ChIP-exonuclease in mammalian cells

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Introduction

ChIP-exonuclease (ChIP-exo) is a high resolution method of genome-wide mapping of DNA-associated proteins that outperforms ChIP-seq by all parameters. We adapted this method created by Frank Pugh and Ho Sung Rhee from SOLiD to the Illumina sequencing platform (Rhee and Pugh, 2011; Sérandour et al, 2014) (*cf* figure 1). We provide here a ChIP-exo protocol, optimized on 60 million cells (4 dishes of 15 cm diameter at confluence). Lower cell number has not been tested.

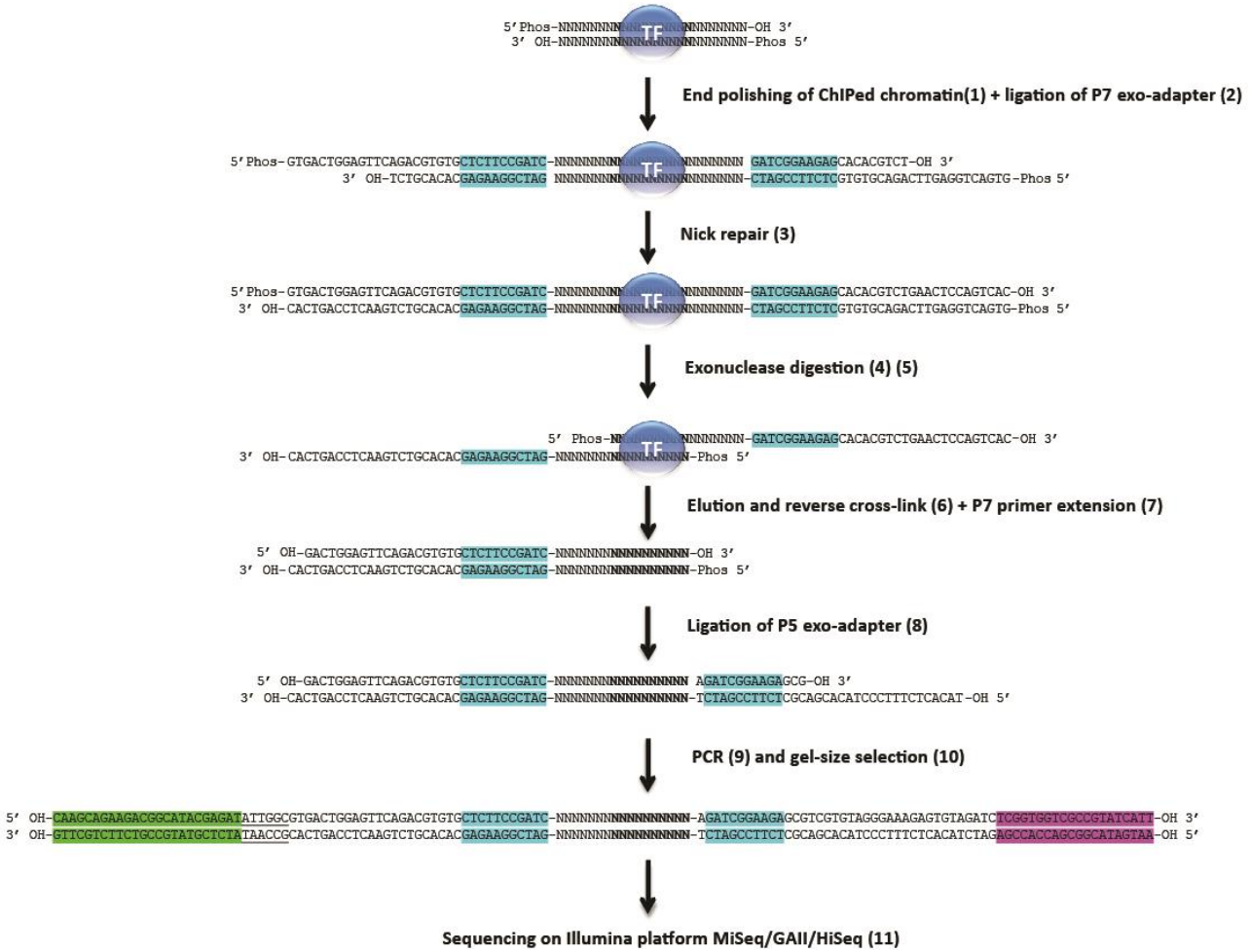

Figure 1: Illustration of the Illumina-adapted ChIP-exo strategy. *To carry out ChIP-exo, the P5 adapter is ligated upstream and downstream of the exonuclease digestion-protected region. The ChIP-exo library is sequenced with single-end reads from the P5 adapter. The reads are mapped on the reference genome. The overlap between the reads mapped on the top and the bottom strands is considered as the exonuclease digestion-protected region. The index sequence is underlined. The P7 flow cell capture sequence is in green. The P5 flow cell capture sequence is in purple. The P5/P7 complementary sequence is in blue.*

Procedure

Cross-linking cells

1. Remove medium from cells and add 1 % formaldehyde PBS 1X warmed to 37^{0}C (15 mL to 15 cm dish).
2. Leave for not more than 10 minutes.
3. Quench with 1 M glycine (filtered): 1.5 mL to 15 mL formaldehyde in dish.

4. Wash cells 2 times with 10 mL of ice cold PBS 1X.
5. Put dishes on a tray of ice and remove the excess of PBS 1X.
6. Add 500 uL PBS 1X + freshly added Protease Inhibitor 1× (cOmplete Protease Inhibitor Cocktail, Roche, ref. 11697498001; 1 tablets PI in 500 uL PBS = 100x) and scrape. Transfer the cells to a 1.5 mL tube.
7. Centrifuge at 2000 g for 3 minutes at 4^0C.
8. Remove the supernatant.
9. Freeze the cell pellet in dry ice and store at -80^0C (up to 2-4 months).

Beads preparation

Choose magnetic beads protein A or G, depending on your antibody of interest (Note 1).

10. Vortex the beads well and add 50 uL of beads to a 2 mL tube, and put in a magnetic rack, on ice.
11. Remove the supernatant and wash 3 times in 1 mL PBS 1X + 5 mg/mL BSA (filtered). Remove the magnet to wash and ensure that the beads are well resuspended. When removing the supernatant, ensure it is clear.
12. Resuspend in 350 uL PBS 1X + 5 mg/mL BSA (filtered) and add 10 ug of antibody.
13. Vortex briefly and rotate at 4^0C overnight or for a least 4 hours.

Nuclei preparation and sonication

14. Resuspend the cell pellet from one dish in 1 mL buffer Lysis Buffer 1 + freshly added PI 1X and rotate for at least 10 minutes at 4^0C.
15. Spin at 2000 g for 3 minutes at 4^0C.
16. Remove the supernatant and resuspend the nuclei pellet in 1 mL Lysis Buffer 2 + freshly added PI 1X and rotate for at least 5 minutes at 4^0C.
17. Spin at 2000 g for 3 minutes at 4^0C.
18. Remove the supernatant. Resuspend the nuclei pellet in 300 uL of Lysis Buffer 3 + freshly added PI 1X.
19. Transfer the lysate to a 1.5 mL TPX sonication tube (Diagenode, ref. C30010010). Sonicate on Bioruptor Standard or Bioruptor Plus (2 times 15 minutes, 30 seconds on, 30 seconds off, High Power setting) (Note 2). Add 1-2 cm of ice in the sonication bath water before each 15 minutes sonication to avoid overheating, or use a cooling system.
20. Add 30 uL of Lysis Buffer 3 containing 10 % Triton X-100 and vortex (final lysate concentration: 1 % Triton X-100). Transfer to a new 1.5 mL tube.
21. Centrifuge at full speed for 10 minutes at 4^0C and transfer the supernatant to a new 1.5 mL tube.

22. Remove 30 uL for the input and keep at -80^{0}C (Note 3).

Chromatin ImmunoPrecipitation

23. Wash the beads 3 times in 1 mL PBS 1X + 5 mg/mL BSA (filtered) to remove unbound antibody.

24. Add the lysate from 4 dishes (about 60 million cells) to the beads in a 2 mL tube.

25. Rotate overnight at 4^{0}C.

26. Wash the beads 6 times in RIPA Buffer. At the last RIPA wash, transfer the beads to a new 2 mL tube.

27. Wash the beads 2 times in 10 mM Tris HCl pH 8. Keep the tube in the magnet rack during the removal of the second Tris wash, to avoid the loss of beads. Then spin briefly the tube, put in the magnet rack and remove the residual Tris buffer. Proceed directly to the step 28. Do not let the beads dry out (see comment 1).

ChIP-exonuclease steps

The beads then undergo 5 successive reactions in a 2 mL tube agitated at 900 rpm in a thermomixer as followed:

28. **End polishing** at 30°C for 30 min in 100 uL 1X NEBuffer 2 (NEB, ref. B7002S) containing 1 mM ATP, 100 uM dNTP, 15 U T4 DNA polymerase (NEB, ref. M0203), 5 U Klenow DNA polymerase (NEB, ref. M0210), 50 U T4 PolyNucleotide Kinase (NEB, ref. M0201).

29. 2 washes of 1 mL RIPA and 2 washes of 1 mL 10 mM Tris HCl pH 8 (Note 4).

30. **Ligation of the P7 exo-adapter** at 25°C for 1 hour in 100 uL 1X NEBuffer 2 containing 1 mM ATP, 150 pmol P7 exo-adapter, 2000 U T4 DNA ligase (NEB, ref. M0202).

31. 2 washes of 1 mL RIPA and 2 washes of 1 mL 10 mM Tris HCl pH 8.

32. **Nick repair** at 30°C for 20 min in 100 uL 1X Nick Repair Buffer containing 150 uM dNTP and 15 U phi29 DNA polymerase (NEB, ref. M0269).

33. 2 washes of 1 mL RIPA and 2 washes of 1 mL 10 mM Tris HCl pH 8.

34. **Lambda exonuclease digestion** at 37°C for 30 min in 100 uL 1X NEB Lambda exonuclease Buffer containing 10 U Lambda exonuclease (NEB, ref. M0262).

35. 2 washes of 1 mL RIPA and 2 washes of 1 mL 10 mM Tris HCl pH 8.

36. **RecJf exonuclease digestion** at 37°C for 30 min in 100 uL NEBuffer 2 containing 30 U RecJf exonuclease (NEB, ref. M0264).

37. 2 washes of 1 mL RIPA and 2 washes of 1 mL 10 mM Tris HCl pH 8.

38. **Elution and reverse cross-linking:** the beads are incubated with 100 ug of Proteinase K in 200 uL of Elution Buffer overnight at 65°C. Vortex well. Remove the 200 uL of supernatant from the beads, transfer to a new tube and dilute in 200 uL TE. The DNA is extracted using 400 uL of Phenol:Chloroform:Isoamyl Alcohol (Sigma, ref. P2069) then ethanol precipitated using glycogen (Note 5). The resulting DNA pellet is dissolved in 20 uL water. The DNA can be stored at this step at −20°C for few days.

Steps 39, 41 and 43 are done using PCR tubes and a thermocycler.

39. **P7 primer extension:** the 20 uL of DNA is denaturated for 5 min at 95°C, then mixed with 5 pmol of the P7 primer and incubated in a final volume of 48 uL 1X NEB Phi29 reaction buffer (NEB, ref. M0269) for 5 minutes at 65°C and 2 minutes at 30°C. After the addition of 10 U Phi29 DNA polymerase and 200 uM dNTP, the mix is incubated 20 minutes at 30°C and then 10 minutes at 65°C.

40. The DNA is purified using AMPure beads (1.8 volume):
    a. Vortex AMPure XP beads (Beckman Coulter, ref. A63880), then add 90 uL to 50 uL sample.
    b. Vortex well.
    c. Incubate at room temperature for 15 minutes.
    d. Place on a magnetic stand at room temperature until liquid appears clear.
    e. Remove carefully the liquid from beads, some liquid may remain in tube.
    f. With the tube on magnetic stand, add 200 uL freshly prepared 80% EtOH to sample.
    g. Incubate for at least 30 seconds then remove all the supernatant.
    h. Repeat the 80% EtOH wash.
    i. Remove tube from magnetic stand and leave at room temperature for 15 mins to dry (Note 6).
    j. Resuspend dried beads in 22 uL Resuspension Buffer and vortex well.
    k. Incubate at room temperature for 2 minutes.
    l. Place tube on magnetic stand until liquid appears clear and transfer 20 uL eluate to a fresh tube.
    m. The DNA can be stored at this step at −20°C.

41. **Ligation of the P5 exo-adapter:** the 20 uL of DNA is mixed with 15 pmol of the P5 exo-adapter, 2,000 U T4 DNA ligase and incubated in 50 uL 1X NEB T4 DNA ligase buffer (NEB, ref. M0202) for 1 hour at 25°C and then 10 min at 65°C.

42. The DNA is purified using AMPure beads (1.8 volume) and eluted in 20 uL of Resuspension Buffer. The DNA can be stored at this step at −20°C.

43. **PCR amplification:** the DNA sample is amplified using 0.5 uM of the universal reverse PCR primer and the forward PCR primer containing the index sequence of choice in 50 uL 1X NEBNext High-Fidelity PCR Master Mix (NEB, ref. M0541): 98°C for 30 secs, then 18 cycles of 98°C for 10 secs / 65°C for 30 secs / 72°C for 30 secs, then 72°C for 5 min. The standard number of PCR cycles is 18, depending on the ChIP efficiency.

44. The PCR product is purified using AMPure beads (1.8 volume) and eluted in 20 uL of Resuspension Buffer. The DNA can be stored at this step at −20°C.

45. **Gel-size selection:** 200 to 350 bp PCR product is purified from a 2% agarose gel using MinElute Gel Extraction Kit (Qiagen, ref. 28604) and eluted in 20 uL of 10 mM Tris HCl pH 8.

46. **Illumina sequencing:** the library is quantified using the KAPA library quantification kit for Illumina sequencing platforms (KAPA Biosystems, ref. KK4824) and single-end sequenced on a MiSeq, GAIIx or HiSeq following the manufacturer's protocol.

Authors Notes

Note 1: the antibodies should be chosen carefully, based on the literature, the providers' validation datasheets and personal experience. At the first instance, it could be wise to test different antibodies, if possible validated in ChIP(-seq) or IHC, polyclonal and made using a long immunogen. A ChIP-exo using a non-immune serum could be done as a negative control.

Note 2: the optimal conditions of sonication depend on cells of interest. The majority of chromatin fragments should be between 200 and 500 bp long. Fragments sizes can be checked after reverse-crosslinking, DNA purification and gel migration.

Note 3: after reverse-crosslinking and DNA purification, the input-seq libraries should be prepared using a standard Illumina library preparation kit like the TruSeq ChIP Sample Prep Kit (Illumina, ref. IP-202-1012). Input-seq libraries are usually prepared using 50-100 ng of input DNA. Library preparation on higher DNA quantity would fail.

Note 4: beads can be less magnetic in the Tris HCl pH 8. Keep the tube in the magnet rack during the removal of the second Tris wash, to avoid the loss of beads. Then spin briefly the tube, put in the magnet rack and remove the residual Tris buffer. Tris washes should be done carefully to eliminate any trace of detergent that can be detrimental for the subsequent enzymatic reaction. Do not let the beads dry out.

Note 5: a DNA purification using AMPure beads (1.8 volume) can be done instead of the phenol-chloroform extraction.

Note 6: any remaining trace of EtOH would inhibit the next enzymatic reaction.

Lists of materials and reagents

Reagents

-Annealing Buffer: 10 mM Tris pH 8, 50 mM NaCl, 1 mM EDTA
-Lysis Buffer 1: 50 mM Hepes KOH, pH 7.5, 140 mM NaCl, 1 mM EDTA, 10 % glycerol, 0.5 % NP-40 or Igepal CA-630, 0.25% Triton X-100
-Lysis Buffer 2: 10mM Tris HCl pH 8, 200mM NaCl, 1mM EDTA, 0.5M EGTA
-Lysis Buffer 3: 10mM Tris HCl pH 8, 100 mMNaCl, 1 mM EDTA, 0.5 mM EGTA, 0.1% Na-Deoxycholate, 0.5% N-lauroylsarcosine
-Nick Repair Buffer: 50 mM Tris HCl pH 7.5, 10 mM MgCl2, 10 mM (NH4)2SO4, 1 mM DTT
-PBS 1X (Fisher Scientific, ref. BP399)
-RIPA Buffer: 50 mM HEPES pH 7.6, 1 mM EDTA, 0.7 % Na deoxycholate, 1 % NP-40, 0.5 M LiCL
-Elution Buffer: 50 mM Tris HCl pH 8; 10 mM EDTA; 1% SDS
-TE: 10 mM Tris, 1 mM EDTA, HCl pH 7.4
-Resuspension Buffer: Tris-Acetate 10 mM pH 8

Materials

-1.5 mL / 2 mL tube rotator in cold room
-Agencourt AMPure XP beads (Beckman Coulter, ref. A63881)
-Bioruptor (Diagenode, ref. B01010001) or equivalent
-Dynabeads Protein A/G (Life Technologies, ref. 10002D/10004D) or equivalent
-Thermocycler
-Thermomixer (Eppendorf, ref. 5384 000.012) or equivalent
-TPX sonication tube (Diagenode, ref. C30010010)
-Tube Magnetic rack

Oligonucleotides

After synthesis, the oligonucleotides are purified by HPLC. The P7 exo-adapter and the P5 exo-adapter were obtained in mixing the couple of complement oligonucleotides in 4 volumes of Annealing Buffer and annealed by heating 5 min at 95°C then let cool slowly to room temperature. The oligonucleotides designed for ChIP-exo are adapted from the oligonucleotide sequences

P7 exo-adapter reverse	5’ Phos-GTGACTGGAGTTCAGACGTGTGCTCTTCCGATC-OH 3’
P7 exo-adapter forward	5’ OH-GATCGGAAGAGCACACGTCT-OH 3’
P5 exo-adapter reverse	5’ OH-AGATCGGAAGAGCG-OH 3’
P5 exo-adapter forward	5’ OH-TACACTCTTTCCCTACACGACGCTCTTCCGATCT-OH 3’
P7 primer	5’ OH-GACTGGAGTTCAGACGTGTGCT-OH 3’
PCR Primer universal reverse	5’ OH-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACG*A-OH 3’
PCR Primer index 2 forward	5’ OH-CAAGCAGAAGACGGCATACGAGATACATCGGTGACTGGAGTTCAGACGTGTGC*T-OH 3’

PCR Primer index 4 forward	5' OH-CAAGCAGAAGACGGCATACGAGATTGGTCAGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 5 forward	5' OH-CAAGCAGAAGACGGCATACGAGATCACTGTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 6 forward	5' OH-CAAGCAGAAGACGGCATACGAGATATTGGCGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 7 forward	5' OH-CAAGCAGAAGACGGCATACGAGATGATCTGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 12 forward	5' OH-CAAGCAGAAGACGGCATACGAGATTACAAGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 13 forward	5' OH-CAAGCAGAAGACGGCATACGAGATTTGACTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 14 forward	5' OH-CAAGCAGAAGACGGCATACGAGATGGAACTGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 15 forward	5' OH-CAAGCAGAAGACGGCATACGAGATTGACATGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 16 forward	5' OH-CAAGCAGAAGACGGCATACGAGATGGACGGGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 18 forward	5' OH-CAAGCAGAAGACGGCATACGAGATGCGGACGTGACTGGAGTTCAGACGTGTGC*T-OH 3'
PCR Primer index 19 forward	5' OH-CAAGCAGAAGACGGCATACGAGATTTTCACGTGACTGGAGTTCAGACGTGTGC*T-OH 3'

5’ Phos = phosphorylated 5’ end

* = Phosphorothioates S-linkage

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References

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Reviewer comments:

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(1) remove a small fraction (5-10%) of the ChIP material. With the input chromatin, process and examine by qPCR to verify a successful ChIP.