Video Article

Chromatin Immunoprecipitation (ChIP) to Assay Dynamic Histone Modification in Activated Gene Expression in Human Cells

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Abstract

In response to a variety of extracellular ligands, the STAT (signal transducer and activator of transcription) transcription factors are rapidly recruited from their latent state in the cytoplasm to cell surface receptors where they are activated by phosphorylation at a single tyrosine residue[1]. They then dimerize and translocate to the nucleus to drive the transcription of target genes, affecting growth, differentiation, homeostasis and the immune response. Not surprisingly, given their widespread involvement in normal cell processes, dysregulation of STAT function contributes to human disease, particularly to cancers[2] and autoimmune diseases[3].

It is well established that transcription is regulated by alterations to the chromatin template[4,5]. These alterations include the activities of ATP-dependent complexes, as well as covalent histone modifications and DNA methylation[6]. Because STAT activation of gene expression is both rapid and transient, it requires specific mechanisms for modulating the chromatin template at STAT-dependent gene loci. To define these mechanisms, we characterize the histone modifications and the enzymatic activities that generate them at gene loci that respond to STAT signaling. This protocol describes chromatin immunoprecipitation, a method that is valuable for the study of STAT signaling to chromatin in activated gene expression.

Video Link

The video component of this article can be found at https://www.jove.com/video/2053/

Protocol

PLANNING

The day before a ChIP experiment is planned, cells should be cultured so that a sufficient number is available. Assume that each ChIP assay will require ~1-1.5 x 10^7 cells. Always plan to do both the negative (IgG) and positive (pan histone antibody) controls and duplicate experiments.
Tip - For 2FTGH cells, each ChIP assay requires about one 15 cm tissue culture dish at 80% confluency.

PART 1: PREPARE CHROMATIN AND PERFORM ChIP

1. Remove culture media and induce cells for the times required with 20 mL of 10% cosmic calf serum (CCS)/DMEM containing IFN-gamma at 5 ng/mL. Replace the media on the uninduced plates with 20 mL of 10% CCS/DMEM as well.
2. In a fume hood, add 540 µL 37% formaldehyde to 20 mL of media (1% final concentration) in the 15 cm dish.
   Tip - Tilt plate and add the formaldehyde to the media pool - then rock plate to spread it evenly.
3. Allow crosslinking to proceed for 10 minutes at room temperature.
4. Add glycine to 0.125 M final concentration to stop crosslinking.
5. Vacuum aspirate the media and wash cells once with 10 mL ice cold phosphate buffered saline (PBS).
6. Collect cells with a cell scraper in ~ 7 mL PBS and combine cells (from 6 plates) that were treated identically in 50 mL conical tube on ice.
7. Centrifuge (Beckman Coutler Allegra 12-R Centrifuge) at 1800 rpm at 4°C for 5 minutes and aspirate the PBS.
   Tip - Snap freeze the cell pellet if stopping here and store at -80°C.
8. Resuspend the cell pellet in 4 mL Swelling Buffer (25 mM HEPES pH 7.8, 1.5 mM $MgCl_2$, 10 mM KCl, 0.1% NP40. Add 1 mM DTT, Complete protease inhibitors and chill on ice prior to use) and transfer to a 15 mL conical tube.
   Tip - This swelling buffer volume is appropriate for a 6 plate pooling (~6-9 x 10^7 cells). More or less buffer might change the douncing efficiency.
9. Incubate the cell suspension on ice for 10 minutes.
10. Transfer suspension to a pre-chilled 15 mL douncer (Wheaton) and dounce 20 strokes with pestle A.
11. Transfer dounced cells back to a 15 mL conical tube on ice.

12. Centrifuge at 2500 rpm for 5 minutes at 4°C and discard the supernatant.
13. Resuspend the nuclear pellet (~ 200 µL from 6 plates) in 3 mL (15 pellet volumes) of Sonication Buffer (50 mM HEPES pH 7.9, 140 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% Sodium deoxycholate, 0.1% SDS. Add Complete protease inhibitors and chill on ice just prior to use).
    Tip - This sonication buffer volume is appropriate for a 6 plate pooling (~6-9 x 10^7 cells). More or less buffer might change the sonication efficiency.
14. Sonicate nuclei suspension on ice for 10 cycles (20 seconds on/40 seconds off /amplitude setting of 8) using a Misonix Sonicator 3000 equipped with the microtip. This typically results in chromatin fragments averaging 200-1000 bps in size.
    Tip - Because sonicators will vary, the sonication efficiency must be determined empirically. To do so, take aliquots of 20 µL before sonication and after each sonication cycle. Heat these samples at 65°C for 4 hours. Add DNA loading buffer and run a standard 1% agarose/TBE gel to visualize DNA fragments.)
15. Transfer sonicated material to an SS34 centrifuge tube and centrifuge for 20 minutes at 4°C at 13000 rpm in an SS34 rotor (Sorvall RC6 Plus Centrifuge) to pellet what is usually a small amount of cell debris.
16. Carefully transfer the supernatant to a 15 mL conical tube on ice.
17. Pre-clear by adding 360 µL Salmon Sperm DNA/Protein A or G agarose beads slurry and rocking at 4°C for at least 30 minutes.
    Tip - This step decreases the noise in the assay that arises from non-specific binding events.
18. Centrifuge at 1000 rpm at 4°C for 5 minutes to pellet the agarose beads.
19. Transfer pre-cleared supernatant to another 15 mL conical tube on ice.
20. Measure the A260 units of the pre-cleared chromatin and adjust the volume with Sonication Buffer to four (4) A260 units (0.2 mg/mL assuming 1 A260 unit = .050 mg/ml)
    Tip - To measure the A260 units, dilute 10 µL of pre-cleared chromatin in 190 µL DDW; blank spectrophotometer with 10 µL sonication buffer in 190 µL DDW.
21. Save 75 µL of the chromatin (at 4 A260 units) as the Input Sample and store at 20°C until ready to reverse crosslinks.
    Tip - This step is crucial for data analysis. Do not neglect to take the input aliquot.
22. Aliquot the prepared chromatin to 1.7 mL microfuge tubes on ice as 1 mL aliquots.
    Tip - If stopping here, snap freeze the prepared chromatin on dry ice and store at -80°C. NB: stored chromatin can degrade, producing less than optimal results, so avoid if possible.
23. Add the ChIP-grade antibodies to the induced and uninduced samples sets in duplicate. Add IgG (2 µg) and pan Histone H3 antibody (15 µL) as the negative and positive controls to induced and uninduced samples sets.
    Tip - The amount of antibody to use must be determined empirically or see the supplier's product sheet for suggested amounts. Typically 1-2 µg of a ChIP-grade antibody works well. The appropriate volume must be determined empirically for antibodies that are supplied as serum.
24. Rock all tubes overnight at 4°C.

PART 2: COLLECT IMMUNOCOMPLEXES

1. Add 60 µL of Salmon Sperm DNA/Protein A or Protein G agarose beads slurry to all tubes.
   Tip Use Protein A for rabbit polyclonal antibodies and Protein G for mouse monoclonal antibodies.
2. Rock at 4°C for at least 60 minutes.
3. Pellet the immunocomplexes (agarose beads) by microfuging gently at 2000 rpm at 4°C for 3 minutes.
4. Aspirate the supernatant.
5. Wash the immunocomplexes with 1 mL of each of the following Wash Buffers for 10 minutes with rocking at 4°C. Microfuge for 3 minutes at 2000 rpm at 4°C between each wash and aspirate supernatant. Keep samples chilled on ice. Add PMSF just prior to use.
   - Low Salt Wash - 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 150 mM NaCl, 1 mM PMSF
   - High Salt Wash - 0.1% SDS, 1% Triton X-100, 2 mM EDTA, 20 mM Tris-Cl, pH 8.0, 500 mM NaCl, 1 mM PMSF
   - LiCl Wash - 0.25 M LiCl, 1% NP-40, 1% Sodium deoxycholate, 1 mM EDTA, 10 mM Tris-Cl, pH 8.0, 1 mM PMSF
   - TE Wash - TWO Washes with TE Buffer -10 mM Tris-Cl, pH 8.0, 1 mM EDTA, 1 mM PMSF
6. Remove the final TE Buffer wash and to the beads, add 500 µL Elution Buffer (50 mM Tris-Cl, pH 8.0, 10 mM EDTA, 1% SDS).
   Tip - The Elution Buffer should be prepared freshly.
7. Vortex to mix agarose beads in Elution Buffer and heat for 30 minutes at 65°C.
   Tip - vortex each tube at least every 10 minutes during this 30 minutes.
8. Microfuge for 30 seconds at 2000 rpm and transfer the eluent to a new microfuge tube.
   Tip - During the elution, thaw your input samples and add 500 µL Elution Buffer to each one. Include these samples in all steps going forward.
9. Add 5 M NaCl to a final concentration of 200 mM to all samples and vortex.
10. Incubate at 65°C overnight (or at least 4 hours).

PART 3: PURIFY ChIP'd AND INPUT DNA

1. Microfuge all samples for 30 seconds to collect condensation on lids.
2. Add 1µL of RNAse A (10 U/µL) to each tube and incubate at room temperature for 15 minutes.
3. Add 10 µL 0.5 M EDTA, 40 µL 0.5 M Tris-Cl pH 6.5, 2 µL Proteinase K (10 mg/mL) and incubate at 45°C for 60 minutes. Tip Prepare a master mix of these reagents and add 52 µL to each tube.
4. Phenol/chloroform/isoamyl alcohol extract and then chloroform/isoamyl alcohol extract the samples.
5. Add 2 µL glycogen (25 µg/µL) and 0.1 volumes of 3M Na Acetate pH 5.2 and vortex.
6. Add 2.5 volumes of 100% ethanol and vortex.
7. Incubate at 20°C overnight.

PART 4: PURIFY ChIP'd AND INPUT DNA CONTINUED

1. Microfuge samples for a minimum of 20 minutes at 13000 rpm at 4°C to precipitate the DNA/glycogen pellets.
2. Wash with DNA/glycogen pellets with 1 mL of 70% Ethanol.
3. Air dry the pellets or quickly dry in DNA Savant speed vacuum centrifuge.
   Tip - Do not over dry the DNA.
4. Resuspend DNA/glycogen pellets in 200 µL TE Buffer.
5. DNA (1 µL) is ready for quantification via real-time PCR. Store remaining DNA at 20°C.
   Tip - Using more than 1 µL of DNA can inhibit the real-time PCR reactions, so scale up with caution.

Part 5: REPRESENTATIVE RESULTS:

ChIP and Input DNA samples are quantified with Real-Time PCR [7,8] (Applied Biosystems 7500 Fast Real-Time PCR System) using primers specific to the gene locus of interest. Primers that target genomic sequence known to be enriched in or lacking in the histone modifications being assayed can be used as positive and negative controls as well. Data is presented as Percent of Input. The ChIP procedure should be wholly repeated with three biological replicates to ensure reported changes in histone modifications are statistically significant.

There are several important issues to consider when using real-time PCR to quantify DNA, including primer design, PCR efficiency, and the appropriate way to calculate the percent of input and normalization procedures. When profiling histone modification occupancy across a gene, the PCR efficiency must be consistent among all primer pairs. The real-time PCR system manufacturer can provide the necessary information and training.

Figure 1 shows representative results of chromatin immunoprecipitated during STAT1 activation of the *IRF1* gene[9], triggered by IFN-γ.

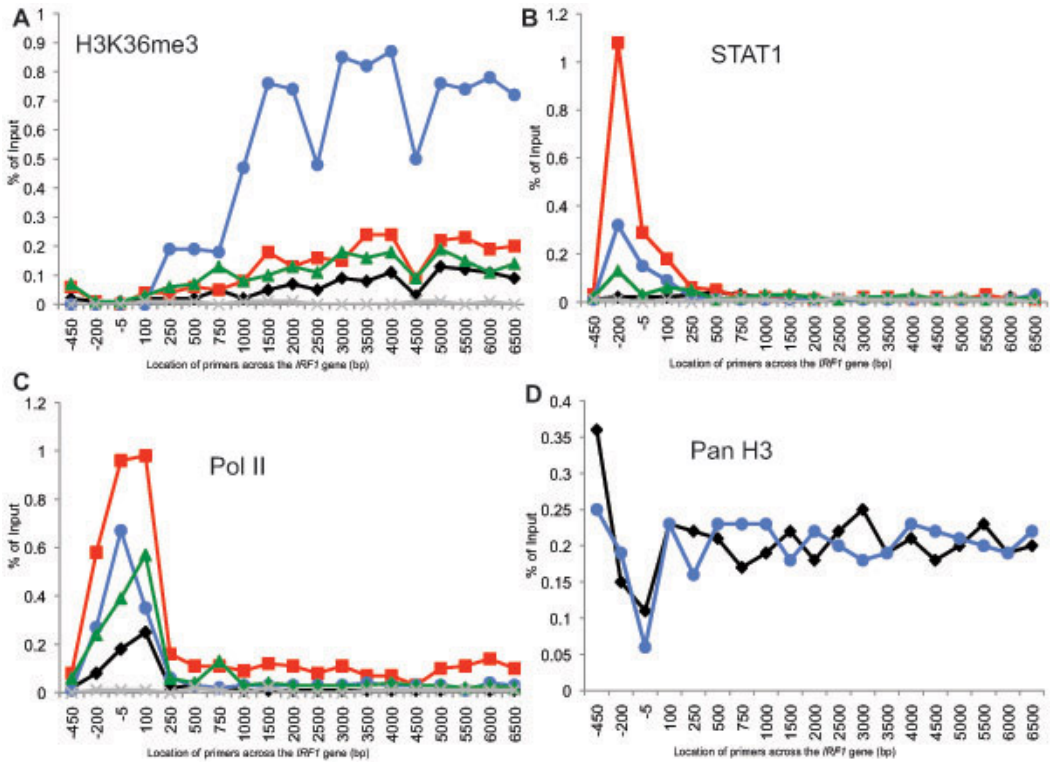

Figure 1. Occupancy of STAT1, RNA Polymerase II and H3K36me3 is dynamic during STAT1 activation of the IRF1 gene. (A, B, C) ChIP with α-H3K36me3, α-STAT1 and α-Pol II of chromatin collected from 2FTGH cells induced with IFN-γ for 30 minutes (red squares), 1.5 hours (blue circles), 5 hours (green triangles) or uninduced (black diamonds). IgG is the negative control (grey crosses). **(D)** Pan H3 is the positive control and shows the histone occupancy across the locus in the induced (blue circles) and uninduced conditions (black diamonds). The dip around -5 bp is due to the nucleosome depletion that is found at transcription start sites.

Discussion

The ChIP protocol outlined has been used successfully in the lab to assay more than twenty different histone modifications. In addition, we have characterized changes in the occupancy of transcription factors, histone-modifying enzymes, proteins that recognize histone modifications and the RNA Polymerase II transcription machinery, at several IFN-γ induced genes. We have also used the procedure to characterize changes in histone modifications during the differentiation of a cancer stem cell[10].

The quality of ChIP data ultimately depends upon the effectiveness of the antibody used and there is considerable variability among antibodies. The failure to ChIP a modification, therefore, cannot be interpreted to mean that said modification is not present. Furthermore, changes in a particular modification observed in a ChIP experiment may simply reflect a change in the recognition of the epitope by the antibody caused by additional histone modifications at nearby residues. Therefore, ChIP results must be validated with approaches that will relate the observed changes in a histone modification to a biological outcome. When this is done, ChIP is a powerful way to study chromatin-based mechanisms that contribute to the regulation of gene expression.

Disclosures

No conflicts of interest declared.

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References

1. Levy, D. & Darnell, J. E. Signalling: Stats: transcriptional control and biological impact. Nature Reviews Molecular Cell Biology 3, 651-662, (2002).
2. Bromberg, J. & Darnell, J. E., Jr. The role of STATs in transcriptional control and their impact on cellular function. Oncogene 19, 2468-2473, (2000).
3. Hu, X. & Ivashkiv, L. B. Cross-regulation of signaling pathways by interferon-gamma: implications for immune responses and autoimmune diseases. Immunity 31, 539-550, (2009).
4. Campos, E. I. & Reinberg, D. Histones: annotating chromatin. Annu Rev Genet 43, 559-599, (2009).
5. Kouzarides, T. Chromatin Modifications and Their Function. Cell 128, 693-705, (2007).
6. Jenuwein, T. & Allis, C. D. Translating the histone code. Science 293, 1074-1080, (2001).
7. Wittwer, C. T., Herrmann, M. G., Moss, A. A. & Rasmussen, R. P. Continuous fluorescence monitoring of rapid cycle DNA amplification. Biotechniques 22, 130-131, 134-138, (1997).
8. Higuchi, R., Dollinger, G., Walsh, P. S. & Griffith, R. Simultaneous amplification and detection of specific DNA sequences. Biotechnology (N Y) 10, 413-417, (1992).
9. Kroger, A., Koster, M., Schroeder, K., Hauser, H. & Mueller, P. P. Activities of IRF-1. J Interferon Cytokine Res 22, 5-14, (2002).
10. Ross, R. A. & Spengler, B. A. Human neuroblastoma stem cells. Seminars in cancer biology 17, 241-247, (2007).

