Chapter 17

ChIP for Identification of p53 Responsive DNA Promoters

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Abstract

Chromatin immunoprecipitation assay (ChIP) has been frequently used to determine whether a transcriptional regulator can bind to a specific DNA element in the chromatin content of cells. Here, we describe a detailed protocol for this assay with hands-on tips based on our own experience in working on the transcriptional regulator and tumor suppressor p53.

Key words: Chromatin immunoprecipitation, p53 responsive DNA element, Transcription

1. Introduction

Although p53 can induce apoptosis in a transcription-independent fashion (1), it has been well established that p53 functions as a tumor suppressor primarily through its transcriptional regulation of a number of target genes, which encode proteins and miRNAs important for executing the p53 response to a variety of stress signals (2–5). This transcriptional activity is exerted via the direct binding of p53 to the canonical p53 responsive DNA elements of its target promoters. Thus, monitoring the binding of p53 to its target gene promoters becomes one critical measurement of p53 transcriptional functions for specific target genes in cells. Chromatin immunoprecipitation (ChIP) was initially developed to define the Polycomb-repressed chromatin domains in cells (6) and later on often applied for the detection of the binding of a number of transcriptional factors to their responsive DNA elements in the chromatin content in cells (7). This method has also been successfully used not only to detect the binding of p53 to its known responsive DNA elements in vivo (8), but also to identify new p53 target promoters, including some of our own studies in the laboratory (9–11). Hence, ChIP has become one of the frequently used

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and important methods in the field of p53 to analyze in vivo transcriptional functions of this tumor suppressor.

In 1991, p53 was identified as a transcription factor that was able to bind to a sequence-specific DNA promoter (12, 13), and the consensus sequence of p53 response elements was resolved by in vitro assays 1 year later (14). By using a global ChIP-sequencing assay, Wei et al. have recently refined the consensus sequence with the pattern 5′-RRRC(A/T)(A/T)GYYY-3′ (15). Although several studies showed that p53 can also bind to some DNAs without the consensus sequence (16, 17), the identification of p53 as a sequence-specific DNA-binding transcription factor is clearly a landmark in the p53 field, since most tumor-associated p53 mutations occur within the DNA-binding domain (18) and more than 150 p53 target genes including those encoding miRNAs have been reported thus far since the *p21* and *mdm2* genes were identified as the first batch of the p53 target genes (19–23). In spite of the possibility that p53-DNA binding may be affected by other factors, like DNA structure and topology, defining the sequence-specific DNA elements that specifically interact with p53 in cells is critical for verifying bona fide target genes for this transcription factor.

It has been debated whether binding of p53 to chromatin DNA in cells is stress-induced or not. While the binding of p53 to the promoter sequences of its target genes was previously believed to require stress induction, later studies showed that p53 is able to bind the promoter DNA element of *p21*, *mdm2*, or *pig3* genes in unstressed cells, though to a lesser extent, when compared to stressed cells (24, 25). These findings reveal that although the inactive form of p53 in unstressed cells could bind to its target promoters, it is not sufficient to induce transcription of target genes. Considering that the procedures of cell culture and cell isolation could cause cellular stress, the basal level of the binding of inactive p53 to its promoter might be due to this type of artificial stress. Thus, to achieve a conclusive result for the definition of a new p53 responsive (p53RE) DNA element in vivo, it is recommended to induce p53 in cells with a specific stressor, such as treatment with 5-FU or actinomycin D, prior to conducting a ChIP assay as detailed below.

In this chapter, we describe a hands-on protocol for conducting a ChIP assay with some tips gained from our own direct experience in studying the binding of p53 to its target promoters in cells. Although we use p53 as a working model here, this protocol can be used for studying other p53 family members, such as p73 or p63, and other transcription factors as well. This protocol could also be used to identify new p53 target DNA elements. In the latter case, putative p53 responsive DNA elements in the promoter region of a putative target gene could be readily identified through bioinformatic analysis of human genome sequences. Two pairs of primers should be designed based on the sequence information, including one that covers the putative p53RE DNA element and another pair of control primers derived from downstream or upstream sequences irrelevant to this

DNA element. In principle, p53-containing cells can be treated with anticancer drugs, such as actinomycin D or 5-FU, for several hours before being harvested to ensure that p53 is induced. Harvested cells are then subjected to cross-linking and sonication prior to being used for immunoprecipitation with anti-p53 antibodies. Immunoprecipitated lysates are then used for Polymerase Chain Reaction (PCR) to amplify the DNA element that may associate with p53. The detailed procedure with all necessary materials is described below.

2. Materials

1. Formaldehyde (Fisher Scientific, F75P1GAL).
2. 1.25 M Glycine (Fisher Scientific, G45-212). Store at 4°C.
3. 20× Phosphate-buffered-saline (PBS) buffer: 160 g NaCl, 4 g KCl, 23 g Na_2HPO_2-$7H_2O$, and 4 g KH_2PO_4. Store at room temperature.
4. 1×PBS: Prepare from 20× stock using water and store at 4°C.
5. Protease inhibitors: 1 μg/mL Aprotinin, 1 μg/mL Leupeptin, 4 μM Pepstatin A, and 0.2 mM Phenylmethylsufonyl-fluoride (PMSF). (Add fresh just before use).
6. Lysis buffer: 150 mM NaCl, 1% NP-40, 0.5% Deoxycholate, 0.1% SDS, 50 mM Tris, and 5 mM EDTA, pH 8.0. Store at 4°C. Add protease inhibitors just before use.
7. ProteinA/GPLUS-Agarose(SANTACRUZBIOTECHNOLOGY, sc-2003).
8. Pre-sheared salmon sperm DNA (Sigma-Aldrich, S3126).
9. Wash buffer: 100 mM Tris, 500 mM LiCl, 1%Np-40, and 1% deoxycholic acid, pH 8.5.
10. 500 mM $NaHCO_3$.
11. 10% SDS (Sigma-Aldrich, 71725).
12. 5 mg/mL RNAse A (Sigma-Aldrich, R4642).
13. 5 M NaCl.
14. Yeast tRNA (Ambion, AM7118).
15. Ethanol.
16. 5× PK buffer: 50 mM Tris, 25 mM EDTA, and 1.25% SDS, pH 7.5.
17. 20 g/L Proteinase K (New England BioLabs, P8102S).
18. 70% ethanol.
19. Agarose gel.
20. Ethidium bromide.

3. Methods

The following protocol is based on the previously described method (26) with some modifications according to our own experience (Fig. 1).

3.1. Chromatin Preparation

1. Culture sufficient cells (at least 10^7 cells per IP) with desired treatments (see Note 1).
2. Add 37% formaldehyde (30 μL/mL media) directly into the plate with media. Gently shake the plates at RT for 10 min (see Note 2).

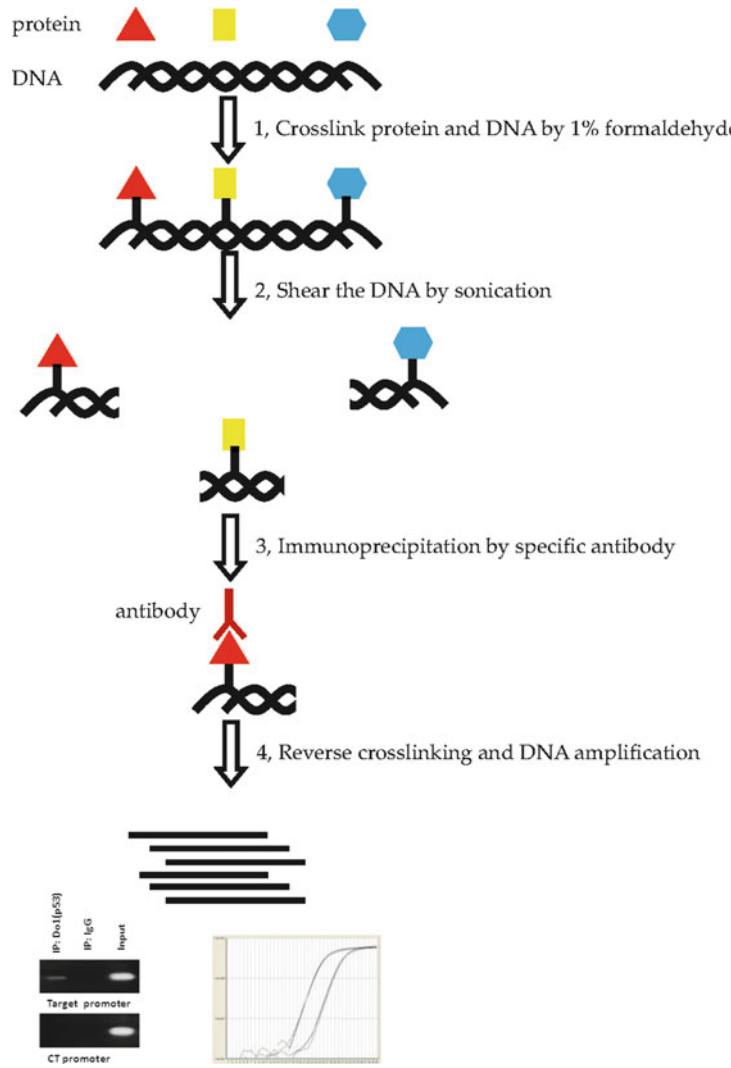

Fig. 1. Schematic diagram showing ChIP.

3. Add glycine to a final concentration of 0.125 M to stop the cross-linking reaction. Incubate at room temperature (RT) for 5 min.
4. Wash cells twice with PBS and scrape 10^7 cells in 1.5 mL PBS with PMSF. Transfer the cells to 1.5 mL tube.
5. Pellet cells for 5 min at 1,000 × *g* at 4°C and aspirate off the supernatant. *The cells can be used for ChIP directly or frozen in liquid N2 and stored at –80°C for months. From this step, keep your sample and reagents at 4°C or in an ice bucket.*
6. Add protease inhibitors into the RIPA buffer (prepare 5 mL per IP).
7. Resuspend cell (do not vortex) pellets with 1 mL RIPA with protease inhibitors (see Note 3).
8. Incubate the mixture in ice for 30 min by reversing the tube occasionally to avoid precipitation.
9. Shear DNA to yield fragments of 300–1,000 bp by sonication (Fig. 2), 10 s, 8 times. We use Fisher Scientific Model 100 Sonic Dismembrator and set up the output power to 14 W (see Note 4).
10. After sonication, spin the lysates for 10 min at 16,000 × *g* and collect the supernatant into a new 1.5 mL tube. *The chromatin can be stored at –80°C at this step.*

3.2. Immunoprecipitation

For the first ChIP experiment, we strongly recommend that you set up both positive and negative controls for both antibodies and primer sets.

1. Take out 50 μL for the input (see Note 5).
2. Prepare DNA–protein A/G slurry (1:1 mixture of 10 g/L pre-sheared salmon sperm DNA and protein A/G).
3. The prepared chromatin was precleared with 50 μL DNA–protein A/G slurry on rotating platform for 30 min at 4°C.
4. Spin at 1,500 × *g* 4°C for 2 min, collect the supernatants to a new 1.5 mL tube (see Note 6).
5. Take 250 μL of mixtures to a new tube for each antibody, add appropriate antibody (1–5 μg) to each sample, and immunoprecipitation was performed on a rotating platform at 4°C overnight (see Note 7).

End of day 1

6. Add 50 μL Protein A/G sepharose beads and incubate for two more hours at 4°C on the rotating platform.
7. Wash the immunocomplexes twice with 1 mL RIPA buffer, four times with IP wash buffer, and twice with RIPA buffer. Between washes, samples are rotated for 5 min and spun at 1,000 × *g* 1 min (see Note 8).

3.3. Elution and Reverse Cross-links

1. Freshly make the IP elution buffer (300 μL/IP).
2. Add 150 μL IP elution buffer, put on a rotating platform at RT for 15 min, spin at 2,000 × *g*, 1 min RT, and transfer supernatants to a new tube. Repeat with another 150 μL IP elution buffer. Combine supernatants and spin at 16,000 × *g* for 5 min to remove any traces of beads.
3. Add 250 μL IP elution buffer into the input sample taken out from step 1 in section 3.2.
4. Add 2 μL 5 g/L RNAse A and 12 μL 5 M NaCl. And incubate at 65°C for overnight. The sample could be stored at –20°C (see Note 9).

End of day 2

5. Add 10 μg tRNA and 650 μL 100% ethanol. Mix and store at –20°C more than 3 h or overnight.

End of day 3

6. Centrifuge at top speed for 20 min. Discard ethanol and dry pellet at RT for 10 min.
7. Resuspend DNA in 100 μL TE buffer and add 25 μL 5× PK buffer. Vortex the sample and add 30 μg proteinase K. Mix well and incubate at 45°C for 2 h (see Note 10).
8. Add 175 μL TE buffer to final volume of 300 μL and add 300 μL 25:24:1 phenol–chloroform–isoamyl alcohol. Vortex vigorously for 1 min. Transfer the top phase to a new tube and extract with 300 μL chloroform and transfer the top phase to a fresh tube.
9. Add 30 μL 5 M NaCl, 10 μg yeast tRNA, and 750 μL 100% ethanol. Mix well and precipitate at –20°C more than 3 h or overnight.

End of day 4

10. Spin samples at top speed for 20 min and wash DNA with 70% ethanol.
11. Dissolve pellets in 100 μL water. The DNA can be stored at –20°C for months.
12. Analyze the pulled down DNA by either a Real-Time PCR assay or regular PCR reaction with relevant or control primers (see Note 11).
13. For Real-Time PCR, use 2 μL DNA as a template and follow the standard qPCR protocol (10). If possible, always do one positive control with the following p21 primers, forward: 5¢-GCTCCCTCATGGGCAAACTCACT reverse: 5¢-TGGC

TGGTCTACCTGGCTCCTCT, with annealing temperature: 60°C and data collecting temperature: 75°C.

14. Or, run regular PCR products on a 2% agarose gel with ethidium bromide staining (Fig. 1) (see Note 12).

4. Notes

1. Start with three confluent 10-cm dishes. A sufficient number of cells are vital to the success of the ChIP assay. Fewer cells may result in weak signal and high background. The required number of cells may vary depending on cell type. Therefore, we suggest measuring the DNA concentration for each cell line and start with 25 μg DNA per IP.
2. The time and formaldehyde concentration may be optimized. However, do not cross-link the proteins to DNA longer than 30 min, for it would affect the antibody binding and the efficiency of sonication or cause cell aggregation.
3. Optional steps to purify the nuclei may reduce the nonspecific binding of primary antibodies. Before adding RIPA buffer, add 1 mL Swelling buffer (5 mM PIPES, 80 mM KCl, and 1% NP-40, pH 8.0) with protease inhibitors to 5×10^7 cells, incubate on ice for 30 min, reverse the tube during the incubation to avoid the precipitation. Centrifuge at $2{,}300 \times g$ at 4°C for 5 min, dump the supernatants, and go to step 7 in this section.
4. Make sure to keep cell lysates cold. Optimizing the conditions for the sonication is crucial, for sonication efficiency varies depending on cell type. Test the pulse duration, intensity, and time course to establish optimal conditions. Make sure to carry on the reverse cross-linking after sonication and apply the DNA fragments to 2% agarose gel as shown in Fig. 2. Do not shear or sonicate DNA for too long, for long time sonication may result in disruption of nucleosome-DNA binding. The fragments shorter than 250 bp could be an indication for too long sonication. Choose the lowest power output that gives ideal fragmentation to avoid overheat.
5. For the first ChIP assay, it is recommended to measure the DNA concentration after reverse cross-linking. Briefly, take the input DNA at step 1 in section 3.2, go to step 3 in section 3.3 directly, and measure the concentration after step 11 in section 3.3. The DNA concentration should be about half of the concentration measured after step 11 in section 3.3.
6. The preclear step is important for reducing the background. Leave 100 μL supernatant in the original tube to avoid any

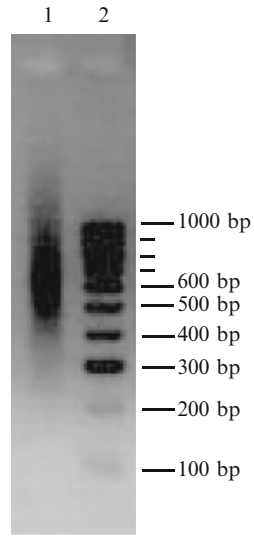

Fig. 2. The sheared DNA fragments from H1299 cells. *Lane 1*, sheared DNA fragments. *Lane 2*, DNA markers.

contamination. The remaining salmon sperm DNA also plays a role as blocking reagent for the immunoprecipitation.

7. Use an equal volume of about 25 μg prepared chromatin for each immunoprecipitation with each antibody. The amount of antibodies may vary, and trial experiments may be needed to determine the amount of antibodies suitable for each immunoprecipitation and PCR. Start with 2 μg antibodies for 25 μg prepared chromatin. If this is the first ChIP assay, do not forget to set up proper positive and negative controls, like anti-pol II and IgG.
8. Additional washes may be needed if the signal–noise ratio is low. The first two washes could be done by inversing the tube several times; but it is recommended to put the tube on a rotating platform at 4°C for 5 min for each wash.
9. Removing RNA by RNAse A treatment will increase the efficiency for the DNA purification, especially when DNA is purified using a PCR purification kit, for the columns could be saturated by a high level of RNA. After this step, the DNA can be purified by using a PCR purification kit, or a miniprep column. For Qiagen QIAprep Spin Miniprep Kit, go through the following steps. Mix 700 μL of buffer PB with 300 μL of elutions and pass through a miniprep column; wash with 750 μL buffer PE; then elute the DNA with 50 μL buffer EB or H_2O and go to step 12 in section 3.3.
10. The predominant site of proteinase K cleavage is the peptide bond adjacent to the carboxyl group of aliphatic and aromatic amino acids with blocked alpha amino groups. The proteinase

K treatment disrupts the binding of protein to DNA and increases the efficiency of DNA purification.

11. The primers could be designed based on the p53RE DNA element within a target gene. The p53RE DNA element could be bioinformatically predicted by p53MH program (27). A good negative control could be the primers encompassing the 3 UTR-coding region of a target gene.
12. It is necessary to optimize the PCR cycle number, for too many cycles will amplify the nonspecific DNA as immunoprecipitated with IgG; however, fewer cycles could not show the DNA pulled down by the p53 antibody used. A titration of immunoprecipitated DNA is recommended to figure out the proper cycle number for a specific PCR reaction.

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References

1. Moll UM, Wolff S, Speidel D, Deppert W (2005) Transcription-independent pro-apoptotic functions of p53. Curr Opin Cell Biol 17(6):631–636
2. Vogelstein B, Lane D, Levine AJ (2000) Surfing the p53 network. Nature 408(6810):307–310
3. Marchenko ND, Moll UM (2007) The role of ubiquitination in the direct mitochondrial death program of p53. Cell Cycle 6(14):1718–1723
4. Vousden KH, Lane DP (2007) p53 in health and disease. Nat Rev Mol Cell Biol 8(4):275–283
5. He L, He X, Lim LP, de Stanchina E, Xuan Z, Liang Y, Xue W, Zender L, Magnus J, Ridzon D, Jackson AL, Linsley PS, Chen C, Lowe SW, Cleary MA, Hannon GJ (2007) A microRNA component of the p53 tumour suppressor network. Nature 447(7148):1130–1134
6. Orlando V, Paro R (1993) Mapping polycomb-repressed domains in the bithorax complex using in vivo formaldehyde cross-linked chromatin. Cell 75(6):1187–1198
7. Orlando V (2000) Mapping chromosomal proteins in vivo by formaldehyde-crosslinked-chromatin immunoprecipitation. Trends Biochem Sci 25(3):99–104
8. Brady CA, Jiang D, Mello SS, Johnson TM, Jarvis LA, Kozak MM, Broz DK, Basak S, Park EJ, McLaughlin ME, Karnezis AN, Attardi LD (2011) Distinct p53 transcriptional programs dictate acute DNA-damage responses and tumor suppression. Cell 145(4):571–583
9. Zeng SX, Dai MS, Keller DM, Lu H (2002) SSRP1 functions as a co-activator of the transcriptional activator p63. EMBO J 21(20):5487–5497
10. Zhang Y, Liao JM, Zeng SX, Lu H (2011) p53 downregulates Down syndrome-associated DYRK1A through miR-1246. EMBO Rep 12(8):811–817
11. Jin Y, Zeng SX, Dai MS, Yang XJ, Lu H (2002) MDM2 inhibits PCAF (p300/CREB-binding protein-associated factor)-mediated p53 acetylation. J Biol Chem 277(34):30838–30843
12. Bargonetti J, Friedman PN, Kern SE, Vogelstein B, Prives C (1991) Wild-type but not mutant p53 immunopurified proteins bind to sequences adjacent to the SV40 origin of replication. Cell 65(6):1083–1091
13. Kern SE, Kinzler KW, Bruskin A, Jarosz D, Friedman P, Prives C, Vogelstein B (1991) Identification of p53 as a sequence-specific DNA-binding protein. Science 252(5013):1708–1711

14. el-Deiry WS, Kern SE, Pietenpol JA, Kinzler KW, Vogelstein B (1992) Definition of a consensus binding site for p53. Nat Genet 1(1):45–49
15. Wei CL, Wu Q, Vega VB, Chiu KP, Ng P, Zhang T, Shahab A, Yong HC, Fu Y, Weng Z, Liu J, Zhao XD, Chew JL, Lee YL, Kuznetsov VA, Sung WK, Miller LD, Lim B, Liu ET, Yu Q, Ng HH, Ruan Y (2006) A global map of p53 transcription-factor binding sites in the human genome. Cell 124(1):207–219
16. Cawley S, Bekiranov S, Ng HH, Kapranov P, Sekinger EA, Kampa D, Piccolboni A, Sementchenko V, Cheng J, Williams AJ, Wheeler R, Wong B, Drenkow J, Yamanaka M, Patel S, Brubaker S, Tammana H, Helt G, Struhl K, Gingeras TR (2004) Unbiased mapping of transcription factor binding sites along human chromosomes 21 and 22 points to widespread regulation of noncoding RNAs. Cell 116(4):499–509
17. Liu Y, Lagowski JP, Vanderbeek GE, Kulesz-Martin MF (2004) Facilitated search for specific genomic targets by p53 C-terminal basic DNA binding domain. Cancer Biol Ther 3(11):1102–1108
18. Hainaut P, Hollstein M (2000) p53 and human cancer: the first ten thousand mutations. Adv Cancer Res 77:81–137
19. el-Deiry WS, Tokino T, Velculescu VE, Levy DB, Parsons R, Trent JM, Lin D, Mercer WE, Kinzler KW, Vogelstein B (1993) WAF1, a potential mediator of p53 tumor suppression. Cell 75(4):817–825
20. Harper JW, Adami GR, Wei N, Keyomarsi K, Elledge SJ (1993) The p21 Cdk-interacting protein Cip1 is a potent inhibitor of G1 cyclin-dependent kinases. Cell 75(4):805–816
21. Barak Y, Juven T, Haffner R, Oren M (1993) mdm2 expression is induced by wild type p53 activity. EMBO J 12(2):461–468
22. Zauberman A, Barak Y, Ragimov N, Levy N, Oren M (1993) Sequence-specific DNA binding by p53: identification of target sites and lack of binding to p53 – MDM2 complexes. EMBO J 12(7):2799–2808
23. Wu X, Bayle JH, Olson D, Levine AJ (1993) The p53-mdm-2 autoregulatory feedback loop. Genes Dev 7(7A):1126–1132
24. Szak ST, Mays D, Pietenpol JA (2001) Kinetics of p53 binding to promoter sites in vivo. Mol Cell Biol 21(10):3375–3386
25. Kaeser MD, Iggo RD (2002) Chromatin immunoprecipitation analysis fails to support the latency model for regulation of p53 DNA binding activity in vivo. Proc Natl Acad Sci USA 99(1):95–100
26. Nelson JD, Denisenko O, Bomsztyk K (2006) Protocol for the fast chromatin immunoprecipitation (ChIP) method. Nat Protoc 1(1):179–185
27. Hoh J, Jin S, Parrado T, Edington J, Levine AJ, Ott J (2002) The p53MH algorithm and its application in detecting p53-responsive genes. Proc Natl Acad Sci USA 99(13):8467–8472