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High Resolution Chromatin Immunoprecipitation: ChIP-sequencing

Roxanne E. Diaz, Aurore Sanchez, Véronique Anton Le Berre and Jean-Yves Bouet

Abstract

Chromatin immunoprecipitation (ChIP) coupled with next generation sequencing (NGS) is widely used for studying the nucleoprotein components that are involved in a variety of cellular processes shaping the bacterial nucleoid. This methodology, termed ChIP-sequencing (ChIP-seq), enables the identification of the DNA targets of DNA binding proteins across genome-wide maps. Here we describe the steps necessary to obtain short, specific, high quality immunoprecipitated DNA prior to DNA library construction for NGS and high resolution ChIP-seq data.

Key words Chromatin immunoprecipitation, next generation sequencing, genome-wide maps, DNA target, DNA fragmentation, DNA sonication, affinity-purified antibody, bacterial nucleoid.

1 Introduction

ChIP-sequencing (ChIP-seq), which combines two methods – chromatin immunoprecipitation and next generation sequencing – has made a tremendous impact in many biological research fields from eukaryotes to prokaryotes. Knowing the locations where proteins interact with DNA is essential in the understanding of their functionality in a system and many methods are available to study their binding sites and specificity of interaction. However, only ChIP-seq can give *in vivo* high-resolution data mapped across the entirety of a genome. Since its first use in eukaryotes (Barski *et al.*, 2007; Robertson *et al.*, 2007; Johnson *et al.*, 2007; Mikkelsen *et al.*, 2007), to its use in prokaryotes (Lun *et al.*, 2009; Kahramanoglou *et al.*, 2011), the cost of sequencing continues to decrease while technology advances, making ChIP-seq an easily accessible method. ChIP-seq has become a widely used technique to investigate nucleoprotein complexes that shape the bacterial nucleoid, giving insight into a range of processes such as local and global organization, replication, segregation, as well as global regulation of gene expression by nucleoid-associated proteins.

Current and widely used methods to capture protein-DNA interactions among a live cell population couple ChIP with the use of covalent and reversible formaldehyde cross-linking, namely X-ChIP. Cross-linked cells are then lysed and DNA is extensively fragmented, through enzymatic digestion or sonication. In this protocol we describe fragmentation through sonication using an automated rotating water bath system. We have found this method to be the most efficient and reproducible that results in a uniform size of DNA among samples with an average of ~200bps required for high resolution ChIP-seq data.

Fragmented protein-DNA complexes are then selectively immunoprecipitated using protein specific antibodies. The quality and specificity of antibodies used for this assay is of utmost importance to prevent non-specific pull downs and datasets with a low signal to noise ratio. Here, we also describe a method for membrane strip affinity purification of antibodies using rabbit polyclonal sera raised against the protein of interest. Following immunoprecipitation, proteins are eliminated using a proteinase K digestion and the samples are reverse cross-linked prior to DNA purification. We have found that using a simple chloroform and isoamyl alcohol purification followed by an isopropanol precipitation, is a cost efficient method that results in a high recovery of quality DNA; however other options such as bead and DNA purification kits are effective alternatives.

The ChIP-seq protocol described here is adapted from Cho *et al.*, 2011 and has contributed to important achievements related to bacterial nucleoids, such as the essential involvement of SImA in *Escherichia coli* in regulating FtsZ ring assembly. More recently, Sanchez *et al.*, 2015 used high-resolution ChIP-seq data that allowed for the physico-mathematical modeling of the ParB_F propagation along the DNA and the proposal of a new model of stochastic self-assembly for the F plasmid partition complex.

2 Materials

Prepare all solutions using Milli-Q[®] or any form of ultra-pure water with a sensitivity of 18.2 MΩ.cm and molecular grade reagents. Unless otherwise specified, filter solutions using a 0.45 μm low protein binding non-pyrogenic membranes and store them at room temperature.

2.1 Antibody purification

Prepare the following buffers:

1. 2x sample buffer: 100 mM Tris pH 6.8, 5% SDS, 20% glycerol, 0.05% Bromophenol blue. Add β-mercaptoethanol (50 μl.ml⁻¹) just before mixing with protein samples.

2. Ponceau S: Mix 50 mg Ponceau S (Sigma-Aldrich), 2.5 ml acetic acid ($\geq 99\%$) with 47.5 ml of water.
3. 10x Tris-Buffered Saline: Mix 20 ml of 1 M Tris pH 7.6, 20 ml of 5 M NaCl with 960 ml of water.
4. TBS-Tween20: Dissolve 0.1% Tween20[®] in 1x TBS.
5. 0.1 M Glycine-HCl pH 2.2: Dissolve 1.5 g of glycine in water, adjust to pH 2.2 with 5 M HCl and add water up to a volume of 200 ml.
6. 10 mM NaPhosphate buffer pH 7.2: for 100 ml, mix 72 ml of 0.1 M Na₂PO₄ with 28 ml of 0.1 M NaH₂PO₄.

2.2 Bacterial culture and cross-linking

1. For *Escherichia coli* and related bacteria, grow cells in LB medium or other appropriate media specific of the tested growth condition.
2. 36% formaldehyde commercial solution.
3. 2.5 M Glycine: Dissolve 92.83 g of glycine powder in 400 ml of water. Transfer solution to a graduated cylinder and add water up to a volume of 500 ml.
4. Tris-Buffered Saline pH 7.6: See antibody purification (§ 2.1.3).

2.3 Cell lysis and sonication

1. 1.5 ml tubes with a low DNA binding grade.
2. Rotating water bath sonicator: We recommend the Bioruptor[®] plus (Diagenode). The M220 Focused-ultrasonicator (Covaris) also provides reproducible and accurate results.
3. Lysis buffer: 10 mM Tris-HCl pH 7.8, 100 mM NaCl, 10 mM EDTA, 20% sucrose, 1 mg/ml lysozyme (see Note 1).
4. 2x IP buffer: 50 mM Hepes-KOH pH 7.5, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF (see Note 2).

2.3 Immunoprecipitation

1. Magnetic rack.
2. Magnetic protein A beads.
3. Rocking tube agitator for use at 4°C.
4. Blocking buffer: 0.1 $\mu\text{g}\cdot\mu\text{l}^{-1}$ BSA, 1 $\mu\text{g}\cdot\mu\text{l}^{-1}$ tRNA.
5. Wash buffer 2: 50 mM Hepes-KOH pH 7.5, 500 mM NaCl, 1 mM EDTA, 1% Triton X-100, 0.1% sodium deoxycholate, 0.1% SDS, 1 mM PMSF (see Note 2).
6. Wash buffer 3: 10 mM Tris-HCl pH 7.8, 250 mM LiCl, 1 mM EDTA, 0.5% IGEPAL CA-630 (Sigma-Aldrich), 0.1% sodium deoxycholate, 1 mM PMSF (see Note 2).
7. TE Buffer: 10 mM Tris pH 7.5, 1 mM EDTA.
8. Elution buffer: 50 mM Tris-HCl pH 7.5, 10 mM EDTA, 1% SDS.
9. 4x reducing buffer (Invitrogen).

2.4 Reverse crosslink

1. RNase: 10 $\text{mg}\cdot\text{ml}^{-1}$.
2. Proteinase K: 1 $\text{mg}\cdot\text{ml}^{-1}$.

2.4 DNA purification

1. 5 M NaCl.
 2. Pure chloroform (CHCl₃) and Isoamyl alcohol solutions.
 3. 20 mg.ml⁻¹ Glycogen.
 4. 70% ethanol and 100% Isopropanol.
 5. 10 mM Tris-Cl pH 8.5.
-

3 Methods

Carry out all procedures at room temperature (23°C) unless otherwise specified.

3.1 Antibody purification

For high-quality ChIP-sequencing data, we recommend to use affinity-purified antibodies raised against the proteins of interest.

1. Recuperate 100 µg of the purified protein of interest and mix with 300 µl of 2x sample buffer (see Note 3).
2. Load the protein sample on an appropriate 1-well SDS-PAGE. Run the gel and transfer subsequently by Western blotting on nitrocellulose membrane.
3. Rinse the membrane twice in 1x TBS for 5 minutes.
4. Cut a vertical strip at one extremity of the membrane. Rinse it with 500 µl of Ponceau S to identify the location of the protein band (see Note 4).
5. Align the colored strip to the full membrane and excise a horizontal strip containing the protein band, as small as possible. Discard the colored strip.
6. Rinse the protein strip in 20 ml of 1xTBS for 10 minutes at room temperature with gentle rocking.
7. Rinse with 20 ml of 1x TBS-0.1% Tween20[®] with 10% milk for 1 hour at room temperature with gentle rocking.
8. Rinse with 6 ml of 1xTBS-tween supplemented with 500 µl of antibody serum, incubate for 3 hours at room temperature on a rocking platform with gentle rocking, or overnight in a cold room with gentle rocking.
9. Wash the strip 5 times for 10 minutes in 1x TBS-0.1% Tween20[®].
10. Wash the strip 2 times for 5 minutes with 10 mM Na-phosphate buffer pH7.2.
11. Place the strip on saran wrap and add 200 ul of 0.1 M Glycine pH 2.2.
12. Recover and place the eluate in a 1.5 ml tube that contains 40 µl 1 M Na₂HPO₄.
13. Repeat step 13 and recuperate the second eluate in the same tube.
14. Rinse the strip with 10 mM Na-Phosphate buffer pH 7.2 for 10 minutes.
15. Store the affinity-purified antibody at 4°C if to be used within a few days or at -20°C for long-term storage.
16. Place the strip on a paper towel and allow to dry for several minutes.
17. The strip can be reused, place in a sterile tube and store at 4°C (see Note 5).

3.2 ChIP-sequencing

All 1.5 ml tubes used are DNA low binding grade.

3.2.1 Bacterial growth culture

1. Inoculate a 10 ml LB pre-culture, containing necessary antibiotics, with an isolated colony on LA of the bacterial strain to be studied and incubate at the appropriate temperature under agitation overnight.
2. Dilute the pre-culture 200-fold in 100 ml of fresh LB medium, and incubate at 37°C under agitation, until OD₆₀₀ ~ 0.6.

3.2.2 Cross-link

1. Aliquot 80 ml of the culture into an Erlenmeyer flask.
2. Add 2.2 ml of 36% formaldehyde solution (final 1%) per sample and incubate at room temperature (23°C) for 30 minutes with gentle agitation (90 rpm).
3. Add 16 ml of 2.5 M glycine (final 0.5 M) to quench the cross-linking reaction and incubate at room temperature for 15 minutes with gentle agitation (90 rpm).
4. Transfer the entire cross-linked samples into appropriate centrifuge tubes.
5. Centrifuge for 10 minutes 6 krpm at 4°C.
6. Discard the supernatant and resuspend the pellet with 96 ml of cold Tris buffered saline pH 7.6.
7. Centrifuge for 10 minutes 6 krpm at 4°C.
8. Discard the supernatant and resuspend the pellet in 1 ml of cold Tris-buffered saline (see Note 6), and aliquot 500 µl into two separate 1.5 ml tubes.
9. Centrifuge for 10 minutes at 6 krpm at 4°C.
10. Carefully discard supernatants, no liquid should remain in the bacterial pellets (see Note 7).

3.2.3 Cell Lysis

1. Resuspend pellet in 500 µl of lysis buffer (see Note 8).
2. Add 50 µl of 10 mg.ml⁻¹ lysozyme and incubate at 37°C for 30 minutes.
3. Add 500 µl of IP buffer and 10 µl of 100 mM PMSF.

3.2.4 Sonication (see Note 9)

1. Aliquot 130 µl of the sample into six 1.5 ml DNA low binding grade tubes (6 tubes per sample).
2. Set aside 100 µl of sample to serve as a non-sonicated control and place on ice.
3. Sonicate samples using the Bioruptor[®] plus (Diagenode) or equivalent apparatus. Sonication conditions: 3 rounds of 28 cycles of 30 seconds on, 30 seconds off (see Note 10).
4. Pool the 6 sonicated samples tubes into 1 tube.
5. Centrifuge the sonicated and the non-sonicated sample for 30 minutes at 13 krpm at 4°C.
6. Recuperate the supernatant into a new 1.5 ml tube.
7. For the sonicated sample, aliquot 500 µl into 1.5 ml tube to serve as the sample to be immunoprecipitated (IP), and aliquot 100 µl into 1.5 ml tube to serve as the input (see Note 11).

8. Test the efficiency of DNA fragmentation by analyzing the DNA size using both bioanalyzer (e.g. Agilent 2100) and agarose gel electrophoresis (see Figure 1).

3.2.5 Immunoprecipitation

1. Combine 500 μ l of IP sample with antibodies (see Note 12), incubate overnight at 4°C with gentle agitation (90 rpm).
1. In a separate tube, add 25 μ l of Protein A magnetic beads to 225 μ l of blocking buffer and incubate overnight at 4°C with gentle agitation (90 rpm).
2. For an IP negative control, prepare a tube containing 500 μ l of sonicated IP sample that will receive IgG antibodies and will go through the same immunoprecipitation process as the IP sample.
3. Recover the blocked Protein A magnetic beads by pelleting using a magnetic rack and discard the blocking buffer.
4. Add the IP sample and antibody mixture to tube containing pre-blocked beads and incubate for 2 hours at 4°C with gentle agitation (90 rpm).
5. Perform consecutive washes of the beads for 10 minutes with gentle agitation at 4°C, precipitating the beads for at least 5 minutes between each wash (see Notes 13, 14, 15).
 - a) 1 wash with 300 μ l 2x IP buffer
 - b) 2 washes with 300 μ l buffer 2
 - c) 1 washes with 300 μ l buffer 3
 - d) 1 wash with 300 μ l TE buffer

3.2.6 IP samples: Elution, RNase and Proteinase K digestion

1. After final wash, resuspend the pellet in 300 μ l of elution buffer (see Note 16 and 17).
2. Add 1 μ l of RNase A at 10 mg.ml⁻¹ and incubate for 1 hour at 37°C.
3. Add 3 μ l of proteinase K at 10 mg.ml⁻¹ (final concentration 0.1 mg.ml⁻¹) and incubate for 2 hours at 37°C.
4. Pellet the magnetic beads using the magnetic rack and recover the supernatant in a new 1.5 ml tube (see Note 18).

3.2.7 Input samples: RNase and Proteinase K digestion

1. On ice, thaw the 100 μ l of input and non-sonicated samples that had been previously frozen (step 7 from 3.2.4; see Note 19).
2. Add 2 μ l of 1 mg.ml⁻¹ RNase A (dilute stock 10-fold) and incubate for 1 hour at 37°C.
3. Add 10 μ l of 1 mg.ml⁻¹ proteinase K (final concentration 0.1 mg.ml⁻¹) and 10 μ l of 10% SDS (final 1%), and incubate for 2 hours at 37°C.

3.2.8 Reverse cross-link:

1. All samples are incubated at 65°C overnight (~16 hours) to reverse the cross-link.

3.2.9 DNA Purification:

1. Add 5 M NaCl to the ~300 μ l of IP and ~120 μ l of input and non-sonicated samples (1 M final concentration).
2. Vortex for 30 seconds.
3. Add one volume of CHCl_3 /Isoamyl Alcohol (24:1) mix.
4. Vortex for 30 seconds.
5. Centrifuge at 13 krpm for 5 min at 4°C.
6. Recupere the aqueous phase and place in a new 1.5 ml tube.
7. Add 1 μ l of Glycogen at 20 $\text{mg}\cdot\text{ml}^{-1}$ and 0.7 volume of cold 100% isopropanol.
8. Incubate at -80°C for 30 minutes.
9. Centrifuge at 13 krpm for 30 min at 4°C and remove the supernatant.
10. Rinse the pellet 2 times with 1 ml of cold 70% ethanol, centrifuging at 13 krpm for 10 min at 4°C between each wash.
11. Resuspend the DNA pellet in 100 μ l of 10 mM Tris-HCl, pH 8.5 (see Note 20 and 21).

3.2.10 DNA library preparation

1. Both immunoprecipitated (ChIP-DNA) and non-immunoprecipitated (input DNA) are used to prepare the DNA library for next generation sequencing. Many new sequencing machines can be used for the sequencing step (Illumina, Ion Torrent...), but the DNA Library preparation always involves the following steps:
 - a. Repair of 3' and 5' ends.
 - b. Preparation of adaptor ligated DNA.
 - c. Size selection.
 - d. Amplification via Polymerase Chain Reaction (PCR) of adaptor ligated DNA.
 - e. Clean up of amplified library.
2. Prepare the libraries according to the sequencing technology used.

3.2.11 Next generation sequencing

1. Prepare templates for sequencing using the DNA libraries. For this step the sequencing workflow differs depending on the sequencing technology used. Briefly,
 - a. For the Ion Torrent technology, the library is clonally amplified by emulsion PCR (emPCR) onto Ion Sphere™ particles. In emulsion PCR methods, the surface of the spheres contains oligonucleotide probes with sequences that are complementary to the adaptors binding the DNA fragments. The spheres are then compartmentalized into water-oil emulsion droplets. Each of the droplets capturing one bead is a PCR microreactor that produces amplified copies of a single DNA template. Then, spheres are inserted into the individual sensor wells by spinning the chip in a centrifuge. Ion Torrent™ Technology directly translates chemically encoded information (A, C, G, T) into digital information (0, 1) on a semiconductor chip to provide the sequences of individual DNA fragments.

- b. For the Illumina technology, the DNA templates are bridge amplified to form clonal clusters inside a flow cell. The library is loaded into a flow cell where fragments are captured on a lawn of surface-bound primers complementary to the library adapters. Each fragment is then amplified into distinct, clonal clusters through bridge amplification. Several million dense clusters of DNA are generated in each channel of the flow cell. Illumina technology utilizes a labeled reversible terminator-based method that detects single bases as they are incorporated into DNA template strands. Successive sequencing cycles provide the sequence of individual DNA fragments.

3.2.12 Software available for ChIP-seq data analysis and publication

1. For peak visualization, convert ChIP-seq data file to BEDGRAPH format. This format allows for its use in Integrated Genome Viewer, which displays the entirety of ChIP-seq data with the possibility to view several datasets simultaneously.
2. Use CLC sequence viewer to determine the exact sequence of peaks on genome with the capability to select and copy sequences. Reference genome of ChIP-seq experiments must be in FASTA format.
3. To analyze specific sections of ChIP-seq data, we have found that Excel provides a user friendly interface to analyze in detail, portions of ChIP-seq data. The limitation of Excel is that it is unable to show the entirety of data, as the file size becomes too large. ChIP-seq data must be in .txt format.
4. R also works as a powerful tool to analyze ChIP-seq data, but requires prior knowledge on the usage of the interface.
5. The Gene Expression Omnibus (GEO) database is a major repository that stores high-throughput functional genomics datasets that are generated using both microarray-based and sequence-based technologies (Clough and Barrett, 2016). Datasets are submitted to GEO to be made freely available to the reviewing process of publication and subsequently for further analysis by the scientific community. In addition to serving as a public archive for these data, GEO has a suite of tools that allow users to identify, analyze and visualize data relevant to their specific interests.

4 Notes

1. Lysozyme must be stored at -20°C, therefore the addition of lysozyme to the lysis buffer is done at the time of the experiment and is added to each sample individually.
2. PMSF must be stored at -20°C, therefore the addition of PMSF to the 2x IP buffer is done at the time of the experiment and is added to each sample individually.
3. A high level (~100 µg) of the protein of interest is required to purify a large amount of specific, polyclonal antibodies from the serum. Proteins can either be produced as a his-tagged recombinant, purified in a one-step procedure using Nickel-affinity chromatography purification, or through a native purification

procedure. In this latter case, enriched side fractions which are usually discarded could be used to avoid wasting the protein of interest. Crude extracts are not suitable for use in this antibody purification procedure.

4. When rinsing the strip with Ponceau S, protein band may be difficult to distinguish. To eliminate the staining of Ponceau S on surrounding areas, rinse with sterile water.
5. Protein strips can be stored at 4°C and re-used for antibody purification up to 7 times. To re-use a protein strip, start antibody purification protocol at step 7.
6. Each sample is separated into two tubes. Tube 1 serves as the experimental sample, and tube 2 serves as a safety sample.
7. This is a freeze point. Flash freezing of pellets must be done with liquid nitrogen and stored at -80°C until needed.
8. Some proteins may be sensitive to proteolysis during the lysis step. In this case, we recommend the addition of Protease Inhibitor Cocktail Tablets (cOmplete™, Mini, EDTA-free Protease Inhibitor Cocktail (Roche)) in the lysis buffer.
9. We recommend using the Bioruptor® Plus, sonication conditions described are adapted to this instrument. Importantly, if using another instrument it is imperative to properly adapt the sonication conditions. Set the cooling device of the sonicator Bioruptor® Plus at 4°C 30 minutes before use.
10. During sonication, the sonicator water bath must be maintained at 4°C, as it is critical to keep the samples at a low temperature. In between sonication rounds let the sonicator cool down for 10 minutes, as Bioruptor sonicator will shut off in the middle of sonicating if overheated.
11. This is a freeze point. Samples can be stored at -20°C or -80°C. Input and non-sonicated samples will rejoin the protocol at the DNA purification step.
12. Antibody concentration used for immunoprecipitation varies based on protein of interest. It is recommended to perform immunoprecipitations using purified protein and several concentrations of antibody to determine the amount needed for ChIP (see Figure 2).
13. When re-suspending magnetic beads between washes, avoid creating bubbles as this will result in inefficient bead pelleting and bead loss.
14. Filter or low retention tips are not required, but can reduce sample loss during re-suspension steps.
15. Bead pelleting can be performed at room temperature.
16. From this point, it is possible to finish the procedure with the IPure kit v2 (Diagenode) to complete the elution, reverse cross-link and purify DNA.
17. To control immunoprecipitation efficiency, recover 5 µl of IP elution sample and add 20 µl of elution buffer. Heat at 65°C for 15 minutes then place sample on magnetic rack, and recuperate cleared sample. Mix with sample buffer and use 10 µl to analyze through Western blotting.
18. Reverse cross-link is performed in the absence of the magnetic beads.
19. As a control of the protein baseline to test for immunoprecipitation efficiency, recuperate 5 µl of input sample and add 20 µl of 2x IP buffer. Heat at 65°C for 15

- minutes then place sample on magnetic rack, and recuperate cleared sample. Mix with sample buffer and use 10 µl to analyze through Western blotting.
20. To determine the sonication profile of the input and non-sonicated samples, purified DNA can be run on a 0.5x TBE, 1.8% agarose gel to observe a DNA smear indicating the average fragment size per sample. In addition, the use of a bioanalyzer is highly recommended for automated sizing with a more precise and digital format of DNA sample fragment sizes.
21. For precise quantification of DNA quantity, we strongly recommend the use of the Qubit[®] 3.0 Fluorometer for Input and IP samples prior to library preparation.

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Figures legends

Figure 1: DNA profiles of sonicated formaldehyde-treated cells prior (A) and after (B) library preparation. (A) Sonicated and purified DNA fragments from the ChIP procedure prior to library preparation were analyzed by 1.8% agarose gel electrophoresis showing fragment sizes between 100-400bps. Molecular weight (base pair) markers are indicated on the right. The brightness and contrast of the image has been modified by linear scaling for amplification of the signal. (B) Following library preparation, the size of DNA fragments, containing an 80 bps barcode and sequencing adaptor, was controlled by Agilent 2100 Bioanalyzer using Agilent High sensitivity DNA kit. Low- and high-DNA markers, noted 35-bp and 10380-bp, respectively, were added in each sample prior to electrophoresis. The average size of the sample after adaptor subtraction was ~170bps.

Figure 2: Optimization step and control of immunoprecipitation. Western blot analysis of ParB_{Vc1} proteins from crude cell extracts of *Vibrio cholerae* (lanes 1-2) or purified ParB_{Vc1} protein (0.38 µg; lanes 3-7) after SDS-PAGE (4-16%). ParB_{Vc1} proteins are from the ParABS system present on chromosome 1 of *V. cholerae*. Protein samples were subjected (+) or not (-) to immunoprecipitation (IP) using the indicated amount (µg) of purified anti-ParB_{Vc1} antibody. The optimal antibody concentration for immunoprecipitation was determined with increasing amount of antibody. The total amount of ParB_{Vc1} contained in the sample was recovered using 7 µg of anti-ParB_{Vc1} antibody (compare lanes 5 and 6). Molecular weight (KDa) markers are indicated on the left.