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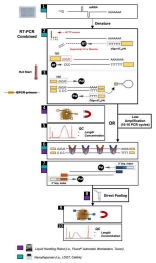
FLASH-seq Low-Amplification protocol (V1) V.1



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Human Cell Atlas Metho... The Single Cell Ninjas

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

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Abstract

Building upon the existing Smart-seq2/3 workflows, we developed FLASH-seq (FS), a new full-length scRNA-seq method capable of detecting a significantly higher number of genes than both previous versions, requiring limited hands-on time and with a great potential for customization.
FLASH-seq Low-Amplification (FS-LA), represents FS quickest iteration, generating sequencing-ready libraries in 4.5 hours by removing intermediate cleanups and QC steps and without sacrificing performance. FS-LA is the best choice when a large number of plates need to be processed in parallel.

Guidelines

FLASH-Seq Low-Amplification (FS-LA) is a variation of FLASH-Seq, where the cDNA after RT is amplified for a limited number of cycles, in order to reduce PCR bias and reduce library preparation time. Three parameters need to be considered when setting up a FS-LA experiment:

1 --- RNA content of the cell type: this is going to determine how many pre-amplification cycles are required to generate enough cDNA for the tagmentation, while minimizing unmapped / intergenic reads.
⇒ The exact number of PCR cycles cannot be determined based on the wet-lab results but rather evaluated based on the sequencing results. We recommend performing a pilot study on a subset of cells processed with varying numbers of PCR cycles and choose the value that minimises the background noise.

2 --- Reaction volume: as the cDNA is not purified prior to tagmentation, it is important to dilute enough the salts and additives of the RT-PCR mix. We recommend diluting the unpurified cDNA 10 times for the best results, although lower dilutions might also work. The increased costs associated with higher reaction volumes are generally negligible when working with in-house Tn5 transposase. When working with the ATM mix contained in the NexteraXT kit we recommend pre-diluting your cDNA 1:10 and performing the tagmentation reaction in 2 µL.

3 --- Amount of Tn5: that needs to be adjusted case by case, depending on the cell type, number of pre-amplification cycles as well as specific activity of each Tn5 batch.
⇒ The exact value can be evaluated at the wet-lab stage based on the library size distribution.

As a guideline, we recommend choosing 10-12 PCR cycles when working with large cells and/or cell lines (i.e. HEK 293 cells) and 14-16 cycles when working with cells containing smaller amounts of mRNA (i.e. PBMC). Refer to FLASH-Seq manuscript and extended files #2 for more information (Hahaut et al).

Materials

REAGENTS - CELL LYSIS MIX

- dNTP-Set 1 **Carl Roth** **Catalog #**K039.2
- Triton X-100 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**X100-100ML
- Recombinant RNase Inhibitor (40 U/uL) **Takara Bio Inc.** **Catalog #**2313B
- dCTP (100 mM solution) **Thermo Fisher Scientific** **Catalog #**10217016

REAGENTS - RT-PCR MIX

- KAPA HiFi HotStart ReadyMix (2x) **Roche** **Catalog #**KK2602
- SuperScript™ IV Reverse Transcriptase **Thermo Fisher Scientific** **Catalog #**18090050
- Magnesium Chloride (1M Solution) **Invitrogen - Thermo Fisher** **Catalog #**AM9530G

REAGENTS - MAGNETIC BEADS SOLUTION PREPARATION

- Polyethylenglycol (MW=8000) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**89510-1KG-F
- Sodium chloride **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**59222C-1000ML
- Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophobic), 15 mL **GE Healthcare** **Catalog #**65152105050250
- Sodium azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-25G
- EDTA (0.5 M), pH 8.0 **Life Technologies** **Catalog #**AM9260G
- Tris-HCl pH 8.0 (1M solution) **Thermo Fisher Scientific** **Catalog #**15568025
- Tween-20 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**P-7949

If a commercial solution for sample cleanup is preferred, choose the following product:

- Agencourt AMPure XP **Beckman Coulter** **Catalog #**A63880

REAGENTS - LIBRARY QC

- Qubit™ 1X dsDNA HS Assay Kit **Invitrogen - Thermo Fisher** **Catalog #**Q33231
- Qubit™ Assay Tubes **Invitrogen - Thermo Fisher** **Catalog #**Q32856
- Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626

REAGENTS - TAGMENTATION WITH IN-HOUSE Tn5 TRANSPOSASE

- KAPA HiFi plus dNTPs **Roche** **Catalog #**KK2102
- NN-Dimethylformamide (DMF) solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**D4551
- SDS, 10% Solution **Life Technologies** **Catalog #**AM9822
- TAPS **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T9659-100G

Sodium Hydroxide (pellet purity 98%) **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**71690-1KG

GENERAL CONSUMABLES

RNase AWAY™ Spray Bottle, RNase in spray bottle; 475mL **Thermo Fisher Catalog #**7002

Adhesive PCR Plate Seals **Thermo Fisher Scientific Catalog #**AB0558

Aluminium foil seals for -80ºC storage **VWR International (Avantor) Catalog #**391-1281

Twin.Tec® PCR plates 384 (LoBind colourless) **Eppendorf Catalog #**EP0030129547

UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023

DNA LoBind® 1.5 mL (PCR clean colourless) **Eppendorf Catalog #**30108051

Ethanol for molecular biology **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**51976-500ML-F

OLIGONUCLEOTIDES - RT-PCR

	A	B	C
	Oligo ID	**Sequence (5′ → 3′)**	**Purification / synthesis scale**
	Smart dT30VN	/5Biosg/AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	desalted or HPLC
	FS TSO	/5Biosg/AAGCAGTGGTATCAACGCAGAGTACrGrGrG	desalted or HPLC

/5Biosg/ = C6-linker biotin

OLIGONUCLEOTIDES - TAGMENTATION

	A	B	C
	Oligo ID	**Sequence (5′ → 3′)**	**Purification / synthesis scale**
	TN5MErev	/5Phos/ CTGTCTCTTATACACATCT	2 µM scale - desalted*
	TN5ME-A	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG	1 µM scale - desalted*
	TN5ME-B	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG	1 µM scale - desalted*

/5Phos/ = Phosphate group

* It is important to follow these recommendations. Ordering oligos at this scale but choosing “HPLC purification” will result in insufficient material for Tn5 loading. The scale indicated here is sufficient for producing 20-25 ml of loaded Tn5.

OLIGONUCLEOTIDES - TAGMENTATION (when not ordering the Nextera Index Kit)

One can order the 4 Nextera XT Index Kit v2 (set A, B, C, D) sets, as described above or, alternatively, get them manufactured by any oligonucleotide provider. Below is the list of 24 N7xx and 16 S5xx adaptors required to multiplex 384 samples.

Prepare working dilution plates containing unique combinations of N7xx + S5xx adaptors, each with a final concentration of 5 µM.

	A	B
	Oligo ID	**Sequence (5′ → 3′)**
	Nextera_v2_N714	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCGTGGGCTCG*G
	Nextera_v2_N715	/5Biosg/CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCGTGGGCTCG*G
	Nextera_v2_N716	/5Biosg/CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCGTGGGCTCG*G
	Nextera_v2_N718	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCGTGGGCTCG*G
	Nextera_v2_N719	/5Biosg/CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCGTGGGCTCG*G
	Nextera_v2_N720	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCGTGGGCTCG*G
	Nextera_v2_N721	/5Biosg/CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCGTGGGCTCG*G
	Nextera_v2_N722	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCGTGGGCTCG*G
	Nextera_v2_N723	/5Biosg/CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCGTGGGCTCG*G
	Nextera_v2_N724	/5Biosg/CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCGTGGGCTCG*G
	Nextera_v2_N726	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTCTTAGGGTCTCGTGGGCTCG*G
	Nextera_v2_N727	/5Biosg/CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCGTGGGCTCG*G
	Nextera_v2_N728	/5Biosg/CAAGCAGAAGACGGCATACGAGATTAGCTGCAGTCTCGTGGGCTCG*G

	A	B
	Nextera_v2_N729	/5Biosg/CAAGCAGAAGACGGCATACGAGATGACGTCGAGTCTCGTGGGCTCG*G
	Nextera_v2_S502	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCTCTCTATTCGTCGGCAGCGT*C
	Nextera_v2_S513	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTCGACTAGTCGTCGGCAGCGT*C
	Nextera_v2_S503	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTATCCTCTTCGTCGGCAGCGT*C
	Nextera_v2_S515	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTTCTAGCTTCGTCGGCAGCGT*C
	Nextera_v2_S505	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGTAAGGAGTCGTCGGCAGCGT*C
	Nextera_v2_S516	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCCTAGAGTTCGTCGGCAGCGT*C
	Nextera_v2_S506	/5Biosg/AATGATACGGCGACCACCGAGATCTACACACTGCATATCGTCGGCAGCGT*C
	Nextera_v2_S517	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGCGTAAGATCGTCGGCAGCGT*C
	Nextera_v2_S507	/5Biosg/AATGATACGGCGACCACCGAGATCTACACAAGGAGTATCGTCGGCAGCGT*C
	Nextera_v2_S518	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCTATTAAGTCGTCGGCAGCGT*C
	Nextera_v2_S508	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCTAAGCCTTCGTCGGCAGCGT*C
	Nextera_v2_S520	/5Biosg/AATGATACGGCGACCACCGAGATCTACACAAGGCTATTCGTCGGCAGCGT*C
	Nextera_v2_S510	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCGTCTAATTCGTCGGCAGCGT*C
	Nextera_v2_S521	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGAGCCTTATCGTCGGCAGCGT*C
	Nextera_v2_S511	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTCTCTCCGTCGTCGGCAGCGT*C
	Nextera_v2_S522	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTTATGCGATCGTCGGCAGCGT*C

All oligonucleotides carry a 5´-biotin (/5Biosg/) and a phosphorothioate bond (*) between the last and the second last nucleotide. For cost reasons, we ordered desalted oligos and not HPLC purified.

OLIGONUCLEOTIDES - TAGMENTATION (when not ordering the Nextera Index Kit)

To increase the multiplex capabilities, we designed an additional set of 32 S5xx and 48 N7xx adaptors (non-UDI).

	A	B
	Oligo ID	**Sequence**
	Nextera_extra_i7_1	/5Biosg/CAAGCAGAAGACGGCATACGAGATGCCTATCAGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 2	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTTGGATGGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 3	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGTCTCACGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 4	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTCATCAGGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 5	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGTACCGTGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 6	/5Biosg/CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 7	/5Biosg/CAAGCAGAAGACGGCATACGAGATCACGTTGTGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 8	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCACAGCAGTCTCGTGGGC TCG*G
	Nextera_extra_i7_ 9	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTACTTGGGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 0	/5Biosg/CAAGCAGAAGACGGCATACGAGATCCTCAGTTGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 1	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCCTACCTGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 2	/5Biosg/CAAGCAGAAGACGGCATACGAGATATGGCGAAGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 3	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTTACCTGGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 4	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTCGATACGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 5	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCCGTGAAGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 6	/5Biosg/CAAGCAGAAGACGGCATACGAGATTAGAGCTCGTCTCGTGGGC TCG*G
	Nextera_extra_i7_1 7	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGACTGACGTCTCGTGGGC TCG*G

	A	B
	Nextera_extra_i7_18	/5Biosg/CAAGCAGAAGACGGCATACGAGATTAGACGTGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_19	/5Biosg/CAAGCAGAAGACGGCATACGAGATCCGGAATTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_20	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTCCTAGAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_21	/5Biosg/CAAGCAGAAGACGGCATACGAGATCAACGGATGTCTCGTGGGCTCG*G
	Nextera_extra_i7_22	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGGCTATCGTCTCGTGGGCTCG*G
	Nextera_extra_i7_23	/5Biosg/CAAGCAGAAGACGGCATACGAGATCGGTCATAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_24	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCCAATCGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_25	/5Biosg/CAAGCAGAAGACGGCATACGAGATGAGCTTGTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_26	/5Biosg/CAAGCAGAAGACGGCATACGAGATGAAGGTTCGTCTCGTGGGCTCG*G
	Nextera_extra_i7_27	/5Biosg/CAAGCAGAAGACGGCATACGAGATATCTCGCTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_28	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGTTACGGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_29	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTGTCTGAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_30	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGACTTCGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_31	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGGATCACGTCTCGTGGGCTCG*G
	Nextera_extra_i7_32	/5Biosg/CAAGCAGAAGACGGCATACGAGATACACCAGTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_33	/5Biosg/CAAGCAGAAGACGGCATACGAGATCAGGTTAGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_34	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGTTGGCTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_35	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCAACTGGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_36	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTGCACTTGTCTCGTGGGCTCG*G

	A	B
	Nextera_extra_i7_37	/5Biosg/CAAGCAGAAGACGGCATACGAGATACACGGTTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_38	/5Biosg/CAAGCAGAAGACGGCATACGAGATAATACGCGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_39	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGCGAACTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_40	/5Biosg/CAAGCAGAAGACGGCATACGAGATGCTGACTAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_41	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTGGTGTTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_42	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTGCTTACGTCTCGTGGGCTCG*G
	Nextera_extra_i7_43	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCAAGGACGTCTCGTGGGCTCG*G
	Nextera_extra_i7_44	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGAACCTGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_45	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGTGTTGGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_46	/5Biosg/CAAGCAGAAGACGGCATACGAGATGTACTCTCGTCTCGTGGGCTCG*G
	Nextera_extra_i7_47	/5Biosg/CAAGCAGAAGACGGCATACGAGATCCGTATCTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_48	/5Biosg/CAAGCAGAAGACGGCATACGAGATCGAAGAACGTCTCGTGGGCTCG*G

All oligonucleotides carry a 5´-biotin (/5Biosg/) and a phosphorothioate bond (*) between the last and the second last nucleotide. For cost reasons, we ordered desalted oligos and not HPLC purified.
Prepare working dilution plates containing unique combinations of N7xx + S5xx adaptors, each with a final concentration of 5 µM.

	A	B
	Oligo ID	**Sequence**
	Nextera_extra_i5_1	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCGACCATTTCGTCGGCAGCGT*C
	Nextera_extra_i5_2	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGATAGCGATCGTCGGCAGCGT*C
	Nextera_extra_i5_3	/5Biosg/AATGATACGGCGACCACCGAGATCTACACAATGGACGTCGTCGGCAGCGT*C

	A	B
	Nextera_extra_i5_4	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCGCTAGTATCGTCGGCAGCGT*C
	Nextera_extra_i5_5	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTCTCTAGGTCGTCGGCAGCGT*C
	Nextera_extra_i5_6	/5Biosg/AATGATACGGCGACCACCGAGATCTACACACATTGCGTCGTCGGCAGCGT*C
	Nextera_extra_i5_7	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTGAGGTGTTCGTCGGCAGCGT*C
	Nextera_extra_i5_8	/5Biosg/AATGATACGGCGACCACCGAGATCTACACAATGCCTCTCGTCGGCAGCGT*C
	Nextera_extra_i5_9	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCTGGAGTATCGTCGGCAGCGT*C
	Nextera_extra_i5_10	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGTATGCTGTCGTCGGCAGCGT*C
	Nextera_extra_i5_11	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTGGAGAGTTCGTCGGCAGCGT*C
	Nextera_extra_i5_12	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCGATAGAGTCGTCGGCAGCGT*C
	Nextera_extra_i5_13	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCTCATTGCTCGTCGGCAGCGT*C
	Nextera_extra_i5_14	/5Biosg/AATGATACGGCGACCACCGAGATCTACACACCAGCTTTCGTCGGCAGCGT*C
	Nextera_extra_i5_15	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGAATCGTGTCGTCGGCAGCGT*C
	Nextera_extra_i5_16	/5Biosg/AATGATACGGCGACCACCGAGATCTACACAGGCTTCTTCGTCGGCAGCGT*C
	Nextera_extra_i5_17	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCAGTTCTGTCGTCGGCAGCGT*C
	Nextera_extra_i5_18	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTTGGTGAGTCGTCGGCAGCGT*C
	Nextera_extra_i5_19	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCATTCGGTTCGTCGGCAGCGT*C
	Nextera_extra_i5_20	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTGTGAAGCTCGTCGGCAGCGT*C
	Nextera_extra_i5_21	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTAAGTGGCTCGTCGGCAGCGT*C
	Nextera_extra_i5_22	/5Biosg/AATGATACGGCGACCACCGAGATCTACACACGTGATGTCGTCGGCAGCGT*C

	A	B
	Nextera_extra_i5_23	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGTAGAGCATCGTCGGCAGCGT*C
	Nextera_extra_i5_24	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGTCAGTTGTCGTCGGCAGCGT*C
	Nextera_extra_i5_25	/5Biosg/AATGATACGGCGACCACCGAGATCTACACATTCGAGGTCGTCGGCAGCGT*C
	Nextera_extra_i5_26	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGATACTGGTCGTCGGCAGCGT*C
	Nextera_extra_i5_27	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGCCTTGTTTCGTCGGCAGCGT*C
	Nextera_extra_i5_28	/5Biosg/AATGATACGGCGACCACCGAGATCTACACTTGGTCTCTCGTCGGCAGCGT*C
	Nextera_extra_i5_29	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCCGACTATTCGTCGGCAGCGT*C
	Nextera_extra_i5_30	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGTCCTAAGTCGTCGGCAGCGT*C
	Nextera_extra_i5_31	/5Biosg/AATGATACGGCGACCACCGAGATCTACACACCAATGCTCGTCGGCAGCGT*C
	Nextera_extra_i5_32	/5Biosg/AATGATACGGCGACCACCGAGATCTACACGATGCACTTCGTCGGCAGCGT*C

All oligonucleotides carry a 5´-biotin (/5Biosg/) and a phosphorothioate bond (*) between the last and the second last nucleotide. For cost reasons, we ordered desalted oligos and not HPLC purified.
Prepare working dilution plates containing unique combinations of N7xx + S5xx adaptors, each with a final concentration of 5 µM.

Before start

The protocol should be carried out in a clean environment, ideally on a dedicated PCR workstation or on a separate bench used only for this purpose. Before starting, clean the bench and wipe any piece of equipment with RNAseZAP or 0.5% sodium hypochlorite. Rinse with nuclease-free water to avoid corrosion of delicate equipment.

Work quickly and preferably on ice.

Reagent mixes should be prepared shortly before use.

Mix thoroughly each mix before dispensing. For higher accuracy use liquid handling robots and/or nanodispensers whenever possible. In FLASH-Seq we use the I.DOT (Dispendix) for all the dispensing steps and the Fluent 780 liquid handling robot (Tecan) for sample cleanup, reagent transfers and pooling.

The protocol described below is meant to be carried out in 384-well plates. When using 96-well plates, we recommend using 5 times larger volume to guarantee successful cell sorting and prevent evaporation issues.

Always use LoBind plates and tubes (especially for long-term storage) to prevent the cDNA/DNA from sticking to plastic.

Prepare lysis mix

15m

1

Prepare the following lysis mix:

	A	B	C	D
	Reagent	**Reaction concentration**	**Volume (µl)**	**384-well plate**
	Triton-X100 (10% v/v)	0.2%	0.020	8.448
	dNTP mix (25 mM each)	6 mM	0.240	101.376
	SMART dT30VN (100 µM)	1.8 mM	0.018	7.603
	RNAse inhibitor (40 U/µl)	1.2 U/µl	0.030	12.672
	DTT (100 mM)	1.2 mM	0.012	5.069
	FS TSO (100 µM)	9.2 µM	0.092	38.861
	dCTP (100 µM)	9 mM	0.090	38.016
	Betaine (5 M)	1 M	0.200	84.480
	Nuclease-free water	-	0.298	125.875
	Total volume (µl)		**1.000**	**422.400**

Add 1 µL lysis mix to each well of a 384-well plate

Seal the plate with a PCR seal and quickly spin it down to collect the lysis mix to the bottom.

Proceed immediately to the next step or store the plate at -20 °C long-term. Plates that are going to be used on the same day can be stored in the fridge or kept on ice.

Note

SAFE STOPPING POINT - Plates containing lysis buffer can be stored for >6 months at -20 °C

Sample collection

10m

2 Sort single cells into 384-well plates containing 1 µL lysis mix.

Seal the plate with an aluminium seal. If processing multiple plates at once, keep each plate on dry ice until ready to transfer them all at -80 °C for long-term storage.
Plates containing single cells should ideally be processed within 6 months.

Cell lysis

3m

3 Remove the plates from the -80 °C freezer and check that the aluminium seal is still intact. If damaged or not sticking to the plate anymore, wait a few minutes for the plate to partially thaw, remove the damaged foil and replace it with a new one.

3m

Place the plate in a thermocycler with a heated lid and incubate for 00:03:00 at 72 °C, followed by a 4 °C hold step.

Spin down any condensation droplets that may have formed during the incubation and return the plate to a cool rack. Proceed quickly to the next step. If not ready with the RT-PCR mix, keep the plate on the cool rack at all times.

RT-PCR reaction

15m

4 While the plate is in the thermocycler, prepare the following RT-PCR mix:

	A	B	C	D
	Reagent	**Reaction concentration**	**Volume (µl)**	**384-well plate**
	DTT (0.1 M)	4.8 mM	0.238	100.531
	MgCl2 (1 M)	9.2 mM	0.046	19.430
	Betaine (5 M)	800 mM	0.800	337.920
	RNAse inhibitor (40 U/µl)	0.8 U/µl	0.096	40.550
	SuperScript IV (200 U/µl)	2.00 U/µl	0.050	21.120
	KAPA HiFi HotStart ReadyMix (2 x)	1 x	2.500	1056.000
	Nuclease-free water	-	0.270	114.048

	A	B	C	D
	Total volume (µl)		**4.000**	**1689.600**

Add 4 µL RT-PCR mix into each well of the 384-well plate.

Seal the plate with a PCR seal, gently vortex and spin down to collect the liquid at the bottom.

Place it in a thermocycler with heated lid and start the following RT-PCR program:

	A	B	C	D	E
	Step		**Temperature**	**Time**	**Cycles**
	RT		50ºC	60 min	1 x
	PCR	initial denaturation	98ºC	3 min	1 x
		denaturation	98ºC	20 sec	10-16 x*
		annealing	67ºC		
		elongation	72ºC		
			15ºC	Hold	

*Adjust the number of cycles according to the cell type. We recommend 10-12 cycles for HEK 293T cells and 14-16 cycles for hPBMC.

Note

SAFE STOPPING POINT - Amplified cDNA before purification can be stored for several months at -20 °C

Tagmentation and enrichment PCR

1h

5 Please note that the Tn5 transposase amount is a suggested starting point only. Optimisation might be necessary, depending on the specific activity of each batch of Tn5 and desired library size.
Indexing primers can be purchased from Illumina (Nextera XT index kit v2) or ordered from your local oligo manufacturer. In the "Materials" section we have added additional sequences for higher multiplexing.

5.1 8m

Note

Please note that the Tn5 transposase amount indicated below is a suggested starting point for tagmenting [M] 150 pg/µl cDNA. Optimisation might be necessary, depending on the specific activity of each batch of Tn5.

Prepare the tagmentation mix as described below:

	A	B	C
	Reagent	**Volume (µl)**	**Final concentration**
	TAPS-Mg buffer, pH=7.3 (5x)	2.000	10 mM TAPS, 5 mM MgCl2
	Dimethylformamide (DMF) (100%)	2.000	20%
	Tn5 transposase (2 µM working dil.)	0.025	5 nM
	Nuclease-free water	4.975	
	Total volume (µl)	**9.000**	

Safety information

Dimethylformamide (DMF) is toxic and should be handled under the hood according to local safety regulations.

Dispense 9 µL tagmentation mix in a new 384-well plate.

Add 1 µL unpurified cDNA to each well containing the tagmentation mix.

Seal the plate, vortex, spin down, and carry out the tagmentation reaction: 55 °C for 00:08:00 , 4 °C hold. Upon completion proceed immediately to the next step.

Add 2.5 µL 0.2% SDS to each well. Seal the plate, vortex, spin down and incubate 5 min at room temperature. Do not put the plate back on ice.

Add 2.5 µL N7xx + S5xx index adaptors ([M] 5 micromolar (µM) each).

Add 10 µL enrichment PCR mix to each well:

	A	B	C
	Reagent	**Volume (µl)**	**Final concentration**
	KAPA HiFi enzyme (1 U/µl)	0.50	0.02 U/µl
	KAPA HiFi Buffer (5 x)	5.00	1 x
	dNTPs (10 mM)	0.75	300 nM
	Nuclease-free water	3.75	
	Total volume (µl)	**10.00**	

Seal the plate, vortex, spin down, and place it in a thermocycler and carry out the enrichment PCR reaction. Adjust the number of PCR cycles according to the number of processed cells AND the number of pre-amplification cycles used in the RT-PCR reaction.

	A	B	C	D	E
	Step		**Temperature**	**Time**	**Cycles**
	gap filling		72ºC	3 min	1 x
	enrichment PCR	initial denaturation	98ºC	30 sec	1 x
		denaturation	98ºC	10 sec	14-16 x
		annealing	55ºC	30 sec	
		elongation	72ºC	30 sec	

	A	B	C	D	E
			15ºC	hold	

Note

SAFE STOPPING POINT - The final unpurified sequencing library can be stored for several months at -20 °C

Library cleanup and quantification

30m

6 Take an aliquot from each sample for the final library cleanup (i.e. 5 µl). and transfer it to a 1.5-ml Eppendorf tube. The rest of the library can be stored long-term at -20 °C . 31m 30s

Remove the Sera-Mag SpeedBeads™ working solution from the 4 °C storage and equilibrate it at room temperature for 00:15:00 .

Add Sera-Mag SpeedBeads™ working solution to a final ratio of 0.8 x and mix well to homogenisation.

Incubate the tube off the magnetic stand for 00:05:00 at Room temperature .

Place the tube on the magnetic stand and leave it for 00:05:00 or until the solution appears clear.

Remove the supernatant without disturbing the beads.

Recommended: wash the pellet with 1 mL 80% v/v ethanol. Incubate 00:00:30 without removing the tube from the magnetic stand.

Remove any trace of ethanol and let the bead pellet dry for 00:02:00 or until small cracks appear. Do not cap the tube or remove it from the magnetic stand during this time. Do not completely air-dry the beads.

Remove the tube from the magnetic stand, add 50 µL nuclease-free water and mix well by pipetting or vortexing to resuspend the beads.

Incubate 00:02:00 off the magnetic stand.

Place the tube back on the magnetic stand and incubate for 00:02:00 or until the solution appears clear.

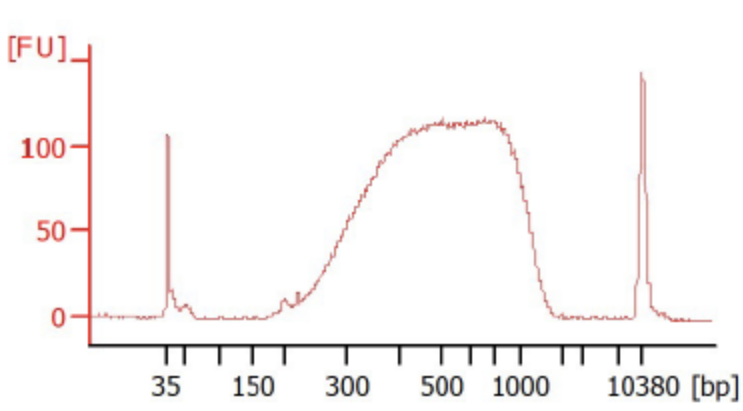
Remove 49 µL of the supernatant and transfer it to a new 1.5-ml LoBind tube. Store the cDNA at -20 °C long-term or until ready for sequencing.

Use Qubit fluorometer to quantify the library. Library yield can vary depending on the number of cells being pooled.

Check the final library size on the Agilent Bioanalyzer.

Use the average size indicated on the Bioanalyzer and the concentration reported after Qubit measurement to determine the exact molarity required for sequencing.

Expected result



HEK293T cells amplified for 10 cycles and tagmented with 0.015 µl of Tn5 transposase

Note

SAFE STOPPING POINT - The final purified sequencing library can be stored for several months at -20 °C

Pooling and sequencing

7 The purified library can be sequenced on any Illumina sequencer. Follow the specifications reported for each instrument. Single-end 75 bp is generally sufficient but longer read modes or paired-end sequencing can be an option, depending on the question at hand.

Data processing

8 These instructions briefly describe the data processing of the sequencing results. The final pipeline will likely have to be adapted to the question at hand. The following lines assume that all the programs and their dependencies are installed on your machine and that the data are single-end reads (75 bp). Some values, such as the number of threads and RAM usage may have to be adapted to your machine settings.

It should be noted that there are many other ways to analyse full-length single-cell RNA-sequencing data. Pseudo-alignment tools (e.g., Salmon or Kallisto) or automatic pipelines (zUMIs) could be used as well.

Requirements (tested version):

- bcl2fastq (v2.20)
- STAR (v2.7.3)
- FeatureCounts (v1.6.5)
- BBMAP (v38.86)
- samtools (v1.9)
- IGV

8.1 **Sample demultiplexing**

Sequencing results will be delivered as demultiplexed FASTQ or raw bcl2 files. To convert bcl2 files to FASTQ, bcl2fastq program (Illumina) can be used.

Command

new command name

```
# 0. Variables
BASECALL_DIR="/path/to/flowcell/Data/Intensities/BaseCalls/"
OUTPUT_DIR="/path/to/output_folder/"
SAMPLESHEET="/path/to/Demultiplexing_SampleSheet.csv"
```

Command

new command name

```
# 1. Bcl2fastq
ulimit -n 10000
cd /path/to/flowcell/
bcl2fastq --input-dir $BASECALL_DIR --output-dir $OUTPUT_DIR --sample-
sheet $SAMPLESHEET --create-fastq-for-index-reads --no-lane-splitting
```

When sequencing on a NextSeq 550 instrument, the sample sheet should contain the following information in a csv file:

```
[Header]
IEMFileVersion          5
Investigator Name       Your Name
Experiment Name         Experiment Title
Date            11/12/2021
Workflow        GenerateFASTQ
Application     NextSeq FASTQ Only
Instrument Type         NextSeq/MiniSeq
Assay           Nextera XT
Index Adapters          Nextera XT Index Kit (24 Indexes 96 Samples)
Chemistry       Amplicon

[Reads]
75

[Settings]
Adapter         CTGTCTCTTATACACATCT

[Data]
Sample_ID  Sample_Name          Sample_Plate          Sample_Well I7_Index_ID  index          I5_Index_ID  index2         Sample_Project
Sample1_A1 Sample1_A1 001       A1          Nextera_extra_i7_25     ACAAGCTC Nextera_extra_i5_17     CAGAACTG
```

Illumina Experiment Manager can be used to assist you in creating the sample sheet.

We recommend exploring the barcode combinations left in the undetermined reads looking to confirm that all the cells have been properly demultiplexed.

Command

new command name

```
zcat Undetermined_S0_I1_001.fastq.gz | awk -F' 1:N:0:' 'NR%4==1{print
$2}' | sort | uniq -c > left_index.txt
sort -k1,1 left_index.txt
```

as well as the read distribution between samples:

Command

new command name

```
for file in ./out/*R1*
do
zcat $file | wc -l
done
```

8.2 **Index the genome**

The reference genome needs to be indexed prior to any mapping. The FASTA and GTF references can be obtained from ENSEMBL, Gencode, UCSC, ...

Command

new command name

```
# 0. Variables
OUTPUTREF="/path/to/STAR_indexed_genome/"
FASTA="GRCh38.primary_assembly.genome.fa"
GTF="gencode.v34.primary_assembly.annotation.gtf"
```

Command

new command name

```
# 1. Genome indexing
# sjdbOverhang should be adapted based on the read length
(read_length - 1)
mkdir $OUTPUTREF
```

Command

new command name

```
STAR --runThreadN 15 --runMode genomeGenerate --genomeDir $OUTPUTREF --genomeFastaFiles $FASTA --sjdbGTFfile $GTF --sjdbOverhang 74
```

8.3 **FASTQ trimming (optional)**

If you observe sequencing primer left-overs the FASTQ files can be trimmed using BBDUK or Trimmomatic.

Command

new command name

```
bbduk.sh -Xmx48g in=sample.fastq.gz out=cleaned.left.fastq t=32 ktrim=l ref=adapters.fa k=23 mink=7 hdist=1 hdist2=0 tbo
bbduk.sh -Xmx48g in=cleaned.left.fastq out=cleaned.fastq t=32 ktrim=r ref=adapters.fa k=23 mink=7 hdist=1 hdist2=0 tbo
```

Command

new command name

```
mv FASTQ/cleaned.fastq FASTQ/sample.R1.fastq.gz
```

8.4 Mapping

The FASTQ file can then be mapped onto the reference genome. Example for one sample, use a loop or parallelise this task to process all the cells:

Command

new command name

```
# 0. Variables
GENOME="/path/to/STAR_indexed_genome/"
FASTQ="/path/to/sample.R1.fastq.gz"
ID=”sample_id”
```

Command

new command name

```
# 1. Mapping
STAR --runThreadN 30 --limitBAMsortRAM 20000000000 --genomeLoad
LoadAndKeep --genomeDir "$GENOME" --readFilesIn "$FASTQ" --
readFilesCommand zcat --limitSjdbInsertNsj 2000000 --
outFilterIntronMotifs RemoveNoncanonicalUnannotated --outSAMtype BAM
SortedByCoordinate --outFileNamePrefix "$ID"_
```

Command

new command name

```
# 2. SAM to sorted BAM
# -F 260 filters out unmapped and secondary alignments
samtools view -@ 30 -Sb -F 260 "$ID"_Aligned.sortedByCoord.out.bam >
"$ID"_Aligned.sortedByCoord.filtered.bam
samtools index "$ID"_Aligned.sortedByCoord.filtered.bam
```

8.5 **Data visualization (optional)**

Once the reads have been mapped we highly recommend using the Integrated Genome Viewer (IGV) to visualise the mapping results and ensure that the results make sense. As a quick check-up visualise a few housekeeping genes (i.e., ACTB, GAPDH, ...) and cell specific markers to look for reads mapping to exon, intron, exon-intron junctions. Look for abnormalities such as read piles falling in intergenic or centromeric regions.
No single-cell RNA sequencing protocol is perfect and non-specific priming, genomic DNA contaminations, ... can happen but should represent rare events.
Recurrent soft-clipping could also indicate the presence of sequencing adaptor left-overs that could affect the mapping rate.

8.6 **Count matrix**

Finally, the number of reads associated with each gene can be obtained as follows:

Command

new command name

```
featureCounts -T 1 -t exon -g gene_name --fracOverlap 0.25 -a "$GTF" -
o "$ID"_ReadCount.featureCounts.gencode.txt
"$ID"_Aligned.sortedByCoord.filtered.bam
```

8.7 **Post-processing**

The post-processing steps will vary depending on the question at hand. The online book “Orchestrating Single-Cell Analysis with Bioconductor” (**https://bioconductor.org/books/release/OSCA/**) is a gold mine of information that can be used to help you design your own pipeline. Alternatively, Seurat (R, **https://satijalab.org/seurat/**) or scanpy (python, **https://scanpy.readthedocs.io/en/stable/**) provide tools compatible with FLASH-seq data. Given their similarities, we currently recommend using Smart-seq2 guidelines when processing FLASH-seq data.