

scifi-RNA-seq step-by-step protocol (v2021-01-25)

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Future updates of the protocol will be shared via <http://scifi-rna-seq.bocklab.org> and announced via <https://twitter.com/BockLab>.

Preparations prior to the experiment

Prepare custom i7 transposome (Appendix A) and corresponding buffers (Appendix F), alternatively Illumina Nextera XT reagents can be used as described in step 10B
For fixing cells, cool a 50 ml aliquot of 100% methanol at 4 °C
Cool a 500 ml bottle of 1x PBS at 4 °C
Thaw an aliquot of 10% BSA at 4 °C overnight in the fridge
Prepare 10% IGEPAL CA-630 solution (5 ml in 15 ml Falcon, vortex some minutes to dissolve, store in the dark at 4°C)
Prepare plates with barcoded reverse transcription primers (1 µl of 25 µM per well, store at -20°)

Buffer preparations on the day of the experiment

For scifi-RNA-seq without fixation, use the tables in Appendix D to prepare:

- PBS-BSA-SUPERase
- Nuclei Preparation Buffer
- Nuclei Wash Buffer

If following the nuclei fixation protocol, use the tables in Appendix E to prepare:

- Nuclei Preparation Buffer without Digitonin and without Tween-20
- Nuclei Wash Buffer without Tween-20
- 1% Formaldehyde in 1xPBS
- PBS-BSA-SUPERase

DAY 1

1A Whole cell fixation (for cell lines)

<Note> Cool centrifuge to 4 °C and pellet cells at 300 rcf for 5 min throughout

Transfer 5 million cells in growth medium to a 15 ml tube and pellet
Wash once with 10 ml of ice-cold 1x PBS (resuspending carefully) and pellet
Fix cells in 5 ml ice-cold methanol at -20 °C for 10 min
Wash 2x with 5 ml ice-cold PBS-BSA-SUPERase
Resuspend cells in 200 µl of ice-cold PBS-BSA-SUPERase
Filter through cell strainer (40 µm or 70 µm depending on cell size)
Use 5 µl of the sample for cell counting (in duplicates), dilute to 5,000 cells/µl with ice-cold PBS-BSA-SUPERase
Proceed immediately to step 2 (reverse transcription)

1B Nuclei isolation (for cell lines)

Transfer 5 million cells in growth medium to a 15 ml tube and pellet (300 rcf, 5 min, room temperature)
Wash once with 10 ml of ice-cold 1x PBS and pellet (300 rcf, 5 min, 4 °C)
Resuspend in 500 µl ice-cold Nuclei Preparation Buffer
Incubate on ice for 5 min to lyse plasma membrane
Add 5 ml of ice-cold Nuclei Wash Buffer and pellet (500 rcf, 5 min, 4 °C)
Resuspend nuclei in 200 µl of ice-cold PBS-BSA-SUPERase
Filter through cell strainer (40 µm or 70 µm depending on cell size)
Use 5 µl of the sample for nuclei counting (in duplicates), dilute to 5,000 nuclei/µl with ice-cold PBS-BSA-SUPERase
Proceed immediately to step 2 (reverse transcription)

1C Nuclei isolation with formaldehyde fixation (for primary cells, allows freezing)

Transfer 5 million cells in growth medium to a 15 ml tube and pellet (300 rcf, 5 min, room temperature)

Wash once with 10 ml of ice-cold 1x PBS and pellet (300 rcf, 5 min, 4 °C)

Carefully resuspend in 500 µl ice-cold Nuclei Preparation Buffer without Digitonin and without Tween-20

Incubate on ice for 5 min to lyse plasma membrane

Add 5 ml of ice-cold Nuclei Wash Buffer without Tween-20 and pellet (500 rcf, 5 min, 4 °C)

Remove supernatant, resuspend in 5 ml of ice-cold 1% Formaldehyde in 1x PBS

<Note> The exact percentage of formaldehyde should be optimized for the cell type and sample processed

Fix on ice for 15 min, then spin immediately (500 rcf, 5 min, 4 °C)

Remove supernatant (pellet is hard to see in this step)

Add 1.5 ml of ice-cold Nuclei Wash Buffer without Tween-20, mix and transfer to 1.5 ml tube

Spin (500 rcf, 5 min, 4 °C)

Wash one more time with 1.5 ml of ice-cold Nuclei Wash Buffer without Tween-20

Spin (500 rcf, 5 min, 4 °C)

Remove supernatant, carefully resuspend pellet in 200 µl of ice-cold Nuclei Wash Buffer without Tween-20

<Note> Do not use stickers to label the tubes. They come off during the snap-freezing.

Snap-freeze in liquid nitrogen, then store at -80 °C <Safe Stopping Point>

Thaw frozen samples in a water bath at 37 °C for exactly 1 min, then immediately place on ice

Spin (500 rcf, 5 min, 4 °C)

Remove supernatant, and resuspend pellet in 250 µl of ice-cold Permeabilization Buffer, incubate for 5 min on ice

Add 250 µl of ice-cold Nuclei Wash Buffer without Tween-20 per sample

Spin (500 rcf, 5 min, 4 °C)

Wash with 250 µl of ice-cold Nuclei Wash Buffer without Tween-20 per sample

Spin (500 rcf, 5 min, 4 °C)

Take up in 100 µl of ice-cold PBS-BSA-SUPERase

Use 5 µl of the sample for nuclei counting (in duplicates), dilute to 5,000 nuclei/µl with ice-cold PBS-BSA-SUPERase

Proceed immediately to step 2 (reverse transcription)

2 Reverse transcription (round 1 indexing)

<Note> Avoid harsh centrifugation, spin down plates for a maximum of 30 sec at 50 rcf

Set thermocycler to 55 °C (incubation mode, with heated lid set to 60 °C)

Thaw plate containing barcoded reverse transcription primers (1 µl of 25 µM per well) and place on ice

Add 10,000 cells or nuclei (2 µl per well) to the pre-dispensed primer, record well assignments

Incubate for 5 min at 55 °C, then place on ice immediately (denaturation of RNA secondary structures)

Prepare Reverse Transcription Master Mix as described below and distribute into 12-tube strip on ice

Reverse Transcription Master Mix							
Component	Per reaction		Number of 96WP				384WP
	Volume (µl)	Amount	1	2	3	4	
Nuclease-free water	3	to 10 µl	330	660	990	1320	1416
RT buffer, 5x	2	1x	220	440	660	880	944
DTT, 100 mM	0.5	5 mM	55	110	165	220	236
dNTPs, 10 mM	0.5	0.5 mM	55	110	165	220	236
RNaseOUT RNase inhibitor (40 U/µl)	0.5	20 U	55	110	165	220	236
Maxima H Minus reverse transcriptase (200 U/µl)	0.5	100 U	55	110	165	220	236
Dispense into 12-tube strip for multichannel			62	123	185	246	250

Add 7 µl of Reverse Transcription Master Mix with a multichannel pipette and slowly pipette two times to mix

Seal and spin plate (maximum of 30 sec at 50 rcf)

Incubate (with heated lid set to 60 °C):

50 °C for 10 min
3 Cycles { 8 °C for 12 sec
15 °C for 45 sec
20 °C for 45 sec
30 °C for 30 sec
42 °C for 2 min
50 °C for 3 min }
50 °C for 5 min
store at 4 °C

3 Pooling and cell counting

Cool two centrifuges suitable for 15 ml tubes and 1.5 ml tubes to 4 °C, thaw 10x Ampligase Buffer (Lucigen)

Remove reverse transcription plate(s) from thermocycler, place on ice

Add 40 µl (96WP) or 20 µl (384WP) of 1xPBS-1%BSA per well, then pool via a tube strip in a 15 ml tube on ice

Wash with an additional 40 µl (96WP) or 20 µl (384WP) of 1xPBS-1%BSA per well, add to pool for maximum recovery

Fill up to 15 ml with 1xPBS-1%BSA

Pellet for 5 min at 500 rcf at 4 °C and carefully remove supernatant

Slowly resuspend nuclei in 1.0 ml of 1x Ampligase Buffer (dilute Lucigen stock 1/10 with H₂O)

Filter through cell strainer (40 µm or 70 µm depending on cell size) into 1.5 ml tube

Pellet for 5 min at 500 rcf at 4 °C and remove supernatant almost completely without disturbing the pellet

<Note> Aim for a remaining volume < 15 µl, which is the maximum input for the Thermocycling Ligation

Centrifuge for 30 sec at 500 rcf at 4 °C to bring the remaining liquid to the bottom of the tube

Dilute sample prior to cell counting (typically a 1:200 dilution of 1 µl sample + 199 µl 1x Ampligase Buffer is countable)

Load 20 µl into a Fuchs Rosenthal counting chamber (Incyto DHC-F01)

Count five squares per sample and calculate the cell concentration as follows

Cells per µl = [(Cells in five squares / 5) x 200 (dilution factor) x 5000 (volume factor)] / 1000 (converts to cells per µl)

4A Thermocycling ligation (round 2 indexing) using Chromium scATAC v1.0 reagents

<Note> Some parts of this section were adapted from the 10x Genomics documentation:

CG000168_ChromiumSingleCellATAC_ReagentKits_UserGuide_RevA.pdf

<Note> scifi-RNA-seq was tested for loading amounts of up to 765,000 nuclei per microfluidic channel

<Note> The number of single-cell transcriptomes recovered depends on quality and transcript content of the sample

Transfer up to 765,000 pre-indexed cells/nuclei to a tube-strip on ice

Bring the volume to 15 µl with 1x Ampligase Buffer

Thaw the following reagents from the 10x Chromium Single Cell ATAC v1.0 kit:

scATAC Gel Beads v1.0, Reducing Agent B

Place Chromium Chip E in 10x Chip Holder and close

For unused channels: add 75 µl (row 1), 40 µl (row 2) and 240 µl (row 3) of 50% glycerol solution

Prepare Thermoligation Master Mix as described below, keep on ice

Thermoligation Master Mix for scATAC v1.0 chips and reagents									
Component	Per reaction Volume (µl)	Number of channels (+ 10% overhead)							
		1	2	3	4	5	6	7	8
Nuclease-free water	47.4	47.4	104.3	156.4	208.6	260.7	312.8	365.0	417.1
Ampligase Buffer, 10x	11.5	11.5	25.3	38.0	50.6	63.3	75.9	88.6	101.2
Ampligase Enzyme (100 U/µl)	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2
Reducing Agent B	1.5	1.5	3.3	5.0	6.6	8.3	9.9	11.6	13.2
Bridge Oligo, 100 µM	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2

Add 65 µl of Thermoligation Master Mix to each sample, bringing the volume to 80 µl
 Mix gently, then load 75 µl into Row 1 of the chip (bottom center, avoid air bubbles), wait > 30 sec to allow priming
 In the meantime, vortex 10x Gel Beads for 30 sec (use 10x Vortex Adapter) and bring contents to bottom of the wells
 Load 40 µl of Gel Beads into Row 2 of the chip (bottom center, avoid air bubbles)
 Dispense 240 µl of Partitioning Oil into Row 3 of the chip (pipette along the wall of the inlet)
 Attach gasket, aligning the notch with the top left-hand corner of the chip
 Run chip on the 10x Chromium Controller (takes ~ 7 minutes, immediately proceed with next step)
 Place 8-tube strip on ice
 Remove chip from instrument, discard gasket and fold back chip holder lid to present wells at 45 degrees
 Transfer 100 µl of the emulsion into the tube strip on ice (very slowly, should take 20 sec in both directions)
 Incubate emulsion droplets in thermocycler (lid: 105 °C, volume: 100 µl) according to the program below:
 12 Cycles { 95 °C for 30 sec
 59 °C for 2 min }
 store at 15 °C

During the incubation:

Thaw Cleanup Buffer for 10 min at 65 °C, check for precipitate, cool to room temperature
 Equilibrate to room temperature: Reducing Agent B, Dynabeads MyOne Silane, SPRIselect beads
 Proceed immediately with the cleanup steps

4B Thermocycling ligation (round 2 indexing) using Chromium scATAC v1.1 (Next GEM) reagents

<Note> Some parts of this section were adapted from the 10x Genomics documentation:

CG000209_Chromium_NextGEM_SingleCell_ATAC_ReagentKits v1.1_UserGuide_RevD.pdf

<Note> We confirmed compatibility with up to 100-fold droplet overloading (1.53 million nuclei per microfluidic channel) and our thermoligation mechanism for the new Next GEM chip (Chromium chip H) and reagents

<Note> The number of single-cell transcriptomes recovered depends on quality and transcript content of the sample

Transfer up to 765,000 pre-indexed cells/nuclei to a tube-strip on ice
 Bring the volume to 15 µl with 1x Ampligase Buffer
 Thaw the following reagents from the 10x Chromium Single Cell ATAC v1.1 (Next GEM) kit:
 scATAC Gel Beads v1.1 (Next GEM), Reducing Agent B
 Place Chromium Chip H in 10x Next GEM Chip Holder and close
 For unused channels: add 70 µl (row 1), 50 µl (row 2) and 40 µl (row 3) of 50% glycerol solution

Prepare Thermoligation Master Mix as described below, keep on ice

Thermoligation Master Mix for scATAC v1.1 (Next GEM) chips and reagents									
Component	Per reaction	Number of channels (+ 10% overhead)							
	Volume (µl)	1	2	3	4	5	6	7	8
Nuclease-free water	41.9	41.9	92.2	138.3	184.4	230.5	276.5	322.6	368.7
Ampligase Buffer, 10x	12	12	26.4	39.6	52.8	66.0	79.2	92.4	105.6
Ampligase Enzyme (100 U/µl)	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2
Reducing Agent B	1.5	1.5	3.3	5.0	6.6	8.3	9.9	11.6	13.2
Bridge Oligo, 100 µM	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2

Add 60 µl of Thermoligation Master Mix to each sample, bringing the volume to 75 µl
 Mix gently, then load 70 µl into Row 1 of the chip (bottom center, avoid air bubbles), wait > 30 sec to allow priming
 In the meantime, vortex scATAC Gel Beads v1.1 (Next GEM) for 30 sec (use 10x Vortex Adapter) and bring contents to bottom of the wells
 Load 50 µl of Gel Beads into Row 2 of the chip (bottom center, avoid air bubbles)
 Dispense 40 µl of Partitioning Oil into the outlet of the chip (labelled as Row3, bottom center, avoid air bubbles)

Attach gasket, aligning the notch with the top left-hand corner of the chip
 Run chip on the 10x Chromium Controller (takes ~ 18 minutes, immediately proceed with next step)
 Place 8-tube strip on ice
 Remove chip from instrument, discard gasket and fold back chip holder lid to present wells at 45 degrees
 Transfer 100 µl of the emulsion into the tube strip on ice (very slowly, should take 20 sec in both directions)
 Incubate emulsion droplets in thermocycler (lid: 105 °C, volume: 100 µl) according to the program below:
 12 Cycles { 95 °C for 30 sec
 59 °C for 2 min }
 store at 15 °C

During the incubation:

Thaw Cleanup Buffer for 10 min at 65 °C, check for precipitate, cool to room temperature
 Equilibrate to room temperature: Reducing Agent B, Dynabeads MyOne Silane, SPRIselect beads
 Proceed immediately with the cleanup steps

5.1 Silane bead cleanup

<Note> From here onwards, the protocol is identical for scATAC v1.0 and v1.1 (Next GEM) reagents

Add 125µl of Recovery Agent to each sample at room temperature

Invert ten times to mix, centrifuge briefly

<Note> This results in a pink oil phase at the bottom and a smaller aqueous phase containing the sample on top

Slowly remove 125 µl of the pink oil phase from the bottom of the tube, discard

Prepare Dynabead Cleanup Master Mix as described below

Dynabead Cleanup Mix									
Component	Per reaction Volume (µl)	Number of channels (+ 10% overhead)							
		1	2	3	4	5	6	7	8
Cleanup Buffer	182	182	400.4	600.6	800.8	1001.0	1201.2	1401.4	1601.6
Dynabeads MyOne SILANE	8	8	17.6	26.4	35.2	44.0	52.8	61.6	70.4
Reducing Agent B	5	5	11.0	16.5	22.0	27.5	33.0	38.5	44.0
Nuclease-free water	5	5	11.0	16.5	22.0	27.5	33.0	38.5	44.0

Add 200 µl of Dynabead Cleanup Mix per sample, pipet mix

Incubate for 10 min at room temperature

Prepare Elution Solution I as described below

Elution Solution I									
Component	Per reaction Volume (µl)	Number of channels (+ 10% overhead)							
		1	2	3	4	5	6	7	8
Buffer EB	49	49	107.8	161.7	215.6	269.5	323.4	377.3	431.2
10% Tween 20	0.5	0.5	1.1	1.65	2.2	2.75	3.3	3.85	4.4
Reducing Agent B	0.5	0.5	1.1	1.65	2.2	2.75	3.3	3.85	4.4

Place tube strip on magnetic separator (position: high) until supernatant clears

Remove and discard supernatant along with white precipitate at the bottom of the tube

Wash with 300 µl of 80% ethanol for 5 seconds, then wash with 200 µl of 80% ethanol for 5 seconds

Spin tube strip, place on magnetic separator (position: low) and remove residual ethanol

Remove from magnet and immediately add 40.5 µl Elution Solution I

<Note> Depending on the sample and number of cells/nuclei, clumps can form. Shear them with a thin pipette tip. If clumps cannot be suspended this way, try the column-based cleanup described in Appendix G.

Incubate for 1 min at room temperature to elute sample from beads

Place on magnetic separator (position: low) and transfer 40 µl of each sample to a fresh tube strip

5.2 SPRI cleanup

Add 40 μ l of SPRIselect beads (ratio 1.0x) per sample

Pipet mix and incubate for 5 min at room temperature to allow DNA binding to the beads

Place on magnetic separator (position: high) until supernatant clears

Wash twice with 200 μ l of 80% ethanol

Spin tube strip briefly, then place on magnetic separator (position: low) and remove residual ethanol

Resuspend beads in 22 μ l of EB Buffer, and incubate 2 min at room temperature to elute sample from beads

Place on magnetic separator (position: low) and transfer 20 μ l of each sample to a fresh tube strip

6 Template switching

Prepare Template Switching Master Mix as described below

Template Switching Master Mix									
Component	Per reaction Volume (μ l)	Number of reactions (+ 10% overhead)							
		1	2	3	4	5	6	7	8
RT buffer, 5x	10	10	22	33	44	55	66	77	88
Ficoll PM-400, 20%	10	10	22	33	44	55	66	77	88
dNTPs, 10 mM	5	5	11	16.5	22	27.5	33	38.5	44
Recombinant Ribonuclease Inhibitor	1.25	1.25	2.75	4.13	5.5	6.88	8.25	9.63	11
Template Switching Oligo, 100 μ M	1.25	1.25	2.75	4.13	5.5	6.88	8.25	9.63	11
Maxima H Minus reverse transcriptase (200 U/ μ l)	2.5	2.5	5.5	8.25	11	13.75	16.5	19.25	22

Add 30 μ l of Template Switching Master Mix per sample, mix and incubate (heated lid: 60 °C):

25 °C for 30 min

42 °C for 90 min

store at 4 °C

<Safe stopping point> Samples can be left in the thermocycler overnight, and stored at 4 °C for several days.

DAY 2

7 SPRI cleanup (1.0x)

Add 50 µl of AMPure XP beads (ratio 1.0x) per sample

Pipet mix and incubate for 10 min at room temperature to allow DNA binding to the beads

Place on magnetic separator until supernatant clears

Wash two times with 100 µl of 80% ethanol

Remove residual ethanol by pipetting

Resuspend beads in 17 µl of EB Buffer, and incubate 2 min at room temperature to elute sample from beads

Place on magnetic separator and transfer 15 µl of each sample to a fresh tube strip

8 cDNA enrichment

Prepare cDNA Enrichment Master Mix as described below

cDNA Enrichment Master Mix									
Component	Per reaction Volume (µl)	Number of reactions (+ 10% overhead)							
		1	2	3	4	5	6	7	8
Nuclease-free water	33	33	72.6	108.9	145.2	181.5	217.8	254.1	290.4
NEBNext High Fidelity 2x Master Mix	50	50	110	165	220	275	330	385	440
Partial-P5 Primer, 100 µM	0.5	0.5	1.1	1.65	2.2	2.75	3.3	3.85	4.4
TSO Enrichment Primer, 100 µM	0.5	0.5	1.1	1.65	2.2	2.75	3.3	3.85	4.4
SYBR Green, 100x in DMSO	1	1	2.2	3.3	4.4	5.5	6.6	7.7	8.8

Add 85 µl of cDNA Enrichment Master Mix per sample, bringing the volume to 100 µl

Run reactions in qPCR cycler (heated lid: 105 °C)

<Note> When samples reach > 1000 RFU, pause run after the plate read and transfer samples to second thermocycler. Then resume run for any remaining samples that need further enrichment. We are using a BioRad C1000 Touch thermocycler and CFX96 qPCR module. RFU values corresponding to sufficient cDNA might be system-specific.

98 °C for 30 sec

Cycle and stop when RFU > 1000 RFU {
98 °C for 20 sec
65 °C for 30 sec
72 °C for 3 min, plate read }

72 °C for 5 min (in second thermocycler)

store at 4 °C

9 cDNA cleanup (0.8x, 0.6x) and quality control

Add 80 µl of AMPure XP beads (ratio 0.8x) per sample

Pipet mix and incubate for 10 min at room temperature to allow DNA binding to the beads

Place on magnetic separator until supernatant clears

Wash two times with 100 µl of 80% ethanol and remove residual ethanol by pipetting

Resuspend beads in 42 µl of EB Buffer, and incubate 2 min at room temperature to elute sample from beads

Place on magnetic separator and transfer 40 µl of each sample to a fresh tube strip

Add 60 µl of nuclease-free water to bring the sample volume to 100 µl

Add 60 µl of AMPure XP beads (ratio 0.6x) per sample

Pipet mix and incubate for 10 min at room temperature to allow DNA binding to the beads

Place on magnetic separator until supernatant clears

Wash two times with 100 µl of 80% ethanol and remove residual ethanol by pipetting

Resuspend beads in 17 µl of EB Buffer, and incubate 2 min at room temperature to elute sample from beads

Place on magnetic separator and transfer 15 µl of each sample to a fresh tube strip

Use 1 µl of each sample to measure the concentration of double-stranded DNA with the Qubit HS assay

Load 1.5 ng on the Bioanalyzer High Sensitivity DNA chip to check the size distribution of the cDNA

<Note> The size distribution of a typical cDNA library is shown in Appendix B, Figure 1a

10A Tagmentation with i7-only transposome

<Note> We have tested the Illumina Nextera XT kit (Illumina #15032350) as replacement for the custom i7-only transposome, and obtained comparable results. The use of Nextera reagents (which are a mix of i5 and i7 transposomes) results in a 50% loss of library fragments, but this does not seem to affect the library quality since cDNA is already enriched at this point. In this step, we describe the i7-only tagmentation, which we consider conceptually cleaner, cheaper and more versatile. In step 10B, we describe the tagmentation with Illumina Nextera XT reagents.

Dilute cDNA to 0.2 ng/μl with nuclease-free water

Per tagmentation reaction, distribute 5 μl (1 ng) into a 96-well plate on ice

<Note> Tagment entire sample for maximum library complexity. Typically, 6-15 tagmentations of 1 ng are required.

Prepare Tagmentation Master Mix as described below

Tagmentation Master Mix		
Component	Per reaction [μl]	Number of reactions (+ 10% overhead) <input type="text"/> x
Nuclease-free water	11.25	
Tn5 Reaction Buffer, 5x	5	
Dimethylformamide	2.5	
Freshly diluted i7-only transposome (1:4.5 in Tn5 Dilution Buffer)	1.25	

Add 20 μl of Tagmentation Master Mix per well and mix by pipetting

Incubate for 10 min at 55 °C

Place reactions on ice for 1 min

Add 2.5 μl of 1% SDS solution, mix by pipetting and incubate for 5 min at room temperature

Add 22.5 μl of nuclease-free water per well

Add 50 μl of AMPure XP beads (ratio 1.0x) per sample

Pipet mix and incubate for 10 min at room temperature to allow DNA binding to the beads

Place on magnetic separator until supernatant clears

Wash two times with 100 μl of 80% ethanol, then remove residual ethanol by pipetting

Resuspend beads in 17 μl of EB Buffer, and incubate 2 min at room temperature to elute sample from beads

Place on magnetic separator and transfer 15 μl of each sample to a fresh plate

Add 5 μl of 10 μM barcoded P7 primer per well

<Note> If processing samples for a 2-color sequencer (such as the Illumina NovaSeq 6000 platform) sequencing complexity of Index1 can be increased by using multiple P7 primers per sample.

Prepare Enrichment PCR Master Mix as described below

Enrichment PCR Master Mix		
Component	Per reaction [μl]	Number of reactions (+ 10% overhead) <input type="text"/> x
NEBNext High Fidelity 2x Master Mix	50	
Partial-P5 Primer, 100 μM	0.5	
SYBR Green, 100x in DMSO	1	
Nuclease-free water	28.5	

Primer name	Sequence	Index*
Partial-P5	AATGATACGGCGACCACCGAGA	No index
P7-001	CAAGCAGAAGACGGCATAACGAGATTCGCCTTAGTCTCGTGGGCTCGG	TAAGGCGA
P7-002	CAAGCAGAAGACGGCATAACGAGATCTAGTACGGTCTCGTGGGCTCGG	CGTACTAG
P7-003	CAAGCAGAAGACGGCATAACGAGATTCTGCCTGTCTCGTGGGCTCGG	AGGCAGAA
P7-004	CAAGCAGAAGACGGCATAACGAGATGCTCAGGAGTCTCGTGGGCTCGG	TCCTGAGC
P7-005	CAAGCAGAAGACGGCATAACGAGATAGGAGTCCGTCTCGTGGGCTCGG	GGACTCCT

P7-006	CAAGCAGAAGACGGCATAACGAGATCATGCCTAGTCTCGTGGGCTCGG	TAGGCATG
P7-007	CAAGCAGAAGACGGCATAACGAGATGTAGAGAGGTCTCGTGGGCTCGG	CTCTCTAC
P7-008	CAAGCAGAAGACGGCATAACGAGATCAGCCTCGGTCTCGTGGGCTCGG	CGAGGCTG

Sample index (i7, Index1); *Index sequences are colored according to the lasers used by the NovaSeq 6000 2-color sequencing chemistry, red: red laser, green: green laser, yellow: both lasers, black: dark cycle.

Add 80 µl of Enrichment PCR Master Mix per well and mix by pipetting

Run reactions in qPCR cycler (heated lid: 105 °C)

<Note> When samples reach > 4000 RFU, pause run after the plate read and transfer samples to second thermocycler. Then resume run for any remaining samples that need further enrichment. We are using a BioRad C1000 Touch thermocycler and CFX96 qPCR module. RFU values corresponding to sufficient library might be system-specific.

72 °C for 3 min (for end fill-in after tagmentation)

98 °C for 30 sec

Cycle and stop when > 4000 RFU { 98 °C for 10 sec
 65 °C for 30 sec
 72 °C for 30 sec, plate read }

72 °C for 5 min (in second thermocycler)

store at 4 °C

10B Tagmentation with Illumina Nextera XT reagents

Dilute cDNA to 0.2 ng/µl with nuclease-free water

Per tagmentation reaction, distribute 5 µl (1 ng) into a 96-well plate on ice

<Note> Tagment entire sample for maximum library complexity. Typically, 6-15 tagmentations of 1 ng are required.

Prepare Tagmentation Master Mix with reagents from the Nextera XT kit (Illumina #15032350) as described below

Tagmentation Master Mix		
Component	Per reaction [µl]	Number of reactions (+ 10% overhead) <input type="text"/> x
Nextera TD buffer	10	
Amplicon Tagment Enzyme	5	

Add 15 µl of Tagmentation Master Mix per well and mix by pipetting

Incubate for 5 min at 55 °C

Add 5 µl of Neutralization Buffer per well (Nextera XT kit, #15032350) and incubate for 5 min at room temperature

Place reactions on ice for 1 min

Add 2.5 µl of 10 µM barcoded P7 primer per well

<Note> If processing samples for a 2-color sequencer (such as the Illumina NovaSeq 6000 platform) sequencing complexity of Index1 can be increased by using multiple P7 primers per sample.

Prepare Enrichment PCR Master Mix as described below

Enrichment PCR Master Mix		
Component	Per reaction [µl]	Number of reactions (+ 10% overhead) <input type="text"/> x
Nuclease-free water	6.75	
Nextera PCR mix	15	
Partial-P5 Primer, 100 µM	0.25	
SYBR Green, 100x in DMSO	0.5	

Add 22.5 µl of Enrichment PCR Master Mix per well and mix by pipetting

Run reactions in qPCR cycler (heated lid: 105 °C)

Appendix A Preparation of the custom i7-only transposome and activity check

<Note> Nextera XT reagents can be used as replacement for the i7-only transposome with comparable results

Oligonucleotides for custom i7-only transposome assembly	
Oligo ID	Oligo sequence [5' > 3']
Tn5-top_ME	[5Phos]CTGTCTCTTATACACATCT
Tn5-bottom_Read2N	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG
Functional elements	
5' phosphorylation (required for transposition mechanism)	
mosaic end for association with Tn5 transposase	
i7 primer binding site for enrichment PCR	

Step 1 Anneal oligonucleotides

Prepare Annealing Buffer as described in Appendix F

Dissolve lyophilized oligonucleotides at 100 μ M in EB buffer

Set up annealing reactions according to the table below

Anneal oligonucleotides for transposome assembly		
Component	Per reaction	
	Volume	Amount
Tn5-top_ME, 100 μ M	22.5 μ l	45 μ M
Tn5-bottom_Read2N, 100 μ M	22.5 μ l	45 μ M
Annealing Buffer, 10x	5 μ l	1x

Anneal in thermocycler: 95 $^{\circ}$ C for 3 min, 70 $^{\circ}$ C for 3 min, ramp to 25 $^{\circ}$ C at a rate of 2 $^{\circ}$ C per minute

Add 180 μ l of nuclease-free water to the annealed oligonucleotides and mix

<Safe stopping point> The diluted oligo cassette can be aliquoted and frozen for future transposome assemblies

Step 2 Load Tn5 transposase

Mix 20 μ l of the diluted oligo cassette from the previous step with 20 μ l of 100% glycerol

<Note> Glycerol is extremely viscous, use slow reverse pipetting

Add 10 μ l of EZ-Tn5 Transposase (Lucigen #TNP92110) and mix well by pipetting

Incubate for 30 min at 25 $^{\circ}$ C in a thermocycler

Store at -20 $^{\circ}$ C until ready to use

Step 3 (Optional) Test activity of custom i7-only transposome in a qPCR assay

<Note> This optional test allows to compare the activity of the custom i7-only Tn5 with that of the ATAC Enzyme provided by 10x Genomics as a reference. Generally, a 1:4.5 dilution of custom Tn5 works well, but in case of problems with the tagmentation step this test can be performed.

<Note> Tagmented DNA flanked by two Illumina i7 adapters is suppressed in PCR reactions due to competition between intramolecular annealing and primer binding. Our custom i7-only transposome is therefore tested in a negative qPCR assay. Briefly, a defined PCR product is subjected to one tagmentation reaction and one no-enzyme control reaction. Both samples are then re-amplified with the same primers in a second qPCR reaction. Since the tagmentation fragments the PCR product, the corresponding reaction should yield higher Ct values. The tagmentation efficiency can then be calculated from the shift of Ct values.

Set up PCR reactions as indicated in the table below

PCR product for tagmentation test		
Component	Per reaction	
	Volume per 6.6 reactions	Amount per reaction
pUC19 plasmid (NEB, 50 pg/μl)	33 μl	250 pg
Q5 HotStart High-Fidelity Master Mix, 2x	165 μl	1x
pUC19-FWD (AAGTGCCACCTGACGTCTAAG), 100 μM	1.65 μl	500 nM
pUC19-REV (CAACAATTAATAGACTGGATGGAGGCGG), 100 μM	1.65 μl	500 nM
Nuclease-free water	128.7 μl	to 50 μl

Distribute 50 μl into six wells of a tube strip

Run PCR in a thermocycler:

98 °C for 30 sec

31 cycles { 98 °C for 10 sec

68 °C for 30 sec

72 °C for 1 min }

72 °C for 2 min, storage at 12 °C

To each 50 μl PCR reaction, add 6.25 μl of CutSmart Buffer (10x) and 6.25 μl of DpnI

Incubate at 37 °C for 1 hour, 80 °C for 20 min, storage at 12 °C

<Note> This step digests the template plasmid

Pool the six PCR reactions in a 2 ml tube, add 5 volumes (1500 μl) of PB Buffer (QiaQuick PCR Purification Kit), mix

Continue with QiaQuick PCR purification using two columns, elute in 30 μl EB buffer each and pool

Measure Qubit HS of a 1/10 dilution and dilute the PCR product to 25 ng/μl with EB buffer

<Note> This typically results in about 400 μl of 25 ng/μl solution

Run 100 ng (4 μl) of PCR product on a 1% agarose gel containing ethidium bromide, next to 500 ng (1 μl) of Gene-

Ruler 1 kb DNA Ladder

<Note> The size of the desired band is 1961 bp

Set up tagmentation reactions as indicated below

Tagmentation Test Master Mix		
Component	Per 3.3 reactions	
	Volume	Amount per reaction
pUC19 PCR product from above (25 ng/μl)	6.6 μl	50 ng
ATAC Buffer from 10x Genomics Single Cell ATAC Kit	23.1 μl	N/A

Transfer 9 μl of Tagmentation Test Master Mix to three wells of a tube strip on ice

Add nuclease-free water and Tn5 enzymes as indicated below

Tagmentation Test Conditions			
Component	Positive Control	Tagmentation Negative Control	Test
ATAC Enzyme from 10x Genomics Single Cell ATAC Kit	3	-	-
Custom i7 transposome	-	-	6
Nuclease-free water	3	6	-

Incubate at 37 °C for 60 min, storage at 4 °C

Add 1.75 μl of 1% SDS solution and incubate at 70 °C for 10 min to dissociate Tn5 from the DNA

Dilute reactions 1/100 with EB buffer

Run qPCR reactions in triplicates, as indicated below

qPCR reactions for transposome activity check		
Component	Per reaction	
	Volume	Amount
Tagmentation reaction (1/100 diluted)	2 µl	
GoTaq qPCR Master Mix, 2x	10 µl	1x
FWD primer (AAGTGCCACCTGACGTCTAAG), 100 µM	0.1 µl	500 nM
REV primer (CAACAATTAATAGACTGGATGGAGGCGG), 100 µM	0.1 µl	500 nM
Nuclease-free water	7.8 µl	to 20 µl

Run qPCR reactions in thermocycler:

95 °C for 2 min

40 cycles { 95 °C for 30 sec

68 °C for 30 sec

72 °C for 2 min + plate read }

stop when plateau is reached

Calculate tagmentation efficiency for each condition as:

$$\text{Tagmentation efficiency [\%]} = 100 - (100 / (2 ^ {(\text{average Ct}^{\text{Sample}} - \text{average Ct}^{\text{Negative Control}})}))$$

Appendix B Expected results

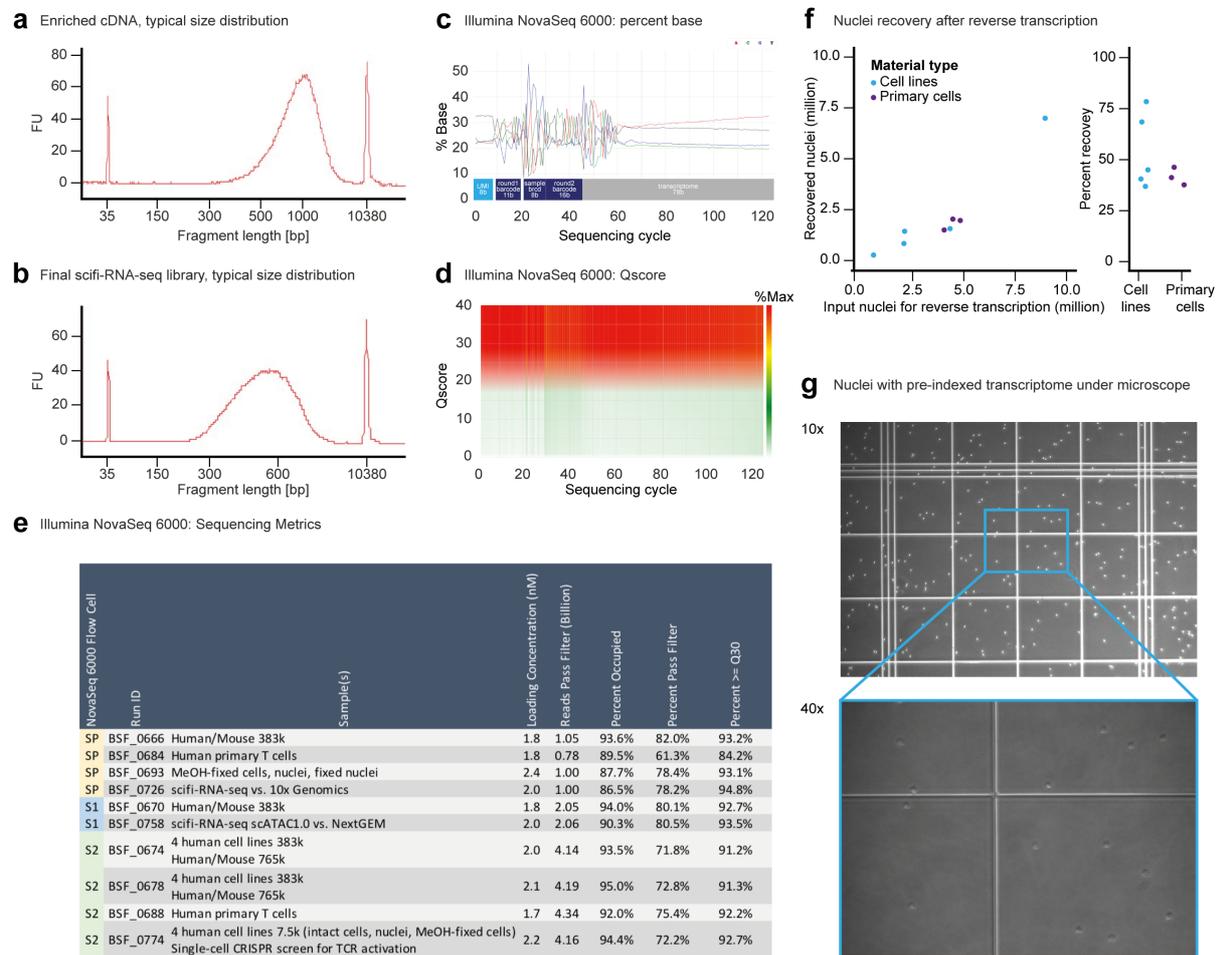


Figure 1 Expected results for scifi-RNA-seq. **a**) Typical size distribution of enriched cDNA obtained using scifi-RNA-seq. **b**) Typical size distribution of a final scifi-RNA-seq library ready for next-generation sequencing. **c**) Distribution of DNA bases along scifi-RNA-seq sequencing reads, showing the characteristic sequence patterns of the UMI, round1 barcode, round2 barcode, sample barcode, and transcript. **d**) Heatmap showing sequencing quality (Qscore) for each sequencing cycle. **e**) Table summarizing all NovaSeq 6000 sequencing runs performed for the scifi-RNA-seq paper. scifi-RNA-seq was thoroughly tested with NovaSeq SP, S1, and S2 reagents. The table also contains information on the type of samples that were pooled for a particular run. **f**) Nuclei recovery after pre-indexing of the whole transcriptome by reverse transcription. scifi-RNA-seq achieves high recovery rates for both cell lines and primary material. **g**) Nuclei with pre-indexed transcriptome, prior to microfluidic device loading, visualized under a microscope in a counting chamber. The selected images show nuclei derived from human primary T cells.

Appendix C Materials

Reagent (alphabetical order)	Supplier	Cat. No.	Comment on use
Agarose	Sigma	A9539-100G	custom i7-only Tn5 activity check
Agilent Bioanalyzer High Sensitivity DNA chip	Agilent	5067-4626	scifi-RNA-seq library prep
Ampligase Enzyme & Buffer, High Concentration (100 U/ul)	Lucigen	A0102K	scifi-RNA-seq library prep
AMPure XP beads	Beckman Coulter	A63880	scifi-RNA-seq library prep
BSA	Sigma	A7030-50G	scifi-RNA-seq library prep
Cell strainer 40 um	A. Hartenstein	Z540	scifi-RNA-seq library prep
Cell strainer 70 um	A. Hartenstein	Z570	scifi-RNA-seq library prep
Chromium Single Cell ATAC Library & Gel Bead Kit, 16 rxns	10x Genomics	1000110	scifi-RNA-seq library prep
Chromium Single Cell E Chip Kit, 48 rxns	10x Genomics	1000082	scifi-RNA-seq library prep
Counting Chamber, Fuchs Rosenthal	Incyto	DHC-F01	scifi-RNA-seq library prep
Digitonin, 2%	Promega	G944A	scifi-RNA-seq library prep
Dimethylformamide	Sigma	D4551-250ML	scifi-RNA-seq library prep
dNTPs, 10 mM	Thermo Fisher Scientific	R0193	scifi-RNA-seq library prep
DpnI	NEB	R0176L	custom i7-only Tn5 activity check
DTT	Sigma	646563-10x.5ML	scifi-RNA-seq library prep
EB Buffer	Qiagen	19086	scifi-RNA-seq library prep
EDTA, 0.5M	Invitrogen	AM9260G	custom i7-only Tn5 activity check
Ethanol, absolute	Merck	603-002-00-5	scifi-RNA-seq library prep
Ethidium bromide	AppliChem	A2273,0015	custom i7-only Tn5 activity check
EZ-Tn5 Transposase	Lucigen	TNP92110	scifi-RNA-seq library prep
Ficoll PM-400	Sigma	F5415-50ML	scifi-RNA-seq library prep
Formaldehyde, 16%	Thermo Fisher Scientific	28908	scifi-RNA-seq library prep
GeneRuler 1kb DNA Ladder	Thermo Fisher Scientific	SM0311	custom i7-only Tn5 activity check
Glycerol	Sigma	G5516-100ML	scifi-RNA-seq library prep
GoTaq qPCR Master Mix	Promega	A600A	custom i7-only Tn5 activity check
IGEPAL CA-630	Sigma	I8896-50ML	scifi-RNA-seq library prep
Maxima H Minus Reverse Transcriptase, 4x 10000 U	Thermo Fisher Scientific	EP0753	scifi-RNA-seq library prep
Methanol	Fisher Scientific	M/4000/17	scifi-RNA-seq library prep
MgCl ₂ , 1M	Ambion	AM9530G	scifi-RNA-seq library prep
MinElute PCR Purification Kit (optional cleanup in case of extreme clumping)	Qiagen	28004	scifi-RNA-seq library prep
NaCl, 5M	Sigma	S5150-1L	scifi-RNA-seq library prep
NaOH, 10M	Sigma	72068-100ML	scifi-RNA-seq library prep
NEBNext High Fidelity 2x Master Mix	NEB	M05415	scifi-RNA-seq library prep
Nextera XT Kit, 24 rxns (optional replacement for custom i7-only transposome)	Illumina	15032350	scifi-RNA-seq library prep
NovaSeq 6000 S1 Reagent Kit (100 Cycles)	Illumina	20012865	next-generation sequencing
NovaSeq 6000 S2 Reagent Kit (100 Cycles)	Illumina	20012862	next-generation sequencing
NovaSeq 6000 SP Reagent Kit (100 Cycles)	Illumina	20027464	next-generation sequencing
PBS, 1x	Gibco	14190-094	scifi-RNA-seq library prep
pUC19 plasmid	NEB	N3041A	custom i7-only Tn5 activity check
Puradisc 25 mm Polyethersulfone Syringe Filter, 0.2 µm, sterile	GE Healthcare	6780-2502	scifi-RNA-seq library prep
Q5 HotStart High-Fidelity 2x Master Mix, 500 rxns	NEB	M0494L	custom i7-only Tn5 activity check
QiaQuick PCR Purification Kit	Qiagen	28106	custom i7-only Tn5 activity check
Qubit dsDNA HS Assay Kit	Thermo Fisher Scientific	Q32854	scifi-RNA-seq library prep
Recombinant RNase inhibitor	Takara	2313A	scifi-RNA-seq library prep
RNaseOUT RNase inhibitor	Invitrogen	10777019	scifi-RNA-seq library prep
SDS solution, 10% in water	Sigma	71736-100ML	custom i7-only Tn5 activity check
SPRIselect beads	Beckman Coulter	B23318	scifi-RNA-seq library prep
SUPERase In RNase Inhibitor	Thermo Fisher Scientific	AM2696	scifi-RNA-seq library prep
SYBR Green, 10000x in DMSO	Life Technologies	S7563	scifi-RNA-seq library prep, custom i7-only Tn5 activity check
TAPS	Sigma	T9659-100G	scifi-RNA-seq library prep
Tris-HCl, pH 7.5, 2M	Sigma	T2944-100ML	scifi-RNA-seq library prep
TritonX-100	Sigma	X100-100ML	scifi-RNA-seq library prep
Tween-20	Sigma	P7949-500ML	scifi-RNA-seq library prep

Appendix D Buffers for isolation of nuclei or whole cells without fixation

PBS-BSA-SUPERase						
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei	Per 5 Mio Whole Cells
PBS, 1x					890	9790
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		100	1100
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		10	110

Nuclei Preparation Buffer					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclease-free water					470.25
Tris-HCl, pH 7.5, 2M	2 M		10 mM		2.75
NaCl, 5M	5 M		10 mM		1.1
MgCl ₂ , 1 M	1 M		3 mM		1.65
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		55
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		5.5
Tween-20	10 % (v/v)		0.1 % (v/v)		5.5
IGEPAL CA-630, 10% (v/v)	10 % (v/v)		0.1 % (v/v)		5.5
Digitonin	2 % (v/v)		0.01 % (v/v)		2.75

Nuclei Wash Buffer					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclease-free water					4785
Tris-HCl, pH 7.5, 2M	2 M		10 mM		27.5
NaCl, 5M	5 M		10 mM		11
MgCl ₂ , 1 M	1 M		3 mM		16.5
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		550
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		55
Tween-20	10 % (v/v)		0.1 % (v/v)		55

Appendix E Buffers for isolation of nuclei with formaldehyde fixation and permeabilization

DAY1: Nuclei isolation, fixation and flash-freezing					
Nuclei Preparation Buffer without Digitonin and without Tween-20					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclease-free water					478.5
Tris-HCl, pH 7.5, 2M	2 M		10 mM		2.75
NaCl, 5M	5 M		10 mM		1.1
MgCl ₂ , 1 M	1 M		3 mM		1.65
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		55
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		5.5
IGEPAL CA-630, 10% (v/v)	10 % (v/v)		0.1 % (v/v)		5.5
Nuclei Wash Buffer without Tween-20					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclease-free water					7937.6
Tris-HCl, pH 7.5, 2M	2 M		10 mM		45.1
NaCl, 5M	5 M		10 mM		18.04
MgCl ₂ , 1 M	1 M		3 mM		27.06
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		902
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		90.2
1% Formaldehyde in 1x PBS					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
PBS, 1x					5156
Formaldehyde, 16%	16 %		1 %		344
PBS-BSA-SUPERase					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
PBS, 1x					890
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		100
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		10
DAY2: Thawing, nuclei permeabilization					
Nuclei Wash Buffer without Tween-20					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclease-free water					748
Tris-HCl, pH 7.5, 2M	2 M		10 mM		4.25
NaCl, 5M	5 M		10 mM		1.7
MgCl ₂ , 1 M	1 M		3 mM		2.55
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		85
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		8.5
Permeabilization Buffer					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
Nuclei Wash Buffer without Tween-20 (from above)					270.875
Digitonin	2 %		0.01 %		1.375
Tween-20	10 %		0.1 %		2.75
PBS-BSA-SUPERase					
Component	Stock	Unit	Final	Unit	Per 5 Mio Nuclei
PBS, 1x					489.5
BSA, 10% (w/v)	10 % (w/v)		1 % (w/v)		55
SUPERase In Rnase Inhibitor (20 U/ul)	20 U/ul		0.2 U/ul		5.5

Appendix F Buffers for transposome assembly and tagmentation

Annealing Buffer, 10x						
Component	Stock	Unit	Final	Unit	Per 1 ml	Unit
Nuclease-free water					875 μ l	
Tris-HCl, pH 7.5, 2M	2 M		10 mM		5 μ l	
NaCl, 5M	5 M		500 mM		100 μ l	
EDTA, 0.5M	0.5 M		10 mM		20 μ l	
Tn5 Reaction Buffer, 5x						
Component	Stock	Unit	Final	Unit	Per 100 ml	Unit
Nuclease-free water					87.5 ml	
TAPS	powder		50 mM		1.216 g	
MgCl ₂ , 1 M	1 M		25 mM		2.5 ml	
adjust pH to 8.5 with 10 M NaOH, then fill up to 100 ml with nuclease-free water filter through 0.2 μ m filter under a cell culture hood and aliquot						
Tn5 Dilution Buffer						
Component	Stock	Unit	Final	Unit	Per 10 ml	Unit
Nuclease-free water					4538 μ l	
Glycerol	100 %		50 %		5000 μ l	
Tris-HCl, pH 7.5, 2M	2 M		50 mM		250 μ l	
NaCl, 5M	5 M		100 mM		200 μ l	
EDTA, 0.5M	0.5 M		0.1 mM		2 μ l	
Triton X-100	100 %		0.1 %		10 μ l	
Before using this buffer, add 1/100 volumes of fresh 0.1M DT	0.1 M		1 mM			

Appendix G Column-based post GEM incubation cleanup

<Note> For some samples, and when loading extremely high numbers of pre-indexed cells/nuclei per microfluidic channel, we have observed clumping of MyOne Silane Dynabeads. This is presumably due to the high amounts of genomic DNA in the sample. As mentioned in step 5.1, shearing the clumps with a thin pipette tip is often sufficient to resolve the problem. If the problem persists or if this is a common problem with a particular type of sample, we recommend to replace step 5.1 with the following instructions.

Replace step 5.1 with instructions below for column-based post GEM incubation cleanup

Add 125µl of Recovery Agent to each sample at room temperature

Invert ten times to mix, centrifuge briefly

<Note> This results in a pink oil phase at the bottom and a smaller aqueous phase containing the sample on top

Slowly remove 125 µl of the pink oil phase from the bottom of the tube, discard

Prepare Cleanup Master Mix without Dynabeads as described below

Cleanup Master Mix without Dynabeads		Number of channels (+ 10% overhead)							
Component	Per reaction Volume (µl)	1	2	3	4	5	6	7	8
Cleanup Buffer	182	182.0	400.4	600.6	800.8	1001.0	1201.2	1401.4	1601.6
Reducing Agent B	5	5.0	11.0	16.5	22.0	27.5	33.0	38.5	44.0
Nuclease-free water	13	13.0	28.6	42.9	57.2	71.5	85.8	100.1	114.4

Add 200 µl of Dynabead Cleanup Mix without Dynabeads per sample, pipet mix

Load sample in Cleanup Master Mix without Dynabeads onto MinElute column, centrifuge for 1 min at 15,000 rcf

<Note> Do not add PB buffer to the sample, the Cleanup Master Mix without Dynabeads is sufficient to allow DNA binding to the column

Discard flow-through

Wash once with 700 µl of PE buffer (containing ethanol)

Discard flow-through, place column in empty collection tube, spin for 1 min at 15,000 rcf to remove residual ethanol

Elute in 11 µl of EB buffer, this should result in an eluate of about 10 µl

Add 30 µl of nuclease-free water to bring the sample volume to 40 µl

Proceed with step 5.2 (SPRI cleanup)

Appendix H Thermoligation in scATAC Barcoding Reagent supplemented with NAD⁺ and magnesium

For some samples, we have observed an unstable droplet emulsion when loading the microfluidic droplet generator with Ampligase Reaction buffer, as described in steps 4A and 4B of this protocol. In these cases we recommend to perform the thermoligation in scATAC Barcoding Reagent supplemented with NAD⁺ and magnesium for compatibility with the Ampligase enzyme.

<Note> This procedure has been tested for scATAC v1.0 reagents only.

Add pre-indexed cells/nuclei to tube strip on ice
Add Barcoding Reagent to a total volume of 9.2 μ l
Prepare Thermoligation Master Mix as described below

Thermoligation Master Mix for scATAC v1.0 chips and reagents									
Component	Per reaction Volume (μ l)	Number of channels (+ 10% overhead)							
		1	2	3	4	5	6	7	8
Barcoding Reagent	61.5	61.5	135.3	203.0	270.6	338.3	405.9	473.6	541.2
Reducing Agent B	1.5	1.5	3.3	5.0	6.6	8.3	9.9	11.6	13.2
Ampligase Enzyme (100 U/ μ l)	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2
Bridge Oligo, 100 μ M	2.3	2.3	5.1	7.6	10.1	12.7	15.2	17.7	20.2
NAD ⁺ , 50 mM	1.6	1.6	3.5	5.3	7.0	8.8	10.6	12.3	14.1
MgCl ₂ , 500 mM	1.6	1.6	3.5	5.3	7.0	8.8	10.6	12.3	14.1

Add 70.8 μ l of Thermoligation Master Mix per sample, and mix by pipetting. This brings the volume to 80 μ l. Continue with microfluidic chip loading as described in step 4A.