Protocol for SIMPLE-seq

mTET1CD preparation

Plasmid Construct Datasheet (gift from Guoliang Xu Lab)

I.	Plasmid name	**pcDNA4-Flag-Tet1CD**	Other name
II.	Purpose	**protein expression**	Epitope
III.	Vector Information		
Name	**pcDNA4-Flag**		
Cloning sites used	**BamHI+NotI**	Resistance	**Ampicillin**
V.	Insert Information		
Gene name	**Tet1CD**	Gene ID	**NM_027384**
Region	**1367-2040 aa**	Organism	**mouse**

Reagents:

□ Expi293F™ Cells (Gibco, A14527)

□ FreeStyle™ 293 Expression Medium (Gibco, 12338018)

□ 2L cell culture roller bottles (e.g. CELLMASTER™, 680068)

□ Polyethylenimine (Polysciences, 23966-1)

□ DPBS, no calcium, no magnesium (Gibco, 14190-094)

□ UltraPure™ 1 M Tris-HCl Buffer, pH 7.5 (Invitrogen, 15567027)

□ 5M NaCl (Invitrogen, AM9759)

□ Triton X-100 (Sigma, T8787)

□ cOmplete™ Protease Inhibitor Cocktail EDTA-free (Sigma, 11873580001)

□ PMSF (ThermoScientific, 36978)

□ 3X Flag Peptide (Sigma, F4799)

□ HEPES (Sigma, H3375)

□ DTT (Fluorochem, M02712)

□ ANTI-FLAG® M2 Affinity Gel (Sigma, A2220)

□ Empty gravity chromatography column (e.g. Econo-Pac® Chromatography Column, Bio-rad, 7321010)

□ 3X Flag Peptide (Sigma, F4799)

- Glycine (Sigma, G7126)
- Amicon® Ultra-4 Centrifugal Filter Unit MWCO = 30 kD (Merck, UFC803024)
- Bio-Spin® P-30 Gel Columns, Tris Buffer (Bio-rad, 7326231)

mTET1CD expression

1. Seed Expi293F™ Cells at 0.5 x 10^6 cells/ml density into final volume of 1000 mL FreeStyle™ 293 Expression Medium in 2 L roller bottle and grow at 37°C, 170 rpm, and 5% CO_2 until cells reach density of 1.0 x 10^6 cells/mL. It takes approximately 24 h.
2. Transfect cells with pcDNA3-Flag-mTET1CD plasmid:

a) Use 1 µg of pCDNA3-Flag-mTET1CD plasmid to transfect 1 million of cells. For 1L culture at density 1.0 x 10^6 cells/ml, dilute 1 mg of plasmid in 100 mL PBS and add 3 mL Polyethylenimine (PEI, stock concentration 1 mg/mL). Final DNA to PEI ratio is 1:3. Vortex DNA:PEI solution for at least 10 sec and incubate at room temperature for 20 min.

b) Slowly add DNA:PEI solution to 1 L cell culture and mix well. Maintain cells at 37°C, 170 rpm, and 5% CO_2 for 48 h.

3. After 48 h, collect cells by centrifugation at 3000 g and 4˚C for 20 min.

Remove supernatant and proceed with purification protocol. Alternatively, pellet can be stored at -80°C for few days without affecting protein activity.

mTET1CD purification

Buffers:

Lysis Buffer: 50 mM Tris-Cl pH = 7.5, 500 mM NaCl, 1X cOmplete™ Protease Inhibitor Cocktail, 1 mM PMSF, 1% Triton X-100.

TBS: 50 mM Tris pH = 7.5, 150 mM NaCl.

Elution buffer: 20 mM HEPES pH = 8.0, 150 mM NaCl, 0.1 mg/mL 3X Flag, 1X cOmplete™ Protease Inhibitor Cocktail, 1 mM PMSF.

Storage buffer: 20 mM HEPES pH = 8.0, 150 mM NaCl, 1 mM DTT.

1. Resuspend 1 L cell pellet in 100 mL lysis buffer. Incubate on ice for 20 min.
   Note: Volume of lysis buffer can be scaled-up if cell pellets from more than 1 L culture are purified.
   2. Clarify the lysate by centrifuging at 30000 g and 4°C for 30 min. Collect clear supernatant to clean glass bottle. Save 5 µL for SDS-PAGE gel.

Note: If larger lysate volumes are used, centrifugation time should be extended to ensure complete removal of cellular debris.

3. Prepare 2.5 mL ANTI-FLAG® M2 Affinity Gel (Sigma, A2220) in 20 mL empty gravity chromatography column. Allow storage buffer to drain and wash the gel according to the protocol below. After every wash allow buffer to drain completely before proceeding to the next step.

a) Wash gel with three volumes (7.5 mL) TBS.

b) Wash column three times with 2 volumes (5 mL) 0.1 M glycine-HCl pH = 3.5. Do not leave resins in glycine buffer for longer than 20 min.

c) Wash gel five times with 2 volumes (5 mL) TBS.

d) Wash gel three times with 2 volumes (5 mL) lysis buffer.

4. Resuspend resins in 10 mL of lysis buffer and transfer to the bottle containing protein supernatant. Incubate at 4°C for 30 min with gentle rotation or shaking.

5. Transfer supernatant with resins back to gravity chromatography column and allow it to pass through the column. Collect flow through. Save 5 µL for SDS-PAGE gel.

6. Wash resins three times with 10 volumes (25 mL) of TBS. Save 5 µL of each wash fraction for SDS-PAGE gel.

7. Elute bound mTet1CD protein. Resuspend beads with 1 volume (2.5 mL) of elution buffer and incubate for 5 min. Then allow buffer to flow though the column and collect the fraction. Repeat 5 times. Save 5 µL of each fraction for SDS-PAGE gel.

8. Concentrate eluted protein on Amicon Ultra 30 kD filter until concentration is around 2.5– 5 mg/mL.

9. Buffer-exchange Bio-Spin® P-30 Gel Column (Bio-Rad, 7326231) with storage buffer and purify concentrated protein fraction. Do not apply more than 70 µL of protein solution to the column. Save 2-5 µL of protein for SDS PAGE gel.

Note: This step is performed to remove residual Flag-peptide and protein buffer into final storage buffer.

10. Add glycerol to the concentration 30%, measure final protein concentration by Nanodrop (Thermo Fisher), aliquot and freeze in liquid nitrogen. Store protein at -80˚C. Avoid multiple freeze-thaw cycles.

11. Run collected fractions on SDS-PAGE gel to check the quality and purity of mTet1CD protein. mTET1CD MW ~ 75 kD.

General notes: Keep all protein-containing fractions on ice during whole purification.

A-1 Freshly prepare the following buffers:

A-2 Cell culture

1. Naive mouse Embryonic Stem Cells (mESCs) were maintained on the pre-gelatinized dishes without feeders, supplemented with two inhibitors (2i): 1 mM PD0325901 (Selleckchem, S1036), 3 mM CHIR99021 (Selleckchem, S1263), 1,000 U/mL leukemia inhibitory factor (LIF, Millipore, ESG1107), 20% fetal bovine serum (FBS, GIBCO, 26140087) in Dulbecco's modified eagle's medium (DMEM/F-12, GIBCO, 11320033). Primed mESCs were cultured with DMEM/F12 with 20% FBS and 1,000 U/mL LIF. Human PBMCs were purchased form *AllCells* (PB003F-C).

A-3 Nuclei preparation

1. Spin-down dissociated cultured cells or PBMCs at 300 g for 5 min; wash with 10 mL fresh no-serum media (DMEM/F-12, GIBCO). Resuspend cell pellet in 10 mL fresh no-serum media;
2. Add 406 µL 37% formaldehyde solution and fix onto a rotator, incubate at RT for 10 min with rotating; Add 800 µL 2.5 M glycine (Sigma-Aldrich, G7126) and mix upside-down, incubate on ice 5 min to quench the fixing reaction;
3. Spin-down at 550 g, 4°C for 8 min; wash with 10 mL ice cold PBS buffer; spin-down at 550 g, 4°C for 8 min;
   Stop point: Cells can be snap-frozen in liquid nitrogen and stored for up to 1.5 years at −80°C.
4. Resuspended in 5 mL ice cold NIB buffer (20 mM HEPES (Sigma, H3375), 10 mM NaCl, 3 mM $MgCl_2$ (Invitrogen, AM9530G), 0.1% Igepal (Thermo Scientific, 85124), 1× cOmplete Protease Inhibitor Cocktail); incubate on ice for 20 min with gentle shaking with a mixer; Spin-down at 550 g, 4°C for 5 min; wash with 1 mL 1× NEBbuffer 2.1;
5. Cell counting using cell counter;
6. Spin-down at 500 g, 4°C for 5 min;
7. Pre-wash 10 individual 1.5 mL tube with 5% BSA in PBS buffer;
8. Resuspend cells in 1× NEBuffer 2.1 with 0.3% SDS, with a cell density of 250K/mL (for limited cell clumps) and divided 800 µL of the suspension into each tube, total 8 mL in 10 tubes; incubate at 42°C for 30 min; immediately, add 80 µL 20% Triton-100X buffer (final

1.8%) and incubate at 42°C for 30 min; pool together and spin-down at 100 g, 4°C for 10 min;

9. Wash with 1mL 1× TTBL buffer (Vazyme, S601, or 1× home-made tagmentation buffer) twice and resuspend with 1× TTBL buffer to ensure 2000K/mL; use cell strainer 20 μm to remove potential nuclei clumps;

   Optional: If there are any cell clumps, brief sonication is optional to make single cell suspension using Covaris M220 at 4°C water bath, the setting is below: Duration:60; peak power: 75; Duty factor 2%; cycles/burst: 200; Average power: 1.5;
10. To generate barcoded Tn5 transposons, barcoded DNA oligos were annealed to a pMENTs oligo in a thermocycler with the following program: 95 °C for 5 min, slowly cooled to 4 °C with a temperature ramp of 1 °C/min. The transposons (9 μL, 10 μM) were then mixed with 10 μL unloaded transposase Tn5 (Vazyme, 0.5 mg/mL, S601) and 17 μL coupling buffer (Vazyme, S601), mixed by pipetting and quickly spin-down, incubated at 30 °C for 30 min. The loaded transposases can be stored at −20 °C.
11. The nuclei were distributed into 96 well plates (10K nuclei/each well, ~6μL); add 3-4 μL assembled Tn5 and 0.4 μL 10 × tagmentation buffer into each well (wells in each column contain the same specific barcoded Tn5 transposase); incubated at 55°C, 300 g for 30 min in a thermomixer. The reaction was quenched by adding final 15 mM EDTA (Invitrogen, AM9260G)
12. Nuclei were pooled together and passed through a 20 μm cell strainer. Nuclei were spun-down at 1,000 g, 4°C for 10 min and resuspended in 1 mL 1× NEBuffer 3.1

A-4 Split-barcoding

1. To prepare the barcode plates (Barcode-plate-R02 and Barcode-plate-R03, **Supplementary Table**), 6 μL of each of the barcoded oligos (100 μM) were distributed into two 96-well plates (Eppendorf, 0030603303). Then, 44 μL of Linker-R02 (for Barcode-plate-R02) or Linker-R03 (for Barcode-plate-R03) (12.5 μM) was added to each well of the two plates. The plates were sealed and annealed with the following program: 95 °C for 5 mins, and slowly cool down to 20 °C with a ramp of −0.1 °C/s (stock solution plates). The stock solution plates were then divided into ten new 96-well plates, with each well of the working plates containing 5 μL of annealed barcoded adaptor ready for ligation reaction.

2. Nuclei resuspended in 1 mL of 1× NEBuffer 3.1 (NEB, B6003S) were transferred to ligation mix (2,262 µL of H_2O, 500 µL of 10× T4 DNA Ligase Buffer (NEB, B0202S), 50 µL of 10 mg/mL BSA, 100 µL of 10× NEBuffer 3.1 and 100 µL of T4 DNA Ligase (NEB, M0202L)).
3. Each 20 µL of the ligation reaction mix was distributed to two Barcode-plate-R02 using a multichannel pipette and incubate at 300 rpm at 37 °C for 30 mins in a ThermoMixer.
4. Then, 5 µL of R02-Blocking-Solution (264 µL of 100 µM Blocker-R02 oligo, 250 µL of 10× T4 DNA Ligase Buffer, 486 µL of H_2O) was added to each well using a multichannel pipette and continued incubation for an additional 30 mins.
5. The nuclei were then pooled and spun down at 1,000g, 4°C for 10 mins. The second round of ligation was then carried out similarly to the first round, except that after 30 mins of the ligation reaction, Termination-Solution (264 µL of 100 µM Quencher-R03, 250 µL of 0.5 M EDTA and 236 µL of H_2O) was added to quench the reaction without additional incubation.
6. Typically, 100,000 to 300,000 nuclei could be recovered after ligation-based barcoding. Nuclei were then resuspended in PBS, counted and aliquoted to sub-libraries containing 5,000 to 10,000 nuclei. Store at -80°C.

A-5 library preparation

1. Potassium perruthenate (Sigma-Aldrich,334537) solution was prepared according to published protocol, briefly: 0.15 mmol Potassium perruthenate (Aldrich, 334537) was added to 0.5 M NaOH solution (1 mL, Alfa Aesar, A18395) and vortexed to make sure all solid was dissolved; incubated the solution at 25°C for 2 days to produce potassium ruthenate solution (K_2RuO_4). Store as 10× oxidant stock.
2. Sub-libraries were diluted to 35 µL with PBS. Then, 5 µL of 4 M NaCl, 5 µL of 10% SDS (Invitrogen, 15553-035) and 5 µL of 10 mg/mL Protease K (NEB, P8107S) were added and nuclei were lysed at 850 rpm at 55 °C for 2 hrs in a ThermoMixer. The lysed solution was cooled down to room temperature and then purified with 1× SPRI beads (Beckman Coulter, B23319) and eluted in 12.5 µL of H_2O. The purified DNA can be stored at −20 °C or −80 °C for up to 4 weeks.
3. For Second adaptor tagging, genomic DNA fragments was incubated at 72 °C for 5 min and then chilled on an ice bath for 3 min. 1× Exonuclease I reaction buffer and 1µL Exonuclease I (NEB, M0293S) were added to DNA solution and incubated at 37 °C for

60 min to remove excess Linker_R02. After 1.8× Ampure XP beads purification, DNA solution was incubated at 95 °C for 1 min in a thermocycler with a heated lid and then chilled on an ice bath. For the 3'-end ligation, Bridge Adapter (final concentration 10 μM), 1× T4 DNA Ligase Buffer, PEG8000 (7.5% w/v) ,30 units T4 DNA ligase (Thermo Scientific, EL0013) and nuclease-free water were added to the mixture on ice, to the total volume of 30 μL. The reaction was performed at 20 °C for 16 h, and purified with 1.8× AMPure XP beads, washed with 80% acetonitrile and eluted with 26 μL nuclease free water.

4. Before 5hmC oxidation, genomic DNA was purified with Micro Bio-Spin P-6 SSC column (pre-washed with ultrapure water 10 times, Bio-rad, 732-6200). The DNA was then denatured in 0.05 M NaOH for 30 min at 37 °C followed by chilling on ice-water bath for 5 min. Next, 1.5 μL 1× oxidant was added to the denatured DNA sample (28.5 μL) and briefly mixed with tapping followed by incubation on ice for 1 h. The oxidized DNA was purified with a Micro Bio-Spin P-6 SSC column (pre-wash). To label the newly generated 5fC, the reaction was carried out in 10 mM pH 7.0 Tris buffer and 150 mM malononitrile (J&K, 261700) in a total volume of 35 μL at 37 °C for 20 h, 850 rpm in a thermomixer.
5. To obtain the 5hmC profiles from the single cells, the 5hmC labeled DNA was subjected to primer extension to record the 5hmC-to-T transition signal. Primer extension mix (10 μL of 5× KAPA HiFi buffer, 1 μL of 10 mM dNTPs, 2 μL of 10 μM P7-indicator primer, 0.8 μL KAPA HF DNA polymerase (Roche)) was added and primer extension was performed with the following program: Step 1: 95 °C × 5 min; Step 2: 65 °C × 60 s, 68 °C × 8 min, and repeat Step 2 an additional 15 times; Step 3: 72 °C × 10 min and hold at 4 °C. Then, 3 μL of 10× Exo I reaction buffer and 1 μL of Exonuclease I were added to the mix, the primer digestion reaction was incubated at 37 °C for 60 min and purified using 1.8× Ampure XP beads and eluted with 10 μL nuclease free water.
6. mTET1CD oxidation reaction was prepared as: 10 μL DNA, 50 mM HEPES pH 8.0, 100 mM NaCl, 1 mM α-Ketoglutaric acid (Sigma-Aldrich, K3752-5G), 2 mM L-Ascorbic acid (Sigma-Aldrich, 95210-50G), 1.2 mM ATP (Sigma, A6419), 2.5 mM DTT (Fluorochem, M02712), 100μM Fe^{2+} (Sigma, 09719) and 8 μM mTET1CD. After that, the oxidation reaction was carried out with at 37 °C for 80 min. Then, 0.8 U QIAGEN Protease (Qiagen,19157) was added to the oxidation reaction and incubated for 1 h at 50 °C. The reaction mixture was purified using 1.8× Ampure XP beads, elute in 10 μL. It is optional to perform TET oxidation twice. 2-picoline-borane (pic-borane, Sigma-Aldrich, 654213-

5G) was dissolved in DMSO to give ~3.26 M solution, 2.5 μL of 3 M sodium acetate solution pH = 5.2 (Sigma-Aldrich, R1181) and 12.5 μL of 3.26 M pic-borane solution were added to 10 μL DNA sample and incubated at 70 °C in a thermocycler for 4 h. Dilute this reaction with 4-fold volume of water. The reaction mixture was purified with Zymo Oligo & Clean Concentrator Kit (Zymo Research, D4060).

7. After the reaction, the final library was amplified by PCR amplification mix (25 μL of 2× Kapa U^+ HiFi Master Mix, 2.5 μL of 10 μM Universal primer, 2.5 μL of 10 μM Index primer) and the reaction was performed with the following program: Step 1: 98 °C × 10 s; Step 2: 98 °C × 10 s, 68 °C × 15 s, 72 °C × 1 min, and repeat Step 2 an additional 3~5 times; Step 3: 72 °C × 10 min and hold at 4 °C. The product was purified with 1.8× Ampure XP beads and eluted with 20 μL nuclease-free water.
8. The purified libraries were quantified by qPCR.