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An integrated and semi-automated micro-scaled approach to profile cis-regulatory elements by histone modification ChIP-Seq for large-scale epigenetic studies.

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Abstract

Chromatin immunoprecipitation followed by sequencing (ChIP-Seq) is the preferred approach to map histone modifications and identify cis-regulatory DNA elements throughout the genome. Multiple methods have been described to increase the efficiency of library preparation and to reduce hands-on time as well as costs. This review describes detailed steps to perform cell fixation, chromatin shearing, immunoprecipitation and library preparation for sequencing a batch of 48–96 samples with small cell numbers. The protocol implements a semi-automated platform to reduce technical variability and improve signal-to-noise ratio as well as reduce hands-on time, thus allowing large-scale epigenetic studies of clinical samples with limited cell numbers.

Keywords

ChIP-Seq; H3K27ac; IP-Star; tagmentation

1 Introduction

Genome-wide profiling of histone modifications in DNA regions by chromatin immunoprecipitation followed by sequencing (ChIP-Seq) represents the preferred method to identify *cis*-regulatory DNA elements (active enhancers, promoters, silencers, insulators) that are playing important roles in gene regulation and cellular development [1–4]. However, large-scale ChIP-Seq experiments in clinical samples, besides from being technically challenging, are often limited by the quantity of cells or tissue of interest [5]. In addition, the heterogeneous cellular composition of clinical samples can confound analyses and mask significant changes in gene regulation. To overcome these hurdles, a number of micro-scaling techniques have been reported [5–7]. We developed a sensitive and robust micro-

scaled ChIP-Seq assay to profile histone modification marks for as little as 10,000 cells [8,9]. We applied the method to a translational research project in which we profiled H3K4me2 marks in three types of circulating CD4⁺ T cells (naive, T_H1 and T_H2 memory T cells) directly isolated from blood samples of a cohort of healthy individuals and asthmatic patients. Looking at epigenetic changes between cell types and disease groups, we have identified a number of new active and poised promoters and enhancers, new potential transcription factor binding sites, and functional SNPs that could play a role in T cell development and asthma pathogenesis [9,10]. In murine cells, we used H3K27ac-ChIP-Seq assay along with RNA-Seq to characterize the different subtypes of developing NKT cells present in the thymus [8]. More recently, a new method called “ChiPmentation” has been described by *Schmidl et al* [11]. It combines chromatin immunoprecipitation with a single-step integration of sequencing-adaptors using Tn5 transposase technology, increasing library preparation efficiency and reducing hands-on time.

In this review, we detail an integrated, high-sensitive and semi-automated approach to perform every step of the procedure (cell fixation, chromatin shearing, immunoprecipitation and library preparation) for up to 48 samples with very low cell numbers (10,000 to 100,000 cells). This approach reduces technical variability and hands-on time, and thus is suited for large-scale epigenetic studies. Fig. 1a displays an overview of the entire procedure. It describes all critical steps, quality control (QC) steps, and an estimation of hands-on time. For clarity purposes, this chapter was subdivided into six sub-sections corresponding to cells fixation; chromatin shearing; chromatin immunoprecipitation; library preparation by fragmentation of DNA fragments; amplification with barcoded adaptors; library purification and size selection as well as the description of DNA quantification using a PicoGreen assay. For each section, materials & methods is described.

2 Materials

2.1 Cell fixation (see Note 1)

1. 37% formaldehyde
2. 5 M NaCl*
3. 0.5 M EDTA pH 8.0*
4. 0.5 M EGTA pH 8.0*
5. 1 M HEPES pH 7.5*
6. 2.5 M glycine*
7. Phosphate-buffered saline (PBS) pH 7.5
8. Complete cell culture medium pH 7.5: Dulbecco's Modified Eagle's Medium (DMEM) complemented with 5 % fetal bovine serum and 2 % human serum (for human cells).
9. Short-term 10x cell fixation buffer: 11 % formaldehyde solution, 100 mM NaCl, 1 mM EDTA, 0.5 mM EGTA, 50 mM HEPES, pH 7.5 completed with nuclease-free ultrapure water. Store at room temperature.

10. 1.5 mL Axygen maxymum recovery tubes
11. Rotating platform
12. Liquid nitrogen

2.2 Chromatin Shearing by sonication (see Note 1)

1. Dry ice
2. 1 M Tris-HCl pH 8.0*
3. 0.5 M EDTA pH 8.0*
4. 10 % Sodium dodecyl sulfate (SDS)*
5. 1 M Sodium butyrate (NaBu)
6. Protease Inhibitor Cocktail 200x (PI) (Sigma Aldrich)
7. Short term complete lysis buffer: 50 mM Tris-HCl pH 8.0, 10 mM EDTA, 0.25 % SDS in nuclease-free ultrapure water. Right before use, add 1 M NaBu (to reach 20 mM) and 200x Protease-inhibitor cocktail (to reach 1x).
8. 0.6 mL Axygen Maxymum Recovery tubes
9. Bioruptor Pico
10. 0.2 mL 8-tube strips
11. PureLink RNase A 20 mg/mL (Invitrogen)
12. Proteinase K Solution 20 mg/mL, RNA grade (ThermoFisher)
13. Thermomixer
14. Agarose
15. 50x Tris-Acetate-EDTA (TAE) buffer*: 2 M Tris base, 1 M Acetate and 50 mM EDTA, pH 8.5–9.0.
16. 6x loading dye buffer
17. SYBR gold nucleic acid gel stain (Invitrogen)
18. Electrophoresis display
19. U.V. transilluminator with a camera
20. 0.5 mL Nunc cryobank vials (ThermoFisher)

2.3 Automated chromatin immunoprecipitation for histone modifications

All buffers for this series of steps are obtained from the True MicroChIP Kit (Diagenode):

1. tC1 buffer.
2. Complete tC1 buffer: right before use, add 20 μ L of protease inhibitor cocktail 200x and 80 μ L of 1 M NaBu to 4 mL of tC1 buffer.

3. tBW1 buffer
4. tW1 buffer
5. tW2 buffer
6. tW3 buffer
7. tW4 buffer
8. tE1 buffer
9. Protease inhibitor (PI) 200x (Sigma Aldrich)
10. 1 M Sodium butyrate (NaBu)
11. ChIP grade antibody: example, H3K27ac (Diagenode)
12. 30 mg/mL ProteinA Dynabeads (Invitrogen)
13. 200 μ L 8-tube strips + cap strips for SX-8G IP Star (Diagenode)
14. 2 mL microtubes for SX-8G IP Star Compact (Diagenode)
15. Medium reagent containers for SX-8G IP Star Compact (Diagenode)
16. Tips (bulk) for SX-8G IP Star (Diagenode)
17. SX-8G IP-Star Compact automated platform (Diagenode).
18. 8-channel micropipette (10 to 100 μ L)

2.4 Library preparation by transposase integration of Illumina library adaptors (see Note 1)

1. 1 M Tris-HCl pH 8.0*
2. 25 mM MgCl₂*
3. N,N-dimethylformamide
4. DNA Tagmentation enzyme from Nextera Kit (Illumina)
5. Short term tagmentation buffer: 10 mM Tris-HCl pH 8.0, 5 mM MgCl, 10 % N,N-dimethylformamide, 1:24 (vol:vol) of DNA Tagmentation enzyme. Keep on the ice.
6. 8-tube strip magnet (Diagenode)
7. Metallic 96-well rack
8. ChIP Buffer tC1 from True Microchip Kit (Diagenode)
9. Tips (bulk) for SX-8G IP Star (Diagenode)
10. 200 μ L 8 tube strips + cap strips for SX-8G IP-Star Compact (Diagenode)
11. 2 mL microtubes for SX-8G IP Star Compact (Diagenode)
12. SX-8G IP-Star Compact automated platform (Diagenode)

13. PureLink RNase A 20 mg/mL (ThermoFisher)
14. Proteinase K Solution 20 mg/mL, RNA grade (ThermoFisher)
15. Thermomixer
16. 8-channel Multichannel pipette (10 to 100 μ L)

2.5 Purification and amplification of the tagmented DNA fragments (see Note 1)

1. 8-tube strips magnet (Diagenode)
2. ChIP DNA Clean & Concentrator column based Kit (Zymo Research), including washing solution to be reconstituted with Ethanol, 200-proof.
3. Ethanol, 200-proof, anhydrous 99.5 %
4. 1.5 mL collection tubes
5. 1 M Tris-HCl pH 8.0*
6. 0.5 M EDTA pH 8.0*
7. 10x Kapa HiFi Hotstart Ready Mix (Kapa Biosystems)
8. SYBR Green dye
9. 50x Rox dye
10. Nextera index primers kit (Illumina)
11. Common CtD reaction mix, per sample: 0.275 μ L of 2 Nextera index primers (25 μ M), 2.75 μ L of pre-heated 2x Kapa HiFi Hotstart Ready Mix, 0.11 μ L of 1:1000 diluted SYBR Green dye and 0.11 μ L of ROX passive dye. Complete the volume to 4 μ L with nuclease-free water.
12. Common Amp reaction mix, per sample: 27.5 μ L of pre-heated 2x Kapa HiFi Hot start ready mix, and complete the volume to 31 μ L with nuclease free water. Then add 2.5 μ L of 2 Nextera index primers (25 μ M) to each sample.
13. 0.2 mL PCR 8-tube strips with individual cap.
14. qPCR plate and seal: 96-well PCR plate, low profile, skirted; qPCR MicroAmp optical adhesive film (Life Technologies).
15. TE buffer 1: 1 mM Tris-HCl pH 8.0 and 1 μ M EDTA (see Note 2)*.
16. Thermomixer
17. Real-time quantitative PCR system

2.6 Purification of DNA post-amplification, size selection and quantification (see Note 1)

1. Ampure XP beads solution (Beckman Coulter).
2. Magnet for 96-well plate (Axygen)
3. Ethanol washing solution: 100 mL of 80 % ethanol solution

4. Plate 1: 96-well semi-skirted PCR plates (BioRad), higher volume capacity.
5. Plate 2: 96-well hard-shell thin-wall 96-well skirted PCR plates.
6. MicroAmp Clear Adhesive Film (Life Technologies)
7. 1 M Tris-HCl pH 8.0*
8. 0.5 M EDTA pH 8.0*
9. TE buffer 1: 1 mM Tris-HCl pH 8.0, 1 μ M EDTA (*see Note 2*)*.
10. TE buffer 2: 10 mM Tris-HCl pH 8.0, 1 μ M EDTA (*see Note 2*)*.
11. 40 ng/ μ L of sonicated standard DNA: sonicate Lambda phage DNA (Life Technologies) to obtain fragments at length ranging between 300 bp and 600 bp. Aliquot and store at -20°C .
12. Quant-iT PicoGreen dsDNA Reagent (Thermofisher)
13. 96-well plate, flat bottom, black for fluorescence measurements

3 Methods

3.1 Cell fixation

To perform good quality ChIP experiments, DNA and histones need to be crosslinked using formaldehyde. The following steps describe how cells are fixed in a 1 % formaldehyde solution, washed and spun to obtain a cell pellet that can be subsequently snap frozen in liquid nitrogen and stored at -80°C for up to a year. (*see Fig. 1a and Note 3*)

The following steps of this procedure occur at room temperature.

1. Bring cell suspension concentration to $1-2 \times 10^6$ cells/mL of complete cell culture medium in a 15 mL tube if less than 10 mL of cell suspension, 50 mL tube for 10–30 mL per tube. If less than 1 million cells, use 0.5 mL of complete cell culture medium in a 1.5 mL tube.
2. Prepare the appropriate amount of 10x Cell Fixation Buffer: total volume of cell suspension (at 1 to 2 million cells per mL) / 10.
3. Place the 2.5 M glycine solution at room temperature and PBS on ice, have a large bucket of ice to accommodate all tubes after fixation. Also, prepare a container with liquid nitrogen (*see Note 4*).
4. Vortex cell suspension at medium speed and add, drop by drop, 1:10 (vol:vol) of 10x cell fixation buffer.
5. Place tubes on a rotating platform at low rpm and incubate the tubes for 10 minutes at room temperature (*see Note 5*).
6. After incubation, vortex the tubes at medium speed, and stop the reaction by adding 1:20 (vol:vol) of 2.5 M glycine solution, invert the tubes twice and place them on ice for at least 5 minutes.

Perform the following steps at 4 °C or on ice.

7. Spin tubes at 800 g-force for 5 minutes at 4 °C, discard supernatant, and resuspend the pellets with 5 mL of ice-cold PBS. Incubate on ice for 2 minutes.
8. Spin tubes at 800 g-force for 5 minutes at 4 °C, discard the supernatant, and carefully resuspend the pellets with 1 mL of ice-cold PBS. Transfer sample to pre-cooled 1.5 mL tubes (*see Note 6*).
9. Spin the tubes for 5 minutes at 1200 g-force at 4 °C and remove as much of the supernatant as possible without affecting the integrity of the cell pellet.
10. Snap freeze the pellets in liquid nitrogen and store in –80 °C freezer (*see Note 7*).

3.2 Chromatin Shearing

This protocol is set up for the preparation of pellets containing 0.3 to 3 million cells (*see Note 8*). It is optimized for the use of the Bioruptor Pico (Diagenode) (*See Note 9*).

1. About 20 minutes before starting the protocol, switch the Bioruptor on to cool the water to 4 °C.
2. Pre-warm the sonicator unit by performing 3 cycles of 16 seconds ON / 32 seconds OFF twice using balancing tubes only (*see Note 10*).
3. Take out the pellets from the freezer and keep them on dry ice. Do not allow thawing of the pellet before adding the lysis buffer (*see Note 11*).
4. Add 70 µL of short term complete lysis buffer, kept at room temperature (RT), to the pellet and allow it to thaw for 1 minute.
5. Carefully resuspend the pellet for 1 minute. Use a 200 µL tip keeping the end of the tip very close to the bottom of the tube to create pressure on cell flow (*see Note 12*).
6. Allow cells to lyse for 1 more minute at room temperature, then put the sample on ice.

From this step, keep the samples on ice (or at 4 °C).

7. To proceed with the sonication, place the samples symmetrically into the tube holder and fill any gaps with balancing tubes.
8. Place the samples on the rack in the chilled water bath and let them incubate for 1 minute.
9. Perform sonication for x cycles (depending on cell type) with the settings 16" ON / 32" OFF (*see Note 13*).
10. Take the tube holder out of sonicator after every 3 cycles, and place it on ice.
11. Carefully vortex and pulse-spin the tubes to collect the samples at the bottom of the tubes (*see Note 14*).

12. Spin the samples at max. speed (>14,000 g-force) for 15 minutes at 4 °C (*see Note 15*).
13. Transfer supernatant (approx. 70 µL) into fresh low binding 0.6 mL tubes and keep on ice.
14. To assess the sonication efficiency, take out 1 to 7 µL (up to 10 % of total volume) of supernatant from the sonicated samples (*see Note 16*) and transfer to fresh 0.2 mL PCR tubes, called QC-tubes.
15. Make up the volume to 10 µL in QC-tubes with short term complete lysis buffer, and add 1 µL of RNase A. Incubate the sample at 37 °C for 30 minutes on a thermomixer with shaking (800 rpm).
16. Add 1 µL of proteinase K to QC-tubes containing 11 µL sample and incubate at 65 °C for 2 hours on a thermomixer with shaking (800 rpm).
17. Take out 2 µL of the decrosslinked sample from the QC-tubes for quantification by PicoGreen assay (*see 3.7, steps 31-41*).
18. Determine the chromatin concentration for each sample. Based on the initial cell numbers, estimate sonication efficiency (*see Note 17*).
19. Mix the rest of the sample (10 µL) with 2 µL of 6x loading dye buffer and load the sample on a 1.2 % agarose – 1x TAE gel. Run electrophoresis for 1 hour at 70 V in 1x TAE buffer. Stain the gel with SYBR Gold dye (1:20,000) in 1x TAE buffer for 20 minutes, wash it twice with 1x TAE buffer for 10 minutes, and read it using a U.V. trans-illuminator.
20. If quantity results given by PicoGreen measurements and gel analysis indicate successful sonication, then proceed with the preparation of chromatin stocks aliquots for storage (*see Fig. 2b*).
21. Spin the sample tube again at max. speed for 15 minutes at 4 °C.
22. Measure the volume using the pipette and dilute the samples to set the chromatin concentration to 25 ng/µL (*see Note 18*).
23. Store all aliquots of sheared chromatin at –80 °C.

3.3 Automated chromatin immunoprecipitation for histone modifications

This protocol is designed to use the automated ancillary liquid handler SX-8G IP-Star from Diagenode (*see Fig. 1a, 1b and Note 19*). Every ChIP reaction sample will contain 500 ng chromatin (20 µL at 25 ng/µL) of sheared DNA equivalent to around 100,000 cells (*see Note 19*). The following steps describe the preparation of the different 8-tube strips required to set-up the automated platform as illustrated in Fig. 3a.

1. Take out 16 chromatin aliquots from –80 °C freezer and place them on ice to allow the chromatin to thaw slowly. After the chromatin tubes are thawed, vortex them briefly and pulse-spin.

2. Take two 8-tube strips and label them appropriately. To avoid a mix-up in samples, color (or number) the left side and right sides of both strips (*see Note 20*).
3. **Chromatin 8-tube strips preparation:** Pipette 100 μ L of complete tC1 buffer (supplemented with protease inhibitor and NaBu) into each tube of two 0.2 mL 8-tube PCR strips.
4. Transfer 20 μ L of each chromatin sample to the 8-tube strips containing 100 μ L of complete tC1 buffer.
5. Wash the chromatin tubes: to each initial chromatin tubes, add 80 μ L of complete tC1 buffer, pipette up and down twice and transfer the volume into the corresponding **tube in the 8-tube strip**. The final volume in **8-tube strips** will be 200 μ L (*see Note 21*).
6. **Antibody 8-tube strips preparation:** Calculate the volume of antibody (Ab) to use based on the formula: $(N \times AbQ)/(AbC)$ in which Antibody Concentration (AbC) = 2.84 μ g/ μ L; Quantity of Ab / ChIP reaction (AbQ) = 0.5 μ g; number of tubes (N)= 16; and the volume of Ab to use (AbV)= $(N \times AbQ)/(AbC) \rightarrow (16 \text{ samples} \times 0.5 \mu\text{g})/2.84 = 2.82 \mu\text{L}$ (*see Note 22*).
7. Pipette 70 μ L of tBW1 buffer into two 8-tube strips.
8. Add the appropriate volume of antibody (AbV) to 500 μ L of buffer tBW1 (*see Note 23*) and dispense 30 μ L of this mix into each tube of the two 8-tube strips containing 70 μ L of tBW1 buffer.
9. **Magnetic bead 8-tube strips preparation:** Vortex the Protein A Dynabeads stock solution thoroughly. For 16 samples, add 5 μ L of beads suspension into a fresh set of two 200 μ L 8-tube strips.
10. Prepare a set of 2 empty “sample” 8-tube strips: to avoid samples mix-ups, color (or number) the left side and right sides of both strips and place them at the last row (row 12) in the IP Star machine (*see Fig. 3a and Note 20*).
11. Place all strips in the IP-Star machine as specified in Fig. 2a and follow IP-Star program “*ChIP-16-IPure-200D.ptd*” with one modification: use tW4 buffer instead of tE1 buffer (*see Note 24*).
12. Organize the day in such a way that the robot will perform the ChIP overnight. At the end of the procedure, proceed with library preparation steps (or – not recommended – store samples at 4 °C for a few days).

3.4 Library preparation by transposase integration of Illumina library adaptors

The following steps are performed on the second day of the procedure (*see Note 25*).

1. Pre-set thermomixer (with shaking option at 500 rpm) to 37 °C, get ice, a metallic 96-well rack and a magnet for 0.2 mL 8-tube strips.
2. Take all reagents out of the freezer and slowly thaw if required (*see Note 26*).

3. For 16 samples, prepare 440 μL of tagmentation buffer and dispense 53 μL into the 8-tube strips. Keep buffer cold in the metallic rack placed on ice.
4. Place 220 μL of tC1 buffer in each tube of an 8-tube strip. Keep cold in the metallic rack placed on ice.
5. Take the Chromatin immunoprecipitation “IP samples” strip tubes **from the IP-Star machine** (see Fig. 3a) and capture the chromatin bound to beads for 2 minutes using the 8-tube strips magnet. Carefully remove the supernatant (see **Note 27**).
6. Take out the “samples” 8-tube strips from the magnet and place in the cold metallic rack.
7. With a multichannel pipette, transfer 25 μL of the tagmentation buffer to both of the “Sample” 8-tube strips on the magnet.
8. Resuspend beads with the 25 μL tagmentation buffer by pipetting gently for 10 seconds (5 moves up & down, with pipette set at 20 μL ; see **Note 28**).
9. Immediately place strips in thermomixer and incubate at 37 °C for 3 minutes with shaking at 500 rpm (see **Note 29**).
10. Quickly transfer the strips to the chilled metallic rack and stop the tagmentation reaction with the addition of 100 μL of ice-cold tC1 buffer. Use multichannel pipette to mix samples well (set at 100 μL).
11. Place the “samples” 8-tube strips back on the IP-Star platform and set-up the platform with plastics to perform a wash program:
“*Washing_for_IP_reacts_16_Ipure.ptg*” (see **Note 30**).

The next set of instructions describes the decrosslinking steps (see **Note 31**).

12. Take out the 8-tube strip “samples” of the IP-Star and add 2 μL RNase A to each sample (use individual tips and add the drop directly into the liquid).
13. Cap tubes and pulse-spin briefly. Open the tubes and mix with a multichannel pipette set to 100 μL . Close tubes and spin briefly.
14. Incubate strips in thermomixer for 30 minutes at 37 °C with shaking at 800 rpm.
15. Take out the samples from the thermomixer and add 2 μL of Proteinase K solution to each sample.
16. Place the 8-tube strips back into the thermomixer and shake at 1250 rpm for 4 hours at 55 °C and then shake overnight at 800 rpm 65 °C.

3.5 Purification and amplification of the tagmented DNA fragments

Purification and amplification with barcoded-adaptor of the tagmented DNA fragments are performed on the third day of the procedure. 16 samples can be batched (see **Note 32**). The following steps describe the purification of de-crosslinked DNA fragments (see **Note 33**).

1. Take out the 8-tube strips that contain the decrosslinked products from the thermomixer to reach room temperature. Do not place the tube strips on ice!
1. Place the strips in to the 8-tube strips magnet to capture the beads.
2. During bead capture, prepare TE buffer 1 and warm it to 60 °C. Then prepare 16 × 1.5 mL tubes appropriately labelled.
3. Add 400 µL of DNA binding buffer provided by the ChIP DNA Clean & Concentrator Kit to all 1.5 mL tubes and transfer the 100 µL of decrosslinked DNA into each of the 1.5 mL tubes.
4. Add 100 µL of the DNA binding buffer to each tube of the 8-tube strips to wash the beads, pipette up and down five times, then add back the 100 µL to the corresponding 1.5 mL tubes containing the de-crosslinked DNA.
5. Close caps and vortex the 1.5 mL tubes for 30 seconds and pulse-spin.
6. Clearly, label 16 columns provided by the ChIP DNA Clean & Concentrator Kit, and load columns with the total amount of liquid in the 1.5 mL tube (600 µL).
7. Close column caps and spin for 20 seconds at 10,000 g-force. Re-load flow through on to the columns.
8. Close column caps and spin for 20 seconds at 10,000 g-force. Discard the flow-through.
9. Wash columns twice with 200 µL of the reconstituted washing buffer provided by the ChIP DNA Clean & Concentrator Kit. Close column caps and spin for 20 seconds at 10,000 g-force. Discard the flow through.
10. Spin columns at 12,000 g-force for 2 minutes.
11. Transfer columns into new labeled 1.5 mL collection tubes.
12. To elute the DNA from the columns, add 9 µL of freshly prepared, pre-warmed TE buffer 1 to the column matrix. Incubate for 1 minute. Close column caps and spin for 1 minute at 10,000 g-force (*see Note 34*).
13. Transfer the collected volume of 9 µL from the collecting tube into an appropriately labeled fresh set of 8-tube strips with individual caps.
14. Add again 8 µL of TE buffer 1 to the columns. Close column caps, place into collecting tubes and spin for 1 minute at 10,000 g-force.
15. Place the collected volume (8 µL) into the appropriate tube of the 8-tube strips.
16. Keep strips with tagmented DNA fragments on ice.

The following steps describe how to set-up the qPCR reaction to determine the number of cycles required for optimal amplification of the fragment of DNA. We refer to it as Ct determination qPCR (CtD).

17. Set up thermocycler to 98 °C to heat activate the kappa polymerase (44 µL for 16 samples) for 1 minute.

18. Prepare a common CtD mix for all samples (*see* **Notes 35** and **36**).
19. Dispense 3.6 μL of CtD mix in qPCR plate wells, add 1.4 μL of tagmented DNA fragment samples ($\approx 10\%$ of total).
20. Perform qPCR with the following program: 72 °C for 5 minutes; 98 °C for 30 seconds; 26 cycles of 98 °C for 10 seconds, 63 °C for 30 seconds, 72 °C for 30 seconds (*see* **Note 37**). Hold at 10 °C for 5 minutes to cool down the samples.
21. Ct values for each sample can be used to estimate the tagmentation efficiency or quantity (requires the use of a standard curve) as shown in Fig. 3b.
22. Determine the number of cycles required for optimal DNA amplification: For each sample capture Ct value when amplification curves reach half of total fluorescence intensity ($\text{Ct}_{1/2}$). Seek a common denominator for all samples ranging between $\text{Ct}_{1/2} + 1$ to 2 cycles (*see* Fig. 3c). Based on the qPCR analysis, perform the amplification of the rest of the tagmented DNA fragments.
23. Set thermocycler to 98 °C and heat activate the kappa polymerase (440 μL for 16 samples) for 1 minute.
24. Prepare a common Amp mix for all samples (amp mix) (*see* **Note 36**).
25. Dispense 31 μL of Amp mix to each “sample” tube containing around 14 μL of tagmented DNA fragments.
26. Add specific Illumina Index Primers, 2.5 μL per primer (*see* **Note 38**), to each “sample” tube. Final reaction volume is 50 μL .
27. With a multichannel pipette, mix samples up and down. Close tubes and pulse-spin.
28. Perform amplification program using the thermocycler for the number of cycles determined previously by the CtD qPCR as follows: 72 °C for 5 minutes; 98 °C for 30 seconds; CtD number of cycles: 98 °C for 10 seconds, 63 °C for 30 seconds, 72 °C for 30 seconds; 72 °C for 5 minutes; hold at 10 °C (*see* **Note 39**).

3.6 Purification of DNA post-amplification, size selection and quantification

The following steps are performed on the last day of the procedure (*see* Fig. 1b). We designed the protocol for 48 samples (*see* **Note 40**). The following steps describe the purification of all amplified DNA fragments using Ampure XP beads solution.

1. Take out the Ampure XP beads solution from the storage location (4 °C) and let the bead suspension equilibrate to room temperature. After equilibration, vortex thoroughly to homogenize solution.
2. Take out the amplified “sample” 8-tube strips from three ChIP runs (total of 48) and warm samples to room temperature. Carefully transfer the amplified samples (50 μL each) into a Plate 1. These should be placed in the wells found in columns 1 to 6 (*see* **Note 41**).

3. Add 90 μ L of Ampure XP beads (1:1.8 ratio) to each well. Mix carefully up & down using a multichannel pipette (set to 100 μ L). Seal the plate and let it sit at room temperature for 2 minutes.
4. Place the plate on the 96-well plate magnet for 5 minutes to capture beads and then discard supernatant.
5. Leave plate on the magnet and add 200 μ L of 80 % ethanol washing solution (made from 100% ethanol) to each well. Be careful not to disrupt the bead pellet. Discard the supernatant.
6. Repeat washing step with 80 % ethanol washing solution twice.
7. Remove all the ethanol washing solution on the last wash. To do this, carefully use 20 μ L tips to eliminate all residues of wash buffer.
8. Leave the beads to dry for about 10 minutes until cracks appear in the bead pellets.
9. Pre-warm 2 mL of nuclease-free water to 60 °C.
10. With the plate still on the magnet, elute the DNA by dispensing 40 μ L of pre-warmed water to each well.
11. Seal the plate with an adhesive film and remove it from the magnet. Vortex the plate thoroughly (careful to avoid spill over on the seal) and briefly pulse-spin the plate at 250 g-force.
12. Place the plate back onto the magnet to capture the beads.
13. Transfer the 40 μ L eluate to a new Plate 1 (called “Sample” plate) - columns 1 to 6.
14. For quality control purposes (see **Note 42**), take out 4 μ L of the samples and transfer to a new Plate 2 (called QC plate) (columns 1 to 6).
15. Add 4 μ L TE buffer 1 to the quality control wells (QC plate - columns 1 to 6) as well as to the samples (in the “samples” plate) setting the volume again to 40 μ L (columns 1 to 6).

At this point, the amplified DNA is purified. The following steps describe the fragment DNA size selection, with an enriched fragment length ranging from 200 to 1000 bp.

16. Add 22 μ L of Ampure XP beads (1:0.55 ratio) to the 40 μ L samples present in the wells of columns 1 to 6. Mix carefully up & down using a multichannel pipette (set to 50 μ L) then seal the plate and let it sit at room temperature for 2 minutes (see **Note 43**).
17. Place the plate on the magnet to capture the beads for 5 minutes. Carefully collect and transfer the supernatants (62 μ L) to empty wells located in columns 7 to 12 (in same “Sample” plate) (see **Note 44**). Remove the plate from the magnet and add 30 μ L of Ampure XP beads to the supernatant (“Sample” plate, columns 7 to 12) to reach a 1:1.3 ratio (40 μ L initial volume: (22+30) μ L of Ampure XP

beads) in these wells. Mix carefully up & down using a multichannel pipette (set to 50 μ L) and seal the plate. Let the plate sit at room temperature for 2 minutes (see **Note 45**).

18. Prepare TE buffer 1 and warm it up to 60 °C.
19. Place the plate on the magnet to capture Ampure XP beads for 5 minutes and discard supernatant.
20. Wash all bead pellets (column 1 to 12) 3 times with 200 μ L of 80 % ethanol washing solution as described in **3.6, Step 5**. After the last wash, use P20 tips to remove all residues of the washing buffer.
21. Leave the Ampure XP beads to dry for about 10 minutes until cracks in bead pellet appear.
22. With the plate still on the magnet, elute the DNA by dispensing 8 μ L of pre-warmed TE buffer 1 to each well.
23. Remove the plate from the magnet once all the pellets are in contact with TE buffer 1. Seal the plate and vortex thoroughly.
24. Let the plate sit at RT for 2 minutes and pulse-spin at 1000 g-force.
25. Place the plate on the magnet to capture the Ampure XP beads and transfer the supernatant to a new Plate 2.
26. Only for the final library wells (column 7 to 12), repeat the elution with another 8 μ L of TE buffer 1 (can be at room temperature). Vortex and spin the plate.
27. Transfer the supernatant to the first 8 μ L eluate volume (16 μ L total for final libraries).
28. At the end of this step, you should have two Plate 2, one (QC plate) with the pre-size-selected fragments for QC (with 8 μ L in 48 wells; see **3.6, Step 14**) and a second plate (sample plate) with the first 48 wells containing the large DNA fragments in 8 μ L of TE buffer 1 and the later 48 wells containing 16 μ L of final libraries ready for sequencing (see **Note 46**).
29. Quantify final libraries and QC samples using PicoGreen fluorescent assay as described here after (see **Note 47**).
30. Determine the volume of TE buffer 2 required (in mL): multiply the number of samples by 0.7 (minimum 20 mL).
31. **Standards:** Prepare a 6-points 4x serial dilution for the standard DNA (sonicated phage Lambda DNA) with starting concentration at 40 ng/ μ L (see **Note 47**). Use 4.4 μ L of standard DNA stock solution (160 ng) per PicoGreen plate and dilute to reach the concentration of 0.4 ng/ μ L (add 39.6 μ L) with TE buffer 2 for the top standard, perform the subsequent 1:4 dilution (11 μ L into 33 μ L) with TE buffer 2.

32. **Samples:** In Plate 2, prepare a 10-fold dilution of samples by adding 3 μ L of purified product to 28 μ L of TE buffer 2 (*see Note 48*).
33. **Blanks:** Use TE buffer 2 as a blank sample.
34. Prepare PicoGreen solution: dilute Quant-iT PicoGreen dsDNA dye 500-fold with TE buffer 2 (*see Note 49*).
35. Function of the number of samples, fill wells of a black 96-well flat bottom plate with 190 μ L PicoGreen solution (*see Fig. 2d and Note 50*).
36. Following the plate layout displayed in Fig. 2d, load 10 μ L of standard and blank conditions; then, load 10 μ L of diluted samples (*see Note 50*).
37. Perform measurement and record data using a top-read plate reader.
38. Analyze by constructing a standard curve to determine sample concentrations and total quantities.
39. Ascertain the loss of samples during size selection comparing to size-selected samples, and estimate what fraction of that loss were >1000 bp fragments (*see Fig. 3d and Note 51*).
40. Run approximately 5 to 10 ng of samples (no more than 10 % of total) on a chip-based capillary electrophoresis machine following manufacturer recommendation to determine the median size of library DNA fragments (*see Fig. 3e*).
41. For each library determine the molar concentration using this formula: $[\text{Library concentration (ng/}\mu\text{l)} * 10^6] / [660 * \text{Median size (bp)}] = \text{Library Molarity (nM)}$
42. Libraries are now ready to be pooled in equimolar amount and sequenced following standard procedure on Illumina sequencing platforms (*see Note 52*).

4 Notes

1. All stock solutions (*) are prepared with ultrapure water and autoclaved prior to use in experimental buffers.
2. TE buffers concentration in Tris-base and EDTA are different from the standard TE buffer commercially available. TE Buffer 1 could be bought as “Low TE” buffer or could be standard TE buffer diluted 10x with ultrapure water.
3. For a batch of 48 samples, the hands-on time is estimated to be 3 hours and the entire procedure will take around 5 hours. The proposed time estimation doesn’t include the sample sorting time that can vary depending on the size of the sample, their number, and the relative abundance of target cells. Take this aspect of the experiment into consideration.
4. Take all safety dispositions to handle liquid nitrogen, wear adequate laboratory personal equipment.

5. Avoid making bubbles, depending on the protein of interest the time of fixation has to be optimized (10 minutes for histone modification, 10 to 30 minutes for transcription factors).
6. Depending on the number of cells, the pellet will look very small.
7. Avoid any pellet thawing afterwards.
8. Sonication is the most efficient way to fragment unbiasedly chromatin.
9. It usually takes 3 to 4 hours of hands-on time to sonicate 12 samples (see Fig. 1a), 24 a day.
10. As others [12], we noticed that using balancing tubes improve the consistency of sonication across the samples.
11. If thawed, the integrity of sample is compromised, the sonication will fail, as a remedy try to refreeze by snap freezing samples in liquid nitrogen and restart the lysis process.
12. Even when used at low concentration, SDS will cause foaming. Avoid the formation of bubbles; air bubbles will affect sonication efficiency.
13. You want to avoid under/over sonication of your samples. This is why it is important to determine an optimal number of sonication cycles specific to every cell type. If you are not sure about the number of cycles, perform a titration of sonication experiment and skip the high-speed spin before the quality control de-crosslinking steps (see Fig. 2a).
14. This is an important step in order to ensure homogenous and more reproducible sonication. To notice that the samples will progressively become clearer during sonication process. A visual inspection of tubes will help to monitor the sonication efficiency.
15. Note that the pellet should be almost invisible; a large white pellet or a cloudy solution indicates the lysis and/or sonication may not have worked properly.
16. Calculate the volume to use for this step: consider 5 ng per cell and aim for 150 ng to 200 ng of chromatin, don't use more than 10 % of the sample.
17. Results interpretation: The sonication failed if any of those 3 facts are true: (i) the recovered quantity of chromatin, determined by PicoGreen, is lower than 50 % of the expected quantity based on cell number, (ii) the presence of a large white pellet size after high centrifugation, (iii) gel analysis post sonication, showing that majority of fragment (>65%) are not located between 100 to 500 bp (see Fig. 2b). Remediation steps will be to sonicate for a few more cycles. Repeat the QC steps as described. Alternatively, repeat the sonication from a new pellet.
18. Based on the results from PicoGreen, we suggest diluting the sample to 25 ng/ μ L (= 500 ng in 20 μ L) with complete lysis buffer (containing proteinase inhibitors and NaBu). We suggest that for each sample, aliquot the chromatin into 3 (or

- more) labeled Nunc storage cryovials with 20 μ L each; pipette any leftovers into an additional Nunc storage cryovial.
19. We optimized the procedure and labor workflow for up to 48 samples organized into 3 rounds of 16 ChIP reactions per week. For technical purposes, in context of largescale project with more than 16 samples, we suggest to run 14 samples (100,000 cells = 500 ng each = 20 μ L aliquot), one technical duplicate control (a 2nd aliquot of 20 μ L) and one permanent sample used for all rounds of ChIP to control for batch effects (e.g. chromatin from a related cell line). We estimate this step will require 1.5 hours of hands-on time.
 20. Color code suggestion: 1 to 8 and 9 to 16; color the left side of the first strip tube blue (#1), the right side of both strip tubes in red (#8 and 16), and the left side of the second strip tube green (#9), this will reduce frequent samples mix-ups.
 21. Optional: if planning to use INPUT (non IP'd samples counterparts): Add 90 μ L of complete tC1 and take out 10 μ L of sample. Store at -20°C . Proceed to section **3.4 step 12** (see **Note 31**).
 22. Numbers are indicative, the concentration of antibody per ChIP experiment has to be validated by specific titration experiments.
 23. We consider that all samples receive the same amount of antibody, if planned differently, then the volumes have to be recalculated accordingly.
 24. tW4 needs to be added to position "A" instead of tE1 buffer (to avoid elution from the beads before tagmentation reaction). The program has the following settings: Ab coating: 2 hours, 4°C , Middle, IP reaction: 10 hours, 4°C , Middle, Washes: 5 minutes, 4°C , Middle. This program takes around 18 hours to complete.
 25. The hands-on estimated time is 1 hour and a total time of around 3 hours for the completion of the experiment (see Fig. 1a and 1b).
 26. The N,N-dimethylformamide is a very unstable reagent that has to be kept in the flammable/hazardous cabinet at room temperature. Handle with care!
 27. Some of the beads can stick to the side of the tubes, don't mix, pipette slowly out the supernatant from the bottom of the tubes.
 28. Tagmentation is a very time sensitive reaction, even if we controlled that the tagmentation reaction was not happening at 4°C , it is reasonable to act promptly.
 29. We have validated the tagmentation time and set it up optimally for 3 min as other have also shown [11]
 30. Select the wash program in IP-Star (3-minute washes). The buffers used are: tE1 in position A, tC1 buffer in positions C&D, and tW4 in positions E&F - this program takes 2 hours and 18 minutes to complete. The machine only has an 8 samples version, contact Diagenode to get the 16 sample.

31. Optional: if planning to use INPUT (non IP'd samples counterparts): Add 90 μ L of TE1 to the 10 μ L inputs samples then follow steps.
32. The hands-on estimated time is 3 hours; the entire procedure will take around 5 hours.
33. Others suggested to using Ampure XP beads for this clean-up step [11]. However, in our hands we observed a lot of variability and loss of material; hence we suggest using a column based purification.
34. We do not know what is the composition of the elution buffer included in the kit, we therefore suggest to use out TE buffer 1 that will stabilize DNA fragment for storage but won't interfere with downstream steps.
35. Use a unique pair of indices for all samples, those samples will not go through sequencing.
36. All volumes are increased by 1.1 to account for volume error during dispensing.
37. Optional: Add a melt curve. We use the qPCR StepOne equipment from Life Technologies and it requires the use of a passive dye to normalize fluorescence signals.
38. All combinations of index primer pairs have to be unique for sequencing purposes, choose wisely, respect color balance, refer to Illumina website.
39. Amplified product can be stored for a few days at -20°C without DNA purification.
40. The hands-on estimated time is 4 hours for a total of a 6-hour procedure. The following steps are written for a batch of 3×16 set of samples. If there are not multiple rounds of ChIP planned, then those steps can be completed on the same day without storage at -20°C .
41. The plate being used needs to have wells with larger capacities (at least 300 μ L) instead of the regular 0.2 mL PCR plate.
42. We advise to perform those steps during early stages, after becoming familiar with the method, those steps could be abandoned.
43. During this step, the fragments $> 1,000$ bp will be captured on beads, smaller fragments will stay in solution as shown in Fig. 3. Ampure XP beads to samples volume ratios have to be validated.
44. Now, all wells in the plate should have a bead pellet.
45. At this step, only the fragments with a size length ranging between 200 to 1000bp will be captured with the beads. The smaller fragments will stay in solution and be discarded, as shown in Fig. 3.
46. Plates can be stored at -20°C or -80°C for long-term storage.
47. We suggest 28 μ L to account for pipetting variance when performing the downstream steps.

48. The volumes shown are for one picogreen 96-well plate as shown in Fig 2.d. quantity for 24 samples will be measured per plate. For a regular set of 96 samples, you will have to prepare 4 picogreen plates. For consistency, it's best to prepare one series of DNA standard points for all plates.
49. This is equivalent to 2 μ L of dye per mL of TE buffer 2.
50. It is very important to add the drop of samples in the liquid and not on the side the wells; the measurement can be affected if so. You can protect the plate from light with aluminum foil (recommended).
51. We should expect no more than 20 % loss. If more than a 20 % loss is measured then verify your bead ratios or elution steps.
52. We usually perform 50 bp single-end read sequencing and aim to generate 15 million mapping reads per sample. Figure 4 shows as an example of consistency for H3K27ac ChIP-Seq enrichment for two different batches of 10 CD4 T cells samples (100,000 cells ChIP-Tagmentation) along different genomic coordinates corresponding to three gene loci with UCSC genome tracks, rpkM: reads per kilobase per million mapped.

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Fig 1. Overview of the method.

(a) Flow-chart connecting all major steps of the procedure including quality controls and timing for easy planning of the experiment. (b) Diagram illustrates ChIP-tagmentation procedure schedule for 48 samples over a week. The sequencing would take place on the following week.

Fig. 2. Sonication quality control steps.

(a) 1.2 % agarose gels DNA electrophoresis stained with SybrGold dye show the progress of chromatin shearing by increase number of cycles (16 seconds on, 32 seconds off) for two different cell types) CD4 naive T cells and monocytes. LD, lanes with 100 bp DNA ladder. Good quality sheared chromatin range in fragment length between 100 to 500 bp (red dotted lines) with max of intensity around 250–300 bp. We consider as passed QC, any chromatin with more than 70% of fragments size ranging between those limits as noted in green. (b) 1.2 % agarose gel DNA electrophoresis stained with SybrGold dye shows Bioruptor pico consistency in sonicating efficiency (12 cycles 16 on/32 off) for 12 different cell pellets with size ranging from 0.3 to 3 million cells for 2 different cell types, CD4 naive T cells and B cell line (GM12878) (only 100 ng or 10% of samples loaded). (c) Correlation plot between the initial cell pellet size and the quantity of sheared chromatin measured by PicoGreen assay after decrosslinking for 96 CD4 naive T cells samples. (d) Picogreen assay plate layout.

Fig. 3. Amplification and final library QCs.

(a) Layout of one of the IP-Star 48 tubes deck, showing initial set up of specific 8-tube strips containing magnetic beads coated with protein A, ChIP-Antibody, sheared chromatin. Every other lane will have empty 8-tube strips, at the end of the automated procedure, the last strip (in red) will contain the immunoprecipitated DNA. (b) Schematic representation of the interpretation of the qPCR amplification curves to determine the number of cycle of amplification for each ChIP samples after fragmentation. The number of cycles is equal to the Ct value corresponding to mid-fluorescence intensity plus one or two depending on the other samples run concomitantly. (c) Correlation plot between the quantities of DNA obtained

after amplification for a given identical number of cycles and the calculated quantities based on CtD qPCR values. **(d)** Quality control plot, showing DNA quantities (in ng; left panel) measured by PicoGreen for a batch of 48 samples after amplification (pre size selection, PSS), and after size selection with values for the large size DNA fractions (LF) and final libraries (FL). The right panel shows relative proportions of LF and FL to PSS in order to identify outliers samples with low quantities and/or failed size selection (red dots; red numbers are indicative of our threshold of elimination). **(e)** Bioanalyzer traces, showing the evolution of the DNA fragment size distribution for two representative samples through the three different steps of final DNA purification and size selection. Any samples that are at the end of the size selection steps have fragments larger than 1000 bp or smaller than 200 bp go either through a second cycle of size selection or are eliminated from sequencing (green values are our suggested values for threshold of elimination).

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Fig. 4. Example of consistency of the method.

Examples of consistency for H3K27ac ChIP-Seq enrichment for two different batches of 10 CD4 T cells samples (100,000 cells ChIP-Tagmentation each) along different genomic coordinates corresponding to three gene loci with UCSC genome tracks. rpkm, reads per kilobase per million mapped.