100 uL Staph A for every 5x10⁷ cells, will use half Day 1 and half Day 2)
DAY 0:

A: Preparation of Cross-Linked Cells:

1. Wipe down bench and pipettes with Alconox
2. Cell cultures should be healthy and not density arrested prior to cross-linking, generally use 1 x 10^7 cells per antibody per ChIP (fewer cells, as low as 2 x 10^6 cells, can be used but may result in lower signal to noise ratio)
3. In fume hood, add formaldehyde (37% stock) directly to tissue culture media to a final concentration of 1%
4. In fume hood, incubate adherent cells on a shaking platform and suspension cells on a stir plate for 10’ at RT (cross-linking for longer periods of ≥ 30’ may cause cells to form aggregates that do not sonicate efficiently)
5. In fume hood, stop the cross-linking reaction by adding glycine to a final concentration of 0.125M continuing to rock/spin for 5’ at RT (for adherent cells use 10X (1.25M) soln and for suspension cells add powdered glycine directly to flask)
6. Wash cells with 1X PBS (for adherent cells, pour off media and rinse plates twice with 1X PBS, after second rinse, stand plate up and let PBS run down and then dump again; for suspension cells, wash twice by centrifuging and resuspend in 1X PBS)
7. Optional: For adherent cultures add an appropriate volume of tissue culture trypsin (e.g. 10 mL per 500 cm^2 dish) to coat monolayer and incubate for 10’ at 37°C (this step is useful for cells that are difficult to swell)
8. Scrape adherent cells from culture dish to 50 mL tube
9. Rinse the adherent cells culture dish with 30 mL PBS, add the remaining cells to the 50 mL tube and mix
10. Centrifuge adherent cells at 1000 rpm for 10’ at 4°C
11. Carefully aspirate supernatant so as to not lose cells
12. Wash cells by resuspending pellet in 50 mL of 1X PBS (thoroughly resuspend and take a small aliquot for a cell count)
13. Pellet again and aspirate the supernatant
14. Cells can be used immediately for ChIP assay or snap frozen in liquid nitrogen and stored in liquid nitrogen or -80°C freezer indefinitely

The following steps will take you through a control ChIP experiment. We recommend that the first ChIP experiment that you do be a comparison of an antibody to PolII vs. an IgG control (ordering info at the end of protocol). We also recommend using the promoter of the gene for the large subunit of RNA polII for the positive control primer set (since this promoter is active in all cells) and the 3’ UTR of the DHFR gene as a negative control primer set (primer sequences at end of protocol). All buffer & solution recipes also found at the end of protocol.
DAY 1:

***Washed/Blocked Staph A cells must be ready at this point***

B. Preparation of Cross-Linked Chromatin and Antibody Incubation

*Prepare everything on ice*

1. Prepare Swelling Solution.
   - Use 1 mL per 5x10^7 cells.

   Prepare from 10X Stock Autoclaved solutions on ice:
   a. Add appropriate amount of Mol. Bio. Grade H_2O
   b. Use 10 x (1M) Tris pH 7.6 to make 1X (0.1M) Tris
   c. Use 10X (100mM) KOAc & (150mM) MgOAc to make 1X (10mM) KOAc & (15mM) MgOAc
   d. protease inhibitors final concentration:
      i. 1mM PMSF
      ii. 0.01mg/mL aprotinin (use aliquot for one day – do not refreeze)
      iii. 0.01mg/mL leupeptin

2. Prepare Nuclei Lysis Buffer.
   - Use 1 mL per 1x10^8 cells

   Prepare Nuclei Lysis Buffer from Stock solutions on ice:
   a. Add appropriate amount of Mol. Bio. Grade H_2O
   b. Use 1M Tris-Cl pH 8.0 to make 50mM Tris-Cl
   c. Use 500mM EDTA pH 8.0 to make 10mM EDTA
   d. Use 20% SDS to make 1% SDS
   e. protease inhibitors final concentration:
      i. 1mM PMSF
      ii. 0.01mg/mL aprotinin (use aliquot for one day – do not refreeze)
      iii. 0.01mg/mL leupeptin

3. Resuspend cells with 1 mL per 5x10^7 cells of prepared Swelling Buffer

4. Incubate on ice for 20’ and flick occasionally to resuspend

5. Dounce cells on ice using a 2 mL B Dounce with 15 strokes to release nuclei & disperse cell clumps (If using larger volume, dounce only 1 mL at a time or use larger dounce)

6. Transfer sample to 1.5 mL tubes

Steps that may vary with cell type
ChIP Protocol

7. Microfuge at 2,500 x g for 5’ at 4°C to pellet nuclei, pour off supernatant

8. Resuspend nuclei in 1 mL per 1x10^8 cells of prepared Nuclei Lysis Solution (If sample was split into two tubes for douncing, use 1 mL to resuspend the first pellet, then transfer to second pellet and resuspend)

9. Incubate on ice for 10’

10. Transfer samples to appropriate tubes for sonication – 15mL polystyrene tube are best (may split samples to avoid going over max volume for tubes to be sonicated)

   (See below for tube ranges)

11. Sonicate:

   a. We use a BioRuptor Sonicator kept in a cold room
   b. Prepare samples with following volume range:
      i. 1.5 mL Tube (polystyrene recommended): 100 - 300 µL
      ii. 15 mL Tube (polystyrene!): 500 µL - 2 mL
   c. Wear ear protection and post signs on doorways stating “sonication is in progress and ear protection is required”
   d. Remove tube holder and check that water level is at blue “water level” mark
   e. Use appropriate tube holder and accessories (check Quick Reference Sheet) for your samples and rinse with ethanol before use
   f. Balance tubes in sonicator (similar to centrifuge)
   g. Input sonication settings: the pulse duration, intensity and number will vary depending on the extent of cross-linking and cell type, you must optimize for your expt. Ideally the least amount of input energy that gives satisfactory fragmentation should be used. We use as starting conditions:
      4 pulses for 15 sec with 1 min rest interval = timer 5 min
      i. LMH dial should be set to High
      ii. Set Interval by adjusting red needle (sonication time, usually 15 sec) and the green needle (rest interval, 1 min standard to allow cooling)
      iii. Then set the Timer (only allows “On” = cycle, 5’, 10’ or 15’, so keeping track with a stopwatch might be necessary for times in between)
   h. Rinse off used tube holder and accessories with water and then ethanol when finished
   i. Run a gel to check sonication
      i. Use 10 µL sample and add 40 µL IP Elution Buffer
      ii. Reverse cross-link by adding 2 µL of 5 M NaCl (0.2M NaCl)
      iii. Boil for 15’
      iv. After returning to RT, add 1 µL of 10 mg/mL RNase A
      v. Clean with QiaQuick PCR Purification Kit
         a. Add 250 µL PBI buffer to each sample
         b. Add 5 µL of 3M NaOAc pH ~5.0, light vortex
         c. Transfer to Qiagen column (purple)
         d. Centrifuge at max speed for 1’ and discard flow through

   Steps that may vary with cell type
ChIP Protocol

e. Place column back in catch tube and add 750 µL PE buffer to each tube
f. Centrifuge at max speed for 1’ and discard flow through
g. Reinsert column into catch tube and spin at max for 1’ more
h. Label tube
i. Place column into a clean/labeled 1.5 mL tube, discard catch tube
j. Add 30 µL of EB to column membrane, let sit 2’
k. Centrifuge at max speed for 1’ and discard column
vi. Load gel with 1 & 4 µL of sonicated sample

12. If using 15 mL tubes, transfer sonicated chromatin to 1.5 mL tubes  
13. Microfuge samples (hinge facing outside) at 14,000 rpm for 10’ at 4°C

14. Transfer supernatant to new 1.5 mL tube. (This sonicated chromatin can be frozen and used at a later time.)

C. Immunoprecipitation of Cross-Linked Chromatin

1. Freshly add ~1 µL of 100mM PMSF per ~100 uL of blocked/washed Staph A cells prepared earlier

2. Preclear chromatin by adding blocked/washed Staph A cells w/PMSF (10 µL per 1x 10^7 cells)

Sample storage info:  
Location =  
Tube Label =

<table>
<thead>
<tr>
<th>Volume B/W Staph A cells in aliquot:</th>
<th>µL</th>
</tr>
</thead>
<tbody>
<tr>
<td>100mM PMSF:</td>
<td>µL</td>
</tr>
</tbody>
</table>

| Volume B/W Staph A w/PMSF added to each tube:  | µL |

*Do NOT throw out remaining Staph A Cells, you will need them for Day 2, Step D2*

3. Incubate on rotating platform for no longer than 15’ at 4°C
4. Microfuge at 14,000 rpm for 5’ at 4°C
5. Transfer supernatant to new tube and measure volume

Volume measured:

| Volume measured:  | µL |

6. Divide volume equally into 2 mL screw-cap tubes representing ~1x10^7 cells for each immunoprecipitation. Include an IgG negative control sample along with your experimental antibodies

Tube Names, volumes & # cells:
7. Make double the total sample volumes of IP Dilution Buffer:

\[(\text{sample volume}) \times 2 \times (\frac{2}{\text{Samples}})\] = _______

with protease inhibitors, by adding:

a. 10 µL/mL PMSF
b. 1 µL per 1 mL of solution - aprotinin (use aliquot for one day – do not refreeze)
c. 1 µL per 1 mL of solution - leupeptin

8. Add double the sample volume of prepared IP Dilution Solution to each sample (excluding “10% Total Input”)

9. Add 1-2 µg of primary antibody to each sample (although the optimal amount for each antibody will need to be determined experimentally, 1 µg is a good starting point for most antibodies)

**Record lot number of antibodies for future reference!**

Tube names & 1º Ab info and 1º Ab amount:

10. Incubate on rotating platform at 4°C overnight

Steps that may vary with cell type
DAY 2:

D. Washing and Cross-Link Reversal

**Use 1.5 mL tubes for washing steps**

1. If you are using monoclonal antibodies or a polyclonal antibody from a species other than rabbit, add 1 ug of appropriate secondary antibody (e.g. rabbit antimouse for mouse monoclonals or rabbit antigoat for goat polyclonals) and incubate for an additional hour at 4°C. Staph A binds rabbit IgG Abs efficiently, therefore a secondary Ab is typically not required if using a rabbit polyclonal.

Tube names & 2° Ab info:

2. Freshly add ~1 µL of 100mM PMSF per 100 uL of blocked/washed Staph A cells prepared earlier

3. Add blocked/washed Staph A cells w/PMSF (10 µL per 1x 10^7 cells)

4. Incubate on rotating platform for no longer than 15’ at RT

5. Transfer to new 1.5 mL tube

Tube names:

6. Microfuge samples at 14,000 rpm for 4’ at 4°C

7. Save all of supernatant from IgG IP for “Input”, and use later in reversing cross-links step D27

8. Pour off supernatant in other samples
**ChIP Protocol**

**Be sure to use appropriate monoclonal or polyclonal dialysis and wash buffers**

9. Wash pellets with 1 mL of 1X Dialysis Buffer (Add 10 µL of 100mM PMSF per 1 mL of buffer, does NOT contain aprotinin/leupeptin)
   (1 mL x 2 washes) x (____ # samples) = ______ mL
   Total volume 1X Dialysis Buffer prepared: ______ mL
   100 mM PMSF: ______ µL

10. Invert tube 20 times by hand at RT

11. Microfuge at 14,000 rpm for 4' at 4°C, pour off supernatant in labeled waste container

12. Repeat 9, 10 and 11 once more

13. Wash pellets with 1 mL of IP Wash Buffer (Add 10 µL of 100mM PMSF per 1 mL of buffer, does NOT contain aprotinin/leupeptin)
   (1 mL x 4 washes) x (____ # samples) = ______ mL
   Total volume IP Wash Buffer prepared: ______ mL
   100 mM PMSF: ______ µL

14. Invert tube 20 times by hand at RT

15. Microfuge at 14,000 rpm for 4' at 4°C

16. Repeat 13, 14 and 15 three more times, pour off supernatant in labeled waste container

17. Microfuge the pellet again orienting the pellet on outside and aspirate the last traces of buffer

18. Prepare IP Elution Buffer at RT (no inhibitors). Need 100 µL for each IP sample plus 100 µL for the “10% Total” sample:
   (50 µL x 2) x (____ # samples) + 100 µL = ______ µL
   Total volume IP EB (1% SDS and 50 mM NaHCO₃) to prepare:
   dH₂O: ______ µL
   20% SDS: ______ µL
   1M NaHCO₃: ______ µL

19. Elute antibody/protein/DNA complexes by adding 50 µL of IP Elution Buffer at RT (no inhibitors)

20. Shake on vortexer for 15’ at setting 3 at RT

21. Microfuge at 14,000 rpm for 3’ at RT

22. Remove supernatant to a new 1.5 mL tube

Tube names:

23. Repeat 19-22 on same pellet, combining supernatant in same new tube (100 µL total)

24. Microfuge samples again at 14,000 rpm from 5’ to remove any traces of Staph A cells

Steps that may vary with cell type
ChIP Protocol

25. Transfer supernatant to new 0.5 mL tube

Tube names:

26. Add 4 µL of 5M NaCl (0.2M NaCl final) to each IP sample tube.

27. From “Input” (IgG supernatant saved earlier in step D7), take 10% of an IP sample volume (see Step C6 to calculate 10% of an IP sample volume) and bring volume up to 100 µL with IP Elution Buffer, then add 4 µL of 5M NaCl (0.2M NaCl final).

\[
\text{____}_\mu\text{L is 10%} + \text{____}_\mu\text{L IP EB} = 100 \mu\text{L of 0.1%}
\]

28. Incubate all samples at 67°C for 4 hrs to overnight to reverse formaldehyde cross-links (recommended) or boil for 15’ if time constraint and then freeze.
DAY 3:

E. Column Purification and PCR Analysis

1. Add 1 µL of 10mg/mL RNase A to each sample (including your “10% Input”) and incubate for 30’ at 37°C

2. Column purify each sample using Qiagen Qiaquick PCR purification Kit
   a. Transfer samples to labeled 1.5 mL tubes
   b. Add 500 µL PBI buffer to each sample
   c. Add 10 µL of 3M NaOAc pH 5-5.2 to make solution less basic, light vortex
   d. Transfer to Qiagen column (purple)
   e. Centrifuge at max speed for 1’ and discard flow through
   f. Place column back in catch tube and add 750 µL PE buffer to each tube
   g. Centrifuge at max speed for 1’ and discard flow through
   h. Reinsert column into catch tube and spin at max for 1’ more
   i. Label 1.5 mL tubes
   j. Place column into a clean/labeled 1.5 mL tube, discard catch tube
   k. Add 50 µL of EB to column membrane, let sit 2’
   l. Centrifuge at max speed for 1’ and discard column

Eluted sample names:

3. Use 2 uL of each ChIP sample for PCR reaction. Make a 1:10 dilution of Total in Qiagen EB and use 2 µL for PCR analysis, as well as, use 2 µL of undiluted Total.

PCR Info for 20 (µL) RXNs:
+ Control = Pol2 Promoter
− Control = DHFR3’ UTR

<table>
<thead>
<tr>
<th>#</th>
<th>DNA (Antibody specific)</th>
<th>Volume DNA (µL)</th>
<th>Primer F/R (10 µM)</th>
<th>Volume Primer (µL)</th>
<th>BSA (µL)</th>
<th>2x AmpliTaq MM (µL)</th>
<th>Mol. Bio. Grade H₂O (µL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pol2</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>2</td>
<td>IgG</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>3</td>
<td>(1:50) Total</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>4</td>
<td>(1:10) Total</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>5</td>
<td>UD Total</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>6</td>
<td>Pol2</td>
<td>2</td>
<td>- Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>7</td>
<td>IgG</td>
<td>2</td>
<td>- Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>8</td>
<td>(1:50) Total</td>
<td>2</td>
<td>+ Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>9</td>
<td>(1:10) Total</td>
<td>2</td>
<td>- Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
<tr>
<td>10</td>
<td>UD Total</td>
<td>2</td>
<td>- Control</td>
<td>1</td>
<td>1</td>
<td>10</td>
<td>6</td>
</tr>
</tbody>
</table>

PCR Cycling Conditions:

95º 3’
95º 30” x33
60º 30” 1’
72º 1’
10º ∞

11 µL + 110.0 µL + 66 µL = 187 µL
17 µL for each sample

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Updated 6/30/06 By Heather N. Witt
http://genomcenter.ucdavis.edu/expression_analysis

* Steps that may vary with cell type
SOLUTIONS:

**Dialysis Buffer**
- 2 mM EDTA
- 50 mM Tris-Cl pH 8.0
- 0.2% Sarkosyl (omit for monoclonal antibodies)

**Swelling Buffer (used in this protocol) Made 10X Tris and 10X KOAc/MgOAc and Autoclaved**
- 0.1 M Tris pH 7.6
- 10 mM KOAc
- 15 mM MgOAc
  
  Before use, add 1% NP40 and protease inhibitors

**Swelling Buffer (alternative) 5 mM PIPES pH 8.0**
- 85 mM KCl
  
  Before use, add 1% NP40 and protease inhibitors

**Nuclei Lysis Buffer**
- 50 mM Tris-Cl pH 8.0
- 10 mM EDTA
- 1% SDS
  
  Before use add protease inhibitors

**IP Dilution Buffer**
- 0.01% SDS
- 1.1% Triton X 100
- 1.2 mM EDTA
- 16.7 mM Tris-Cl pH 8.0
- 167 mM NaCl
  
  Before use add PMSF

**IP Wash Buffer**
(Use stated Deoxycholic Acid, Sodium Salt; this product from other companies can have a hard time going into solution)
- 100 mM Tris-Cl pH 9.0 (8.0 for monoclonal antibodies)
- 500 mM LiCl
- 1% Igepal (aka NP40)
- 1% Deoxycholic Acid, Sodium Salt (Fisher Scientific MW 414.5 Cat. # BP349-100)
  
  Before use add PMSF

**IP Elution Buffer – Made fresh from 1M NaHCO₃ and 20% SDS**
- 50 mM NaHCO₃
- 1% SDS

* Steps that may vary with cell type
Protease Inhibitors – PREPARE WITH CAUTION
100 mM PMSF in ethanol, use at 1:100, use mask when weighing out or use fume hood (prepared 100 µL aliquots)
10 mg/mL aprotinin in 0.01 M HEPES pH 8.0, use at 1:1000 (prepared 5 µL aliquots)
10 mg/mL leupeptin in water, use at 1:1000 (prepared 5 µL aliquots)

Primers Info
+ Control Pol2 F 5’ AGATGAAACCGTTGTCCAAACT 3’
+ Control Pol2 R 5’ AGGTTACGGCAGTTTGCTCTCTC 3’
- Control DHFR3’ UTR F 5’ CTGATGTCCAGGAGGAAAGG 3’
- Control DHFR3’ UTR R 5’ AGCCCGACAATGTCAGGACTG 3’

Antibodies Info
IgG from Rabbit Serum, Sigma Cat#15006-10MG
Rabbit Anti-Mouse IgG, MP Biomedical Cat#55436
RNA Polymerase II 8WG16 Monoclonal Antibody, Covance Cat#MMS-126R

EQUIPMENT/MATERIALS
Knotes Dounce Tissue Grinder 2 mL Capacity, VWR# KT885300-0002
Refrigerated Microcentrifuge/Centrifuge with 1.5/2.0 mL Tube Rotor
Rotator
PCR Machine

Steps that may vary with cell type
**[Since the input material is sonicated chromatin, the initial series of fragmentation steps is skipped and one can go right to library preparation]**

**A. Library Preparation**

1. Add 2 ul 1X Library Preparation Buffer to 10 ul of input material

[For the “input” sample, measure the concentration of reverse crosslinked, QIAquick purified DNA and add 10 ng to a total volume of 10 ul with H2O. For the ChIP sample, the concentration of nucleic acid is usually too low to get an accurate quantitation. Typically the entire 50ul of reverse crosslinked, QIAquick purified DNA is lyophilized and resuspended in 10 ul of H2O]

Transfer samples to strip tubes or individual thin walled 0.2 ml PCR tubes

2. Add 1 ul Library Stabilization Solution, vortex or mix by pipetting. Quick spin and place at 95° for 2 minutes in thermal cycler

3. Immediately cool on ice, quick spin again

4. Add 1 ul Library Preparation Enzyme, vortex or mix by pipetting and quick spin if necessary

5. Incubate in thermal cycler as follows:
   - 16° for 20’ (cycler should be precooled to this temperature)
   - 24° for 20’
   - 37° for 20’
   - 75° for 5’
   - 4° hold

9. Quick spin if necessary and either proceed to first amplification or freeze at –20° for up to three days

**B. Amplification (round 1)**

10. Prepare master mix for each sample containing:

7.5 ul of 10X Amplification Master Mix
47.5 ul Nuclease-free H20
5 ul WGA DNA polymerase
11. Add 60 ul master mix to each sample, vortex or mix by pipetting and quick spin if necessary

12. Incubate in thermal cycler as follows:
   - 95° for 3’, then 14 cycles of
     - 94° for 15”
     - 65° for 5’, then
     - 4° hold

At this point, amplified material is stable and can be stored at –20 ° indefinitely

13. Purify samples using QIAquick PCR cleanup columns or analogous product. It is important to elute the samples in water so that the subsequent labeling reactions are efficient.

   [Since the amplified material contains both single- and double-stranded DNA that can be effectively labeled, the column purification method used should recover both.]

   [At this stage, the purification column eluates for total and immunoprecipitated samples should be readily quantifiable by nanodrop, spectrometer, or dye intercalation, eg, picogreen (dye intercalation may underestimate amount due to single strand product). Optimally, total recovery for immunoprecipitated samples will be in the 1-4 ug range. This gives enough material for several labelings for downstream microarray analysis. If yields are less, or more product is desired, re-amplify material using Sigma GenomePlex WGA Reamplification Kit]

**C. Reamplification (round 2)**

1. Add 15 ng purified amplification product in 10 ul volume to strip tubes or individual thin walled 0.2 ml PCR tubes

   [For input material start with the high concentration primary amplified stock]

2. Prepare master mix for each sample containing:

   - 7.5 ul of 10X Amplification Master Mix
   - 47.5 ul Nuclease-free H20
   - 5 ul WGA DNA polymerase

   For multiple samples, multiply above volumes by the number of samples then add 1/10 volume extra of each component

3. Add 60 ul master mix, vortex or mix by pipetting and quick spin if necessary
4. Incubate in thermal cycler as follows:
   95° for 3’, then 14 cycles of
   94° for 15”
   65° for 5’, then
   4° hold

At this point, amplified material is stable and can be stored at –20 ° indefinitely

5. Purify samples using QIAquick PCR cleanup columns or analogous product.

[Since the amplified material contains both single- and double-stranded DNA that can be effectively labeled, the column purification method used should recover both.]