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In devel.

scTHS-seq

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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.

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

https://www.nature.com/articles/nbt.4038

PROTOCOL STATUS

In development

We are still developing and optimizing this protocol

MATERIALS

NAME	CATALOG #	VENDOR
HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns	E2040S	New England Biolabs
Taq 5X Master Mix - 500 rxns	M0285L	New England Biolabs
Pierce™ Dimethylformamide (DMF), Sequencing grade	20672	Thermo Fisher Scientific
2x Kapa HiFi Hotstart Readymix	KK2602	Kapa Biosystems
Tango Buffer	BY5	Thermo Fisher Scientific
Ultrapure SMART MMLV Reverse Transcriptase for RT-PCR	639524	Clontech
Advantage® UltraPure dNTP Combination Kit (100 mM each dNTP)	639125	Clontech
RNAse H	Y9220L	Enzymatics
KAPA Pure Beads (60 mL)	07983298001	Roche
twin.tec PCR Plate 96 LoBind semi-shirted clear 25 pcs.	30129504	Eppendorf

STEPS MATERIALS

NAME	CATALOG #	VENDOR
KAPA Pure Beads (60 mL)	07983298001	Roche
Taq 5X Master Mix - 500 rxns	M0285L	New England Biolabs
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MATERIALS TEXT

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

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 x 200 uL, 1 x 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 x 96-wells plate for sorting

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 x 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)
by 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
by 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)
by 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

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**

Incubate in thermocycler

54 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

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)
by 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

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)
by 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

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

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)
by 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.