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Detection of E2F-DNA Complexes Using Chromatin Immunoprecipitation Assays

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Abstract

Chromatin immunoprecipitation (ChIP), originally developed by John T. Lis and David Gilmour in 1984, has been useful to detect DNA sequences where protein(s) of interest bind. ChIP is comprised of several steps: (1) cross-linking of proteins to target DNA sequences, (2) breaking genomic DNA into 300–1000 bp pieces by sonication or nuclease digestion, (3) immunoprecipitation of protein bound to target DNA with an antibody, (4) reverse cross-linking between target DNA and the bound protein to liberate the DNA fragments, and (5) amplification of target DNA fragment by PCR. Since then, the technology has evolved significantly to allow not only amplifying target sequences by PCR, but also sequencing all DNA fragment bound to a target protein, using a variant of the approach called the ChIP-seq technique (1). Another variation, the ChIP-on-ChIP, allows the detection of protein complexes bound to specific DNA sequences (2).

Keywords

Chromatin immunoprecipitation (ChIP); E2Fs; Nek2 promoter; Plk4 promoter; Her2+ breast cancer cell lines

1 Introduction

Chromatin immunoprecipitation (ChIP) was originally developed by John T. Lis and David Gilmour in 1984, in order to test the binding of RNA polymerase from *E. coli* to a constitutively expressed, *lambda cI* gene, and to the isopropyl beta-D-thiogalactoside (IPTG) uninduced and induced *lac* operon [1]. They tested this method in vivo by detecting the binding of RNA polymerase II-DNA interactions in untreated or heat-shocked *Drosophila melanogaster* cells, where they demonstrated that binding of this protein increased at specific DNA sequences in response to heat shock [2]. Since then, it has been heavily used to identify DNA sequences where target proteins, mostly transcription factors, bind to regulate transcription.

⁷The amount of antibody needs to be optimized. Generally speaking, you need to add more if you use polyclonal antibody compared to monoclonal antibody. When you test an antibody for the first time in a ChIP protocol, it is better to include an antibody previously tested in your laboratory and shown to work in your hands.

⁸This protocol was performed with the following antibodies: E2F1, Cat. No. 3742, Cell Signaling; E2F2, c-633; Santa Cruz Biotechnology; and E2F3, Cat. No. sc-878, Santa-Cruz biotechnology. However, a standard ChIP protocol such as the one described here can be adapted to a variety of antibodies.

⁹Normal rabbit IgG, we use Cell Signaling Cat. No. 2729.

ChIP is comprised of several steps: (1) cross-linking proteins to target DNA sequences, (2) breaking genomic DNA into 300–1000 bp pieces by sonication or nuclease digestion, (3) immunoprecipitation of protein bound to target DNA with antibody, (4) reverse cross-linking between target DNA and a protein to liberate the DNA fragment, and (5) amplification of target DNA fragment by PCR. The technology has evolved significantly, and it now allows not only the amplification of target sequences by PCR, but also sequencing all DNA fragment bound to a specific target protein, in a variation of the approach called the ChIP-seq technique [3]. Another variation, the ChIP-on-ChIP, allows the detection of protein complexes bound to specific DNA sequences [4].

ChIP and its variations have been used to identify novel targets of the transcription factor family collectively called E2Fs. For example, Dynlacht et al. used this powerful technique in combination with DNA sequencing to identify transcripts directly regulated by the E2F transcription factors in human cells [5]. These genes included previously identified genes involved in canonical functions of the E2Fs, such as DNA replication, but also previously unidentified target genes involved in other processes, including mitosis, chromosome condensation and segregation, DNA damage checkpoints, and DNA repair. This work was critical to the Rb/E2F field, since it suggested that E2Fs could regulate several important cellular functions besides the control of S phase. For example, their RT-PCR analyses identified elevated levels of the mitotic and spindle assembly checkpoint regulators TTK, Mad2L, Hec1, and Nek2 in mouse embryonic fibroblasts lacking the Rb family members p130 and p107, which antagonize E2F function. Farnham et al. used ChIP to identify novel E2F promoters with consensus, and nonconsensus E2F sequences [6], and combined ChIP with a CpG array to show that E2Fs bound non-consensus sites to regulate expression of genes that are involved in recombination and DNA repair [7]. Nevins et al. used ChIP to show that E2Fs transcription factors coordinate G1/S and G2/M by differential binding of E2F repressors (E2F4) and activators (E2F1, E2F2 and E2F3) to negative or positive E2F sites within promoters of genes that are involved in mitotic entry and exit, including cdc2 [8].

Our laboratory has used ChIP to show that E2F1, E2F2, and E2F3 bind the promoters of the centrosome and mitotic regulators Nek2 and Plk4 to regulate their expression [9]. We uncovered these findings by adapting a protocol from The Gudas lab [10]. We also found that the E2Fs were sufficient to induce centrosome amplification and chromosome instability in mammary epithelial and breast cancer cells in part by maintaining high levels of Nek2. An unpublished example of how the E2F activators E2F1, E2F2, and E2F3 bind to the Plk4 promoter in Her2⁺ JIMT-1 and SKBR3 breast cancer cells is presented in Fig. 1. This figure also shows the limitation of shRNA-mediated knockdown and ChIP, since, while E2F3 occupancy on the promoter was clearly reduced relative to input, there was still some E2F3 bound to the promoter. While shRNA-mediated knockdown results in reduced protein levels, there is always some left, and that is why technologies that have achieved complete knockdown of proteins, including Cas9/CRISPR have emerged [11, 12]. Also, the presence of E2F3 on the promoter may also reflect the inability of E2F3 protein to be degraded upon binding to a promoter.

2 Materials

2.1 Reagents and Solutions

1. RIPA buffer: 50 mM Tris-HCl (pH 8.0), 150 mM NaCl, 5 mM EDTA, 1% NP-40, 0.5% sodium deoxycholate, 0.1% SDS (*see Note*¹).
2. ChIP wash buffer: 50 mM Tris-HCl (pH 8.5), 500 mM LiCl, 1% NP-40, 1% sodium deoxycholate.
3. TE buffer: 10 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0).
4. ChIP elution buffer: 50 mM Tris-HCl (pH 8.0), 1% SDS, 1 mM EDTA (pH 8.0).
5. Protein A Sepharose.
6. Qiagen QIAquick PCR Purification Kit (Cat. No. 28106).
7. 1× Phosphate Buffered Saline (PBS): 137 mM NaCl, 2.7 mM KCl, 10 mM Na₂HPO₄, 2 mM KH₂PO₄ (pH 7.4).
8. Formaldehyde 37%.
9. 1.25 M Glycine stock: dissolve 46.92 g of glycine in 400 mL sterile H₂O. After the glycine is completely dissolved, complete to a final volume of 500 mL with sterile H₂O.
10. Protease inhibitor cocktail.
11. 5 M NaCl: dissolve 292 g of NaCl in 800 mL H₂O, after complete dissolution, complete to 1 L final volume with H₂O.
12. Antibodies: The procedures described in this chapter use antibodies for E2F1, E2F2 and E2F3 as well as nonimmune normal rabbit IgG (all of these are described in the appropriate Methods subsection with accompanying notes). However, this procedure can be adapted to work with a variety of antibodies.
13. Cell lines: We have successfully applied the ChIP protocol in this chapter to JIMT1 (breast carcinoma) and SKBR3 (breast adenocarcinoma) cell lines, but the procedure described below can be adapted for any cell line. Our cell lines are described below in the appropriate Methods subsection. Culture your cell lines in culture media and conditions appropriate for them, and follow the guidelines described in Subheading 3 below regarding the cell density and percent of confluence recommended for the procedure.
14. Goat anti-mouse IgM.
15. Primer sets: according to target sequence of interest.

2.2 Equipment and Labware

1. Orbital shaker.

¹Adding SDS is optional since some antibodies may not be compatible with SDS.

2. Cell scrapers.
3. 15 mL tubes.
4. Probe sonicator.
5. 1.5 mL microcentrifuge tubes.
6. Heat block.

3 Methods

In preparation, and before starting the procedure, you need to plate $\sim 2.5 \times 10^6$ cells in p150 mm culture dishes for each treatment or group. Culture medium depends on the cell type. In this protocol, we used JIMT1 (breast carcinoma) and SKBR3 (breast adeno-carcinoma) cell lines. We cultured the parental cell lines, as well as their derivatives expressing empty vector -PLKO.1-, or stably knocked down for E2F3 by lentiviral mediated transduction (as a negative control in our experiments). We keep JIMT1 cells in DMEM with 10% FBS, 1 $\times$ Penicillin/Streptomycin, with 2 μ g/mL puromycin, while we keep SKBR3 in RPMI1640 with 10% FBS, 1 $\times$ Penicillin/Streptomycin, with 2 μ g/mL puromycin. However, it must be noticed that the procedure described below is applicable to a great variety of cell lines. You must culture cells until they reach confluence.

3.1 Chromatin Preparation and Antibody Binding to Cross-Linked Chromatin (Day 1)

1. To start your experiments, be sure to have at least 2×10^7 cells in your cultures at the time of harvest (this should be about 80–90% confluence). You test this by setting a pilot experiment where you plate various numbers of cells and harvest cells for counting when they become confluent.
2. Add formaldehyde (37% solution) to a final concentration 1% directly to the media (540 μ L in 20 mL media) to cross-link cells, and put plates on shaker and shake vigorously for 10 min at room temperature.
3. Add glycine to a final concentration of 0.15 M to quench the cross-linking reaction, and incubate on shaker for at least 5 min at room temperature.
4. Discard media from plates and wash cells with ~ 10 mL cold 1 $\times$ PBS twice.
5. Add 1–2 mL of cold 1 $\times$ PBS to each plate, scrape cells off the plate, and transfer cells to a labeled 15 mL tube stored on ice.
6. Centrifuge cells for 5 min at 4 $^{\circ}$ C at $5000 \times g$.
7. Discard supernatant by aspiration, suspend pellet in 350 μ L of RIPA buffer containing protease inhibitors by gently pipetting pellet up and down, and transfer to labeled prechilled 1.5 mL microcentrifuge tube on ice (*see Note²*).

²We used complete mini protease inhibitor cocktail (Cat. No. 11 836 153 001) from Roche. It comes in the form of a tablet and we add 1 tablet/10 mL lysis buffer.

8. Sonicate samples for 15 s using a probe sonicator. Choose the sonicator setting such that the output should be ~6–7 amp. Keep samples on ice after sonication and repeat sonication one more time (*see Note*³).
9. Harvest sonicated samples by centrifuging for 10 min at 4 °C at $12,000 \times g$ (*see Note*⁴).
10. Transfer soluble supernatant which should contain the chromatin to a clean 1.5 mL microcentrifuge tube and adjust the volume to 50 μ L with RIPA buffer, so each tube contains material from an estimated $3\text{--}3.5 \times 10^6$ cells. Each of these tubes will be used in an immunoprecipitation (IP) reaction in subsequent steps.
11. To make one IP reaction, to the 50 μ L volume from **step 10**, add 450 μ L of RIPA buffer with protease inhibitors (*see Note*⁵).
12. To preclear the chromatin-containing soluble supernatant, add 75 μ L of 50% Protein A sepharose/1 $\times$ PBS (v:v) slurry in each reaction tube and incubate on a rocker at 4 °C for 15 min to several hours (*see Note*⁶).
13. Spin down the precleared chromatin by centrifuging at $3000 \times g$ for 30 s.
14. Transfer 475 μ L of precleared soluble chromatin to a clean microcentrifuge tube. Save extra precleared soluble chromatin for input control during PCR.
15. Add 2 μ g antibody of interest to each immunoprecipitation (IP) tube and incubate at 4 °C overnight on shaker. For our particular purpose, we used antibodies against E2F1, E2F2, and E2F3. Set a negative control by using normal nonimmune rabbit IgG (*see Notes 7–9*).

3.2 Immuno-precipitation and Reversion of Cross-Link (Day 2)

1. Spin down each IP tube by brief centrifugation, add 50 μ L of 50% Protein A slurry to each tube, and incubate for 1 h on a rocker at 4 °C. When using monoclonal antibodies, add 2.5 μ g of goat anti-mouse IgM to each tube and incubate 1 h before adding Protein A Sepharose slurry).
2. Centrifuge tubes at $3000 \times g$ for 30 s and discard supernatant by aspiration.
3. To wash pellet, add 1 mL of RIPA buffer, and incubate on rocker for 5 min at room temperature and spin down at $3000 \times g$ for 30 s.
4. Repeat **step 3** one more time.
5. Wash pellet twice with ChIP wash buffer.
6. Wash pellet with TE buffer twice and after second wash, remove supernatant as much as possible.

³It most likely happens that samples can be overheated or overflowed during sonication. You can avoid or minimize these possibilities by either not putting the sonicator at the surface of the samples or by sonicating samples on ice.

⁴You will see slightly black debris pellet at the bottom after centrifugation, which is expected and normal.

⁵You need to set-up at least two IPs, one for Ab of your interest and one for negative control. To make multiple IP reactions, for example 3 IPs, add 150 μ L of chromatin from **step 10** into 1350 μ L of RIPA buffer containing protease inhibitor.

⁶We used protein Sepharose CL-4b from GE Healthcare life science (cat#17-0780-01) and prepared slurry following manufacturer's guide (according to the manual, 1 g of protein Sepharose CL-4b powder usually generates 4–5 mL of slurry after swelling).

7. Add 100 μL of ChIP elution buffer to each IP tube. Elution buffer should be made fresh and used within a month after preparation.
8. Incubate at 65 $^{\circ}\text{C}$ in a heat block for 10 min.
9. Vortex all samples for 15 s and spin down samples at $12,000 \times g$ for 30 s (*see Note*¹⁰).
10. Transfer 100 μL of supernatant to a new tube containing 4 μL of 5 M NaCl and reverse the cross-linking by incubating samples at 65 $^{\circ}\text{C}$ in a heat block overnight. Set up an input control tube by adding 75 μL of elution buffer containing 5 μL of 5 M NaCl per 25 μL of precleared soluble chromatin.

3.3 DNA Isolation and PCR Amplification of Target Sequence (Day 3)

To isolate DNA fragments, we use Qiagen QIAquick PCR purification kit (Cat. No. 28106, Qiagen), following manufacturer's instructions.

1. Remove tubes from heat block and spin down condensation.
2. Add 500 μL of PB buffer from the Qiagen kit to each tube and vortex it for a couple minutes.
3. Spin down for 30 s at $12,000 \times g$.
4. Transfer solution to Qiagen column and centrifuge for 1 min at $12,000 \times g$.
5. Discard flow-through and wash column with 750 μL of Qiagen PE wash buffer.
6. Spin down for 30 s at $12,000 \times g$ and discard flow-through.
7. Remove any residual wash buffer by centrifuging tube for an additional 1 min.
8. Remove the Qiagen column and place it a new microcentrifuge tube.
9. Add 50 μL of the kit's elution buffer to the center of column and centrifuge for 1 min at $12,000 \times g$ to collect DNA fragments.
10. Use this for semiquantitative or real-time PCR. *See Table 1* for the primers that we used for our particular target genes [9].
11. Evaluate results by loading the PCR product in a 2% agarose gel. Figure 1 depicts a typical result from a ChIP experiment, in this case, for the target genes of our interest.

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¹⁰Vortex speed sets up between 5 and 7 to avoid breaking the interactions between the antibody and DNA.

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Fig. 1.

ChIP on putative E2F binding site on Plk4 promoter. Immunoprecipitation (IP) was performed on two Her2+ breast cancer cell lines, JIMT1 and SKBR3 knocked down for E2F3 using E2F1, E2F2, or E2F3 antibody. Control IgG was used as a negative control for IP. Then, PCR was performed with primer set that covers tentative E2F binding sites on Plk4 promoter region. Input was used as a positive control for PCR

Table 1

Primers used in ChIP for Nek2 and Plk4 target genes

Sequences	
Nek2_F	5'-TTG GCG ATC TCT ATC AGA GGG-3'
Nek_R	5'-AAA GTG TCA CTA GGC AAC CGC-3'
Plk4_F	5'-AGT GTC CCG AGG CAC TGC GGC TT-3'
Plk4_R	5'-AGA TAA CCG CCA TCC CCT TGG A-3'