Acta Biologica Hungarica 67(4), pp. 373–378 (2016)
DOI: 10.1556/018.67.2016.4.3

ChIP – DOES IT WORK CORRECTLY? THE OPTIMIZATION STEPS OF CHROMATIN IMMUNOPRECIPITATION

Małgorzata Kus-Liśkiewicz*

Faculty of Biotechnology, Biotechnology Centre for Applied and Fundamental Sciences, University of Rzeszow, Sokołowska 26, 36-100 Kolbuszowa, Poland

(Received: April 1, 2016; accepted: June 20, 2016)

The proteins interaction with DNA is one of the key regulatory elements of many biological processes; including gene transcription, epigenetic modification or cell differentiation. Immunoprecipitation of chromatin; ChIP; is a method used to assess the interaction of the protein with a DNA sequence, and determines the localization of specific locus in the genome. The main steps of this method are fixation, sonication, immunoprecipitation and analysis of DNA. Although the immunoprecipitation assay is a multipurpose tool applied in biochemistry and biotechnology, it requires optimization. This paper describes several critical parameters that should be taken into account when immunoprecipitation assay is applied.

Keywords: Chromatin – immunoprecipitation – transcription factor binding

INTRODUCTION

Chromatin immunoprecipitation (ChIP) is a very useful technique, which is implicated in many studies focused on determining the interaction between proteins and the genomic DNA [1, 3]. Those interactions are crucial for the functioning of the cell/organism. Protein–DNA interactions occur during DNA repairing, chromosome segregation or in the regulation of gene expression [3, 9]. Thus, the different effects of epigenetic modifications, such as the determining of the methylation sites in the DNA or the post-translational alterations of histones, can be carried out using ChIP. Nowadays, different analytical tools are also combined with ChIP, e.g. ChIP-chip [6], ChIP-seq [7] and ChIP-display [1] as well as ChIP-PET [13], which enable researchers to map protein binding in a truly genome-wide manner with high resolution. Development of those techniques has occurred over a last years, however, ChIP has still remained a cumbersome protocol requiring a few steps of optimization (Fig. 1).

To determine the optimal conditions for implementation of chromatin immunoprecipitation, the consideration of the optimization of the cells fixation with formalde-

*E-mail address: mkus@ur.edu.pl

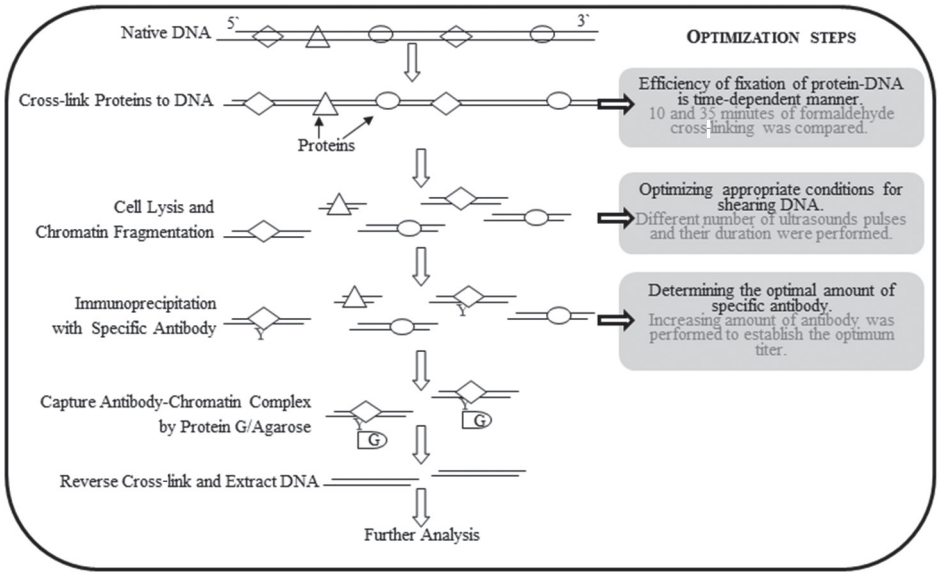

Fig. 1. The chromatin immunoprecipitation assay and the optimization steps

hyde, the sonication as well as the amount of the antibody used is required. The cross-linking of the DNA-protein complex with formaldehyde, enables to hold and stabilize all the *in vivo* interaction. However, formaldehyde may cause masking of the epitopes while preventing the specific binding of antibodies [10]. Too long fixation of the cells also leads to a partial losing of the precipitated material due to the denaturation proteins and hinders subsequent fragmentation. Obtaining chromatin fragments of the appropriate length (from 200 to 1000 base pairs, the limit to 4000 bp) is important to an extent that allows more precisely determine the transcription factor binding to the DNA.

The procedures outlined in this report describe and show the optimization of the most important steps of the chromatin immunoprecipitation. To carry out the ChIP method, EZ ChIP™ 17-371, MerckMillipore, Upstate kit was used. In subsequent stages, the efficiency of the cross-linking, the DNA fragmentation and the selection of the optimal antibody concentration were assessed.

MATERIALS AND METHODS

Cell/tissue culture and reagents

Hepatocytes were obtained from the liver of FVB/N 10 to 16-week-old male mice by collagenase perfusion method [5]. After isolation, the cells were cultivated in DMEM

(Sigma-Aldrich) with high glucose in CO_2 atmosphere. Usually yield of 1×10^6 cells was used, unless stated otherwise. Due to the heat shock is one of the best-studied exogenous cellular stresses, one part of cells was exposed to 42 °C in water bath for 1 h, hereinafter referred as HS, while the control cells (C) were not heat-treated. Standard chemicals were purchased from Sigma-Aldrich (USA), POCH (Poland), Roche Biochemicals (Switzerland), Merck (Germany), (Boehringer, Germany). For immunohistochemistry polyclonal an anti-HSF1 antibody (SPA-901, Stressgen, Victoria, Canada) was used. Reagents for PCR reaction and agarose gel electrophoresis were obtained from Fermentas or SeaKem (USA). All buffers and solutions were prepared with Millipore water. Labware was always autoclaved. The animal experiments were carried out according to Polish legislation, and were approved by the Local Committee of Ethics and Animal Experimentation at the Medical University of Silesia in Katowice, Poland.

Time optimization of protein-DNA crosslinking

Cell culture was divided into two parts and incubated at presence of 1% formaldehyde for 10 and 35 min at room temperature, respectively. Subsequent quenching was achieved by adding 1/20 volume of 2.5 M glycine, followed by 5 min incubation. The culture was washed and suspended in the Lysis Buffer, homogenized with Teflon Potter's homogenizer and centrifuged for 10 min at 900×*g* at 4 °C. Pellet was resuspended in 10 mL of Nuclear Lysis Buffer and then sonicated (Ultrasonic Processor, Cole Parmer, 8454) by testing different conditions: time of the impulse (3×10 sec and 7×20 sec) with 30% amplitude. After fragmentation, the sample was centrifuged for 10 min at 4 °C at 14,000×*g*. The efficiency of the cross-linking was evaluated by reversing the protein-DNA cross-links as described in the protocol (EZ ChIP™ 17-371, Merck Millipore, Upstate). After the DNA isolation, the length of the resulting fragments was assessed by performing electrophoresis on a 1% agarose gel, applying 0.5 ug of DNA per lane. DNA concentration in the sheared chromatin was determined with a Nanodrop spectrophotometer (Thermoscientific).

Fragmentation of DNA

Upstate protocol recommends shearing chromatin to 200–1000 base pairs in length. Cell lysate was transferred to the conical 1.5 mL test tube in the ice-water bath. The sonicator probe was submerged at to the depth to prevent foaming. The samples were sonicated as follows: 5, 10 or 8 rounds of 20, 10 or 15 seconds, respectively, with 1 minute break between each round. Then, the sheared DNA was decross-linked and analyzed on an agarose gel.

Immunoprecipitation conditions and antibody titration

Antibody performance should be optimizing by determining its optimal concentration. For analyses of HSF1 binding kinetics, 0.3, 1 and 3 µg of rabbit anti-HSF1 antibody per sample of 5×10^6 cells were conducted.

RESULTS AND DISCUSSION

To ensure a successful ChIP protocol, it is crucial to optimize the following steps: fixation, chromatin fragmentation, immunoprecipitation with a specific antibody, as well as analysis of the immunoprecipitated DNA [6]. Therefore, it is important to properly select the parameters for an experiment, because only in this case we would be able to assess the existence of a specific protein-DNA interactions. At the beginning, it has been checked whether the longer time of fixation will increase the efficiency of obtaining good quality of the DNA. Insufficient time of fixing can result in the lack of interaction of protein to DNA, whereas, overcross-linking may cause the denaturation of the protein. The samples were incubated for 10 or 35 minutes with formaldehyde, and were sonicated at various conditions. In both cases of sonication types, the quality of DNA fragmentation are better when exposing to only 10 minutes fixation (Fig. 2A). As shown on the left gel, the better shearing conditions (smaller DNA fragments) corresponds to 10 minutes fixation. This agrees with the results of Ray and Das [8] and confirmes that longer incubation causes permanent cross-linking. Sonication attends two roles, solubilization and shearing of chromatin, both of which are essential for successful ChIP analysis. It is known that this step is the most variable part in the whole chromatin immunoprecipitation process. Therefore, all the factors affecting on sonication effectiveness were taken into account; i.e. the volume of the sample, the amplitude of the ultrasound, the number and duration of pulses as well as the intervals between them, and optionally the presence of glass beads. Similarly, each cell type may behave differently and could be more resistant to sonication [2].

Sheared DNA from hepatocytes following 10 sonication pulses show the optimal size range for immunoprecipitation: 200–1000 bp with the majority of DNA fragments between 300–700 bp (lane 2 in Fig. 2A right). While, the sonication efficiency using other conditions (lane 1, 3 on Fig. 2A right) are insufficient and unsatisfactory. DNA fragments observed in lane 1 are definitely too large with range between 300–13,000 bp; whereas, the lane 3 is abundant in too small chromatin fragments (smaller than 300 bp). The length of the chromatin fragments influences the final result. Smaller DNA fragments allow a more precise localization of a specific binding event, as a smaller region of DNA will be pulled down in the immunoprecipitation [4].

An essential step of the ChIP method is the antibody immunoprecipitation. Due to various antibody specificity, affinity and quality; its amount may need to be titrated to achieve the optimal sample enrichment. In this experiment, the interaction of heat

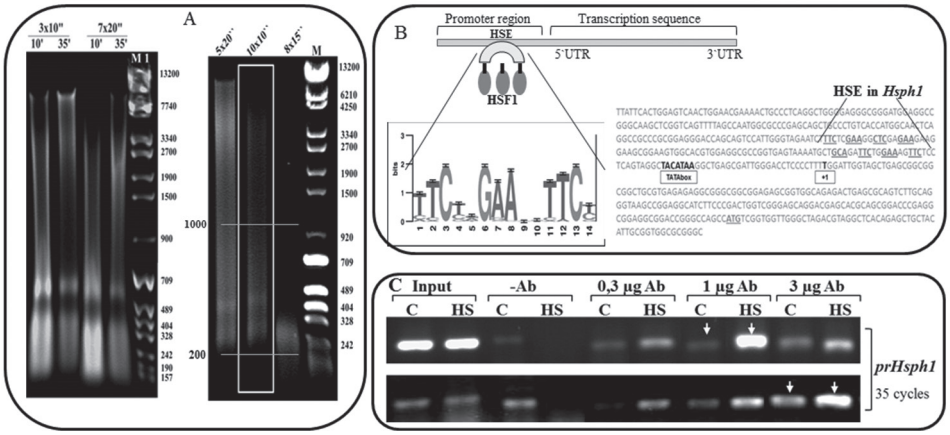

Fig. 2. (A) Time optimization of fixation. Fragmented chromatin of hepatocytes cross-linked with formaldehyde for 10 or 35 minutes (left) and efficient of chromatin shearing depending on sonication conditions (right). Details are described in the section Materials and Methods. DNA (0.1 µg/lane) was separated on a 1% agarose gel. Lane M – DNA marker (13200-157 bp). (B) A schematic representation of the mouse Hsph1 gene locus. Putative regulatory elements (HSE, heat shock elements) binding by HSF1 are shown (12). (C) HSF1 binding to the promoter Hsph1, detected using ChIP-PCR technique with different amounts of antibody. DNA without immunoprecipitation step (Input), DNA immunoprecipitated with (Ab) and without (–Ab) antibody were analyzed. C and HS represent untreated hepatocytes and cells exposed to 1 h heat stress at 42 °C, respectively. The maximum binding of HSF1 after heat shock was observed with 1 µg (upper panel) or 3 µg (lower panel) of antibody (arrows)

shock transcription factor 1 (HSF1) to DNA was evaluated. HSF1 is well known as the transcription factor responsible for the activation of heat shock genes following stress [11]. Activated form of HSF1 binds to the heat shock elements in the promoter regions of Hsp genes (Fig. 2B) [5]. To find out the optimal concentrations of the antibody amount, the ChIP experiment was performed with the mouse *Hsph1* gene (Fig. 2C), already known to be activated by HSF1 [11].

During the determination of the interaction site of the DNA and the protein of interest with ChIP, it is important to know some reference gene/genes, which can clearly identify the presence of specific protein interactions. Figure 2C shows the result of two independent experiments. Comparing to the control; in both cases; the significant enrichments are observed when the cells were exposed to the heat shock. It has been result of the HSF1 regulation of the promoter region of *Hsph1* gene, already known as stress activated. Increasing of the specific DNA target, the best amplification were noticed for 1 ug or 3 µg antibody used for the immunoprecipitation.

When the ChIP technique is applied, it is important to optimize the procedure, before making the actual experiment. It should be noted, that each stage is equally essential to obtain reliable and repeatable results. Especially, in view of increased use of chromatin immunoprecipitation in combination with various, global molecular technique. Herein, it is concluded what is the best ChIP strategy, when specific pro-

tein (HSF1) and/or the cells (hepatocytes) will be an object of the research. However, these results may provide the basic rule of how chromatin immunoprecipitation should be processed in other applications.

REFERENCES

1. Barski, A., Frenkel, B. (2004) ChIP display: novel method for identification of genomic targets of transcription factors. *Nucleic Acids Res. 32,* e104.
2. Browne, J. A., Harris, A., Leir, S. H. (2014) An optimized protocol for isolating primary epithelial cell chromatin for ChIP. *PLoS One 9,* e100099.
3. Collas, P., Dahl, J. A. (2008) Chop it, ChIP it, check it: the current status of chromatin immunoprecipitation. *Front Biosci. 13,* 929–943.
4. Das, P. M., Ramachandran, K., Vanwert, J., Singal, R. (2004) Chromatin immunoprecipitation assay. *Biotechniques 37,* 961–969.
5. Kus-Liskiewicz, M., Polanska, J., Korfanty, J., Olbryt, M., Vydra, N., Toma, A., Widlak, W. (2013) Impact of heat shock transcription factor 1 on global gene expression profiles in cells which induce either cytoprotective or pro-apoptotic response following hyperthermia. *BMC Genomics 14,* 456.
6. Kus-Liskiewicz, M., Widlak, W. (2011) [Finding targets of transcriptional regulators-chromatin immunoprecipitation assay (ChIP)]. *Postepy Biochem. 57,* 418–424.
7. Mundade, R., Ozer, H. G., Wei, H., Prabhu, L., Lu, T. (2014) Role of ChIP-seq in the discovery of transcription factor binding sites, differential gene regulation mechanism, epigenetic marks and beyond. *Cell Cycle 13,* 2847–2852.
8. Ray, S., Das, S. K. (2006) Chromatin immunoprecipitation assay detects ERalpha recruitment to gene specific promoters in uterus. *Biol Proced Online 8,* 69–76.
9. Saiz, L. (2012) The physics of protein-DNA interaction networks in the control of gene expression. *J Phys. Condens Matter 24,* 193102.
10. Spencer, V. A., Sun, J. M., Li, L., Davie, J. R. (2003) Chromatin immunoprecipitation: a tool for studying histone acetylation and transcription factor binding. *Methods 31,* 67–75.
11. Trinklein, N. D., Chen, W. C., Kingston, R. E., Myers, R. M. (2004) Transcriptional regulation and binding of heat shock factor 1 and heat shock factor 2 to 32 human heat shock genes during thermal stress and differentiation. *Cell Stress Chaperones 9,* 21–28.
12. Trinklein, N. D., Murray, J. I., Hartman, S. J., Botstein, D., Myers, R. M. (2004) The role of heat shock transcription factor 1 in the genome-wide regulation of the mammalian heat shock response. *Mol Biol Cell 15,* 1254–1261.
13. Wei, C. L., Wu, Q., Vega, V. B., Chiu, K. P., Ng, P., Zhang, T., Shahab, A., Yong, H. C., Fu, Y., Weng, Z., Liu, J., Zhao, X. D., Chew, J. L., Lee, Y. L., Kuznetsov, V. A., Sung, W. K., Miller, L. D., Lim, B., Liu, E. T., Yu, Q., Ng, H. H., Ruan, Y. (2006) A global map of p53 transcription-factor binding sites in the human genome. *Cell 124,* 207–219.