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Chromatin Immunoprecipitation (ChIP) for Analysis of Histone Modifications and Chromatin-Associated Proteins

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Summary

Disruption of epigenetic regulators of transcription is a central mechanism of oncogenesis. Many of the advances in the understanding of these mechanisms are attributable to the successful development of chromatin immunoprecipitation (ChIP) for in vivo detection of histone modifications as well as chromatin binding regulatory proteins. This is a powerful technique for analyzing histone modifications as well as binding sites for proteins that bind either directly or indirectly to DNA. Here we present two ChIP protocols. The first is particularly useful for identifying histone modifications or binding at specific, known genomic sites. The second, employing serial analysis of gene expression, is particularly powerful for the discovery of previously unidentified sites of modification or binding.

Keywords

Chromatin immunoprecipitation; ChIP; Histone modification; Acetylation; Methylation; Quantitative PCR; SAGE

1. Introduction

Chromatin immunoprecipitation (ChIP) is a powerful technique for analyzing histone modifications as well as binding sites for proteins that bind either directly or indirectly to DNA (1, 2) (Fig. 1). These techniques are based on the ability of formaldehyde to form protein–protein and protein–DNA Schiff’s base cross-linkages that can be reversed by acid or increased temperature. The immunoprecipitated DNA can then be detected by a variety of ways, including Southern blotting, conventional PCR, quantitative PCR, hybridization to arrays (“Chip-on-chip”), or cloning and sequencing (ChIP-serial analysis of gene expression – “ChIP-SAGE”). Although straightforward in principle, the ChIP technique is dependent on many variables, such as the specific antibody, cross-linking conditions, and sonication conditions that must be empirically determined. Here we present protocols for ChIP with PCR or Q-PCR detection as well as ChIP-SAGE approaches. A number of reviews have been published on ChIP-on-chip (3, 4) and a number of companies now offer ChIP-on-chip

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solutions. One excellent source for protocols and an overview of this approach is given in www.chiponchip.org.

2. Materials

2.1. ChIP with PCR Detection

Most buffers and reagents used are from the Upstate Chromatin Immunoprecipitation (ChIP) kit (Cat No. 17-295) or EZ ChIP™ (Cat No.17-371) kit (Upstate Biotechnology, Charlottesville, VA).

1. Sterile PBS.
2. 10% Paraformaldehyde stock: Microwave 18 mL of PBS in a 50-mL tube placed in a beaker with water until water starts to boil (leave lid of tube slightly ajar). Do the subsequent steps in a hood. Add 2 g of paraformaldehyde (wear a mask when weighing out) and 140 µL of 1 M KOH. Vortex carefully until the paraformaldehyde dissolves. Add additional PBS to 20 mL (about 500 µL). Cool to 37°C before use. Can be stored as frozen aliquots indefinitely.
3. DMP/DMSO/PBS: 20 mM DMP solution in 25% dimethyl sulfoxide (DMSO)/PBS (77.75 mg DMP + 3.75 mL DMSO + 11.25 mL PBS).
4. Dimethyl pimelimidate dihydrochloride (DMP, Sigma D8388).
5. Elution buffer prepared fresh either from EZ kit™ components or by dissolving 0.5 g SDS and 0.42 g NaHCO₃ (1% SDS, 0.1 M NaHCO₃) in 50 mL sterile Milli-Q™ water.
6. Protease inhibitors (generally use 1:100 dilution of protease inhibitor cocktail (Sigma No. P8340).
7. DNase-free Proteinase K.
8. Qiagen QIAquick™ PCR purification kit (cat no. 28104).
9. Chromatin IP kit (Upstate Biotechnologies: Cat no. 17-295) or EZ kit (Upstate Biotechnologies: cat no. 17-371)

2.2. ChIP Coupled with SAGE

2.2.1. Linker and Primer Sequences—

1. Linker WL1: 5'-[biotin]GCGGTGACCCGGGAGATCT-GAATTC-3' (PAGE-purified).
2. Linker WL2: 5'-GAATTCAGATC-3' (PAGE-purified).
3. SAGE Linker 1A: 5'-TTTGGATTTGCTGGTGCAGTACAA CTAGGCTTAAT-ATCCGACATG-3' (PAGE-purified).
4. SAGE Linker 1B: 5'-TCGGATATTAAGCCTAGTTGTACTGCACCAGCAAA-TCC[amino mod.C7]-3' (PAGE-purified).

5. SAGE Linker 2A: 5'-TTTCTGCTCGAATTCAAGCTTCTA
ACGATGTACGTCC-GACATG-3' (PAGE-purified).
6. SAGE Linker 2B: 5'-TCGGACGTACATCGTTAGAAGCTTGAATTCGAGC-
AG[amino mod.C7]-3' (PAGE-purified).
7. SAGE Primer 1: 5'-[dual
biotin]GTGCTCGTGGGATTTGCTGGTGCAGTACA-3' (PAGE-purified).
8. SAGE Primer 2: 5'-[dual biotin]GAGCTCGTGCTGCTC-GAATTCAAGCTTCT.

2.2.2. Other Reagents—

1. DNA ENDTM-repair kit (Epicentre Biotechnologies, Cat No. ER0720).
2. 10× END-Repair buffer: 330 mM Tris–acetate, pH 7.8, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM dithiothreitol.
3. T4 DNA ligase (400 U/ μ L, New England Biolabs, catalog no. M0202S).
4. 10× T4 DNA ligase buffer: 500 mM Tris–HCl, pH 7.5, 100 mM magnesium chloride, 100 mM dithiothreitol, 10 mM ATP, 250 μg/mL bovine serum albumin.
5. Dynabeads binding buffer: 10 mM Tris–HCl, pH 7.5, 1 M sodium chloride.
6. 1× TAE buffer: 40 mM Tris–acetate, 1 mM EDTA, pH 8.0.
7. Pfu enzyme (Stratagene, cat no. 600135).
8. Nla III (New England Biolabs, cat no. R0125S).
9. DynabeadsTM M280 Strepavidin (Invitrogen, cat. no. 1112-05D).
10. Sph I-digested pZero-1TM (Invitrogen).
11. ElectromaxTM DH10Bs bacteria (Invitrogen).

2.3. Antibodies for ChIP

The success of ChIP is highly dependent on the antibodies used, which must recognize formalin-fixed epitopes. One way of assessing the likelihood that antibodies will work for ChIP is to perform immunofluorescent staining of cells fixed under the same conditions used for ChIP. In addition, if there is a question of whether the immunoprecipitation is successful the ChIPped chromatin can be boiled in SDS loading buffer and analyzed by western blotting. Some of the antibodies that have been successfully used for ChIP are listed below.

2.3.1. Histone Modifications—

1. Histone H3 dimethyl lysine 4 (Abcam: #ab7766).
2. Histone H3 trimethyl lysine 4 (Abcam: #ab8580).
3. Histone H3 trimethyl lysine 4 (Upstate Biotechnologies: #07-473).
4. Histone H3 dimethyl lysine 79 (Upstate Biotechnologies: #05-835).
5. Histone H3 trimethyl lysine 27 (Upstate Biotechnologies: 07-449).

6. Histone H3, polyacetylated (Upstate Biotechnologies: #06-599).
7. Histone H3 acetyl lysine 9 (Upstate Biotechnologies: #06-942).
8. Histone H3 acetyl lysine 14 (Upstate Biotechnologies: #07-353).
9. Histone H3 acetyl lysine 27 (Upstate Biotechnologies: #07-360).
10. Histone H4, polyacetylated (Upstate Biotechnologies: #06-866).
11. Histone H4 acetyl lysine 5 (Upstate Biotechnologies: #07-327).
12. Histone H4 acetyl lysine 8 (Upstate Biotechnologies: #07-328).
13. Histone H4 acetyl lysine 12 (Upstate Biotechnologies: #07-323).
14. Histone H4 acetyl lysine 16 (Upstate Biotechnologies: #07-329).

2.3.2. Antibodies for Chromatin-Associated Proteins—

1. Rbbp5 (Bethyl Laboratories, Montgomery, TX: cat no. A300-109A).
2. Menin (Bethyl: A300-105A).
3. Estrogen receptor (ER) Ab10 (Lab Vision-Neomarkers, Fremont, CA, TE111.5D11, #MS-315-PO).
4. Estrogen receptor (ER) Ab3 (Lab Vision-Neomarkers AER308, MS-170-PO).
5. MLL1 antibodies (Bethyl: A300-086A).

3. Methods

This protocol is a minor modification of the Upstate Biotechnology protocol that accompanies their chromatin IP kit.

3.1. ChIP and PCR Detection

3.1.1. Fixation

General Fixation:

1. For fibroblasts, use 5×10^5 cells per ChIP experiment. For 293 cells, hematopoietic cells or primary human cells, use 5×10^6 cells per ChIP experiment.
2. For histone ChIP or standard transcription factor ChIP (*see* Note 1), fix cells for 10 min with 1% freshly made or frozen paraformaldehyde in media at 37°C. For proteins that are members of multisubunit or nucleosome remodeling complexes (*see* Note 2), fix cells using the alternate DMP fixation protocol outlined below.
3. For adherent cells, remove media/paraformaldehyde and rinse cells 1× with PBS. Scrape cells into 5 mL PBS with protease inhibitors. Alternatively the protease inhibitor cocktail from EZ ChIP™ kit can be used. Pellet the cells at $364 \times g$ for 5 min. Continue with **step 5**.
4. For nonadherent cells, fix in a small volume of 1% paraformaldehyde in media (~5 mL). When fixation is complete, dilute cells 10× with 45 mL of RPMI. Spin at 364

$\times g$, 5 min, remove the majority of the RPMI. Transfer the remainder to a new tube (10 mL). Rinse with PBS and continue with **step 5**.

5. The pellet can be flash frozen on dry ice at this point and stored at -80°C or you can continue with the protocol at this point. If continuing at this point, freeze/thaw the cells once to facilitate cell disruption. Fixed pellets can be stored frozen for several months without loss of signal.

Alternate Fixation Protocol with Dimethyl Pimelimidate Dihydrochloride (DMP)
(Based on Robyr et al. 2004) (5):

1. Remove a bottle of dimethyl pimelimidate dihydrochloride from the freezer and allow it to warm to room temperature before opening (it is highly hygroscopic).
2. Rinse cells gently with PBS and then fix cells either directly on the plate or in a 1.5-mL tube by rocking at room temperature for 1 h (for a 10-cm dish use 5 mL DMP/DMSO/PBS, for a 1.5-mL tube use 400 μL DMP/DMSO/PBS).
3. Add 1% paraformaldehyde (in PBS) directly to the plate/tube for a final concentration of 2/3% paraformaldehyde (i.e., add 10 mL 1% paraformaldehyde to 5 mL of DMP solution or 800 μL to 400 μL solution).
4. Seal the plates with Parafilm™ or rotate in 1.5-mL tubes at room temperature in a fume hood for 5–20 h (empirically determine time for different proteins).
5. Spin down at $364 \times g$, 5 min and rinse cells with PBS being careful not to lose material on the walls of the tube.
6. Add lysis buffer and begin ChIP protocol. Alternatively, the pellet may be frozen at this point. The samples should be aspirated $3\times$ through a 27G needle. The sonication conditions will also need to be increased empirically (for e.g., Bioruptor™ for 20 min on high with a 0.5-min cycle). Continue with **step 4** in **Subheading 3.1.2**.

3.1.2. Lysis and Sonication—

1. Add 100 μL of chromatin lysis buffer (+ protease inhibitors) per 5×10^5 MEF cells (or 5×10^6 or more cells per ChIP for “smaller” cell types) and place in 1.5-mL microfuge tube on ice for 10 min.
2. Syringe passage $3\times$ with a 27-gauge needle; centrifuge briefly between passages if mixture gets foamy. Do the first and second passes and spin briefly at $15,000 \times g$, then follow with third pass.
3. Sonication conditions must be empirically determined depending upon the specific sonicator and the fixation conditions used. As an example, for a 10 min, 1% paraformaldehyde fixation, a Diagenode™ Bioruptor set at high for 10 min (0.5 min cycle) is usually sufficient for sonication of DNA to 200–700 bp. If using the DMP fixation outlined below, sonication should be increased to two cycles of 10-min each.

4. Spin at $15,000 \times g$ for 20 min at 4°C. Transfer supernatant to a 15-mL tube and dilute 10× with chromatin dilution buffer plus protease inhibitors. Save several 50-μL aliquots of diluted chromatin. These aliquots represent input and comprise about 5% of the total chromatin used per ChIP. These should be flash frozen and stored at −20°C to be processed along with the immunoprecipitated samples.

3.1.3. Immunoprecipitation—

1. Add antibodies to tubes (usually 1–5 μL/mL, depending on the antibody). One tube without antibody (or pre-immune serum if available) should be used as a no antibody control. We have not found a preclearing step to be necessary.
2. Rotate at 4°C overnight.
3. Collect immune complexes with 60 μL of a 50:50 slurry of agaroseA (or agaroseG for mouse monoclonal antibodies) and salmon sperm DNA slurry (from kit) by rotating for 1 h at 4°C. The slurry is viscous and should be gently mixed immediately before using and transferred using wide bore pipette tips.
4. Pellet beads by centrifugation. Spin tubes briefly until the centrifuge gets up to speed, or by spinning at $94 \times g$ for 1 min. Longer spins may crush the beads. Wash the beads for 3–5 min on a rotating platform with 1 mL of each of the following kit buffers (labeled wash buffers 1–5) (1) Low salt immune complex buffer; (2) High salt immune complex buffer; (3) LiCl immune complex wash buffer; (4) 1× TE; 5. 1× TE. All washes should be performed at 4°C.
5. Elute immune complexes by adding 100 μL of elution buffer to pelleted beads. Vortex briefly at $15,000 \times g$ to mix and incubate at RT for 15 min with rotation. Spin down the beads; carefully transfer supernatant (eluate) to another tube and repeat elution. Combine eluates.
6. Add 8 μL of 5 M NaCl (from kit) to the combined eluates and reverse crosslinks at 65°C overnight. At this point, thaw the input DNA (50 μL) and add 150 μL of elution buffer and 8 μL of 5 M NaCl. Process along with IPs (also 65°C overnight). The tubes can be flash frozen after reversing the crosslinks if desired.
7. The next day, add 4 μL of 0.5 M EDTA, 8 μL of 1 M Tris-HCl, pH 6.5 and 1 μL of 10 mg/mL Proteinase K to the eluate. Incubate for 1 h at 45°C.
8. Recover DNA using a Qiagen Qiaquick™ PCR kit according to the manufacturer's basic protocol. Alternatively, DNA can also be purified either by phenol:chloroform extractions or with the Upstate EZ™ ChIP kit.
9. Analyze ChIPped chromatin using quantitative PCR or ChIPSAGE (*see* Notes 3 and 4).

3.2. Analysis of ChIP DNA Using the Long-SAGE Protocol

A different approach is required if the gene targets of interest are not known. One approach is the use of ChIP–on-chip on genome-wide arrays. Serial analysis of gene expression (SAGE), a technique for high-throughput analysis of gene expression, has also been used for

high-throughput analysis of histone modifications and DNA binding proteins. SAGE is based on the principle that short sequence tags contain sufficient information for the identification of a transcript and the number of tags from a particular transcript reflects the absolute abundance of the transcript in the cell. In the modified SAGE protocol (Long-SAGE) (6), the cDNA synthesized from the mRNA isolated from the cells is digested with Nla III restriction enzyme and ligated to a linker containing a class II restriction enzyme sequence (Mme I). Digestion with Mme I cleaves the DNA 21 bp away from its recognition site and releases a 21-bp tag from the cDNA. The short tags are concatenated to 1–2 kb molecules, which are then cloned and sequenced.

ChIP coupled with SAGE has been used to analyze genome-wide histone modification patterns in yeast and human cells (7–9). Because of the quantitative nature of SAGE, this procedure requires minimal normalization. Moreover, it does not require preselected probes and, therefore, can identify any genomic loci that are modified. The combination of this method with the recently developed high throughput sequencing techniques, such as massive parallel signature sequencing (MPSS), provides a comprehensive, quantitative, and economical method for analyzing genome-wide epigenetic modifications of the human genome.

1. After ChIP (**Subheading 3.1.3**), use a DNA ENDTM-repair kit to blunt the DNA ends by mixing 0.1–0.3 µg of ChIP DNA, 5 µL of 10× END-RepairTM buffer, 5 µL of 2.5 mM each dNTPs, 5 µL of 10 mM ATP, 1 µL of END-RepairTM Enzyme mix (T4 DNA polymerase and T4 polynucleotide kinase). Adjust to 50 µL of volume and incubate at room temperature for 45 min. The DNA is purified using phenol–chloroform extraction and ethanol precipitation.
2. Ligate the annealed biotinylated WL1 + 2 linker to the repaired DNA ends by mixing 38 µL of the ChIP DNA, 5 µL of 10× T4 DNA ligase buffer, 5 µL of 8 µM of the WL1 + 2 linker, 2 µL of T4 DNA ligase (400 U/ µL). Incubate at 14°C for 14 h. Purify the DNA using the QIAquickTM column.
3. Amplify the DNA with linkers using the 5' biotinylated WL1 as the primer by mixing 34 µL of DNA, 5 µL of 10× Pfu buffer, 5 µL of 1 mM dNTPs, 5 µL of 10 µM of WL1, and 1 µL of Pfu enzyme. First incubate the reaction mixture at 74°C for 10 min to synthesize the complementary strand of the linker region. Then cycle as follows: 94°C, 30 s; 58°C, 30 s; 74°C, 45 s; repeat the cycle 17×. Purify the DNA using the QIAquickTM column.
4. Digest the DNA with 10 U of Nla III at 37°C for 2 h. Purify the DNA with phenol–chloroform extraction and ethanol precipitation.
5. Ligate with the SAGE linkers 1(A + B) and 2(A + B): Resuspend the DNA in 66 µL of 1× T4 DNA ligase buffer and dispense it into two tubes (33-µL each). Add 5 µL of 8 µM SAGE linker 1(A + B) to tube 1 and 5 µL of 8 µM long-SAGE linker 2(A + B) to tube 2. Heat the mixture at 50°C for 2 min and incubate at room temperature for 10 min. Add 2 µL of T4 DNA ligase to each tube and incubate at 16°C for 16 h.

6. Combine the two ligation mixtures and dilute with 160 μ L of binding buffer. Add the mixture to 1 mg of Dynabeads™ M280 Streptavidin. Incubate at room temperature for 15 min with occasional mixing. Discard the supernatant and wash beads twice with 200 μ L of binding buffer and once with 1 $\times$ TE.
7. Digest beads-bound DNA with Mme I by adding 244 μ L of H₂O, 30 μ L of 10 $\times$ New England buffer 4, 3 μ L of 5 mM *S*-adenosylmethionine, 3 μ L of 100 $\times$ BSA, and 20 μ L of 2 U/ μ L Mme I. Incubate at 37°C for 3 h with occasional mixing.
8. Collect the supernatant and purify the DNA using phenol–chloroform extraction and ethanol precipitation.
9. Resuspend the DNA in 9 μ L of 1 $\times$ T4 DNA ligase buffer. Add 1 μ L of T4 DNA ligase and incubate at 16°C for 16 h.
10. Amplify the ditags using SAGE primers 1 and 2. Mix 100 μ L of 10 $\times$ Pfu buffer, 100 μ L of 1 mM dNTPs, 50 μ L of DMSO, 35 μ L of 10 μ M primer 1, 35 μ L of 10 μ M primer 2, 650 μ L of H₂O, 10 μ L of the ligation mixture, and 20 μ L of 2.5 U/ μ L of Pfu polymerase. Dispense into aliquots of 50 μ L. Cycle as follows: 94°C, 20 s; 55°C, 30 s; 74°C, 40 s; repeat 22 $\times$.
11. Purify the PCR products using phenol–chloroform extraction. Digest the DNA with Nla III by mixing 320 μ L of DNA, 40 μ L of 10 $\times$ buffer 4, 4 μ L of 100 $\times$ BSA, and 40 μ L of Nla III (10 U/ μ L) and incubating at 37°C for 2 h. Stop the reaction by adding 400 μ L of 1 $\times$ TE + 2 M NaCl.
12. Transfer the reaction mixture to an Eppendorf tube containing 1 mg of Dynabeads™ M280 (streptavidin). Mix at room temperature for 15 min.
13. Transfer the supernatant to a new tube containing 1 mg of Dynabeads™ M280 (streptavidin). Mix at room temperature for 15 min.
14. Repeat **step 13** three times.
15. Transfer the supernatant to a new tube and purify the DNA using phenol–chloroform extraction and ethanol precipitation.
16. Resuspend the DNA in 30 μ L of 1 $\times$ TE. Load the DNA into six lanes of a 12% mini acrylamide gel and resolve the DNA fragments by electrophoresis at 100 V for 1 h.
17. Visualize the DNA bands by ethidium bromide staining and excise the band of approximately 38 bps.
18. Spin the gel slices through a 21-gauge hole at the bottom of an Eppendorf™ tube and extract the DNA from the gel with 500 μ L of 1 $\times$ TE + 100 μ L of 7.5 M NH₄ OAc by incubation at 37°C for 2 h with shaking. Purify the DNA with ethanol precipitation.
19. Resuspend the DNA in 9 μ L of 1 $\times$ ligase buffer. Add 1 μ L of T4 DNA ligase and incubate at 16°C for 1 h.

20. Resolve the ligation products on 1.4% per 1× TAE agarose gel. Excise the DNA fragments from 500 to 1,000 bp. The DNA is purified using the QIAEX II™ gel extraction kit.
21. Ligate to a sequencing vector by mixing 7 µL of the concatenated DNA, 1 µL of 20 ng/ µL of Sph I-digested pZero-1™, 1 µL of 10× T4 DNA ligase buffer, and 1 µL of T4 DNA ligase. Incubate at 16°C for 16 h. Transform Electromax™ DH10Bs bacteria using the ligation mixture by electroporation. Plate the transformation mixture with ampicillin selection. Prepare DNA from individual colonies and analyze the insert by standard enzymatic sequencing reaction.
22. Alternatively, the concatemers can be directly sequenced by the MPSS-like high throughput sequencing services provided by companies such as 454 Lifesciences (Branford, CT). Each sequencing run can generate around 2×10^7 bp of DNA sequences.
23. The sequences obtained either by the traditional sequencing method or the 454 high throughput technique are analyzed using the SAGE2000 v.4.5 program developed by the Johns Hopkins University to extract the 21 bp sequence tags and to quantify the tags. The sequence tags are then compared with a reference library generated from Nla III cutting sites in the human genomic sequence available at UCSC or NCBI databases.

4. Notes

1. Fixation conditions for ChIP must be determined empirically. The optimal conditions appear to vary depending on whether the protein of interest (a) binds directly to a specific DNA sequence such as transcription factors, (b) binds directly to DNA but in a sequence-independent manner such as histones, or (c) binds to DNA only indirectly via interactions with complexes of proteins such as histone methyltransferases or members of nucleosome remodeling complexes (Fig. 1). Generally the fixation conditions should be minimized, especially when dealing with histones as lysine residues are structurally altered by fixation. For histone or histone modifications we have had good results with ChIP when cells are fixed for 10 min with 1% formaldehyde at 37°C. The same conditions work well with transcription factors that are directly bound to DNA as the efficiency for DNA:protein cross-linking is very high (Fig. 1b).
2. When doing ChIP with proteins that are subunits of large molecular weight complexes, multiple protein:protein as well as protein:DNA fixation steps may be required (Fig. 1c). In this case, the signal can often be increased with a multistep fixation procedure employing both a protein:protein crosslinker, such as DMP and then a long formaldehyde crosslinking step. These conditions can increase signal intensity nearly tenfold; however, the conditions need to be optimized because the background signal also increases. Sonication conditions must also be empirically determined. The longer the fixation protocol, the more difficult it will be to sonicate the DNA efficiently. We have found that passage of lysed chromatin through a 27G needle before sonication improves the efficiency of sonication. We

recommend analyzing the sonicated DNA on agarose gels. For optimal ChIP experiments, DNA fragments should be between 200 and 1,000 bp with a mean fragment size of approximately 500 bp.

3. Although conventional PCR may be used for detection in ChIP, in practice, this approach only works well when the differences between signal and noise are significant. For subtle changes or for weaker ChIP signals, quantitative PCR offers many advantages including analysis in the linear range of the PCR reaction and for a rigorous analysis of IP efficiency. We routinely use TaqMan™ primer/probe sets or SYBR green™. Primer Express™ software (ABI) is helpful for designing primers or primer/probe sets, but care must be taken to visually inspect primers to make sure the GC content is between 20 and 80% and to make sure that the TaqMan probe does not start with a G. Both SYBR green™ and Taqman™ reactions generate curves that can be analyzed as discussed below. There is no uniformly agreed approach for quantification of ChIP results. The most common approach is to quantitate immunoprecipitation efficiency as an IP:Input ratio. The two main ways of doing this include using standard curves with a dilution series of an input sample, or comparing input vs. IP signal directly. The approaches yield similar results. For brevity we discuss the second approach. The amount of genomic DNA coprecipitated with antibody is calculated as a percentage of total input using the following formula $C_T = C_T(\text{input}) - C_T(\text{chromatin IP})$, % total = $2^{-C_T} \times 5.0\%$ (Fig. 2). A 50-μL aliquot taken from each of 1 mL of sonicated, diluted chromatin before antibody incubation serves as the input (see protocol). The signal from the input samples represents 5% of the total chromatin used in each ChIP. C_T values are determined by choosing threshold values in the linear range of each PCR reaction (see Fig. 2 for a sample calculation).
4. In using Q-PCR for ChIP analysis, it is important to recognize that quantified values are not direct measures of absolute protein levels. The strength of the ChIP signal is dependent on multiple factors including the strength of the antibody:epitope interaction, the accessibility of the epitope after fixation, as well as the absolute amount of protein present at the locus.

A second important factor is that background signal is inherent to ChIP experiments even in the absence of antibody, raising the question of how to distinguish between background and “real” signal. Contamination by cloned DNA representing target sequences when these are also being handled can be a major impediment to successful ChIP. If at all possible, the pre- and post-PCR areas should be separated. Ideally, ChIP experiments should include the following controls:

- a. A cell line lacking the protein of interest to use as an antibody control
- b. A set of gene targets that are not bound or regulated by the protein of interest
- c. A set of positive gene targets that do not change upon experimental manipulation

For example, in Fig. 3a, MLL ChIP gives a very strong signal in *MLL*^{+/+} cells at the *Hoxa9* locus (0.65%). In *MLL*^{-/-} cells, this signal is reduced to below 0.05%, validating the ChIP assay and establishing this as the background range for this antibody (Fig. 3a). At the *Gapdh*

locus, which microarray experiments suggest is not regulated by Mll, the signal is below 0.05% in both *Mll*^{+/+} and *Mll*^{-/-} cells, indicating that this locus is not bound by Mll.

Conditional models are particularly powerful for ChIP experiments (Figs. 3 and 4). As an example, when fused to the estrogen receptor (ER), the MLL–ENL fusion protein is only active in the presence of tamoxifen (4-OHT). When ChIP is performed with the antibody Ab10, which recognizes the ER present in MLL–ENL, a strong signal is seen at *Hoxa9* (Fig. 3b, black bar) in the presence, but not absence, of 4-OHT (Fig. 3b, striped bar, about 0.2%). A small amount of residual binding is seen in the absence of 4-OHT. Two different antibodies that do not recognize the fusion protein (Ab1 and general IgG) give a signal again below 0.05% (white and grey bars) and Ab10 in both + and – 4-OHT treated cells gives a signal below 0.05% at *Gapdh*, indicating that the residual binding seen at *Hoxa9* is likely “real.”

It is not always possible to include all of these controls. Our general experience is that different antibodies with different fixation conditions generally give a background, nonspecific ChIP signal in the range 0.01–0.08% of input (see Fig. 5). As a rule of thumb, any signal over 0.1% of input is likely to be significant, while below that is likely in the range of background signal.

Another approach that is particularly informative is to generate a series of primers across the locus to be examined. The graphical representation of the quantitative ChIP data can provide additional information and a higher degree of confidence that small changes in binding seen are significant (see Fig. 4).

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Fig. 1.

(a) Nucleosomes (*gray shaded circles*) are intimately associated with DNA at multiple contact points. A transcription factor (*grey circle*) can bind DNA directly but may have only a single interaction point. Other proteins (*black circles*) may interact with DNA only indirectly through interactions with DNA bound proteins. (b) A short fixation efficiently crosslink's (*black "X"*) histones and transcription factors to DNA, but the indirectly bound proteins are less likely to be efficiently cross-linked (c). Longer fixation efficiently

crosslinks (*white* “X”) the indirectly bound proteins, but histone proteins may become masked by excessive cross-linking to other proteins or by alterations of lysine residues.

Fig. 2.

(a) Quantitative PCR curve for input (*black curve*) and chromatin IP (*grey curve*) samples. The threshold value (*horizontal line*) is chosen in a region where the curves are linear. C_T is the difference in cycle # at the threshold (i.e., between the input and IP curves there is 5 cycle difference) (b) A sample calculation. The difference in cycle number between the input and IP samples is -5 (the higher the cycle number, the lower the amount of starting material). Since each cycle indicates a doubling of material, there is 2^{-5} or about 3/100 the amount of material in the IP sample compared to the input. The input represents 5% of the chromatin used in the ChIP experiment (see protocol); therefore the IP is about $3/100 \times 5\%$ or 0.15% of the total chromatin.

Fig. 3.

Comparison of ChIP signal vs. background signal. **(a)** ChIP with antibodies against MLL was performed in MEF cells that were either wild type for *Mll* (*Mll*^{+/+}, black bars) or were *Mll* knockout cells (*Mll*^{-/-}, grey bars). A strong signal is seen at the *Hoxa9* locus in *Mll*^{+/+} cells and this signal is reduced to below 0.05% in *Mll*^{-/-} cells indicating that this is in the background range for this antibody. Binding at *GAPDH* is below this threshold in both cell types indicating that it is negative for Mll binding, at least at the specific region of *GAPDH* examined. **(b)** MLL-ENL-ER, a MLL fusion protein fused to the estrogen receptor, is active only in the presence of 4-OHT (10). Ab10 recognizes the fusion protein and shows a strong binding signal at *Hoxa9* in the presence of 4-OHT (black bar). In the absence of 4-OHT, signal is drastically reduced but not completely abolished (striped bar). Ab1, (grey bars) which does not recognize the fusion protein, gives a similar signal as IgG (white bars).

Fig. 4.

Using the same experimental conditions as in Fig. 3, but with a series of Q-PCR primers (1–7) across the *Hoxa9* locus allows for graphical representation of MLL-ENL-ER binding (reprinted with permission from ref. 10).

Fig. 5.

Background ChIP signals in control cell lines and with control antibodies. 1 MLL ChIP in *Mll*^{-/-} cells, 2 MLL ChIP in *Mll*^{-/-} cells using a Bethyl antibody (cat. no. A300-086), 3 menin ChIP in *menin*^{-/-} cells, 4 rabbit anti-mouse IgG ChIP in *Mll*^{+/+} cells, 5 anti-FLAG ChIP in 293 cells. Each point on the graph represents a separate ChIP experiment quantified for a “positive” gene target (e.g., *HOXA9*) in a control cell line or with a control antibody. Cells were fixed for 10 min in 1% paraformaldehyde. The two MLL antibodies tested in *Mll*^{-/-} cells (1 and 2) produce background signal up to about 0.05%. The menin antibody produces a higher range of background signal (up to 0.08%) in *menin*^{-/-} cells, but all antibodies are below 0.1%.