Mar 07, 2019

Working

Bench top CUT&Tag

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dx.doi.org/10.17504/protocols.io.wnufdew

Human Cell Atlas Method Development Community

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ABSTRACT

Here we describe a single-tube bench top protocol for Cleavage Under Targets and Tagmentation (CUT&Tag), an enzyme-tethering strategy that provides efficient high-resolution sequencing libraries for profiling diverse chromatin components. In CUT&Tag, a chromatin protein is bound in situ by a specific antibody, which then tethers a proteinA-Tn5 transposase fusion protein. Activation of the transposase efficiently generates fragment libraries with high resolution and exceptionally low background. All steps from live cells to sequencing-ready libraries can be performed in a single tube on the benchtop or a microwell in a high-throughput pipeline (see the Kaya-Okur high-throughput protocol), and the entire procedure can be performed in one day. We have demonstrated the utility of CUT&Tag by profiling histone modifications, RNA Polymerase II and transcription factors on low cell numbers and single cells.

PROTOCOL STATUS

Working

We use this protocol in our group and it is working

GUIDELINES

CUT&Tag for efficient epigenomic profiling of small samples and single cells

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MATERIALS TEXT

- Standard (not 'lobind') microfuge tubes (1.5 ml and 2 ml).
- Cell suspension. We have used human K562 cells and H1 hESCs.
- Concanavalin-coated magnetic beads (Bangs Laboratories, ca. no. BP531).
- Antibody to an epitope of interest.
- Positive control antibody to an abundant epitope, *e.g.* α-H3K27me3 rabbit monoclonal antibody (Cell Signaling Technology, cat. no. 9733)
- Secondary antibody, *e.g.*guinea pig α-rabbit antibody (Antibodies online ABIN101961)
- 5% Digitonin (EMD Millipore, cat. no. 300410)
- Dimethyl sulfoxide (DMSO; Sigma-Aldrich cat. no. D4540)
- Protein A–Tn5 (pA-Tn5) fusion protein Store at -20 °C.
- Double-stranded adapters with 19mer Tn5 mosaic ends (Sequence information was derived from Picelli, S. et al. Genome Res 24, 2033-2040 (2014), and ordered through Eurofins, 100 µM in TE buffer)
- Mosaic end_reverse [PHO]CTGTCTCTTATACACATCT
- Mosaic end_Adapter A TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG
- Mosaic end_Adapter B GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG

- Distilled, deionized or RNAse-free H_2O (dH_2O e.g., Promega, cat. no. P1197)
- 1 M Manganese Chloride ($MnCl_2$; Sigma-Aldrich, cat. no. 203734)
- 1 M Calcium Chloride ($CaCl_2$; Fisher, cat. no. BP510)
- 1 M Potassium Chloride (KCl; Sigma-Aldrich, cat. no. P3911)
- 100 mM Magnesium Chloride ($MgCl_2$; Sigma-Aldrich, cat. no. M8266-100G)
- 1 M Hydroxyethyl piperazineethanesulfonic acid pH 7.5 (HEPES (Na+); Sigma-Aldrich, cat. no. H3375)
- 5 M Sodium chloride (NaCl; Sigma-Aldrich, cat. no. S5150-1L)
- 0.5 M Ethylenediaminetetraacetic acid (EDTA; Research Organics, cat. no. 3002E)
- 2 M Spermidine (Sigma-Aldrich, cat. no. S2501)
- 30% Bovine Serum Albumen (BSA,
- Roche Complete Protease Inhibitor EDTA-Free tablets (Sigma-Aldrich, cat. no. 5056489001)
- 2 mg/ml Glycogen (1:10 dilution of Sigma-Aldrich, cat. no. 10930193001)
- 10% Sodium dodecyl sulfate (SDS; Sigma-Aldrich, cat. no. L4509)
- Proteinase K (20 mg/ml Thermo Fisher Scientific, cat. no. EO0492)
- Phase-lock tubes (Qiagen MaXtract High Density cat. no. 129046)
- Phenol-chloroform-isoamyl alcohol 25:24:1 (PCI) Invitrogen - Thermo Fisher, cat. no. 15593049)
- Chloroform 366919-1L Sigma
- Agencourt AMPure XP paramagnetic beads (Beckman Coulter, cat. no. A63880)
- 1 M Tris-HCl pH 8.0
- Ethanol (Decon Labs, cat. no. 2716)
- 0.5 ml PCR tubes (LabSource T54-252)
- NEBNext HiFi 2x PCR Master mix
- PCR primers: A universal i5 primer and 16 i7 primers with unique barcodes [Buenrostro, J.D. et al. Nature 523:486 (2015)].

SAFETY WARNINGS

Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin.

BEFORE STARTING

Prepare reagents (STEP 1)

REAGENT SETUP (for 16 samples)

1 **Digitonin (5%)** Dissolve 50 mg Digitonin in 1 ml DMSO.
Caution: Digitonin is toxic and care should be taken especially when weighing out the powder. Use full PPE including a mask, lab coat and gloves while handling any amount of digitonin. Be aware that DMSO can penetrate through the skin.

Binding buffer Mix 400 µL 1M HEPES pH 7.5, 200 µL 1M KCl, 20 µL 1M $CaCl_2$ and 20 µL 1M $MnCl_2$, and bring the final volume to 20 mL with dH2O. Store the buffer at 4 °C for 6 months.

Wash buffer Mix 1 mL 1 M HEPES pH 7.5, 1.5 mL 5 M NaCl, 12.5 µL 2 M Spermidine, bring the final volume to 50 mL with dH_2O, and add 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 1 week.

Dig-wash buffer Mix 400 µL 5% Digitonin with 40 mL Wash buffer. Store the buffer at 4 °C for up to 2 days.

Antibody buffer Mix 8 µL 0.5 M EDTA and 6.7 µL 30% BSA with 2 mL Dig-wash buffer and place on ice.

Dig-300 buffer Mix 1 mL 1 M HEPES pH 7.5, 3 mL 5 M NaCl and 12.5 µL 2 M Spermidine, bring the final volume to 50 mL with dH_2O, and add 500 µL 5% Digitonin and 1 Roche Complete Protease Inhibitor EDTA-Free tablet. Store the buffer at 4 °C for up to 2 days.

Tagmentation buffer Mix 5 mL Dig-300 buffer and 50 µL 1 M $MgCl_2$.

Tn5-adapter complex formation:
1. Anneal each of Mosaic end - adapter A (ME-A) and Mosaic end - adapter B (ME-B) oligonucleotides with Mosaic end – reverse oligonucleotides.
2. Mix 16 µL of 100 uM equimolar mixture of preannealed ME-A and ME-B oligonucleotides with 100 µL of 5.5 µM protein A - Tn5 fusion protein.
3. Incubate the mixture on a rotating platform for 1 hour at room temperature and then store at -20 °C.

Note pA-Tn5 aliquots received from the CUT&RUN team are pre-loaded with adapters suitable for single- or dual-indexing on a paired-end Illumina flow-cell platform.

Bind cells to Concanavalin A-coated beads

2 Gently resuspend and withdraw enough of the ConA bead slurry such that there will be 10 µL for each final sample. The following is for 16 samples.

3 Transfer 170 µL ConA bead slurry into 1.6 mL Binding buffer in a 2 mL tube and mix by pipetting. Place the tube on a magnet stand to clear (30 s to 2 min).

4 Withdraw the liquid completely, and remove from the magnet stand. Add 1.5 mL Binding buffer, mix by pipetting, remove liquid from the cap and side with a quick pulse on a microcentrifuge.

5 Resuspend in 1.7 mL Binding buffer (100 µL per sample) and hold until cells are washed and ready.

6 **CRITICAL STEP:** All steps prior to the cell permeabilization are performed at room temperature to minimize stress on the cells. We recommend that cavitation during resuspension and vigorous vortexing be avoided.

Harvest fresh culture(s) at room temperature and count cells. The same protocol can be used for up to ~100,000 mammalian cells per sample to be sequenced.

7 Centrifuge 3 min 600 x g at room temperature and withdraw liquid.

8 Resuspend in at least 1 volume Wash buffer at room temperature, centrifuge 3 min 600 x g at room temperature and withdraw liquid.

9 Resuspend in 1.5 mL Wash buffer and transfer to a 2 mL tube. While vortexing gently (1100 rpm) add bead slurry dropwise. Place on end-over-end rotator for 5-10 min.

10 After a quick spin to remove liquid from cap (<100 x g), place the tubes on a magnet stand to clear and withdraw the liquid.

Bind primary antibody

11 Resuspend cells in 800 µL ice-cold Antibody buffer with gentle vortexing, place on ice, and divide into 16 1.5 mL tubes, 50 µL each (for 16 samples; scale up or down as needed).

12 Mix each antibody in 50 µL antibody buffer and add to sample aliquots with gentle vortexing for each final sample.

CRITICAL STEP: We use 1:50 - 1:100 by default or the manufacturer's recommended concentration for immunofluorescence.

13 Place on nutator at 4 °C and incubate at overnight to several days at 4 °C. Alternatively, nutate for 2 hr at room temperature. Liquid should remain in the bottom and side of the tube while rocking.

CRITICAL STEP: To evaluate success of the procedure without requiring library preparation, include in parallel a positive control antibody (*e.g.* α-H3K27me3), and optionally a negative control antibody (e.g. rabbit α-mouse IgG).

Bind secondary antibody

14 After a quick spin to remove liquid from cap (<100 x g), place each tube on the magnet stand to clear and pull off the liquid.

15 Mix secondary antibody 1:100 in Dig-wash buffer and squirt in 50 µL per sample while gently vortexing to allow the solution to dislodge the beads from the sides.

16 Place the tubes on a nutator at room temperature for 30–60 min. **00:30:00**

17 After a quick spin, place the tubes on a magnet stand to clear and withdraw the liquid. Resuspend beads in 200 µL Dig-wash buffer.

18 Repeat Step 17.

Binding pA-Tn5 adapter complex

19 Mix pA-Tn5 adapter complex in Dig-300 buffer to a final concentration of 1:250 for 100 µL per sample.

CRITICAL STEP: pA-Tn5 aliquots received from the CUT&RUN team are pre-loaded with adapters suitable for single- or dual-indexing on a paired-end Illumina flow-cell platform.

20 Place the tubes on the magnet stand to clear and pull off the liquid.

21 Squirt in 100 µL of the pA-Tn5 mix while gently vortexing to allow the solution to dislodge most or all of the beads. Small clumps may appear in Dig-300 buffer (300 mM NaCl), but this does not affect efficiency of incubations or washes.

22 Place the tubes on a nutator at room temperature for 1 hr. **01:00:00**

23 After a quick spin, place the tubes on a magnet stand to clear and pull off the liquid.

24 Add 800 µL Dig-300 buffer. Invert 10x or gently vortex to allow the solution to dislodge most or all of the beads.

25 Repeat steps 23-24 twice.

Tagmentation

26 After a quick spin, place the tube on the magnet stand to clear and pull off the liquid.

27 Add 300 µL Tagmentation buffer while gently vortexing.

28 Incubate at 37 °C for 1 hr. **01:00:00**

DNA extraction

29 To stop tagmentation, add 10 µL 0.5M EDTA, 3 µL 10% SDS and 2.5 µL 20 mg/mL Proteinase K to each sample at room temperature.

30 Incubate 1 hr 50 ºC or 37 ºC overnight to digest. 01:00:00

31 Add 300 µL PCI and mix by full-speed vortexing ~2 s.

32 Transfer to a phase-lock tube, and centrifuge 5 min room temperature at 16,000 x g.

33 Add 300 µL Chloroform and invert ~10x to mix. Allow to separate.

34 Remove aqueous layer by pipetting to a fresh tube containing 750 µL 100% ethanol while pipetting up and down to mix.

35 Chill on ice and centrifuge 15 min at 4 °C 16,000 x g.

36 Carefully pour off the liquid and drain on a paper towel. There is typically no visible pellet.

37 Rinse in 1 mL 100% ethanol and centrifuge 1 min at 4 °C 16,000 x g.

38 Carefully pour off the liquid and drain on a paper towel. Air dry.

39 When the pellet is dry, dissolve in 25-30 µL 1 mM Tris-HCl pH8 0.1 mM EDTA.

PCR

40 Mix 21 µL DNA + 2 µL Universal i5 primer (10 µM) + 2 µL uniquely barcoded i7 primers (10 µM), using a different barcode for each sample*. Save remaining DNA as a backup.

*Nextera primers or indexed primers described by Buenrostro, J.D. et al. Single-cell chromatin accessibility reveals principles of regulatory variation. Nature 523:486 (2015).

41 Add 25 µL NEBNext HiFi 2x PCR Master mix.

42 1. Mix, quick spin and place in Thermocycler and begin cycling program with heated lid.

Cycle 1: 72 °C for 5 min (gap filling)

Cycle 2: 98 °C for 30 sec

Cycle 3: 98 °C for 10 sec

Cycle 4: 63 °C for 30 sec

Repeat Cycles 3-4 13 times

72°C for 1 min and hold at 8 ^{O}C

CRITICAL STEP: To minimize the contribution of large DNA fragments and excess primers, PCR cycles should be at least 12-14 cycles, preferably with a 10 s 60-63^{O}C combined annealing/extension step.

Post-PCR Clean-up

43 After tubes have cooled, remove from cycler and add 1.1 volume (55 µL) Ampure XP beads, and vortex briefly on full.

44 Quick spin and let sit at room temperature 10 min. **00:10:00**

45 Place on magnet and allow to clear before carefully withdrawing liquid. On magnet and without disturbing the beads, add 1 mL 80% ethanol.

46 Withdraw liquid with a pipette to the bottom of the tube and add 1 mL 80% ethanol.

47 Withdraw liquid and remove the remaining liquid with a 20 µL pipette and allow to dry for 4-5 min. **00:05:00**

48 Remove from magnet stand, add 25 µL 10 mM Tris-HCl pH 8 and vortex on full.

49 After 5 min place on magnet stand and allow to clear.

50 Remove liquid to a fresh tube with a pipette.

DNA sequencing and data processing

51 Determine the size distribution and concentration of libraries by capillary electrophoresis using an Agilent 4200 TapeStation or equivalent.

52 Mix libraries to achieve equal representation as desired aiming for a final concentration as recommended by the manufacturer.

53 Perform paired-end Illumina sequencing on the barcoded libraries following the manufacturer's instructions.

54 We align paired-end reads using Bowtie2 version 2.2.5 with options: --local --very-sensitive-local --no-unal --no-mixed --no-discordant --phred33 -I 10 -X 700. For mapping spike-in fragments, we also use the --no-overlap --no-dovetail options to avoid cross-mapping of the experimental genome to that of the spike-in DNA.

CRITICAL STEP: Separation of sequenced fragments into ≤120 bp and ≥150 bp size classes provides mapping of the local vicinity of a DNA-binding protein, but this can vary depending on the steric access to the DNA by the tethered Tn5 transposase.

Because of the very low background with CUT&Tag, typically 3 million paired-end reads are sufficient for nucleosome modifications, even for the human genome. For maximum economy, we mix up to 48 barcoded samples per 2-lane flow cell, and perform paired-end 25x25 bp sequencing.

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