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Genome-wide approaches to determining nucleosome occupancy in metazoans using MNase-Seq

Kairong Cui and Keji Zhao

Laboratory of Molecular Immunology, National Heart, Lung and Blood Institute, NIH, 9000 Rockville Pike, Bethesda, MD 20892

1. Introduction

Nucleosomes consisting of approximately 146 base pairs (bp) of DNA wrapped around a histone octamer are the fundamental structural units of chromatin in metazoans (1, 2). The translational positioning of nucleosomes along DNA is implicated in profoundly influencing gene expression (3-6). Thus, defining the nucleosome positioning and occupancy is critical to understand the mechanisms of regulation of transcription by chromatin.

Nucleosome structure is resistant to micrococcal nuclease (MNase) digestion, leaving a footprint of about 150bp that reflects the position of a nucleosome (7). Therefore, determining the boundaries of these footprints can indicate the positions of nucleosomes in the genome. Since the genomic sequences of most model organisms are already available, sequencing a short tag from DNA at each end of the nucleosome is sufficient to determine its position in the genome. Thus the next generation sequencing techniques are perfectly suited for this purpose (8).

We have generated genome-wide maps of nucleosome positions in both resting and activated human CD4+ T cells by direct sequencing of nucleosome ends using the Illumina Genome Analyzer Platform (MNase-Seq) (9). As the next generation sequencing techniques improve, the capacity and cost of sequencing become lower. For example, one sequencing run on the Illumina Genome Analyzer II can produce 100 to 200 millions of sequencing reads, which is sufficient to reach a 10x coverage for all nucleosomes in the human genome.

We describe two different methods to prepare nucleosome templates used for sequencing. One is digestion of native chromatin and the other is digestion of formaldehyde-crosslinked chromatin by MNase. The native nucleosome protocol works well to reveal stable nucleosome structure and avoid crosslinking of non-histone proteins; the crosslinking protocol may stabilize “unstable” nucleosomes but may also stabilize non-nucleosome structure that is resistant to MNase digestion.

2. Materials

2.1 Materials

1. 1×PBS: 137mM NaCl, 2.7mM KCl, 10mM Na₂HPO₄, 2mM KH₂PO₄, without calcium & magnesium, pH7.4
2. 1×PBS + 0.5% Triton X-100 solution

3. Stop Buffer: 20mM EDTA, 20mM EGTA, 0.4% SDS and 0.5mg/ml Proteinase K.
4. Phenol/chloroform
5. 70% ethanol
6. 1×TE Buffer: 10mM Tris, 1mM EDTA, pH7.4
7. 10× End Repair Buffer: 330 mM Tris-acetate, pH7.8, 660 mM potassium acetate, 100 mM magnesium acetate, 5 mM DTT
8. 2.5mM dNTPs
9. 10mM ATP
10. 10× Tag Buffer: 200mM Tris-HCl, 100mM (NH₄)₂SO₄, 100mM KCl, 20mM MgSO₄, 1% Triton X-100, pH8.8
11. 1mM dATP
12. 10× T4 DNA Ligase Buffer: 500mM Tris-HCl, 100mM MgCl₂, 100mM DTT, 10mM ATP, pH7.5
13. E-Gel iBase Power System (Invitrogen, Cat#: G6400)
14. E-Gel Safe Imager Real-time Transilluminator (Invitrogen, Cat#: G6500)
15. E-Gel EX 2% agarose (Invitrogen, Cat#: G4020-02)
16. The gel knife (Invitrogen, Cat. no. EI9010)
17. Genome Analyzer IIX (Illumina)
18. Lysis Buffer: 10mM Tris, pH7.5, 10mM NaCl, 3mM MgCl₂, 0.5% NP-40, 0.15mM spermine, 0.5mM spermidine
19. MNase digestion buffer: 10mM Tris-HCl pH7.4, 15mM NaCl, 60mM KCl, 0.15mM spermine, 0.5mM spermidine

2.2 Reagents

1. 5M NaCl
2. Sodium dodecyl sulphate (SDS, 10% [w/w] solution).
3. QIAquick PCR Purification Kit (Qiagen, Cat#: 28104)
4. QIAquick Gel Extraction Kit (Qiagen, Cat#: 28704)
5. Epicentre DNA END-Repair kit (Epicentre Biotechnologies, Tel 800-284-8474, Cat# ER0720)
6. 2X Phusion HF Master Mix (NE Biolabs, Cat#: F-531)
7. Formaldehyde Solution for Molecular biology (Sigma, Cat#: F8775)
8. Glycogen (stock solution) (Roche, Cat#: 10901393001)
9. 100mM dATP Solution PCR Grade (Invitrogen, Cat#: 10216-018)
10. PE Adapter Oligo Mix, PCR Primer PE 1.0 and 2.0 (Illumina)
11. Proteinase K, recombinant PCR Grade (Roche, Cat#: 03115828001)

2.3 Enzymes

1. Nuclease micrococcal (Sigma, Cat#: N3755-500UN)

2. Taq DNA Polymerase with ThermoPol Buffer (NE Biolabs, Cat#: M0267S)
3. T4 DNA Ligase (NE Biolabs, Cat#: M0202S)

3. Methods

3.1. Preparation of Nucleosome DNA Templates

1. Native nucleosomes: Harvest cells (10 - 20 million). Wash the cells 2X with ice cold 1XPBS (5 – 10 ml). Spin down the cells at 2000rpm (350×g) for 5 min at 4°C. Lyse the cells in 1ml of ice-cold lysis buffer, incubate for 5min on ice. Crosslinked nucleosomes: Crosslink 10 – 20 million cells by adding formaldehyde to 1% and incubating for 10min. Wash 2X with 10 – 20ml 1XPBS. Spin down the cells at 2000rpm (350×g) for 5 min at 4°C. Lyse cells in 1 ml 1XPBS+0.5% Triton X-100 for 3 min on ice.
2. Pellet the nuclei by spinning at 2000rpm (350×g) for 5min at 4°C. Wash the nuclei with 1 ml MNase digestion buffer, spin down at 2000rpm for 5 min at 4°C and resuspend the pellet in 800μl of the same buffer (at a concentration of 10-20 million nuclei per ml). Adjust the final Ca²⁺ concentration to 1mM with 1M CaCl₂.
3. Aliquot the nuclei suspension into eight tubes (100μl each), to which 0, 0.01, 0.03, 0.05, 0.1, 0.3, 0.5, and 1 units of MNase are added, respectively. Incubate the reaction mixture at 37°C for 5min (**Note 1**), then stop the reaction by adding 150μl of Stop Buffer.
4. Incubate the mixture at 65°C for 6 hrs or overnight.
5. Extract the mixture using an equal volume of phenol/chloroform.
6. Add 20μg of Glycogen from a stock solution to the aqueous phase, precipitate the DNA with 750μl of ethanol and 75μl of 3M NaAc, pH5.3, wash the DNA pellet once with 750μl of ice-cold 70% ethanol, and dissolve the pellet in 30μl of 1X TE buffer.
7. Load 10 μl DNA from each reaction onto a 2% E-Gel EX(Invitrogen). Run 10min at EX Gel condition (**Note 2**).
8. Using E-Gel Safe Imager Real-time Transilluminator (Invitrogen), identify and excise the mononucleosome bands (**Note 3**).
9. Purify the DNA using Qiagen gel extraction kit (**Note 4**), and elute the DNA in 30 μl EB from Qiagen gel extraction kit (**Note 5**).

¹Incubate the reaction for 8-10min at 37°C for formaldehydecrosslinked nuclei.

²The E-Gel® agarose gel electrophoresis system is a complete bufferless system for agarose gel electrophoresis of DNA samples. It provides fast, safe, consistent, high-resolution electrophoresis and minimizes sample contamination. E-Gel® EX pre-cast agarose gels are generally used gels which contain a proprietary fluorescent nucleic acid stain with high sensitivity, allowing: (1)Detection of down to 1 ng/band of DNA, (2)Compatibility with blue light transillumination to dramatically reduce DNA damage, (3)Easy opening of cassette with gel knife. If this system is not available, traditional gel purification methods can be used, but cross-contamination may result.

³Unlike UV-transilluminators, the E-Gel® Safe Imager™ Real-time Transilluminator does not produce UV light and does not require UV-protective equipment during use. Blue light transillumination also results in dramatically increased cloning efficiencies compared to UV transillumination.

⁴Dissolve gel slices at room temperature with frequent mixing, but not at elevated temperature. This helps to preserve AT-rich DNA that can be easily denatured at higher temperature and could then be lost at the column binding step.

⁵Warm up EB at 65°C. Ensure that the EB is dispensed directly onto the QIAquick membrane for complete elution of bound DNA.

3.2. Preparation of DNA for sequencing

- 1 Repair DNA ends using the Epicentre DNA END-Repair kit This step generates blunt-ended DNA. Mix ingredients as follows:

- 1-34 μl DNA (0.1 to 0.5 μg)
- 5 μl 10 $\times$ End repair buffer
- 5 μl 2.5 mM each dNTPs
- 5 μl 10 mM ATP
- x μl H₂O to adjust the reaction volume to 49 μl
- 1 μl End-Repair Enzyme mix (T4 DNA Pol + T4 PNK)

Incubate the reaction mixture at room temperature for 45 min. Purify the DNA using QIAquick PCR Purification Kit. Elute DNA in 30 μl of EB (Note 5)

- 2 Add “A” to 3’ ends of DNA templates. Mix ingredients as follows:

- 30 μl DNA from above
- 2 μl H₂O
- 5 μl 10 $\times$ Taq buffer
- 10 μl 1 mM dATP
- 3 μl 5 U/ ml Taq DNA polymerase

Incubate the reaction mixture at 70°C for 30 min. Purify the DNA as before. Elute DNA in 20 μl of EB (**Note 5**).

- 3 Adaptor ligation. Mix ingredients as follows:

- 20 μl DNA (300ng)
- 3.9 μl H₂O
- 3 μl 10 $\times$ T4 DNA ligase buffer
- 0.1 μl Adaptor oligo mix (mixture of two adaptors from Illumina)
- 3 μl T4 DNA ligase (400 units/ml)

Incubate the reaction mixture at room temperature for 30 min.

- 4 Size selection using 2% E-Gel

Load 30 μl adaptor ligated DNA onto a 2% E-Gel EX (Invitrogen). Run 10min at EX Gel condition. Using E-Gel Safe Imager Real-time Transilluminator (Invitrogen), cut the gel around 200-400 bp region (DNA may not be visible). Extract the DNA using QIAquick Gel Extraction Kit(Note 4). Elute in ~23 μl EB (**Note 5, Note 6**).

- 5 Amplify the DNA using Illumina PCR primers and Phusion HF Master Mix. Mix ingredients as follows:

- 23 μl of DNA
- 25 μl of master mix

⁶NEVER run PCR-amplified samples of Step 7 with the linker-ligated products of Step 5 together on the same gel because the latter can be contaminated.

1 µl of PCR primer 1 (2× diluted, Illumina)

1 µl of PCR primer 2 (2× diluted, Illumina)

Total volume: 50 µl

Denature at 98°C for 30 sec.

98°C, 10"; 65°C, 30"; 72°C, 30".

Try 18 cycles first, check 2.5 µl of product on 1.8% gel. If the band is not clearly visible, do 3 more cycles. Check again.

6 Purify the amplified products on 2.5% agarose gel.

Excise the band near 220 bp and purify the DNA using Qiagen gel extraction kit. Measure the DNA concentration using Qubit fluorometer (**Note 4**).

3.3. Sequencing and data analysis

1. Purified DNA is used directly for cluster generation and sequencing analysis on Illumina IIX Genome Analyzer following manufacturer protocols.
2. Data Analysis: Sequenced reads of mostly 25 bp are obtained using the Illumina Analysis Pipeline. All reads are mapped to the human genome (hg18) or other reference genomes and all uniquely matching reads are retained. Nucleosome profiles are obtained by applying a scoring function to the sequenced reads. A sliding window of 10 bp is applied across all chromosomes and at each window all reads mapping to the sense strand 80 bp upstream of the window and reads mapping to the antisense strand 80 bp downstream of the window contribute equally to the score of the window.

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