CoTACIT step by step Protocol

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Buffers:

Reagents	Composition
Wash Buffer	1 mL 1 M HEPES-KOH pH 7.5, 1.5 mL 5 M NaCl, 12.5 μL 2 M spermidine, 500 μL sodium butyrate, 100 μL 5% digitonin, 125 μL 20% Triton X-100, 1 × cocktail, and the final volume to 50 mL with ddH_2O
High-salt Wash Buffer	1 mL 1 M HEPES-KOH pH 7.5, 3 mL 5 M NaCl, 12.5 μL 2 M spermidine, 500 μL sodium butyrate, 100 μL 5% digitonin, 125 μL 20% Triton X-100, 1 × cocktail, and the final volume to 50 mL with ddH_2O
Antibody Buffer	Mix 4 μL 0.5 M EDTA with 1 mL Wash-buffer
Tagmentation Buffer	20 mM TAPS-NaOH pH 8.3, 20 mM $MgCl_2$, 1 × cocktail, 20 mM sodium butyrate, and 2 mM PMSF
Lysis Buffer	10 mM Tris-HCl pH 8.5, 0.05% SDS and 0.1 mg/mL proteinase K

Notes: The hyperactivity of protein A-Tn5 (PAT) is most critical in this protocol to achieve extremely high genome coverage in single cells. Most commercially available PAT products are not so good in our hands. He lab is happy to provide a reasonable amount of in-house PAT upon request.

1. Take out methanol-fixed cells from -80°C freezer and put on ice for at least 15 minutes for rehydration.
2. Wash cells twice with 180 μL Wash Buffer (or 0.1% BSA/PBS) to remove residual methanol. For each time, cells were collected at an appropriate speed for 3 minutes at 4°C and 10-20 μL Wash Buffer was retained to reduce cell loss.

First round barcoding:

3. Resuspend cells with 100 μL Antibody Buffer containing 0.5 μg primary antibodies

(for example, anti-H3K27ac antibody).

4. Put the tube on a rotator and incubate at 4°C for 4 hours.
5. Collect cells at an appropriate speed for 3 minutes at 4°C.
6. Resuspend cells with 180 μL Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free antibodies.
7. Collect cells at an appropriate speed for 3 minutes at 4°C.
8. Resuspend cells with 100 μL High-salt Wash Buffer containing 3 μg/ml PAT-T5-1/T7-1 enzyme.
9. Put the tube on a rotator and incubate at 4°C for 60 minutes.
10. Wash cells twice with 180 μL High-salt Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free PAT enzyme.
11. Collect cells at an appropriate speed for 3 minutes at 4°C. Aspirate the supernatant and retain about 10 μL High-salt Wash Buffer.
12. Resuspend cells with 10 μL Tagmentation Buffer and incubate at 37°C for 60 minutes.
13. Add 20 μL 40 mM EDTA and incubate at room temperature for 15 minutes to stop the reaction.
14. Wash cells three times with 0.1% BSA/PBS (or Wash Buffer) and rotate cells 4°C for 5 minutes to wash out free PAT enzyme.

Second round barcoding:

15. Resuspend cells with 100 μL Antibody Buffer containing 0.5 μg primary antibodies (for example, anti-H3K27me3 antibody).
16. Put the tube on a rotator and incubate at 4°C for 4 hours.
17. Collect cells at an appropriate speed for 3 minutes at 4°C.
18. Resuspend cells with 180 μL Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free antibodies.
19. Collect cells at an appropriate speed for 3 minutes at 4°C.
20. Resuspend cells with 100 μL High-salt Wash Buffer containing 3 μg/ml PAT-T5-2/T7-2 enzyme.
21. Put the tube on a rotator and incubate at 4°C for 60 minutes.
22. Wash cells twice with 180 μL High-salt Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free PAT enzyme.
23. Collect cells at an appropriate speed for 3 minutes at 4°C. Aspirate the supernatant and retain about 10 μL High-salt Wash Buffer.
24. Resuspend cells with 10 μL Tagmentation Buffer and incubate at 37°C for 60

minutes.

25. Add 20 μL 40 mM EDTA and incubate at room temperature for 15 minutes to stop the reaction.
26. Wash cells three times with 0.1% BSA/PBS (or Wash Buffer) and rotate cells 4°C for 5 minutes to wash out free PAT enzyme.

Third round barcoding:

27. Resuspend cells with 100 μL Antibody Buffer containing 0.5 μg primary antibodies (for example, anti-H3K9me3 antibody).
28. Put the tube on a rotator and incubate at 4°C for 4 hours.
29. Collect cells at an appropriate speed for 3 minutes at 4°C.
30. Resuspend cells with 180 μL Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free antibodies.
31. Collect cells at an appropriate speed for 3 minutes at 4°C.
32. Resuspend cells with 100 μL High-salt Wash Buffer containing 3 μg/ml PAT-T5-3/T7-3 enzyme.
33. Put the tube on a rotator and incubate at 4°C for 60 minutes.
34. Wash cells twice with 180 μL High-salt Wash Buffer and rotate cells at 4°C for 5 minutes to wash out free PAT enzyme.
35. Collect cells at an appropriate speed for 3 minutes at 4°C. Aspirate the supernatant and retain about 10 μL High-salt Wash Buffer.
36. Resuspend cells with 10 μL Tagmentation Buffer and incubate at 37°C for 60 minutes.
37. Add 20 μL 40 mM EDTA and incubate at room temperature for 15 minutes to stop the reaction.
38. Wash cells three times with 0.1% BSA/PBS (or Wash Buffer) and rotate cells 4°C for 5 minutes to wash out free PAT enzyme.

Library construction:

39. Pre-rinse the 96-well plates with 0.1% BSA/PBS to avoid loss of DNA fragments.
40. And add 2 μL Lysis Buffer to each well of the pre-rinsed plates.
41. Pick single cell into a well of a 96-well plate with a mouth pipette under a microscope.
42. Add 5 μL mineral oil (Sigma) to each well and incubate the plates at 55°C for 15 minutes to release DNA fragments.
43. Add 0.5 μL of 10 mM PMSF and 1 μL of 0.9% Triton X-100 to each well to deactivate protease K and quench SDS.

44. Add 17 μL PCR mix (0.2 μL KAPA HiFi HotStart DNA polymerase, 4 μL 5× KAPA High-GC buffer, 0.5 μL 10 mM dNTP mix, 0.5 μL 25 mM $MgCl_2$, 11.3 μL H_2O and 0.5 μL 50 μM first-round primer mix) to each well.
45. Perform PCR with the following program: 1 cycle of 72°C for 5 minutes; 1 cycle of 95°C for 3 minutes; 17 cycles of 98°C for 20 seconds, 65°C for 30 seconds, 72°C for 1 minutes; 1 cycle of 72°C for 5 minutes; and hold at 4°C.
46. Add 0.25 μL ExoI (NEB) enzyme and incubate plates at 37°C for 60 minutes to digest excess primers.
47. Incubate plates at 72°C for 20 minutes to deactivate ExoI enzyme.
48. 10 μL second-round PCR mix (0.1 μL KAPA HiFi HotStart DNA polymerase, 2 μL 5 × KAPA High-GC buffer, 0.25 μL 25 mM $MgCl_2$ and 6.4 μL ddH2O) was added to each well containing 0.5 μL 10 mM Truseq i5 and 0.5 μL Truseq i7 index primers.
49. Perform PCR with the following program: 1 cycle of 95°C for 3 minutes; 5 cycles of 98°C for 20 seconds, 65°C for 30 seconds, 72°C for 1 minutes; 1 cycle of 72°C for 5 minutes; and hold at 4°C.
50. Purify the DNA fragments with 1× AMPure XP beads (Beckman) once, and 200-1,000 bp fragments were selected with 0.5 × + 0.5 × AMPure XP beads.
51. The libraries were sequenced with paired-end 150-bp reads on a NovaSeq 6000 platform (Illumina) or BGI T7 or MGI2000 platform.

Table 1. The sequences of PAT-T5/T7 used in CoTACIT.

Primer	Sequence
T5-1	TCGTCGGCAGCGTCTCCACGCTATAGCCTGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
T5-2	TCGTCGGCAGCGTCTCCACGCATAGAGGCGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
T5-3	TCGTCGGCAGCGTCTCCACGCCCTATCCTGCGATCGAGGACGGCAGATGTGTATAAGAGACAG
T7-1	GTCTCGTGGGCTCGGCTGTCCCTGTCCCGAGTAATCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
T7-2	GTCTCGTGGGCTCGGCTGTCCCTGTCCTCTCCGGACACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
T7-3	GTCTCGTGGGCTCGGCTGTCCCTGTCCAATGAGCGCACCGTCTCCGCCTCAGATGTGTATAAGAGACAG
common annealing primer	5Phos/CTGTCTCTTATACACATCT

Table 2. The sequences of Truseq i5 and Truseq i7 index primers used in CoTACIT.

Primer	Sequence
Truseq-501	AATGATACGGCGACCACCGAGATCTACACTATAGCCTACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-502	AATGATACGGCGACCACCGAGATCTACACATAGAGGCACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-503	AATGATACGGCGACCACCGAGATCTACACCCTATCCTACACTCTTTCCCTACACGAC GCTCTTCCGATCT
Truseq-504	AATGATACGGCGACCACCGAGATCTACACGGCTCTGAACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-505	AATGATACGGCGACCACCGAGATCTACACAGGCGAAGACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-506	AATGATACGGCGACCACCGAGATCTACACTAATCTTAACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-507	AATGATACGGCGACCACCGAGATCTACACCAGGACGTACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-508	AATGATACGGCGACCACCGAGATCTACACGTACTGACACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-509	AATGATACGGCGACCACCGAGATCTACACTTGCTTGCACACTCTTTCCCTACACGAC GCTCTTCCGATCT
Truseq-510	AATGATACGGCGACCACCGAGATCTACACGAGAGGTTACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-511	AATGATACGGCGACCACCGAGATCTACACACCTGGTTACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-512	AATGATACGGCGACCACCGAGATCTACACAAGCGGAAACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-513	AATGATACGGCGACCACCGAGATCTACACCGGAACAAACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-514	AATGATACGGCGACCACCGAGATCTACACGGTAAGCTACACTCTTTCCCTACACGA CGCTCTTCCGATCT
Truseq-515	AATGATACGGCGACCACCGAGATCTACACTGTGGCATACACTCTTTCCCTACACGA CGCTCTTCCGATCT

Primer	Sequence
Truseq-516	AATGATACGGCGACCACCGAGATCTACACACTACGGAACACTCTTTCCCTACACGACGCTCTTCCGATCT
Truseq-701	CAAGCAGAAGACGGCATACGAGATCGAGTAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-702	CAAGCAGAAGACGGCATACGAGATTCTCCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-703	CAAGCAGAAGACGGCATACGAGATAATGAGCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-704	CAAGCAGAAGACGGCATACGAGATGGAATCTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-705	CAAGCAGAAGACGGCATACGAGATTTCTGAATGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-706	CAAGCAGAAGACGGCATACGAGATACGAATTCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-707	CAAGCAGAAGACGGCATACGAGATAGCTTCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-708	CAAGCAGAAGACGGCATACGAGATGCGCATTAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-709	CAAGCAGAAGACGGCATACGAGATCATAGCCGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-710	CAAGCAGAAGACGGCATACGAGATTTCGCGGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-711	CAAGCAGAAGACGGCATACGAGATGCGCGAGAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-712	CAAGCAGAAGACGGCATACGAGATCTATCGCTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-713	CAAGCAGAAGACGGCATACGAGATAGGAGGAAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-714	CAAGCAGAAGACGGCATACGAGATAGCAAGCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-715	CAAGCAGAAGACGGCATACGAGATTCATCACCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT

Primer	Sequence
Truseq-716	CAAGCAGAAGACGGCATACGAGATCGTAGGTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-717	CAAGCAGAAGACGGCATACGAGATTCAGATCCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-718	CAAGCAGAAGACGGCATACGAGATCGTGATCAGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-719	CAAGCAGAAGACGGCATACGAGATAGTCGCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-720	CAAGCAGAAGACGGCATACGAGATGAACGCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-721	CAAGCAGAAGACGGCATACGAGATTACGCCTTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-722	CAAGCAGAAGACGGCATACGAGATCTCATCAGGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-723	CAAGCAGAAGACGGCATACGAGATTCTTCTGCGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT
Truseq-724	CAAGCAGAAGACGGCATACGAGATGCTGGATTGTGACTGGAGTTCAGACGTGTGCTCTTCCGATCT