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Using Intra-ChIP to Measure Protein–DNA Interactions in Intracellular Pathogens

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Abstract

Chromatin immunoprecipitation is used to measure the binding of transcription factors to target DNA sequences in order to better understand transcriptional regulation. Here, we describe a process to analyze bacterial transcription factor binding in the context of an infected eukaryotic host cell. Using this approach, we measured the binding kinetics of three *Chlamydia trachomatis* transcription factors within infected cells, and demonstrated temporal changes in binding.

Keywords

Chromatin immunoprecipitation; Intracellular pathogen; Transcriptional regulation; *Chlamydia*

1 Introduction

Chromatin immunoprecipitation (ChIP) utilizes antibody specificity to selectively capture protein–DNA complexes for analysis [1]. Cells and tissues are first treated with a crosslinking agent, such as formaldehyde, to covalently link proteins that are bound to DNA. Specific antibodies are then used to preferentially isolate a particular protein cross-linked to its DNA targets. Bound DNA is subsequently dissociated from the protein by reversing the crosslinks, and can be analyzed using a variety of techniques.

ChIP is a critical component of modern molecular biology, but has only recently been used to probe gene regulation in microbial pathogens within an infected cell. Here, we demonstrate a ChIP method, called Intra-ChIP [2, 3], to measure binding of a bacterial transcription factor to its target genes in the context of an intracellular infection. We have used *Chlamydia trachomatis* as a representative intracellular pathogen, and describe our approach to detect binding of multiple chlamydial transcription factors to their regulatory DNA target sequences. *C. trachomatis* utilizes an unusual developmental cycle for intracellular growth [4], which involves temporal control of transcription [5]. We used the Intra-ChIP approach to measure intracellular binding of the chlamydial transcription factors EUO, HrcA, and Sigma-28.

We were able to determine that EUO binds its target DNA early in the chlamydial developmental cycle, and that binding is lost over time (Fig. 1a). We were also able to demonstrate that HrcA binds its target DNA at middle and late times of the chlamydial developmental cycle, and that HrcA binding is minimal at earlier times (Fig. 1b). Finally, we

observed that Sigma-28 binds to its target DNA exclusively at late times in the developmental cycle (Fig. 1c). Thus, we have shown that transcription factor binding can be measured in an intracellular pathogen. Furthermore, temporal changes in transcriptional regulation can be monitored over time. In addition, the method described is broadly applicable to other intracellular microbes.

2 Materials

Prepare solutions using sterile ultrapure water and analytical grade reagents. Prepare and store all materials at room temperature, unless otherwise specified. Follow all relevant regulations and guidelines to dispose of waste appropriately.

2.1 Cell Culture

1. Cell Culture Medium: RPMI with 25 mM HEPES and 5% FBS (heat-inactivated). Prepare by combining 925 mL of RPMI 1640 with 25 mL of 1 M HEPES and 50 mL FBS. Filter through a 0.22 μm Corning filter. Store at 4 °C.
2. 500 mL spinner flask.
3. L929 mouse fibroblast cells (ATCC).
4. *Chlamydia trachomatis* Serovar L2, strain 434/Bu (ATCC).

2.2 Crosslinking

1. Crosslinking Solution: 1% formaldehyde in RPMI 1640 lacking HEPES and FBS. Prepared by adding an appropriate volume of fresh 37% formaldehyde directly to RPMI 1640.
2. Quenching Solution: 2.5 M glycine pH 7.5.

2.3 Lysate Preparation

1. Lysis Buffer: 25 mM Tris–HCl pH 7.5, 150 mM NaCl, 0.5% sodium deoxycholate, 1% NP-40, 0.1% SDS, protease inhibitor cocktail.
2. Branson Digital Sonifier.

2.4 Immunoprecipitation

1. GE Healthcare Life Sciences Protein G Sepharose, stored at 4 °C.
2. Antibody: Antigen-specific Serum and Control Serum, stored at −20 °C.
3. Blocking buffer: 5% BSA with 200 μg/mL Salmon sperm DNA, stored at 4 °C.
4. Phosphate Buffered Saline (PBS).
5. Wash buffer: 40 mM HEPES, 4 mM $MgCl_2$, 70 mM KCl, 7.5% glycerol.
6. Elution buffer: 0.1 M glycine pH 2.5.
7. Neutralization buffer: 1 M Tris–HCl pH 8.5.
8. Machery-Nagel Nucleospin Gel and PCR Clean-up Kit.

2.5 Quantitative Real-Time PCR Analysis

1. Bio-Rad SYBR Green master mix, stored at −20 °C.
2. Bio-Rad iCycler Real-Time PCR Detection System.
3. Primers to amplify Target DNA and Non-target DNA.

3 Methods

3.1 Cell Culture and Infection

1. Grow L929 cells in Cell Culture Medium at 37 °C in 5% CO_2. To produce sufficient cells for this protocol, we grow them in suspension culture in a 500 mL spinner flask at a density of 6×10^5 cells/mL (*see* Note [1]).
2. Infect cells by directly adding *C. trachomatis* at a multiplicity of infection (MOI) of 3 to L929 suspension culture (*see* Note [2]).
3. At desired time point post-infection, collect 6×10^7 infected cells (~100 mL) with centrifugation at 2500 × *g* for 10 min.

3.2 Crosslinking

1. Resuspend cells in 30 mL Crosslinking Solution and incubate gently, rocking for 30 min at room temperature (*see* Note [3]).
2. Add 3.3 mL Quenching Solution to crosslinked cells and incubate gently, rocking for 15 min at room temperature (*see* Note [4]).
3. Pellet crosslinked cells with centrifugation at 2500 × *g* for 10 min.
4. Aspirate supernatant and store infected cell pellets at −80 °C, or proceed directly to Lysate Preparation.

3.3 Lysate Preparation

1. Resuspend crosslinked infected cells in 4 mL Lysis Buffer in a 15 mL conical tube.
2. Sonicate on ice with an output setting of 22% for a total of 2 min in 15 s bursts. Sonication conditions should result in fragmentation of DNA to a size range between 300 and 1200 bp (*see* Note [5]).

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[1] The use of a biosafety cabinet is required to maintain sterility when performing tissue culture.
[2] *Chlamydia trachomatis* is a human pathogen and requires a Biosafety Level 2 facility and precautions.
[3] Crosslinking solution must be made up fresh just prior to adding to infected cells. A new 37% formaldehyde solution should also be prepared approximately every 1–2 months due to instability in aqueous solution.
[4] After crosslinking, it is critical to quench and remove excess formaldehyde. If formaldehyde is carried over to the immunoprecipitation step, it will result in nonspecific crosslinking of protein–DNA complexes to the Antibody-beads.
[5] Sonication conditions that result in DNA fragmentation between 300 and 1200 bp must be determined empirically, and are dependent on a number of factors including; sonifier, cell density, and buffer. Care must be taken to ensure the sample does not heat up during sonication, because this can result in reversal of crosslinking. Fragmentation to this size range allows for resolution of binding events to individual loci that are at least 1200 bp apart on the bacterial genome. Because both host DNA and bacterial DNA are present in the same sample, the desired size range is actually a composite of all total DNA. In our experience, achieving this size range with total DNA has proven sufficient for detection of bacterial binding events.

3. Centrifuge at 10,000 × *g* for 30 min to clarify lysate.
4. Carefully remove lysate supernatant without dislodging pellet.
5. Pre-clear crosslinked lysate by adding 50 μL (bed volume) of Protein G Sepharose and incubating with gentle rocking at 4 °C for 2 h (*see* Note [6]).
6. Pellet Protein G Sepharose by centrifuging at 10,000 × *g* for 5 min.
7. Carefully remove lysate without disturbing Protein G Sepharose pellet.
8. Separate lysate into 250 μL aliquots (Input DNA) and store at −80 °C until ready to proceed with immunoprecipitation (*see* Note [7]).

3.4 Immunoprecipitation

1. In two separate 1.5 mL microcentrifuge tubes combine 25 μL Protein G Sepharose (bed volume) and 100 μL of PBS.
2. Add 5 μL of Antigen-specific Serum to the first tube, and 5 μL of Control Serum to the second tube.
3. Incubate Antibody-beads with rotation at 4 °C for 1 h (*see* Note [8]).
4. Add 500 μL of Blocking Buffer to each tube and incubate with rotation at 4 °C for 1 h.
5. Pellet Antibody-beads with centrifugation at 10,000 × *g* for 2 min.
6. Wash twice with 500 μL PBS by resuspending Protein G Sepharose and pelleting with centrifugation at 10,000 × *g* for 2 min.
7. Add 250 μL of crosslinked infected cell lysate (Input DNA) to each tube and incubate with rotation at 4 °C overnight.
8. The following day, place Wash Buffer on ice to cool.
9. Pellet Antibody-beads with centrifugation at 10,000 × *g* for 2 min.
10. Aspirate and discard supernatant.
11. Add 500 μL ice-cold Wash Buffer to each tube and incubate with rotation at room temperature 3 min.
12. Pellet Antibody-beads with centrifugation at 10,000 × *g* for 2 min.
13. Repeat **steps 10–12** three additional times for a total of four washes.
14. Prepare two new 1.5 mL microcentrifuge tubes by adding 80 μL of Neutralization Buffer to each tube.

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[6]Pre-clearing lysate with Protein G Sepharose aids in removing nonspecific interactions that may occur during the immunoprecipitation step.

[7]Lysates are very stable when stored at −80 °C, and we have successfully performed ChIP after over a year of storage.

[8]We pre-bind antibodies to Protein G Sepharose to ensure that Protein G Sepharose is not a limiting factor. If excess antibodies are added directly to the lysate, protein–DNA interactions may be missed if there is insufficient Protein G Sepharose to bind all the antibodies.

15. Add 100 μL of Elution Buffer to Antibody-beads and incubate at room temperature for 1 min.
16. Pellet Antibody-beads with centrifugation at 10,000 × *g* for 2 min.
17. Remove supernatant and add directly to a new tube containing Neutralization Buffer (*see* Note [9]).
18. Repeat **steps 15–17** three additional times for a total of four elutions.
19. Discard tubes containing Antibody-beads.
20. Incubate tubes containing neutralized eluate at 95 °C for 1 h to reverse crosslinks (*see* Note [10]).
21. Allow eluate to cool to room temperature.
22. Use Nucleospin Gel and PCR Clean-up Kit according to the manufacturer's instructions to purify DNA from eluate, with a final volume of 100 μL.
23. Store purified DNA at −20 °C.
24. To determine the amount of input DNA used in each Intra-ChIP reaction, incubate a separate 250 μL aliquot of cross-linked lysate (Input DNA) at 95 °C for 1 h to reverse crosslinks.
25. Perform **steps 21–23** to obtain purified Input DNA in a final volume of 100 μL.

3.5 Quantitative Real-Time PCR

1. Prepare PCR master mixes by combining primers for Target DNA or Non-target DNA (final 250 nM concentration) with SYBR Green master mix according to the manufacturer's instructions (*see* Note [11]).
2. Add 20 μL of master mix to optically clear PCR tubes.
3. For each primer pair, set up triplicate reactions by adding 5 μL of the following templates:
   a. Control Serum Immunoprecipitated DNA.
   b. Antigen-specific Serum Immunoprecipitated DNA.
   c. Purified Input DNA.
   d. H_2O (negative control).

[9]The low pH of the Elution Buffer is responsible for dissociating antibodies from Protein G Sepharose, and for dissociating protein–DNA complexes from antibodies. However, this low pH can be damaging to DNA, so it is important to rapidly neutralize the eluate.
[10]It is critical to reverse the protein–DNA crosslinks prior to analysis; otherwise the primers for Real-Time PCR will be unable to bind the template. It is also possible to reverse cross-links by incubating at 65 °C overnight instead of using 95 °C. Crosslinking reversal conditions should be determined empirically by running DNA on an agarose gel with ethidium bromide staining. Successful reversal of crosslinks will result in a smear of DNA within the desired size range (300–1200 bp). Insufficient reversal of crosslinks will result in a DNA smear that extends all the way up to the well.
[11]Primer design is an important part of the Real-Time PCR analysis. Primers should be designed to have a melting temperature of ~60 °C and should amplify ≤100 bp of Target or Non-target DNA. It is also important to verify that the primers only amplify a single product from Purified Input DNA, as assessed with gel electrophoresis and ethidium bromide staining. Non-specific interactions between primers and host-cell DNA can also result in reduced amplification efficiency. Therefore, it is also important to determine amplification efficiency for each primer pair using Purified Input DNA, which contains a mix of host and bacterial DNA.

4. Use a Bio-Rad iCycler to perform Real-Time PCR, with 40 cycles of 95 °C for 30 s and 60 °C for 30 s, with detection occurring during the 60 °C step.

3.6 Data Analysis

1. To facilitate direct comparison of the Ct values for Target DNA, Non-target DNA, and Input DNA, 5% of each sample is used as a template in each Real-Time PCR reaction (5 μL out of 100 μL).

2. Calculate the amount of Target DNA or Non-target DNA isolated with either Control Serum or Antigen-specific Serum relative to Purified Input DNA (Input) using the formula below (*see* Note [12]):

$$\%\text{Input recovered} = 100\% \times 2^{(\text{Ct}_{\text{Input}} - \text{Ct}_{\text{ChIP}})}.$$

3. Calculate Fold Enrichment of Target DNA or Non-target DNA using the formula below (*see* Note [13]):

$$\text{Fold Enrichment} = \frac{\text{Antigen-specific Serum \% Input Recovered}}{\text{Control Serum \% Input Recovered}}.$$

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[12]For the formula given, $2^{(\text{Ct\_Input} - \text{Ct\_ChIP})}$ assumes an amplification efficiency of 100%, leading to a twofold difference in copies between each Ct value. However, primer amplification efficiency can vary significantly. Amplification efficiency can be determined by generating standard curves with DNA templates at known copy numbers, or by using linear regression analysis directly on sample data [6].

[13]Fold Enrichment is used to determine the amount of DNA isolated with a specific antibody relative to a nonspecific antibody. This calculation indicates the specificity of the Intra-ChIP procedure. When used in combination with a Non-Target DNA control, Fold Enrichment accounts for antibody specificity and Target DNA specificity.

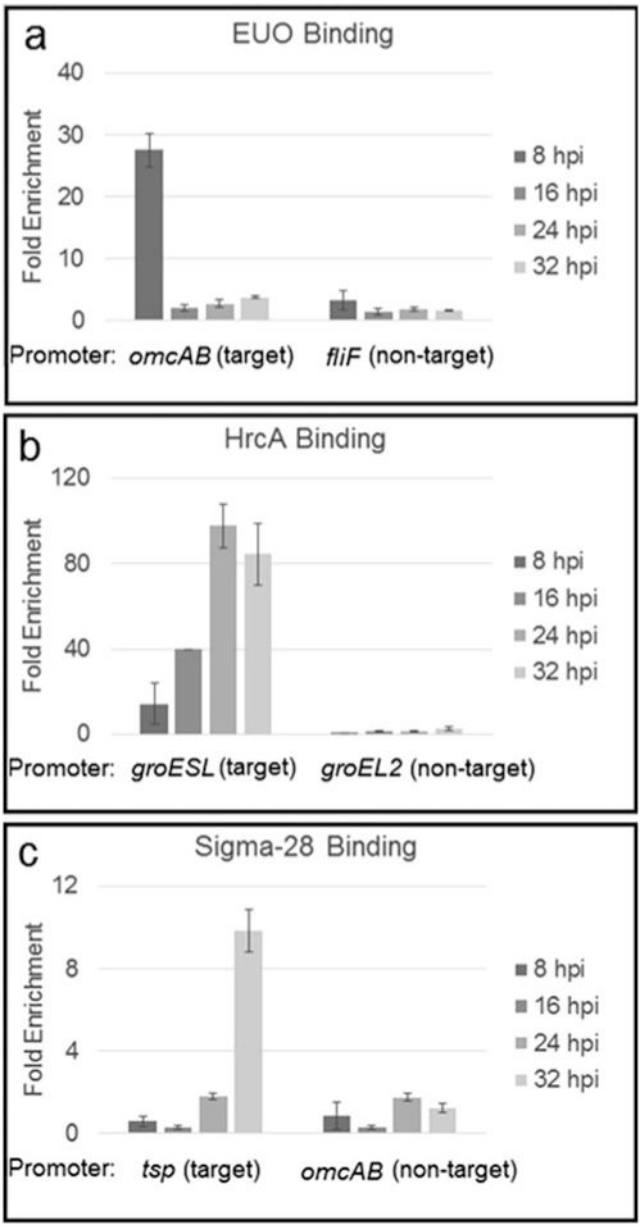

Fig. 1.

Measurement of chlamydial transcription factor binding to target promoters using Intra-ChIP. The transcription factors EUO (**a**), HrcA (**b**), and Sigma-28 (**c**) were immunoprecipitated at multiple times after infection (hours post-infection, hpi) to measure binding to target or non-target DNA sequences. Binding is expressed as Fold Enrichment relative to immunoprecipitation with Control Serum