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scTHS-seq V.1

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Protocol status: In development
We are still developing and optimizing this protocol

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Abstract

Single cell transposome hypersensitive site sequencing (scTHS-seq) combined transposome hypersensitive site sequencing (THS-seq) with combinatorial cellular indexing using customized barcoded transposomes. Compared to a similar method, ATAC-seq, THS-seq have improved coverage of distal enhancer sites which is due to the utilization of an engineered super-mutant Tn5 transposase as well as in vitro transcription to perform linear amplification from a single insertion site of the Tn5 transposase instead of two insertion sites required in ATAC-seq. scTHS-seq was used to generate single nucleus accessibility profile of 36,869 cells with an average of 10,168 unique reads per cell that are also associated with a predicted accessible region. The doublet rate of scTHS-seq is estimated to be between 20-25% which is comparable to similar combinatorial indexing methods.

Materials

MATERIALS

HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs** **Catalog #**E2040S

Taq 5X Master Mix - 500 rxns **New England Biolabs** **Catalog #**M0285L

Pierce™ Dimethylformamide (DMF), Sequencing grade **Thermo Fisher Scientific** **Catalog #**20672

2x Kapa HiFi Hotstart Readymix **Kapa Biosystems** **Catalog #**KK2602

Tango Buffer **Thermo Fisher Scientific** **Catalog #**BY5

Ultrapure SMART MMLV Reverse Transcriptase for RT-PCR **Takara Bio Inc.** **Catalog #**639524

Advantage® UltraPure dNTP Combination Kit (100 mM each dNTP) **Takara Bio Inc.** **Catalog #**639125

RNAse H **Enzymatics** **Catalog #**Y9220L

KAPA Pure Beads (60 mL) **Roche** **Catalog #**07983298001

twin.tec PCR Plate 96 LoBind semi-shirted clear 25 pcs. **Eppendorf** **Catalog #**30129504

STEP MATERIALS

-

KAPA Pure Beads (60 mL) **Roche** **Catalog #**07983298001

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For purification of engineered super-mutant Tn5-059, see previous publications (https://genomebiology.biomedcentral.com/articles/10.1186/s13059-016-0882-7) for details.

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Tagmentation

1 Make 10 mL 75% glycerol with 0.3% tritonX-100

- 9.45 g Glycerol (100%)
- 300 µL TritonX-100, 10%
- 2200 µL nuclease free water

2 Make 3.045 mL transposons dilution buffer

- 2345 µL 75% glycerol/0.3% TritonX-100
- 700 µL Qiagen EB

3 Make 1000 uL transposase dilution buffer

- 667 µL 75% glycerol/0.3% TritonX-100
- 1 µL DTT, 1M
- 6.25 µL Tris-HCl, 4M, pH 7.5
- 325.75 µL nuclease free water

4 Make 10 mL of 1.5X D6 buffer

- 1500 µL Tango Buffer, 10X
- 2400 µL DMF, 100%
- 6100 µL nuclease free water

5 Make 10 mL of 1X lysis buffer

- 25 µL Tris-HCl, 4M, pH 7.5
- 20 µL Sodium chloride, 5M
- 15 µL Magnesium chloride, 2M
- 100 µL IGEPAL-630, 10%

2000 µL BSA, 10%

1000 µL PBS, 10X

6840 µL nuclease free water

Finally, add 1 protease inhibitor tablet (EDTA-free).

6 Make 10 mL of 2X FACS buffer

1000 µL PBS, 10X

2000 µL BSA, 10%

80 µL EDTA, 0.5M

6920 µL nuclease free water

7 Generate 384 annealed transposons

7 µL Indexed Tn5 top strand (100uM)

7 µL 5' phosphorylated mosaic end sequence bottom strand (100uM)

Combine top and bottom strand oligos in a PCR tube. In a thermocycler, incubate for 2 minutes at 95 °C then ramp to 14 °C at a rate of -0.1 °C per second.

8 Dilute annealed transposons

1 µL Annealed transposons

6.7 µL transposon dilution buffer

9 Make 420 uL of 4.8 uM transposase Tn5-059

252 µL transposase, Tn5-059, 8 uM

168 µL transposase dilution buffer

10 Load Tn5-059

1 µL 4.8 uM transposase Tn5-059

1 µL diluted annealed transposons (6.5 uM)

11 Incubate

22 °C Thermocycler 00:30:00

12 Wash nuclei or cells suspension with ice-cold PBS

13 Count cells

14 Pellet 400,000 cells / nuclei by centrifuging at 330 RCF (cells) or 900 RCF (nuclei)

15 Resuspend 400,000 cells/nuclei in lysis buffer. Use 100 uL per 100,000 cells/nuclei

16 Incubate

4 °C On ice 00:05:00

17 Pellet by centrifuging at 900 RCF

18 Resuspend nuclei in 100 uL 1.5X D6 buffer initially. Count and add more 1.5X D6 buffer to create ~250 nuclei/uL

19 Aliquot 4.0 uL of cells in 1.5X D6 buffer into wells containing 2.0 uL of prepared transposomes

20 Incubation without shaking

37 °C Thermocycler 00:30:00

21 Terminate the tagmentation reaction with EDTA

4 µL EDTA, 50 mM

22 Incubate

37 °C Thermocycler 00:15:00

23 Store cells at -20C overnight or until ready to sort

FACS (BD Influx)

24 Coat 12 × 200 uL, 1 × 15 mL conical using 1X FACS buffer

01:00:00 Let tubes sit in FACS buffer

25 Add 10 uL PBS to each well of 10 to 15 × 96-wells plate for sorting

Note

Cover and store all plates on ice.

26 Add 1 volume (10 uL) ice cold 2X FACS buffer to each sample well with cells in tagmentation mix

27 When 15 mL conical have been coated for 1 hr, pool all samples together using multichannel pipette and adding to 12 × 200 uL tubes

28 Centrifuge at 500 RCF 4 °C

29 Resuspend nuclei in 1.5 mL 1X FACS buffer

30 Add stain for sorting

75 µL Propidium Iodide

31 Sort 100 cells into each well using BD Influx, remove doublets using forward and side scatter plots and selecting for PI-staining events

In vitro transcription (10 plates of sorted nuclei)

32 Add 11 uL of 8M guanidine chloride to each well using multi-channel to lyse nuclei and release DNA

14 mL Guanidine hydrochloride, 8M

33 Add 30 uL of KAPA Pure Beads to each well to bind DNA

42.24 mL KAPA Pure Beads

KAPA Pure Beads (60 mL) **Roche Catalog #**07983298001

34 Mix well and bind for at least 8 minutes

00:08:00 Room temperature

35 Place plate on magnet for at least 3 minutes then discard the supernatant

00:03:00 Room temperature

36 Prepare fresh 80% ethanol solution. Wash beads (while still on magnet) twice with 170 uL of 80% ethanol

384 mL Freshly prepared 80% ethanol

37 After last wash, completely remove all ethanol and transfer to a heated thermomixer to dry

37 °C 00:01:00

38 Add 8 uL of nuclease free water to each well using a multichannel pipette

8640 µL nuclease-free water

39 Add 2 uL of 5X Taq Master Mix to perform gap filling

2160 µL 5X Taq Master Mix

Taq 5X Master Mix - 500 rxns **New England Biolabs Catalog #**M0285L

40 Incubate plates for gap filling

72 °C 00:08:00

41 Add 2 uL of HiScribe T7 IVT reaction components to each well

2112 µL T7 buffer, 10X

2112 µL UTP

2112 µL GTP

2112 µL ATP

2112 µL CTP

2112 µL T7 RNA Polymerase

42 Incubate

37 °C Thermocycler 16:00:00

43 Add 40 uL of KAPA Pure Beads to each well to bind DNA

42.24 mL KAPA Pure Beads

KAPA Pure Beads (60 mL) **Roche Catalog #**07983298001

44 Mix well and bind for at least 8 minutes

00:08:00 Room temperature

45 Place plate on magnet for at least 3 minutes then discard the supernatant

00:03:00 Room temperature

46 Prepare fresh 80% ethanol solution. Wash beads (while still on magnet) twice with 170 uL of 80% ethanol

384 mL Freshly prepared 80% ethanol

47 After last wash, completely remove all ethanol and transfer to a heated thermomixer to dry

37 °C 00:01:00

48 Add 9 uL of nuclease free water to each well using a multichannel pipette

9720 µL nuclease-free water

cDNA synthesis (10 plates of sorted nuclei)

49 Add 2.5 uL of random hexamers

3000 µL random hexamers, 20 uM

50 Incubate

70 °C Thermocycler 00:03:00

Note

Cool immediately on ice after.

51 Add 9.5 uL of MMLV RT mixture

4224 µL First strand buffer, 5X

2112 µL Advantage UltraPure dNTPs, 100 uM each

2112 µL DTT, 100mM

528 µL MMLV RT

52 Incubate in a thermocycler

22 °C 00:10:00
42 °C 01:00:00
70 °C 00:10:00

53 Add 1 uL RNase H

1127.52 µL nuclease-free water
125.28 µL RNase H, 5 Units/uL

54 Incubate in thermocycler

37 °C 00:20:00

55 Add 2.5 uL second strand synthesis primer

3000 µL sss_scnXTv2, 20 uM

56 Incubate in thermocycler

65 °C 00:02:00

Note

Cool immediately on ice after incubation

57 Add 5.9 uL 5X Taq Master Mix

7363 µL Taq Master Mix, 5X

58 Incubate in thermocycler

72 °C 00:08:00

Tagmentation on beads (10 plates of sorted nuclei)

59 Add 40 uL of KAPA Pure Beads to each well to bind DNA

42.24 mL KAPA Pure Beads

KAPA Pure Beads (60 mL) **Roche Catalog #**07983298001

60 Mix well and bind for at least 8 minutes

00:08:00 Room temperature

61 Place plate on magnet for at least 3 minutes then discard the supernatant

00:03:00 Room temperature

62 Prepare fresh 80% ethanol solution. Wash beads (while still on magnet) twice with 170 uL of 80% ethanol

384 mL Freshly prepared 80% ethanol

63 After last wash, completely remove all ethanol and transfer to a heated thermomixer to dry

37 °C 00:01:00

64 Add 4 uL of 1.5X tagmentation buffer to each well using a multichannel pipette

3172 µL nuclease-free water

780 µL Tango Buffer, 10X

1248 µL DMF, 100%

65 Generate annealed transposons

40 µL i7 top strand (100uM)

40 µL 5' phosphorylated mosaic end sequence bottom strand (100uM)

Combine top and bottom strand oligos in a PCR tube. In a thermocycler, incubate for 2 minutes at 95 °C then ramp to 14 °C at a rate of -0.1 °C per second.

66 Dilute annealed transposons

74 µL Annealed transposons

1026 µL transposon dilution buffer

67 Make 420 uL of 2.4 uM transposase Tn5-059

330 µL transposase, Tn5-059, 8 uM

770 µL transposase dilution buffer

68 Load Tn5-059

1100 µL 2.4 uM transposase Tn5-059

1100 µL diluted annealed transposons (6.5 uM)

69 Incubate

22 °C Thermocycler 00:30:00

70 Add 2 uL of loaded transposomes to each well

71 Incubate

55 °C Thermocycler 00:06:00

Note

Cool immediately on ice after.

72 Add 6 uL guanidine hydrochloride

7488 µL Guanidine hydrochloride, 8M

73 Add 30 uL of KAPA Pure Beads to each well to bind DNA

31.68 mL KAPA Pure Beads

KAPA Pure Beads (60 mL) **Roche Catalog #**07983298001

74 Mix well and bind for at least 8 minutes

00:08:00 Room temperature

75 Place plate on magnet for at least 3 minutes then discard the supernatant

00:03:00 Room temperature

76 Prepare fresh 80% ethanol solution. Wash beads (while still on magnet) twice with 170 uL of 80% ethanol

384 mL Freshly prepared 80% ethanol

77 After last wash, completely remove all ethanol and transfer to a heated thermomixer to dry

37 °C 00:01:00

78 Add 16 uL nuclease free water

21.6 mL nuclease-free water

Test amplification (10 plates of sorted nuclei)

79 Preheat hotstart polymerase mix

250 µL KAPA HiFi Hotstart ReadyMix, 2X

98 °C Thermocycler 00:00:45

Note

Cool immediately on ice

80 Add primers to make a master mix to 417 nM final each

42 µL i5XX, 5 uM

42 µL i7XX, 5 uM

81 Add SYBR Green for qPCR to 0.8X final

4 µL SYBR Green, 100X

82 Aliquot 8 uL of amplification mix to each well to qPCR plate

83 Sample 5 wells from each plate, adding 4 uL of each sample. Include an no nuclei control well.

84 Perform qPCR. Monitor the sample progress, terminate when all samples appears to be in exponential phase (usually 6-8 cycles). No nuclei control may appear to amplify but majority of those products are primer dimers.

1. 72C x 5 min

2. 98C x 30 s
3. 59C x 30 s
4. 72C x 1 min
Plate read
5. Go to 2 for 20 cycles
6. 72C for 5 min

85 Assess PCR products on a 6% TBE PAGE gel. If lack of products, repeat test amplification on a different sampling of wells but adding 3-4 more cycles of amplification. If samples appear to be over-amplified, reduce the number of cycles by 1.

Library amplification (10 plates of sorted nuclei)

86 Preheat hotstart polymerase mix

12000 µL KAPA HiFi Hotstart ReadyMix, 2X
98 °C Thermocycler 00:00:45

Note

Cool immediately on ice

87 Aliquot 12 uL of preheated KAPA HiFi ReadyMix to each well of a new PCR plate

88 Aliquot 2 uL of 16 different s5XX primers to each set of 2 plates (one per row)

150 µL each s5XX primer, 5 uM

89 Aliquot 2 uL of 12 different s7XX primers to each column of every plate (one per column)

100 µL s7XX primer, 5 uM

90 Transfer 8 uL of sample to each well of PCR plate

91 Perform PCR using the number of cycles determined previously with test PCR

1. 72C x 5 min
2. 98C x 30 s
3. 59C x 30 s
4. 72C x 1 min
5. Go to 2 for XX cycles
6. 72C for 5 min

92 Pool 4 uL of each well from 2 plates with non-overlapping s5XX primers. Final volume is ~800 uL per pool. 5 pools total

93 Add 800 uL of KAPA Pure Beads to each pool to bind DNA

3 mL KAPA Pure Beads

KAPA Pure Beads (60 mL) **Roche Catalog #**07983298001

94 Mix well and bind for at least 8 minutes

00:08:00 Room temperature

95 Place plate on magnet for at least 3 minutes then discard the supernatant

00:03:00 Room temperature

96 Prepare fresh 80% ethanol solution. Wash beads (while still on magnet) twice with 1000 uL of 80% ethanol

12 mL Freshly prepared 80% ethanol

97 After last wash, completely remove all ethanol and transfer to a heated thermomixer to dry

37 °C 00:01:00

98 Add 20 uL of nuclease free water to each pool

100 µL nuclease-free water

99 Quantify each pool using dsDNA HS Qubit

100 Perform gel size selection for 220-1000 bp using 6% TBE PAGE. Use 5-wells gel, and loading ~1 ug of library per well.