Chromatin Immunoprecipitation (ChIP) of heat shock protein 90 (Hsp90)

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Abstract

Chromatin immunoprecipitation followed by sequencing (ChIP-seq) is a widely used technique for genome-wide mapping of protein-DNA interactions and epigenetic marks *in vivo*. Recent studies have suggested an important role of heat shock protein 90 (Hsp90) at chromatin. This molecular chaperone assists other proteins to acquire their mature and functional conformation and helps in the assembly of many complexes. In this chapter we provide specific details on how to perform Hsp90 ChIP-seq from Drosophila Schneider (S2) cells. Briefly, cells are simultaneously lyzed and reversibly cross-linked to stabilize protein–DNA interactions. Chromatin is prepared from isolated nuclei and sheared by sonication. Hsp90-bound loci are immunoprecipitated and the corresponding DNA fragments are purified and sequenced. The described approach revealed that Hsp90 binds close to the transcriptional start site of around one-third of all Drosophila coding genes and characterized the role of the chaperone at chromatin.

Key words ChIP-seq, Drosophila Schneider (S2) cells, Hsp90

1. Introduction

Heat shock protein 90 (Hsp90) is a specialized molecular chaperone that facilitates complex formation, stability and activity of many protein kinases and transcription factors. Predominantly localized and characterized in cellular cytosol Hsp90 has a well-established role in proteostasis, cell signaling and carcinogenesis (1). It has emerged as a promising target in cancer therapeutics with 13 Hsp90 inhibitors undergoing clinical evaluation (2).

Recent reports suggest an important function of Hsp90 in nuclear events (3). Nuclear Hsp90 has been found to regulate the function of both transcription factors as well as the general transcription machinery thus contributing to regulation of gene expression (4). One of the first examples of Hsp90 regulating transcription factor function was shown in studies of steroid hormone receptors (5, 6) where it modulates their assembly, disassembly, and activity (7). In addition to hormone receptors, Hsp90 physically interacts and affects the activity of a variety of TFs, such as p53, c-myc, STAT3, NF-kB, HSF1 (4). Another intriguing role for Hsp90 in the nucleus appears to

be its interaction with the general transcription machinery. It not only mediates Pol II assembly in the cytoplasm and nuclear import of the fully assembled holoenzyme (8) but regulates Pol II pausing via stabilization of the negative elongation factor complex (9). In addition, the chaperone is known to affect the packaging of DNA into chromatin and thus contributes to epigenetic regulation of gene expression (10). It induces chromatin accessibility changes through interactions with chromatin-modifying/remodeling complexes that could either activate (SMYD3, trithorax/MLL, RSC complex) (11-13) or repress (EZH2) (14) gene expression.

Thus several studies link Hsp90 to transcription regulation at different levels. A comprehensive characterization of the nuclear clients of Hsp90 and the mechanism by which the chaperone controls gene expression has recently been done (22). Such efforts have enhanced our understanding of the nuclear functions of Hsp90, traditionally considered to be a cytosolic chaperone. An important question remains largely unclear: does Hsp90 modulate the nuclear translocation and consequent activity of soluble, non-DNA-bound forms of clients or does Hsp90 directly interact with chromatin-bound forms? Further studies employing genome-wide occupancy analysis will be required to reveal Hsp90-bound chromatin loci to clearly understand its role at chromatin.

Identification of genome-wide occupancy of transcription factors, components of the basal transcription machinery and modified histones has been critical in understanding the mechanisms of transcriptional regulation. Several widely used methods contribute to the study of how proteins interact with chromatin, and some of the popular techniques are detailed below:

- Chromatin Immunoprecipitation (15) – it has become an indispensable tool for studying gene regulation and epigenetic mechanisms. The first step involves reversible cross-linking of cells in their growth media in order to stabilize protein–DNA interactions. Following chromatin preparation and sonication, DNA fragments bound to protein of interest are enriched using an antigen-specific antibody. It allows the genome-wide localization of not only transcription (co)/factors but specific posttranslational modifications of proteins such as histones for example. The major issues here originate from the cross-linking and immunoprecipitation steps. Cross-linking is very likely to mask some of the antigen epitopes and thus prevent antibody binding. The choice of a specific ChIP-grade antibody of a very good quality is critical.

The following three approaches offer an important advantage over ChIP – they allow mapping the binding site of proteins for which a good ChIP-seq-grade antibody is not available, as is the case with many chaperones. However all of them have a significant disadvantage – they do not allow specific mapping of posttranslationally modified proteins such.

- Chemical affinity capture followed by sequencing (Chem-seq) (16) – this recent technique relies on small chemical compounds binding with a high affinity to the protein of interest to enrich it from cross-linked or uncross-linked chromatin. The small size of chemical components makes antigen recognition easier and is not that much hampered by the cross-linking of protein-DNA complexes. It is the first method that allows researchers to directly determine the location of cellular factors targeted by small molecules throughout the genome. The successful outcome of the protocol however relies on the existence of a chemical that binds to an antigen with a high specificity and efficiency. Therefore it has been applied to a limited number of proteins. The increasing number of highly specific and potent Hsp90 inhibitors could soon allow the genome-wide Hsp90 localization through Chem-seq (17).

- Biotin-mediated ChIP (BioChIP) (18) - it involves endogenous expression of critical chromatin/DNA binding factors tagged with biotin. This allows their affinity capture by streptavidin. Biotin-streptavidin interaction is highly specific and one of the strongest non-covalent interactions in nature. These features significantly reduce the non-specific binding background and favor a highly efficient target pull-down. A major disadvantage here is the need of recombinant biotin-tagged protein expression which is more time-consuming, elaborate and could introduce artefacts.

- DNA adenine methyltransferase identification (DamID) (19) – this alternative method involves expression of the DNA-binding protein of interest as a fusion protein with DNA methyltransferase. This enzyme mediates in vivo DNA adenosine methylation in the region of the binding site. By using methylation-sensitive restriction enzymes to enrich methylated DNA, this technique allows an identification of protein-bound DNA independent of immunoprecipitation.

In the following protocol we focus on ChIP and provide specific details on how to identify Hsp90-bound chromatin loci in Drosophila Schneider (S2) cells. It could also be applied to other cell types and (co)chaperones such as p23 (13).

2. Materials

Reagents

Prepare all solutions with molecular biology grade water (Milli-Q™) and supplement them with protease inhibitors.

2.1 Cells Preparation and Cross-linking

1. Protease inhibitors at a final concentration of: 3 µg/ml aprotinin, 10 µg/ml leupeptin, 1 µg/ml pepstatin, 0.1 mM PMSF.
2. Methanol-free formaldehyde 16% (Thermo Scientific, Prod. Nr. 28906).
3. 10X cell lysis buffer: 50 mM HEPES, 50 mM NaCl, 10 mM EDTA, 5 mM EGTA.
4. Glycine 1 M.
5. Wash buffer: 50 mM Tris, pH 8.0, 15 mM NaCl, 0.5 mM EGTA, 60 mM KCl.

2.2 Chromatin Extraction and Sonication

1. Sonication buffer: 10 mM Tris, pH 8.0, 1 mM EDTA, 0.5 mM EGTA.

2.3 Chromatin Quality Check

1. RNase A 10 mg/ml (DNase and protease free; AppliChem, Prod. Nr. A3832).

2. Proteinase K 20 mg/ml (Sigma Aldrich, Prod. Nr. P2308).

3. TE buffer: 1 mM EDTA pH 8.0, 10 mM Tris–HCl pH 8.0.

4. Phenol–chloroform–isoamyl alcohol 25:24:1 (ROTH, Prod. Nr. A156.3).

5. Glycogen 5 mg/ml (Roche, Prod. Nr. 13741729).

6. 100% Ethanol.

7. EB buffer: 10 mM Tris-Cl, pH 8.5.

8. 1X TAE buffer: 40 mM Tris–acetate, 1 mM EDTA, pH 8.0.

9. 2 % agarose gel in TAE buffer.

10. GelRed Nucleic Acid Gel Stain, 10 000X in water.

11. 100 bp DNA ladder.

12. DNA loading dye.

13. High Sensitivity DNA Kit (Agilent).

2.4 Immunoprecipitation

1. ChIP dilution buffer: 10 mM Tris, pH 8.0, 1 mM EDTA, 1 % Triton X-100, 0.1 % sodium deoxycholate, 0.1 % SDS, 140 mM NaCl.

2. Specific antibody against a protein of interest (anti-Hsp90 polyclonal serum used in this case).

3. Protein A-Dynabeads (Novex by Life technologies, Prod. Nr. 10002D).

4. LiCl buffer: 10 mM Tris, 250 mM LiCl, 1 mM EDTA, 0.5 % NP-40, 0.5 % sodium deoxycholate.

5. Elution buffer: 1 % SDS, 100 mM NaHCO3.

Equipment

1. End-over-end rotator.
2. Refrigerated tabletop centrifuge.
3. Protein Low Binding tubes.
4. DNA Low Binding tubes.
5. BioRuptor (Diagenode).
6. Magnetic rack.
7. Thermomixer.
8. Laboratory heating oven.
9. Vortex mixer.
10. Qubit fluorometer.
11. Agarose gel electrophoresis equipment.
12. UV-transilluminator.
13. 2100 Bioanalyzer.

3. Methods

3.1 Cells Preparation and Cross-linking

1. Use 50 x 10^6 exponentially growing cells per reaction in 10 ml of growth media. Add 10X cell lysis buffer and Triton X-100 directly in the media to reach 1X and 0.5 % final concentration, respectively.

2. Immediately add formaldehyde to a final concentration of 1 % to facilitate cells cross-linking (*see* **Note 1** and **2**).

3. Incubate for 15 min with end-over-end rotation at room temperature (*see* **Note 3**).

4. Stop the cross-linking reaction by adding glycine to a final concentration of 100 mM. Incubate for 5 min with end-over-end rotation at room temperature.

5. Spin down the isolated and cross-linked nuclei at 750 x g for 5min at 4 °C and discard the cross-linking solution. (*see* **Note 4**)

6. Wash twice with ice cold wash buffer (*see* **Note 5**).

3.2 Chromatin Extraction and Sonication

All the following procedures should be performed at 4 °C with ice cold buffers containing protease inhibitors.

1. Resuspend the nuclei pellet thoroughly in 200 µl of sonication buffer and transfer to a protein low binding tube (*see* **Note 6** and **7**).

2. Sonicate with Bioruptor (Diagenode) in cold water with the following settings: 30 cycles with intervals of 30s ON/OFF at maximum power. The optimal size range of the acquired DNA fragments for ChIP-seq analysis should be between 200 and 600 base pairs appearing as a smear (Figure 1) (*see* **Note 8)**.

3. Spin down the sonicated material at 16 000 x g for 10 min (*see* **Note 9** and **10**).

4. Transfer the supernatant to a fresh protein low binding tube (*see* **Note 11**).

3.3 Chromatin Quality Check

For optimal results confirm sonication efficiency and chromatin yield prior to IP. The following steps could be skipped once the protocol is optimized.

1. Take a 20 µl aliquot (1 %) of the sheared chromatin. Add 1 µl of RNase A and 1 µl of proteinase K and incubate for 30 min at 37 °C.

2. Reverse cross-link overnight in a 65 °C oven (*see* **Note 12)**

3. Bring the volume of both IP and Input samples to 400 µl with TE buffer.

4. Add an equal volume of Phenol–chloroform–isoamyl alcohol (25:24:1) mixture and vortex (*see* **Note 13)**.

5. Incubate at room temperature for 1 min.

6. Spin down at 20 000 x g for 10 min at 4 °C.

7. Transfer the nucleic-acid-containing aqueous upper phase to a DNA low binding tube. Add sodium chloride and glycogen to 200 mM and 50 µg/mL final concentration, respectively (*see* **Note 14).**

8. Incubate the mixture for 2h at −20 °C for 1h at −80 °C (*see* **Note 15).**

9. Spin down at 20 000 x g for 10 min at 4 °C. Discard the supernatant.

10. Wash with 1 mL of 80 % EtOH (precooled at −20 °C).

11. Spin down at 20 000 x g for 10 min at 4 °C. Discard supernatant.

12. Air-dry the pellet around 5 min.

13. Resuspend in 20 µl of EB buffer.

14. Measure DNA amount by Qubit following manufacturer's protocol - expected yield around 1 µg of DNA per 1 x 10^6 cells).

15. Assess chromatin shearing efficiency either on a 2 % agarose gel or on Bioanalyzer 2100 using High Sensitivity DNA chip according to manufacturer's protocol. The optimal size range of DNA for ChIP-seq analysis should be between 200 and 600 base pairs (Figure 1).

3.4 Immunoprecipitation

1. Use 20-50 µg of chromatin for ChIP-seq and 5-10 µg for qPCR per reaction - one for the specific antibody (IP) and one for an appropriate negative control (mock-IP; pre-immunization serum or IgA/IgG antibody could be used) (*see* **Note 16)**.

2. Dilute it 1:10 in ChIP dilution buffer.

3. Take a 5 % aliquot as input DNA. Freeze at -20 ºC until needed.

4. Add 5 µg of Hsp90 or control antibody to the IP and mock-IP reactions, respectively (*see* **Note 17)**.

5. Incubate overnight at 4 °C with end-over-end rotation.

6. Prepare 25 µl of Protein A-Dynabeads per reaction. Wash in ChIP dilution buffer (*see* **Note 18).**

7. Capture the antigen-antibody complexes by adding 25 µl of Protein A-Dynabeads per reaction and incubate for 4h.

8. Separate the beads on a magnetic rack and discard the supernatant.

9. Transfer the beads in ChIP dilution buffer to a new tube.

10. Wash the beads five times per 5 min with 1 ml of ChIP dilution buffer.

11. Wash once per 5 min with 1ml of LiCl buffer.

12. Wash twice per 5 min with 1ml of TE buffer.

13. Elute by adding 50 µl of Elution buffer and incubating in a thermomixer at 65 °C for 15 min under vigorous shaking (1400 rpm) (*see* **Note 19)**.

At this point, thaw the input DNA and process along with the IP samples.

14. Reverse cross-link overnight at 65 °C in a laboratory oven (*see* **Note 20).**

15. Add 200 µL of TE buffer and 8 µL of RNaseA. Incubate for 2 hs at 37 °C in a thermomixer.

16. Add 4 µL of Proteinase K, mix and incubate overnight at 55 °C in a laboratory oven.

17. Purify the DNA using Phenol–chloroform–isoamyl alcohol (25:24:1) as previously described (Methods steps 3.3.4 to 3.3.12). DNA purification kits are not recommended due to loss of material and size selection.

18. Resuspend the DNA pellet in 50 µl of EB buffer and transfer to a fresh tube - expected yield around 15-30 ng per 50 x 10^6 cells.

IP DNA can then be sent for next generation sequencing (20) (*see* **Note 21) .**

4. Notes

1. Always use methanol-free formaldehyde for optimal and reproducible results (methanol reduces the fixing power of formaldehyde). Ampoule-sealed solutions are highly recommended as they are well-protected from both air oxidation and light. Formaldehyde is highly toxic and has to be handled carefully under a fume hood.

2. Simultaneous cell lysis and cross-linking achieved at this step generates isolated cross-linked nuclei - a much purer substrate for subsequent steps than whole cells. That initial cellular fractionation improves significantly the chromatin shearing process. A recently published protocol (21) offers an alternative – cross-linking of cells in growing media followed by ultrasound-based nuclear extraction. Nuclei isolation prior to chromatin preparation offers an additional advantage for proteins that are highly abundant in the cytosol (like Hsp90). By eliminating the cytosolic pool the efficiency of chromatin-bound Hsp90 pull-down is increased.

3. Cross- linking is a time- and temperature-dependent process. It should not exceed 15 min performed at room temperature. Extended cross-linking could lead to poor results due to reduced sonication efficiency, reduced antibody accessibility to antigen and epitope masking.

4. Discard formaldehyde-containing solution according to the institutional safety rules.

5. **Pause point**: cross-linked nuclei/cells could be snap frozen in liquid nitrogen and stored at -80 °C for at least one month.

6. Avoid foaming since it dramatically reduces cross-linking efficiency. If foaming occurs, centrifuge for 3 min at 20 000 x g. Resuspend the material gently leaving no foam bubbles.

7. Use non-siliconized protein low binding tubes to prevent proteins sticking to tube walls and thus protein loss.

8. One could use alternative sonication devices - Branson tip sonicator or Covaris ultrasonicator, for example. Sonication conditions however must be carefully optimized depending upon the specific sonicator to ensure the optimal quality of sheared chromatin. The optimal size range of the acquired DNA fragments for ChIP-seq analysis should be between 200 and 600 base pairs (Figure 1). DNA fragments out of the range may not be sequenced later on.

9. Proper sonication will generate a clear solution. Turbidity could indicate insufficient sonication.

10. A minimal pellet should be visible at this point. If a larger pellet appears, the sonication did not work properly.

11. **Pause point**: sheared chromatin could be stored at 4 °C for up to one week.

12. Use a laboratory oven for longer thermal incubations to prevent sample condensation on the cap of the tube and ensure uniform heating.

13. Phenol–chloroform is highly toxic and should be handled under the fume hood. Avoid inhalation and skin contact. Wear protective clothing and gloves.

14. Glycogen increases the quantitative recovery of DNA.

15. **Pause point**: incubation at -20 °C or -80 °C could also be done overnight.

16. An isotype matched control immunoglobulin (a negative control) is necessary to determine the non-specific DNA enrichment (background).

17. The choice of antibody is crucial. To test whether an antibody is ChIP-grade, follow the ChIP protocol until the IP washes are done. At this point boil the beads in 1X SDS loading buffer for 30 min (a longer boiling time is necessary to reverse cross-link) and perform a western blot.

    The amount of antibody per IP and the incubation time depend mainly on the the affinity properties of the antibody and the abundance of the protein of interest. Test each new antibody to determine optimal conditions.

18. Protein A/G agarose beads could also be used but magnetic beads give a lower background, do not require blocking and are easier to handle.

19. Always use fresh Elution buffer. **Pause point**: the eluted material could be frozen at -20 °C and stored for up to a week.

20. Do not reverse cross-link for more than 18h.

21. **Pause point**: the DNA could be stored frozen at -80 °C and stored for at least one month.

5. References

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Figure 1. Not sonicated (1) and sonicated (2) chromatin from Drosophila S2 cells.