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SNARE-seq2 with Nuclei Hashing

Forked from [SNARE-seq2](#)

Blue

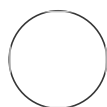
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DISCLAIMER

This is a modification of the original published protocol.

ABSTRACT

To study the heterogeneity of complex tissues by joint profiling of gene expression and its regulation, we require an accurate and high-throughput method. Here we described improved high-throughput combinatorial indexing-based single-nucleus chromatin accessibility and mRNA expression sequencing 2 (SNARE-Seq2) co-assay. This protocol involves fixing and permeabilizing the nucleus followed by fragmentation, chromatin barcode ligation, reverse transcription, pooling and splitting for the next rounds of cell barcode ligation into cDNA and accessible chromatin (AC) on the same nucleus. The captured cDNA and AC are co-amplified before splitting and enrichment into single-nucleus RNA and single-nucleus AC sequencing libraries. The protocol can also be applied to both nuclei and whole cells to capture mRNA in the cytoplasm. This improvement allows us to generate hundreds of thousands of data set of each assay and can be scaled up to half a million cells from a single experiment. The entire procedure can be complete in 3.5 d for generating joint single-nucleus RNA and single-nucleus ATAC sequencing libraries.

ATTACHMENTS

[SNARE-seq2 Oligos.xlsx](#)

MATERIALS

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Protocol status: Working
We use this protocol and it's working

Created: Jul 28, 2023

Last Modified: Jul 28, 2023

PROTOCOL integer ID:
85647

MATERIALS

In-house Tn5 transposase or alternatively,

-  Tagmentase (Unloaded) Diagenode Catalog #C01070010-20
-  Tagmentase Dilution Buffer Diagenode Catalog #C01070010
-  T4 DNA Ligase Reaction Buffer - 6.0 ml New England Biolabs Catalog #B0202S
-  NEBuffer 3.1 - 5.0 ml New England Biolabs Catalog #B7203S
-  Hemo KlenTaq - 1,000 rxns New England Biolabs Catalog #M0332L
-  T7 DNA Ligase - 750,000 units New England Biolabs Catalog #M0318L
-  PMSF Merck MilliporeSigma (Sigma-Aldrich) Catalog #P7626
-  T4 DNA Ligase New England Biolabs Catalog #M0202
-  Tween 20 Merck MilliporeSigma (Sigma-Aldrich) Catalog #P9416-50ML
-  Microseal® 'B' Adhesive Seals Bio-Rad Laboratories Catalog #MSB-1001
-  DNA LoBind Tube 1.5ml Eppendorf Catalog #022431021
-  RNase Inhibitor Enzymatics Catalog #Y9240L
-  Tango Buffer Thermo Fisher Scientific Catalog #BY5
-  twin.tec PCR Plate 96 LoBind semi-shirted clear 25 pcs. Eppendorf Catalog #30129504
-  Dynabeads MyOne Streptavidin C1 Invitrogen - Thermo Fisher Catalog #65001
-  Ficoll PM-400 20% in H2O Merck MilliporeSigma (Sigma-Aldrich) Catalog #F5415-50ML
-  Qubit assay tubes Thermo Fisher Scientific Catalog #Q32856
-  Qubit dsDNA HS Assay Kit Thermo Fisher Scientific Catalog #Q32854
-  cOmplete™ Protease Inhibitor Cocktail Merck MilliporeSigma (Sigma-Aldrich) Catalog #11697498001
-  Low DNA Mass Ladder Thermo Fisher Catalog #10068013

	UltraPure™ DNase/RNase-Free Distilled Water Thermo Fisher Catalog #10977015
	Pierce™ 16% Formaldehyde (w/v), Methanol-free Thermo Fisher Catalog #28906
	SUPERase• In™ RNase Inhibitor (20 U/μL) Thermo Fisher Catalog #AM2696
	GlycoBlue™ Coprecipitant (15 mg/mL) Thermo Fisher Catalog #AM9515
	ATP Solution (100 mM) Thermo Fisher Catalog #R0441
	Bovine Albumin Fraction V (7.5% solution) Gibco, ThermoFisher Catalog #15260037
	PBS Gibco - Thermo Fischer Catalog #10010023
	Polyethylene Glycol 6000 (PEG 6000) Merck MilliporeSigma (Sigma-Aldrich) Catalog #81255-1KG
	Tris hydrochloride (1M) pH 8.0 Thermo Fisher Scientific Catalog #15568025
	Magnesium chloride solution for molecular biology (1.00 M) Merck MilliporeSigma (Sigma-Aldrich) Catalog #M1028
	Potassium chloride (2M) Thermo Fisher Scientific Catalog #AM9640G
	5 M Sodium chloride (NaCl) Merck MilliporeSigma (Sigma-Aldrich) Catalog #S5150-1L
	NN-Dimethylformamide (DMF) Merck MilliporeSigma (Sigma-Aldrich) Catalog #227056
	Advantage UltraPure dNTP combination kit (100 mM each dNTP) Clontech Catalog #639132
	DL-Dithiothreitol Merck MilliporeSigma (Sigma-Aldrich) Catalog #10708984001
	Sodium dodecyl sulfate solution Thermo Fisher Scientific Catalog #AM9822
	Maxima H Minus Reverse Transcriptase (200 U/uL) Thermo Fisher Scientific Catalog #EP0753
	Bovine Serum Albumin (20 mg/mL) Molecular Biology Grade New England Biolabs Catalog #B9000S
	EDTA (0.5 M) pH 8.0 Merck MilliporeSigma (Sigma-Aldrich) Catalog #20158

- ✕ Triton X-100 Merck MilliporeSigma (Sigma-Aldrich) Catalog #X100-100ML
- ✕ Proteinase K solution (20mg/mL) RNA grade Thermo Fisher Scientific Catalog #25530049
- ✕ SSC Buffer (20X) Merck MilliporeSigma (Sigma-Aldrich) Catalog #S6639
- ✕ KAPA HiFi HotStart ReadyMix (2X) Kapa Biosystems Catalog #KK2602
- ✕ EvaGreen® Dye, 20x in Water Gold Biotechnology Catalog #E-670
- ✕ SYBR Gold (10000x) Thermo Fisher Scientific Catalog #S11494
- ✕ Sodium acetate (3M) pH 5.5 Thermo Fisher Scientific Catalog #AM9740
- ✕ 2-Propanol or Isopropanol for molecular biology Merck MilliporeSigma (Sigma-Aldrich) Catalog #I9516-500mL
- ✕ Ethanol Pure 200 proof for molecular biology Merck MilliporeSigma (Sigma-Aldrich) Catalog #E7023-500mL
- ✕ DNA Clean & Concentrator-5 (capped) Zymo Research Catalog #D4014
- ✕ Tris-borate-EDTA buffer (10X) Thermo Fisher Scientific Catalog #15581044
- ✕ DNA LoBind Tubes 2.0 mL Eppendorf Catalog #30108078
- ✕ DNA LoBind Tubes 5mL Eppendorf Catalog #30108310
- ✕ 8-strip PCR tube without cap (0.2mL) VWR International Catalog #20170-002
- ✕ 8-strip PCR tube with individually attached bubble VWR International Catalog #53509-304
- ✕ Corning polypropylene tube (15mL) Fisher Scientific Catalog #0553859B
- ✕ Corning polypropylene tube (50mL) Fisher Scientific Catalog #0553868
- ✕ Reservoir for 8 channel pipettors (25mL) individually wrapped Contributed by users Catalog #28-132
- ✕ Millex-GP syringe filter unit (0.22 µm) Merck Millipore (EMD Millipore) Catalog #SLGP033RS
- ✕ Nanosep 0.2 µm column Pall Catalog #ODM02C35
- ✕ CELLTRICS 30 µm strainer Fisher Scientific Catalog #NC9682496



Scalpel blade Integra Biosciences Catalog #4-410



Dual-chambered counting slide Bio-Rad Laboratories Catalog #145-0011

Equipments List:

- Eppendorf ThermoMixer C with Thermo Top (Eppendorf, cat. no.2231000574)
- IKA MS3 digital orbital shaker, with MS 1.32 tube insert (Coleparmer, cat. no. UX-04304-04)
- Tube revolver/rotator (Thermo Fisher Scientific, cat. no. 88881001)
- 0.2 mL PCR Strip / 1.5 mL Microfuge magnetic separator (Permagen Labware, cat. no. SKU: MSR1224B)
- MiSeq (Illumina)
- MiSeq reagent kit v2 (300 cycles) (Illumina, cat. no. MS-102-2002)
- MiSeq reagent kit v3 (150 cycles) (Illumina, cat. no. MS-102-3001)
- Qubit 3.0 fluorometer (Thermo Fisher Scientific, cat. no. Q33216)
- E1-ClipTip multichannel pipette, 12 channel, 0.5-12.5 μ L, 1-30 μ L and 2-125 μ L (Fisher Scientific, cat. no. 14-387-972TI, 14-387-973TI and 14-387-974TI)
- ClipTip 384 12.5 μ L, 30 μ L and 125 μ L multichannel pipette tip, racked, filter, sterile (Thermo Fisher Scientific, cat. no. 94420053, 94420103 and 94420153)
- Refrigerated centrifuge (Eppendorf)
- Bench top centrifuge (Eppendorf)
- mySPIN mini centrifuge (Thermo Fisher Scientific, cat. no. 75004061)
- CFX96 Touch deep well real-time PCR detection system (BIO-RAD)
- T100 Thermocycler (BIO-RAD, cat. no. 1861096)
- TC20 Automated cell counter (BIO-RAD, cat. no.1450102)
- Dual-chambered counting slide (BIO-RAD, cat. no. 145-0011)
- Eppendorf PCR cooler (Eppendorf, cat. no. 022510525)
- XCell SureLock mini-cell electrophoresis system (Thermo Fisher Scientific, cat. no. EI0001)
- UV transilluminator

Reagent setup

3h

- 40% (wt/vol) PEG 6000.** Weigh 16.0 g of PEG 6000 in 50 mL tube. Add nuclease-free water and bring the total volume to 40 mL. Rotate the tube at room temperature until PEG 6000 completely dissolved. Spin down the tube at 200 g for 2 min, at room temperature to remove the tiny bubble. CRITICAL: 40% (wt/vol) of PEG 6000 is very viscous and difficult to filter through a 0.22 μ m filter. We suggest preparing 40% PEG freshly before making GLR buffer. When PEG is diluted in 4x GLR buffer, it is easier to filter.
- 4x GLR buffer.** To prepare 40 mL of 4x GLR buffer, Add 2.64 mL of nuclease-free water, 10.56 mL of 1 M Tris-HCl, pH 8.0, 0.8 mL of 1 M MgCl₂ and 4 mL of 2 M KCl into 50 mL tube. Gently mix well by vortexing. Add 22 mL of 40% (wt/vol) PEG 6000 and gently mix well by vortexing. Filter through 0.22 μ m into a new 50 mL tube and briefly spin the tube at room temperature for 30 s.

Aliquot 1.8 mL into 2 mL tubes to minimize contamination from each use and store at 4 °C.

- 3 **10% (vol/vol) Triton X-100.** Slowly aspirate 2.0 mL of Triton X-100 liquid with low retention pipette tip and slowly dispense into 18.0 mL nuclease-free water in 50 mL tube. Dissolve Triton X-100 by slowly rotate the tube until the solution is clear. Filter 10% Triton X-100 solution through 0.22 µm syringe filter into a new 50 mL tube and store at room temperature. **CRITICAL:** If it's difficult to pipette Triton X-100 accurately as it's a viscous liquid, we may warm it at 37 °C before pipetting
- 4 **10% (vol/vol) Tween 20.** Tween 20 is very viscous liquid and difficult to pipette accurately. We convert the volume in cm³ into grams using the density of tween 20 at 25 °C is 1.1 cm³. To prepare 20 mL of 10% (vol/vol) Tween 20, weigh 2.2 g of Tween 20 in 50 mL tube. Add 18.0 mL of nuclease-free water and invert or rotate the tube slowly at room temperature until Tween 20 is completely dissolved in water. Filter 10% Tween 20 solution through 0.22 µm syringe filter into a new 50 mL tube and store at room temperature.
- 5 **25 mM dNTP mix.** Mix 250 µL each of 100 mM dATP, dCTP, dGTP and dTTP in 1.5 mL tube. Mix well by vortexing and briefly spin the tube at room temperature for 5 s. Aliquot 250 µL into each of 1.5 mL tube and store at -20 °C for a couple of years
- 6 **2x Lysis buffer.** To prepare 25 mL of 2x Lysis buffer, add 6.5 mL of nuclease-free water into 50 mL tube. Add 0.5 mL of 1 M Tris-HCl, pH 8.0, 2 mL of 5 M NaCl, 5 mL of 0.5 M EDTA and 11 mL of 10% (wt/vol) SDS. Gently mix and aliquot 1.8 mL into 2 mL tubes and store at room temperature.
- 7 **1x B&W-T buffer.** To prepare 40 mL of 1x B&W-T, add 31.56 mL of nuclease-free water into 50 mL tube. Add 200 µL of 1 M Tris-HCl, pH 8.0, 8 mL of 5 M NaCl, 40 µL of 0.5 M EDTA and 200 µL of 10% (vol/vol) Tween 20. Gently mix by vortexing and store at room temperature.
- 8 **2x B&W buffer.** To prepare 25 mL of 1x B&W, add 14.7 mL of nuclease-free water into 50 mL tube. Add 250 µL of 1 M Tris-HCl, pH 8.0, 10 mL of 5 M NaCl, and 50 µL of 0.5 M EDTA. Mix well by vortexing and store at room temperature.
- 9 **0.1 M PMSF.** Weigh 34.8 mg of PMSF and transfer into 2 mL microtube. Add 100% isopropanol to 2 mL and vortex vigorously to dissolve PMSF. Quick spin the tube down and transfer all solution into 3 mL syringe. Filter through a 0.22 µm syringe filter to a new 2 mL tube. Aliquot 50 µL per 0.2 mL PCR tube and store at -20 °C for up to 4 months. **CRITICAL:** To maintain the activity of PMSF in solution, store PMSF in single-use aliquots.
- 10 **Transposon preparation.** Resuspend Nextera adapter 1, 5P-Nextera adapter 2 and mosaic end (ME) oligos with nuclease-free water to 100 µM. Mix 500 µL of 100 µM Nextera adapter 1 and

500 µL of 100 µM ME in 1.5 mL DNA LoBind microtube, aliquot 30 µL of non-annealed transposons into each of 0.2 mL PCR tube and store at -20 °C for up to 1-2 years. Prepare 5P-Nextera adapter 2 and ME the same way as Nextera adapter 1. All sequences of oligos can be found in the attached tables.

- 11 **Samples hashing barcodes preparation.** Resuspend oligonucleotides barcodes with nuclease-free water to 10 pM final concentration and make 20 µL aliquots of each oligo in 0.2 mL PCR tubes.

Oligo name	Sequence
SNARE2- Truhash001	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAAGTGAGTTBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash002	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCCTTCCGGTABAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash003	ACACTCTTTCCCTACACGACGCTCTTCCGATCTTCCGACGCATBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash004	ACACTCTTTCCCTACACGACGCTCTTCCGATCTAGGTAGCACABAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash005	ACACTCTTTCCCTACACGACGCTCTTCCGATCTGAATGCCGGTBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash006	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTCAGGTCTBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash007	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCGTACAGAGCBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA
SNARE2- Truhash008	ACACTCTTTCCCTACACGACGCTCTTCCGATCTCTATATCGTCBAAAAAA AAAAAAAAAAAAAAAAAAAAAAAAAAAA

Hashing Oligos

Round 1 DNA barcoding plates generation

1h

- 12 Accessible chromatin (AC) Round 1 barcoded oligos preparation (Plate A). Prepare 50 µL of 25 µM accessible chromatin (AC) Round 1 barcoded oligos annealed with 23 µM accessible chromatin (AC) Round 1 linker oligos (BC_0100)
 - 12.1 Resuspend AC Round 1 linker (BC_0100) with nuclease-free water to final concentration of 1 mM

- 12.2** Prepare 2.5 mL of 30.67 μ M AC Round 1 linker by adding 76.68 μ L of 1 mM of AC Round 1 linker to 2,423.32 μ L of nuclease-free water in 5 mL tube and mix well by vortexing
- 12.3** Add 12.5 μ L of 100 μ M AC Round 1 barcoded oligos into 96-well plate (total 48 wells, rows A - D) with multichannel pipette
- 12.4** Transfer AC Round 1 linker oligos into 25 mL reservoir
- 12.5** Add 37.5 μ L of 30.67 μ M AC Round 1 linker into each well of AC Round 1 barcodes with multichannel pipette and mix well by pipetting 12 times (mixing volume 45 μ L)
- 12.6** CRITICAL STEP: Seal and spin down the plate on 96-well plate swinging bucket rotor at 160 g for 1min, 4 °C.
- 12.7** Anneal AC Round 1 barcoded oligos and AC linker oligos on thermocycler using the following annealing program: 95 °C for 2 min, slowly cool down to 20 °C (0.1 °C/s) and hold at 4 °C. CRITICAL STEP: After annealing oligonucleotides in the plate, we recommend spinning down the plate and use a sterile needle to punch the holes on the sealing film to release the pressure in every single well. Otherwise, the liquid inside the well will be pulled up to the top of the well when the film is unsealed, and this can lead to barcode cross-contamination. PAUSE POINT: If do not want to continue to mix these annealed oligos in the next step, store that plate at -20 °C
- 13** RNA reverse transcription (RT) Round 1 barcoded oligos preparation (Plate B). Prepare 50 μ L of 25 μ M of oligo (dT)15 and 25 μ M of random hexamer (N6) RT barcoded oligos (see attached tables) mix in each of 48 wells. CRITICAL STEP: 100 μ M of dT and N6 reverse transcription barcoded oligos are ordered in row A-D and row E-H of oligo plate, respectively.
- 13.1** Transfer 12.5 μ L of 100 μ M of rows A-D (dT) in the RT barcoded oligos plate to rows A-D of a new 96-well DNA LoBind plate. CRITICAL STEP If use electronic multichannel pipette, add 1 μ L of air after aspirating to avoid cross-contamination of barcoded oligos. This can be

applied to other steps when we have to transfer barcoded oligos from stock plate to a new plate. Make sure that oligos are delivered to the bottom of the well.

- 13.2** Transfer 12.5 μL of 100 μM of rows E-H oligos (N6) in RT barcoded oligos plate to rows A-D (row E to row A, row F to row B, row G to row C, row H to row D) of 96-well plate that contains dT barcoded oligos above
- 13.3** Pipette 2 mL of nuclease-free water into 25 mL reservoir
- 13.4** Add 25 μL of nuclease-free water to row A-D of RNA Round 1 stock plate and mix well by pipetting 12 times (mixing volume 45 μL)
- 13.5** Seal and spin down the plate at 160 g for 1 min, 4 °C and leave the plate on ice or PCR cool rack
- 14** AC Round 1 barcoded oligos and RT Round 1 barcoded oligos mix. The final concentration of each oligo (dT, N6, AC) in the oligo mix is 12.5 μM .
- 14.1** Transfer 50 μL of oligos in plate B (RT Round 1 barcoded oligos) into plate A that contain 50 μL of annealed AC Round 1 barcoded oligos and linker at identical well IDs (rows A-D) and mix well by pipetting 12 times (mixing volume 90 μL)
- 14.2** Spin down the plate at 160 g for 1 min, 4 °C and put the plate on PCR cool rack
- 14.3** Aliquot 4 μL of mixed Round 1 barcoded oligos (rows A-D) into 10-25 of 96-well plates as “working plates”

- 14.4** Spin down working plates at 160 g for 1 min, 4 °C and store at -20 °C for up to a couple of years. Store the left over Round 1 stock plate at -20 °C.

Round 2 DNA barcoding plates generation

1h

- 15** Ligation Round 2 barcoded oligos. Prepare stock plate of 100 μ L of 18 μ M ligation Round 2 barcoded oligos annealed with 16.5 μ M ligation Round 2 linker (BC_0215).
- 16** Resuspend ligation Round 2 linker (BC_0215) with nuclease-free water to final concentration of 1 mM
- 17** Prepare 9 mL of 20.12 μ M ligation Round 2 linker by adding 181.08 μ L of 1 mM round 2 linker to 8,818.9 μ L of nuclease-free water in 15 mL tube and mix well by vortexing
- 18** Add 18 μ L of 100 μ M of ligation round 2 barcoded oligos into 96-well plate (rows A-H)
- 19** Transfer ligation Round 2 linker into 25 mL reservoir
- 20** Add 82 μ L of 20.12 μ M ligation Round 2 linker to each well of ligation Round 2 barcoded oligos with and mix well by pipetting 10 times (mixing volume 90 μ L)
- 21** Seal and spin down the plate at 160 g for 1min, 4 °C

- 22 Anneal ligation Round 2 barcoded oligos and Round 2 linker on thermocycler using the annealing program for Round 1 barcoded oligos and keep on ice
- 23 Spin down the plate at 160 g for 1min, 4 °C and keep on ice
- 24 Aliquot 10 µL of annealed ligation Round 2 barcoded/linker oligos into 10 of 96-well plate as “working plate”
- 25 Spin down the working plate at 160 g for 1min, 4 °C before store at -20°C for up to a couple of year

Round 3 DNA barcoding plates generation

1h

- 26 Ligation Round 3 barcoded oligos. Prepare stock plate of 100 µL of 21 µM Round 3 barcoded oligos annealed with 19.5 µM ligation Round 3 linker (BC_0060).
- 27 Resuspend ligation Round 3 linker (BC_0060) with nuclease-free water to final concentration of 1 mM
- 28 Prepare 8.5 mL of 24.68 µM ligation Round 3 linker by adding 209.8 µL of 1 mM Round 3 linker to 8,290.2 µL of nuclease-free water in 15 mL tube and mix well by vortexing
- 29 Add 21 µL of 100 µM of ligation Round 3 barcoded oligos into 96-well plate (rows A-H)

- 30** Transfer ligation Round 3 linker into 25 mL reservoir
- 31** Add 79 μL of 24.68 μM ligation Round 3 linker to each well of ligation Round 3 barcoded oligos and mix well by pipetting 12 times (mixing volume 90 μL)
- 32** Seal and spin down the plate at 160 g for 1min, 4 °C
- 33** Anneal ligation Round 3 barcoded oligos and Round 3 linker on thermocycler using the annealing program for Round 1 barcoded oligos and keep on ice
- 34** Spin down the plate at 160 g for 1min, 4 °C and keep on ice
- 35** Aliquot 10 μL of annealed ligation Round 3 barcoded/linker oligos into 10 of 96-well plate as “working plate”
- 36** Spin down the working plate at 160 g for 1min, 4 °C before store at -20°C for up to a couple of year

Transposase preparation

2h

37


Note


Convert Tn5 transposase (Diagenode Tagmentase, Diagenode #C01070010-20, EZ-Tn5 Transposase, Lucigen #TNP92110, or expressed/purified with an in house protocol). For example, if the concentration of unloaded Tn5 is 0.40 mg/mL, the monomer concentration in μM is $7.55 \mu\text{M}$ based on molar mass of monomer Tn5 $\sim 53,000 \text{ g/mol}$ [$0.4 \text{ mg/mL} * (1 \text{ mol}/53,000 \text{ g})$]. **CRITICAL STEP:** Anneal transposons and load Tn5 on the day of experiment 1-2 h prior experiment starts. Combine transposons and mix well before adding Tn5 to make sure that both transposons are sufficiently homogeneous prior to mixing with Tn5. Avoid generating bubbles when mixing Tn5 with transposons by pipetting slowly and do not fill pipette tip with air. If the experiment is not ready, store loaded Tn5 at -20°C .


38 Thaw non-annealed transposons (Nextera adapter 1/ME and 5P-Nextera adapter 2/ME) on ice. Briefly vortex and quick spin the tube. Anneal transposons on thermocycler using following program: 95°C 5 min, slowly cool down to 65°C (0.1°C/s), 65°C 5 min, slowly cool down to 4°C (0.1°C/s) and hold at 4°C .


39 Load transposons 1.5x molar ratio to Tn5. The amount below is sufficient for tagmenting ~ 1.8 million nuclei/cells.

30m


 13 μL 37.72 μM monomer Tagmentase (2mg/mL)

 7.35 μL 50 μM Annealed Nextera adapter 1/ME

 7.35 μL 50 μM Annealed 5P-Nextera adapter 2/ME

 39 μL Tagmentase Dilution Buffer

Add annealed Nextera adapter 1/ME and annealed 5P-Nextera adapter 2/ME into the bottom of 1.5 mL DNA LoBind microtube, mix well by pipetting 10 times or gently vortexing and briefly spin the tube on mini centrifuge for 3 s. Add unloaded Tn5 and mix by gently pipetting 20 times (set the volume of p200 pipette to 80 μL). Quick spin the tube and incubate at 25°C for 0.5 h, 350 rpm. **The final concentration of loaded Tn5 is 7.35 μM** (monomer Tn5 concentration).

 00:30:00

Nuclei isolation and fixation

3h


40 Isolate nuclei from tissue following tissue-specific nuclei extraction protocol (dx.doi.org/10.17504/protocols.io.ufketkw) with 0.1 U/ μL of SUPERase In RNase Inhibitor and 0.2 U/ μL of Enzymatics RNase Inhibitor. For cell lines, nuclei can be extracted with ATAC Lysis buffer with 0.1% NP-40 as previously described with the addition of RNase inhibitors, and


increase lysis volume proportional to the number of input cells.

41 To each tube of 0.5 mL volumes of nuclei suspensions, add 15 μ L of hashing oligos. Make a note of which oligo was added to which sample. Mix briefly, centrifuge at 500 g for 8 minutes at 4C, remove 400 μ L of supernatant. Then pool up to 8 barcoded samples

42 Prepare 1x PBS + RI (1 mL per sample) and keep on ice.


 1000 μ L PBS, pH 7.4

 2.5 μ L SUPERase In (20 U/ μ L)

 1.25 μ L Enzymatics Rnase In (40 U/ μ L)


43 Prepare 1% (wt/vol) formaldehyde in 1x PBS (1 mL per sample) and keep on ice. CRITICAL: formaldehyde solution should be in 1x PBS and methanol free.

 937.5 μ L PBS, pH 7.4

 62.5 μ L Formaldehyde, 16% wt/vol

44 Resuspend 1-2 million nuclei with 1 mL 1x PBS + RI and keep on ice. To each tube of 1 mL volumes of nuclei suspensions, add 15 μ L of hashing oligos. Make a note of which oligo was added to which sample. Mix briefly.

45 Add 1 mL of 1% formaldehyde to nuclei suspension and pipette gently 8 times. Leave the tube on ice for 10 minutes.

 00:10:00 Fixation

46 Pellet nuclei at 900 g for 8 min at 4C using bucket rotor centrifuge.

47 Prepare 1x PBS + 0.1% (wt/vol) BSA + RI (1 mL per sample) and leave on ice.

 1000 μ L PBS, pH 7.4

13.4 μ L BSA, 7.5%
wt/vol

1.5 μ L SUPERase In (20
U/ μ L)

0.75 μ L Enzymatics RNase In (40
U/ μ L)

48 Prepare 1x Tango Buffer + RI (1 mL per ~ 3 million nuclei/cells) and leave on ice.

100 μ L Tango Buffer,
10x

160 μ L DMF,
100%

5 μ L SUPERase In
(20U/ μ L)

2.5 μ L Enzymatics RNase In (40
U/ μ L)

732.5 μ L Nuclease-free water

49 Aspirate the supernatant, resuspend pelleted nuclei with 0.1 mL of 1x PBS + 0.1% BSA + RI to wash. Pool up to 8 samples, ~800 μ L total max. Add wash buffer to 1 mL total volume.

50 Pellet nuclei at 900 g for 8 min at 4C using bucket rotor centrifuge.

51 Aspirate the supernatant and resuspend with 1x Tango Buffer + RI to have a minimum concentration of 3,400 nuclei per microliter.

52 Count the nuclei using cell counter and resuspend the nuclei solution with additional 1x Tango Buffer + RI to obtain 3,400 nuclei per microliter.

Tagmentation

1h

- 53** Set up tagmentation mix per reaction as follows (1 reaction per 150,000 nuclei/cells). Minimum 4 reactions for 1 sample per plate (48 wells of round 1).

3 µL Tango Buffer,
10x

4.8 µL DMF,
100%

4.95 µL Loaded Tn5,
7.35uM

1.5 µL SUPERase In (20
U/uL)

0.75 µL Enzymatics RNase In (40
U/uL)

15 µL Nuclease-free water

- 54** Prepare 150,000 nuclei in 45 µL of 1x Tango Buffer and mix with 30 µL of tagmentation mix. The final concentration of Tn5 and DMF in final reaction is 0.8 µM and 16% (vol/vol), respectively. The ratio of nuclei suspension and tagmentation mix is 3 : 2 or 45 µL : 30 µL. Add nuclei into 1.5 mL DNA LoBind tube then add tagmentation mix and mix gently by pipetting 10 times. Briefly spin the tube on mini centrifuge at room temperature for 3 s and aliquot 75 µL of tagmentation reaction into 1.5 mL DNA LoBind tube.

- 55** CRITICAL STEP: Set up 4 tubes of tagmentation reactions to have enough nuclei for Round 1 barcoding (8,000 nuclei/well x total 48 wells = 384,000 nuclei). Set up the reactions on ice. Do not incubate tagmentation reaction in large volume to make sure that nuclei are distributed evenly in the reaction not sitting on the bottom of the tube when incubating during tagmentation.

- 56** Place the tubes on thermomixer and incubate at 37 °C for 30 min, 300 rpm.

00:30:00 Tagmentation

- 57** Before incubation is complete, prepare 1x PBS + 0.1% (wt/vol) BSA + RI (1 mL per 400 µL tagged nuclei) and keep on ice

1000 µL PBS, pH 7.4

13.33 µL BSA, 7.5%
wt/vol

1.5 µL SUPERase In (20
U/uL)

0.75 μ L Enzymatics RNase In (40 U/ μ L)

- 58 Remove the tubes from thermomixer and place on ice. Then pool tagmented nuclei of the same sample into the same tube.
- 59 Add 2.5x volume of 1x PBS + 0.1% (wt/vol) BSA + RI (1000 μ L to 400 μ L tagmented nuclei) to pooled tagmented nuclei and mix by pipetting gently 5 times and centrifuge at 900 g for 8 min, 4 °C with swinging bucket rotor
- 60 During centrifugation, prepare 0.5x PBS + RI (1 mL per 1 million nuclei/cells) and keep on ice
- 500 μ L PBS, pH 7.4
 - 500 μ L Nuclease free water
 - 2.5 μ L SUPERase In (20 U/ μ L)
 - 1.25 μ L Enzymatics RNase In (40 U/ μ L)
- 61 CRITICAL STEP Aspirate supernatant and resuspend nuclei with 300 μ L of 0.5x PBS + RI to have a minimum concentration of nuclei not lower than 1,000 nuclei/ μ L. If different numbers of nuclei in tagmentation are used, adjust suspension volume. Pipetette gently to resuspend
- 62 Count nuclei concentration with cell counter and dilute nuclei to 1,000 nuclei/ μ L with 0.5x PBS + RI.

Accessible chromatin (AC) oligo ligation

45m

- 63 Thaw Round 1 AC/RT oligo working plate on ice and spin the plate on swinging bucket at 200 g for 1 min, 4 °C and leave the plate on ice. Note: Can leave in 4C at the beginning of Day 1 to thaw.
- 64 Prepare GLR-A mix following table below. CRITICAL STEP: Prepare GLR-A mix during washing tagmented nuclei, but add ATP, RNase inhibitor and T7 DNA Ligase just before ready to load into

Round 1 barcoding plate

🧴 260 μ L GLR Buffer, 4x

🧴 20.8 μ L ATP,
100mM

🧴 52 μ L dNTPs, 25 mM
each

🧴 13 μ L SUPERase In
(20U/ μ L)

🧴 6.76 μ L Enzymatics RNase In,
(40U/ μ L)

🧴 10.4 μ L DTT, 1M

🧴 78 μ L T7 DNA Ligase
(3000U/ μ L)


🧴 1.04 μ L Nuclease-free water

- 65 Add 8 μ L of nuclei to each well (row A – D) with a multichannel pipette.
- 66 Aliquot 73 μ L of GLR-A into 6 tube-strip on PCR cool rack and add 8.5 μ L of GLR-A mix to each well with a multichannel pipette.
- 67 Seal and quick spin the plate at 160 g for 15 s, 4 °C. Gently mix reaction mix, Round 1 barcoded oligos and nuclei 5 times (mixing volume 18 μ L)
- 68 Seal and quick spin the plate at 160 g for 10 s, 4 °C and incubate the plate on thermomixer at 25 °C for 30 min, 300 rpm
- 🕒 00:30:00 AC oligo ligation

Reverse transcription

45m

- 69 Aliquot 20 μ L of Maxima H Minus Reverse Transcriptase into 6 tubes of PCR strip tube

 120 μ L Maxima H Minus RT (200 U/ μ L)


- 70** Remove Round 1 barcoding plate from thermomixer, put on PCR cool rack, and add 2.2 μ L of Maxima H Minus Reverse Transcriptase to each well with a multichannel pipette. **CRITICAL STEP:** To get accurate volumes, set the speed of multichannel pipette to be very slow for aspirating and dispensing as the enzyme is very viscous
- 71** Seal and quick spin the plate at 160 g for 15 s, 4 °C to bring enzyme to the bottom of the well and mix by gently pipetting 5 times (mixing volume 18 μ L). Seal and quick spin the plate at 160 g for 10 s, 4°C
- 72** Incubate the plate on thermocycler using the program: 50°C for 10 min, 3 cycles of (8°C for 12 s, 15°C for 45 s, 20°C for 45 s, 30°C 30 s, 42°C for 2 min, 50°C for 3 min), 50°C for 5 min.


Round 2 DNA barcoding


1h 30m

- 73** Before reverse transcription finishes, prepare 3 mL of 1x PBS + 0.1% BSA + RI

 3000 μ L PBS, pH 7.4


 40 μ L BSA, 7.5% wt/vol

 4.5 μ L SUPERase In (20 U/ μ L)

 2.25 μ L Enzymatics RNase In (40 U/ μ L)

- 74** Remove the plate from thermocycler and put on ice or PCR cool rack then pool all reactions into a chilled 25 mL reservoir and transfer pooled reaction into a chilled 5 mL DNA LoBind tube. **CRITICAL STEP:** Before pooling, pipette Round 1 barcoding plate 2 times to kick up nuclei from the bottom of the well and make sure that you transfer all reactions from the well by aspirating slowly and set pipette volume to 26 μ L. This technique should be done for all pooling steps. Also keep reactions on ice all the time when handling samples or reaction mixes to prevent RNA degradation and preserve enzyme activity or temperature sensitive reagents like ATP.
- 75** Add 2.8 mL of 1x PBS + 0.1% (wt/vol) BSA + RI (2.5x volume) to rinse the basin and transfer buffer to the tube


- 76** Add 19.5 μL of 10% (vol/vol) Triton X-100 (final concentration of Triton X-100 is 0.05% (vol/vol)) and mix by inverting the tube 5 times before centrifuge at 900 g for 8 min, 4 °C


 19.5 μL Triton X-100, 10%
vol/vol

- 77** Thaw Round 2 barcoded oligos working plate on ice and spin the plate at 200 g, for 1 min, 4 °C and leave the plate on ice. Note: Can leave in 4C at the beginning of Day 1 to thaw.


- 78** Prepare 1x Buffer 3.1 as follows:


 210 μL NEBuffer 3.1,
10x


 21 μL Enzymatics RNase In
(40U/ μL)


 1890 μL Nuclease free
water


- 79** Prepare Ligation Mix as follows:


 510 μL T4 DNA Ligase Buffer,
10x

 40.8 μL Enzymatics RNase In
(40U/ μL)

 12.75 μL SUPERase In
(20U/ μL)

 51 μL BSA (20
mg/mL)

 127.5 μL T4 DNA Ligase
(400U/ μL)


 1338.8 μL Nuclease free
water

- 80** Remove supernatant as much as possible (~20 μL left) and resuspend nuclei with 2.02 mL of 1x Buffer 3.1 and add 2.04 mL of Ligation mix and mix by pipetting 10 times


81 Transfer nuclei in ligation mix into 25 mL reservoir and add 40 μ L of nuclei suspension into each well of Round 2 barcoding plate and mix gently by pipetting 5 times (mixing volume 45 μ L)


82 Seal the plate with sealing film and quick spin the plate at 160 g for 10 s, 4 °C


83 Incubate the plate on thermomixer at 37 °C for 30 min, 300 rpm

 00:30:00 Round 2
barcoding

84 Prepare Round 2 blocking solution as follows:


 47.52 μ L BC_0216, 1000
uM

 300 μ L T4 DNA Ligase Buffer,
10x

 852.5 μ L Nuclease free
water

85 Remove Round 2 DNA barcoding plate from thermomixer and quick spin at 160 g for 10 s, 4 °C. Add 10 μ L of Round 2 blocking solution to each well with multichannel pipette and mix by pipetting gently 5 times (mixing volume 55 μ L)

86 Seal the plate with sealing film and quick spin at 160 g for 10 s, 4°C then incubate the plate on thermomixer at 37 °C for 30 min, 300 rpm


 00:30:00 Round 2
blocking

Round 3 DNA barcoding

1h


87 Thaw Round 3 barcoded oligos working plate on ice, spin the plate on swinging bucket at 200 g for 1 min, 4 °C and leave the plate on ice

- 88** Place Round 2 DNA barcoding plate on PCR cool rack and pool into 25 mL reservoir. Add 100 μ L of T4 DNA Ligase (400 U/ μ L) into the basin with nuclei from Round 2 barcoding plate and mix well by gently pipetting 10 times and rock the basin from side-to-side 10 times


 100 μ L T4 DNA Ligase, (400 U/ μ L)

- 89** Add 50 μ L of nuclei suspension to each well of Round 3 DNA barcoding plate and mix gently by pipetting 5 times (mixing volume 55 μ L).


- 90** Seal the plate with sealing film and briefly spin the plate at 160 g for 10 s, 4°C. Incubate the plate on thermomixer at 37 °C for 30min, 300 rpm

 00:30:00 Round 3 barcoding

- 91** Prepare Round 3 blocking solution as follows:

 41.4 μ L BC_0066, 1000 μ M

 600 μ L EDTA, 500mM


 1758.6 μ L Nuclease free water


- 92** Remove Round 3 DNA barcoding plate from thermomixer and add 20 μ L of Round 3 blocking solution to each well and gently mix by pipetting 3 times (mixing volume 75 μ L)

- 93** Without incubation, pool the reaction into 25 mL reservoir placed on ice, transfer supernatant into 15 mL tube and centrifuge at 1,000 g for 8 min, 4°C. OPTIONAL: EDTA in the reaction inhibits ligase activity, therefore there is no need to change the pipette tips when pooling Round 3 ligation reaction. Set pipetting volume 85 μ L when pooling nuclei.

- 94** In parallel, prepare chilled wash buffer as follows.

 4000 μ L PBS, pH 7.4

 40 μ L Triton X-100, 10% vol/vol


 10 μ L SUPERase In
(20U/ μ L)


94.1 Also in parallel, thaw 2X Lysis buffer at 37°C for ~15 minutes if previously prepped. If not see step 6 for recipe.

95 Remove supernatant and add 4 mL of wash buffer. Pipette gently 5 times with p1000 pipette then centrifuge at 1,000 g for 8 min, 4 °C

96 Carefully remove supernatant as much as possible and resuspend nuclei with 300 μ L of 1x PBS + RI

 500 μ L PBS, pH 7.4

 5 μ L SUPERase In (20
U/ μ L)


 2.5 μ L Enzymatics Rnase In (40
U/ μ L)

97 Count nuclei concentration with cell counter and aliquot nuclei at required number in each pool in 1.5 mL DNA LoBind tube and adjust total volume of nuclei to 50 μ L with 1x PBS + RI. CRITICAL STEP: To sequence all nuclei, recommend using $\leq 20,000$ cells per pool by splitting 50 μ L of nuclei suspension into 6 - 12 tubes per plate.

Nuclei lysis


2h


98 Add 50 μ L of 2x Lysis buffer and 10 μ L of 20 mg/mL Proteinase K to each pool, mix well by gently vortexing and brief spin the tube down at room temperature for 10 s to collect all nuclei suspension to the bottom of the tube and incubate on thermomixer at 55 °C for 2 h, 350 rpm to lyse nuclei and reverse crosslink formaldehyde fixation.

 02:00:00 Nuclei
lysis


99 Put nuclei lysate at -80 °C to inactivate Proteinase K before continue to day 2 experiment. CRITICAL STEP: Lysis buffer tends to precipitate at room temperature or low temperature. Redissolve by incubating the tube at 37 °C until it completely dissolves before adding to nuclei suspension. PAUSE POINT: Nuclear or cell lysate can be store at -80 °C for up to 6 months before continue to day 2 experiment.

100 Prepare 3.5 mL of 1x B&W-T + RI for bead washing:

 3500 µL B&W-T,
1x

 5 µL SUPERase In
(20U/µL)

101 Vortex the bottle of Dynabeads MyOne C1 thoroughly and aliquot required volume (44 µL/pool * number of pool) into 1.5 mL tube. Add 800 µL of 1x B&W-T + RI, mix by vortexing and pulse spin on mini centrifuge at room temperature for 3 s

 44 µL MyOne C1
beads


102 Place the tubes onto the magnetic rack until liquid is clear; Remove supernatant with p1000 pipette


103 Resuspend the beads with 800 µL of 1x B&W-T + RI, vortex and pulse spin on mini centrifuge at room temperature for 3 s

104 Place the tubes onto the magnetic rack until solution is clear and remove supernatant with p1000 pipette

105 Repeat washes two more times (total of 3 washes)


106 Resuspend the beads with 100 µL of 2x B&W + RI per pool:


 100 µL 2x B&W-
T

 2 µL SUPERase In
(20U/µL)


107 During bead preparation, take the tubes of lysate out of -80 °C and place onto thermomixer set at 55 °C for 2 min until lysate is completely thawed

108 Add 5 µL of 0.1 M PMSF (from -20 °C) to each tube, pulse vortex for 10 s and pulse spin on mini centrifuge at room temperature for 5 s and incubate at room temperature for 10 min with no shaking

 5 µL PMSF, 100 mM


 00:10:00


109 Add 100 µL of streptavidin beads in 2x B&W + RI to each tube of lysate (no pipetting required) then agitate the tubes on mixer at room temperature for 1 h, 1,200 rpm. CRITICAL STEP: We recommend to use orbital shaker for microtubes. The speed of mixer can be adjusted as long as the beads do not settle on the bottom of the tube

 01:00:00 Binding to beads


110 Pulse spin the tubes on mini centrifuge at room temperature for 5 s, place onto magnetic rack until solution is clear and remove supernatant with p200 pipette. CRITICAL STEP: Every time before placing the tube back to the magnet, quick spin the tubes on mini centrifuge for 3-5 s to collect all supernatant/lysate/buffer and beads to the bottom of the tube. Use p200 pipette to remove supernatant to avoid disturbing the beads and prevent bead loss.

111 Prepare 1 mL of 1x B&W-T + RI (750 uL per lysate) for bead washing as follows:

 1000 µL B&W-T, 1x

 1.4 µL SUPERase In (20U/µL)

112 Add 250 µL of 1 x B&W-T + RI and agitate the bead at room temperature for 5 min, 1,500 rpm to wash the beads

 00:05:00 wash with 1x B&W-T + RI

113 Place the tube onto magnetic rack until solution is clear and remove supernatant. Continue to


template switching oligo blocking on AC DNA immediately.


Blocking template switching oligo

30m

- 114** During working on Steps 109-110, prepare Nextera adapter 1 blocking solution per pool:

 250 μ L SSC, 6x

 2.5 μ L Nextera adapter 1 blocker, 100 μ M

 1 μ L SUPERase In (20U/ μ L)

- 115** Add 250 μ L of 6x SSC to each tube without bead suspension and wait until the supernatant is clear then remove supernatant with p200 pipette


 250 μ L SSC, 6x


- 116** Add 250 μ L of Nextera adapter 1 blocking solution to each tube and agitate the tubes on mixer at room temperature for 1 min, 1,500 rpm then reduce the speed to 500 rpm for 14 min. CRITICAL STEP: Agitate the tubes at high speed 1,500 rpm for 1 min to make sure the beads are resuspended well, then shake gently at 500 rpm for 14 min to allow hybridization of Nextera adapter 1 blocker and Nextera adapter 1 on AC DNA and make sure that the beads do not settle.

 00:15:00 incubate with Nextera Blocking Solution

- 117** Place the tube onto magnetic rack until solution is clear and remove supernatant with p200 pipette.

- 118** Wash the beads twice with 1x B&W-T + RI as described above at room temperature for 5 min each round.

 00:05:00 wash with 1x B&W-T + RI

 00:05:00 wash #2 with 1x B&W-T + RI

- 119** In parallel, prepare Tris-T + RI during second bead wash

250 µL Tris-HCl, pH 8.0, 10 mM

2.5 µL Tween 20, 10% vol/vol

0.63 µL SUPERase In (20U/µL)

- 120** Wash the beads with 250 µL of Tris-T + RI (5 min) the same way as 1x B&W-T + RI wash (at room temperature for 5 min, 1,500 rpm). In parallel, prepare GLR-B mix. CRITICAL STEP: If GLR-B mix is not ready, leave the beads in the tube with Tris-T + RI on ice until GLR-B mix is ready

00:05:00 wash with Tris-T + RI

Gap filling, ligation and complete reverse transcription 2h 15m

- 121** During washing, prepare GLR-B mix as follows:

50 µL GLR buffer, 4x

4 µL ATP, 100mM

20 µL Ficoll PM 400, 20% wt/vol

10 µL dNTPs, 25 mM each

5 µL SUPERase In (20U/µL)


2 µL DTT, 1M

12.5 µL Hemo Klentag

2.5 µL T7 DNA Ligase (3000U/µL)


89 µL Nuclease free water

- 122** Place the tubes onto magnetic rack until solution is clear, remove supernatant with p200 pipette and add 250 µL of nuclease-free water to each tube without bead suspension


 250 µL Nuclease free water


123 Remove water and resuspend the beads with 195 µL of GLR-B mix by gently vortexing and quick spin the tubes on mini centrifuge for 3 s

124 Rotate the tubes in incubator at 37 °C with slow speed for 15 min to allow for gap filling on AC DNA and ligate Nextera adapter 1 blocker to the AC DNA. OPTIONAL: For any step required rotator, thermomixer can be alternatively used as long as the tube is shaken gently and the beads do not settle.


 00:15:00 Gap filling and ligation

125 Remove the tubes from incubator and add each tube with 5 µL of 100 µM TSO and 5 µL of Maxima H Minus reverse transcriptase and mix well by gently vortexing.


 5 µL TSO oligo, 100 µM

 5 µL Maxima H Minus RT, (200 U/µL)

126 Continue to incubate at room temperature for 30 min with slow rotation.

 00:30:00 complete reverse transcription








127 Incubate at 42 °C for 90 min with slow rotation.

 01:30:00 complete reverse transcription

128 PAUSE POINT: The beads can be stored in Tris-T buffer at 4 °C overnight before continuing to 1st PCR (Remove supernatant and replace with Tris-T buffer). However, we recommend to continue to the 1st PCR immediately if possible.

First PCR, cDNA/DNA amplification

1h 30m

- 129** Before finishing 42°C incubation, set up the first PCR mix to amplify both cDNA and accessible chromatin (AC) DNA and prepare PCR strip tubes with individual hinged cap (4 tubes per pool):
-  110 µL KAPA HiFi HotStart ReadyMix, 2x
 -  6.9 µL BC_0108, 10 uM
 -  17.6 µL BC_0062, 10 uM
 -  6.9 µL BC_0082, 10 uM
 -  6.9 µL Hash_PCR, 10 uM
 -  74.8 µL Nuclease free water
- Note:
Sequence of Hash_PCR: ACACTCTTTCCTACACGACGCTCTTCCGATCT
- 130** After finishing incubation at 42 °C for 90 min, place the tubes onto the magnetic rack until liquid is clear and remove supernatant with p200 pipette
- 131** Add 250 µL of nuclease-free water to each tube without bead resuspension. Once liquid is clear, remove supernatant.
-  250 µL Nuclease free water
- 132** Resuspend the beads with 220 µL of first PCR mix, quick spin and aliquot 55 µL of bead suspension in PCR mix to each of 4 PCR strip tubes. Transfer all the leftover beads to 4 PCR tubes equally. CRITICAL STEP: Transfer the beads in PCR mix directly to the bottom of the tube, so there is no need to spin the tube before placing on thermocycler. Try to transfer PCR mix with the beads into PCR strip tubes as quick as possible to minimize bead settling before the reaction starts.
- 133** Place the tubes on thermocycler and run following program: 95 °C for 3 min, 9 cycles of (98 °C for 20 s, 58 °C for 45 s, 72 °C for 3 min), 72 °C for 5 min, 4 °C hold.
- 134** PAUSE POINT PCR reaction can be stored at -20 °C for a month or 4 °C for a week.


- 135** Place strip tubes onto 0.2 mL magnetic rack until supernatant is clear and pool 1st round PCR products from 4 strip tubes of the same pool into 1.5 mL DNA LoBind tube
- 136** Vortex the tube and quick spin on mini centrifuge for 3 s before splitting PCR products into two parts (100 μ L each), “AC” for chromatin accessibility (AC) library preparation and “R” for RNA library preparation. Note: AC and R in this step are the same PCR products but will be bead size-selected at different bead volume ratio.


sn/scATAC libraries: purification and validation

1h


- 137** Perform one round of 1.2x KAPA Pure Beads purification following manufacturer’s instructions by using 120 μ L of KAPA Pure Beads with 100 μ L of PCR products and elute with 40 μ L of nuclease-free water


1. Binding

 120 μ L KAPA Pure Beads


 00:08:00


2. Washing

 180 μ L Ethanol, 100%


 00:00:30

3. 2nd wash

 180 μ L Ethanol, 100%


 00:00:30


4. Drying

 00:01:00

 37 °C

5. Elution

 40 μ L Nuclease free water

 00:10:00

 37 °C

- 138** Transfer eluent into new PCR strip tubes. The resultant is called AC-A.


- 139** Use 2 μ L of AC-A product to determine DNA concentration with Qubit dsDNA HS assay kit following manufacturer’s instruction

- 140** Verify ~10 ng of AC-A in 6% TBE gel and run in 1x TBE buffer at 250 volts for 23 min with 0.5 μ L of Low DNA Mass Ladder as reference


sn/scATAC libraries: 2nd PCR and library preparation

6h 30m


- 141** In PCR strip tubes, use 5 ng of AC-A as template for enrichment of AC DNA over cDNA and adjust volume of template to 10 μ L with nuclease-free water and quick spin down the tubes. **CRITICAL STEP:** If the concentration of PCR product is higher than 5 ng/ μ L, it tends to have high error to pipette the volume smaller than 1 μ L. We recommend to aliquot AC-A, dilute into 0.5 ng/ μ L in total 30-50 μ L and use 10 μ L as template for AC 2nd PCR.


 10 μ L AC-A DNA (total 5 ng)

- 142** Prepare PCR mix as follows:


 25 μ L KAPA HiFi HotStart ReadyMix, 2x

 2.5 μ L SPLiT_N701, 10 μ M

 2.5 μ L EvaGreen, 20x

 7.5 μ L Nuclease free water

- 143** Add 37.5 μ L of PCR mix and 2.5 μ L of 10 μ M Ad1_N50X (attached tables) into the tube with AC-A template

 2.5 μ L Ad1_N5XX, 10 μ M

- 144** Mix by gently vortexing, quick spin PCR tubes on mini centrifuge at room temperature for 3 s and run qPCR on real-time PCR machine: 95 $^{\circ}$ C for 3 min, 12 (or fewer) cycles of (98 $^{\circ}$ C for 20 s, 58 $^{\circ}$ C for 45 s, 72 $^{\circ}$ C for 1 min), 72 $^{\circ}$ C for 5 min, 4 $^{\circ}$ C hold.

- 145** PAUSE POINT: PCR reaction can be stored at -20 $^{\circ}$ C for a month or 4 $^{\circ}$ C for a week.

- 146** Purify PCR product with DNA Clean & Concentrator following manufacturer's instructions and elute with 40 μ L of DNA Elution Buffer. Resultant is called AC-B
- 147** Use 2 μ L of AC-B to determine DNA concentration with Qubit dsDNA HS assay kit following manufacturing's instruction
- 148** Verify ~10 ng of AC-B in 6% TBE gel and run in 1x TBE buffer at 250 volts for 23 min with 0.5 μ L of Low DNA Mass Ladder as a reference in separate lane. CRITICAL STEP: We expect to see nucleosome pattern with larger size (~125 bp larger) compare to standard ATAC-seq due to the presence of cell barcodes and linker sequences
- 149** Pool equimolar ratio of AC-B libraries (~200-250 ng/pool) and perform PAGE size-selection at the size between 300-1,000 bp.
- 150** Use 2 μ L of AC sequencing libraries to determine for DNA concentration with Qubit dsDNA HS assay
- 151** Verify ~5 -10 ng of AC sequencing libraries in 6% TBE gel and run in 1x TBE buffer at 250 volts for 23 min or by TapeStation

sn/scATAC libraries: Quality validation by MiSeq sequencing^{1d}

- 152** Validate AC sequencing libraries with MiSeq sequencing using v2 reagent kit by loading at 20 pm based on Qubit dsDNA HS quantification with at least 5% PhiX spike in following Illumina loading guide. The sequencing configuration is 75 cycles for read 1, 94 cycles for index 1, 8 cycles for index 2, and 75 cycles for read 2.

Note

CRITICAL STEP: For MiSeq sequencing, quantification of sequencing library concentration by Qubit and determination of average library size is sufficient. For high-throughput sequencing, we recommend to run sequencing libraries on Bioanalyzer, TapeStation or equivalent instrument that can determine accurate average library size and contamination of adapter dimers peaks at approximately 125-175 bp. We recommend to run qPCR to quantify sequencing library concentration using the average library size derived from TapeStation. For a two-channel sequencing system such as NovaSeq, we recommend to spike-in with minimum of 10% PhiX or consult sequencing core.

- 153** Mix 1:1 ratio of "SN2-AC R1" sequencing primer pairs for sequencing read 1 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).

3 µL SNARE2_Read1, 100
uM

3 µL PhiX_Read1, 100
uM

594 µL Hybridization buffer,
HT1

- 154** Mix 1:1 ratio of "SN2-AC R2" sequencing primer pairs for sequencing index 1 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).

3 µL SNARE2-AC_BCread, 100
uM

3 µL PhiX_Read1, 100
uM

594 µL Hybridization buffer,
HT1

- 155** Mix 1:1 ratio of "SN2-AC R4" sequencing primer pairs for sequencing read 2 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).


3 µL SNARE2-AC_Read2, 100
uM


3 µL PhiX_Read2, 100
uM

594 µL Hybridization buffer,
HT1


- 156** Perform 1 round of 0.8x KAPA Pure Beads purification following manufacturer's instructions by mixing 100 μ L of 1st PCR product + with 80 μ L of KAPA Pure Beads and elute with 40 μ L of nuclease-free water. Resultant is called R-A.


1. Binding

 80 μ L KAPA Pure Beads


 00:08:00


2. Washing

 180 μ L Ethanol, 100%


 00:00:30

3. 2nd wash

 180 μ L Ethanol, 100%


 00:00:30


4. Drying

 00:01:00

 37 $^{\circ}$ C

5. Elution

 40 μ L Nuclease free water

 00:10:00

- 157** Use 2 μ L of R-A to determine DNA concentration with Qubit dsDNA HS assay following manufacturer's instructions


- 158** Verify 5-10 ng of R-A in 6% TBE gel and run in 1x TBE buffer at 250 volts for 23 min with 0.5 μ L of Low DNA Mass Ladder as a reference


sn/scRNA libraries: 2nd PCR, purification, and validation


2h

- 159** In PCR strip tubes, use 5 ng of R-A as template for enrichment of cDNA over AC DNA and adjust the volume of template to 10 μ L with nuclease-free water and quick spin down the tubes. CRITICAL STEP: If the concentration of PCR product is higher than 5 ng/ μ L, it tends to have high error to pipette the volume smaller than 1 μ L. We recommend to aliquot R-A, dilute into 0.5 ng/ μ L in total 30-50 μ L and use 10 μ L as template for RNA 2nd PCR


- 160** Prepare PCR mix for second round amplification of cDNA as follows:

 25 μ L KAPA HiFi HotStart ReadyMix, 2x

 2 μ L BC_0108, 10 uM

 2 μ L BC_0062, 10 uM

 2.5 μ L EvaGreen, 20x

 8.5 μ L Nuclease free water



161 Add 40 μ L of PCR mix to each tube, gently vortex and quick spin PCR tubes on mini centrifuge at room temperature for 3 s

162 Run the reactions on real-time PCR using following program: 95 °C for 3 min, 12 (or fewer) cycles of (98 °C for 20 s, 67 °C for 45 s, 72 °C for 3 min), 72 °C for 5 min, 4 °C hold.



163 PAUSE POINT PCR reaction can be stored at -20 °C for a month or 4 °C for a week.

164 Purify PCR products with 1 round of 0.8x KAPA Pure Beads and elute with 40 μ L of nuclease-free water. The resultant purified DNA is called R-B



1. Binding

 40 μ L KAPA Pure Beads  00:08:00

2. Washing

 180 μ L Ethanol, 100%  00:00:30

3. 2nd wash

 180 μ L Ethanol, 100%  00:00:30

4. Drying

 00:01:00  37 °C

5. Elution

40 µL Nuclease free water

00:10:00

37 °C

165 Determine DNA concentration with Qubit dsDNA HS assay using 2 µL of R-B

166 Verify ~5-10 ng of R-B in 6% TBE gel by running at 250 volts for 23 min with 0.5 µL of Low DNA Mass Ladder as a reference. CRITICAL STEP: If the smear of R-B is between 375 bp and above, continue to cDNA tagmentation. If there is strong smear smaller than 375 bp, repeat another round of 0.8x KAKA Pure Beads and elute with 40 µL of nuclease-free water.

Hash libraries: 2nd PCR, purification, and validation

2h

167 In PCR strip tubes, use 5 ng of R-A as template for enrichment of hashing libraries and adjust the volume of template to 10 µL with nuclease-free water and quick spin down the tubes. CRITICAL STEP: If the concentration of PCR product is higher than 5 ng/µL, it tends to have high error to pipette the volume smaller than 1 µL. We recommend to aliquot R-A, dilute into 0.5 ng/µL in total 30-50 µL and use 10 µL as template for 2nd PCR

168 Set up 2nd PCR mix during incubation

25 µL KAPA Hifi Hotstart ReadyMix, 2X

2.5 µL HASH_L0X, 10 uM

2.5 µL EvaGreen, 20X

7.5 µL Nuclease free water

Sequences of HASH_L0X (pick one of L01-3):

HASH_L01:

AATGATACGGCGACCACCGAGATCTACACGTAACATGCGACACTCTTCCCTACACGACG

HASH_L02:

AATGATACGGCGACCACCGAGATCTACACGTGGATCAAAACACTCTTCCCTACACGACG

HASH_L03:

AATGATACGGCGACCACCGAGATCTACACCACTACGAAAACACTCTTCCCTACACGACG

169 Add 37.5 µL of PCR mix and 2.5 µL of 10 µM SPLiT_N7XX (attached tables) primers into the tube with R-A template


 2.5 µL SPLiT_N7XX, 10
uM


170 Run PCR on thermocycler using program follows: 72 °C for 3 min, 98 °C for 30 s, 9-12 cycles of (98 °C for 30 s, 63 °C for 30 s, 72 °C for 45 s), 72 °C for 3 min, 4 °C hold. Terminate the reaction when it reaches mid-exponential growth.

171 Pool all reactions together, purify an aliquot of 50 uL with 1X beads. Elute purified hash libraries with 30 µL of nuclease-free water.


20m


1. Binding

 50 µL KAPA Pure
Beads


 00:08:00


2. Washing

 180 µL Ethanol,
100%


 00:00:30

3. 2nd wash

 180 µL Ethanol,
100%


 00:00:30


4. Drying

 00:01:00

 37 °C

5. Elution

 30 µL Nuclease free
water

 00:10:00

 37 °C

172 Use 1 µL of hash sequencing libraries to determine for DNA concentration with Qubit dsDNA HS assay

173 Verify ~ 5 ng of hash libraries in 6% TBE gel at 250 volts for 23 min with 0.5 µL of Low DNA Mass Ladder as a reference.

sn/scRNA libraries: cDNA tagmentation and library preparat..

3h

174 Mix ME and Nextera Adapter 1 (Ad1) oligos 1:1 for final 50 uM each. Briefly vortex and quick spin the tube. Anneal transposons on thermocycler using following program: 95 °C 5 min, slowly cool down to 65 °C (0.1 °C/s), 65 °C 5 min, slowly cool down to 4 °C (0.1 °C/s) and hold at 4 °C.

- 175** Load Tn5 (In-house, 11.32 uM)
- 20 µL Tn5, (In-house, 11.32 uM)
 - 6.8 µL 50 uM, Nextera Adapter 1/ME
- 176** Incubate 1 hour at room temperate with gentle shaking. 1h
- 01:00:00
- 177** Prepare Tagmentation mix as follows in 1.5 mL tube and aliquot 18 µL per pool into 0.2 mL PCR strip tubes with hinged cap.
- 2 µL Tango Buffer, 10X
 - 2 µL DMF, 100%
 - 12 µL Nuclease free water
 - 2 µL Loaded Tn5, 8.44 uM
- 178** Transfer 2 µL of diluted cDNA (total 10-20 ng) with multichannel pipette into tagmentation mix, mix by pipetting 10 times, gently vortex and quick spin on mini centrifuge at room temperature for 5 s
- 179** Place the tubes on thermocycler that set at 55 °C for 7 min with the heated lid 7m
- 00:07:00
- 180** Remove the tube from thermocycler and stop reaction by adding 5 µL of 0.2 % (wt/vol) SDS, mix by pipetting 5 times, gently spin down the tubes on mini centrifuge for 5 s and incubate at room temperature for 5 min
- 5 µL 0.2% SDS
- 00:05:00

181 Set up Tagmentation PCR mix during incubation

25 µL KAPA HiFi Hotstart ReadyMix,
2X

2.5 µL BC_0118, 10
uM

2.5 µL EvaGreen,
20X

7.5 µL Nuclease free
water

182 Add 2.5 SPLiT_N7XX (attached tables) primers to 10 uL tagmented cDNA, then 37.5 of Tagmentation PCR mix respectively, mix well and gently spin down the tube on mini centrifuge for 3 s

2.5 µL SPLiT_N7XX, 10
uM

10 µL tagmented cDNA

37.5 µL Tagmentation PCR
Mix

183 Run PCR on thermocycler using program follows: 72 °C for 3 min, 98 °C for 30 s, 9-12 cycles of (98 °C for 30 s, 63 °C for 30 s, 72 °C for 45 s), 72 °C for 3 min, 4 °C hold. Terminate the reaction when it reaches mid-exponential growth.

20m

184 Purify PCR products at least two rounds of 0.7x KAPA Pure beads to make sure that no adapter dimers are leftover in the sequencing libraries. Elute the last round of bead purified RNA libraries with 30 µL of nuclease-free water. CRITICAL STEP: Adapter dimers can cluster more efficiently than regular sequencing libraries and is more sensitive on the patterned flow cell such as NovaSeq S4. This can lead to the reduction of sequencing output and sequencing quality

1. Binding

35 µL KAPA Pure
Beads

00:08:00

2. Washing

180 µL Ethanol,
100%

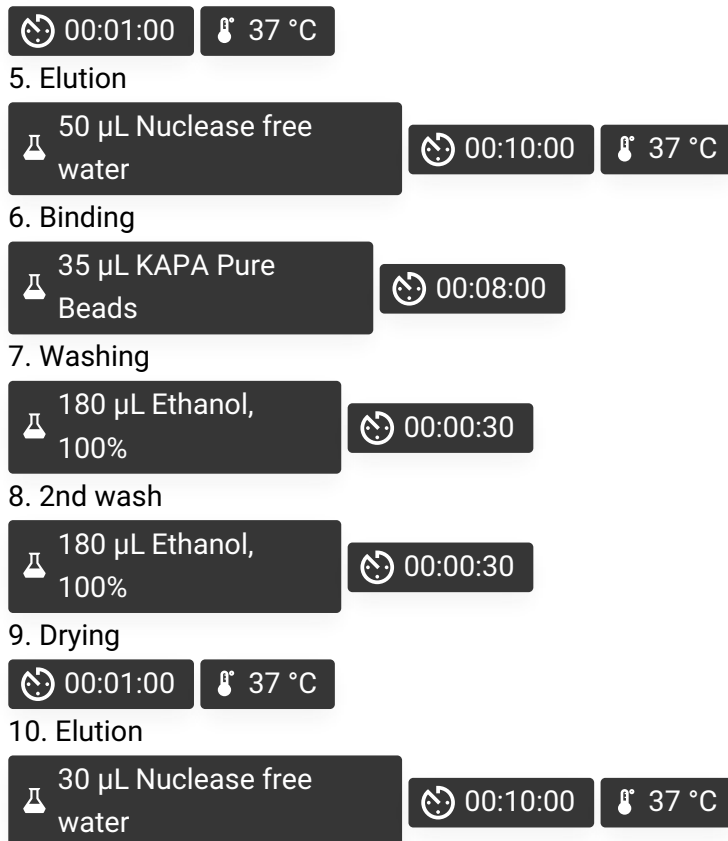
00:00:30

3. 2nd wash

180 µL Ethanol,
100%

00:00:30

4. Drying



- 185** Use 1 μ L of RNA sequencing libraries to determine for DNA concentration with Qubit dsDNA HS assay
- 186** Verify ~ 5 ng of RNA libraries in 6% TBE gel at 250 volts for 23 min with 0.5 μ L of Low DNA Mass Ladder as a reference

sn/scRNA and hash libraries: Quality validation by MiSeq sequencing ^{1d}

- 187** Validate sequencing libraries with MiSeq sequencing using v3 reagent kit by loading at 22 pm based on Qubit dsDNA HS quantification with at least 5% PhiX spike. The sequencing configuration is 70 cycles for read 1, 6 cycles for index, and 102 cycles for read 2.a

Note

CRITICAL STEP: For MiSeq sequencing, quantification of sequencing library concentration by Qubit and determination of average library size is sufficient. For high-throughput sequencing, we recommend to run sequencing libraries on Bioanalyzer, TapeStation or equivalent instrument that can determine accurate average library size and contamination of adapter dimers peaks at approximately 125-175 bp. We recommend running qPCR to quantify sequencing library concentration using the average library size derived from TapeStation. For a two-channel sequencing system such as NovaSeq, we recommend spike-in with a minimum of 10% PhiX or consult sequencing core.

- 188** Mix 1:1 ratio of "SN2-R R1" sequencing primer pairs for sequencing read 1 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).

3 µL SNARE2_Read1, 100 uM

3 µL PhiX_Read1, 100 uM

(Hash libraries uses the same Read 1 sequencing primer as PhiX)

594 µL Hybridization buffer, HT1

- 189** Mix 1:1 ratio of "SN2-R Index1" sequencing primer pairs for sequencing index 1 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).

3 µL SNARE2-R_Index1

3 µL PhiX_Read1

594 µL Hybridization buffer, HT1

- 190** Mix 1:1 ratio of "SN2-R R2" sequencing primer pairs for sequencing read 2 on Illumina workflow A (HiSeq 2500, MiSeq, NovaSeq 6000 platforms).

3 µL SNARE2-R_Read2

3 µL PhiX_Read2

594 µL Hybridization buffer, HT1