Bulk FLASH-seq V.2

Rebecca A Siwicki[1], Vincent Hahaut[1], Simone Picelli[1]

[1]Institute of Molecular and Clinical Ophthalmology Basel (IOB)

SCG_IOB

Rebecca A Siwicki

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

ABSTRACT

Bulk RNA sequencing has revolutionized the study of transcriptomes, enabling the analysis of gene expression in complex tissues and heterogenous cell populations. While single cell RNA sequencing (scRNA-seq) has gained popularity due to its ability to profile individual cells, it comes with limitations such as high costs and reduced sensitivity for detecting low-abundance transcripts.

Here, we present bulk FLASH-seq (FS), a full-length RNA-seq method based on the single cell FLASH-seq workflow (Hahaut *et al*, 2022, https://www.nature.com/articles/s41587-022-01312-3), updated for bulk RNA analysis. FS bulk generates high quality data while requiring minimal hands-on time and offering a greater degree of customization. As a homebrew protocol, it is inexpensive compared to commercial kits allowing you to invest in greater sequencing depths or in a higher number of sequenced samples.

Our protocol enables comprehensive transcriptome analysis of bulk RNA samples, providing an alternative approach to scRNA-seq for gene expression when single-cell RNA-sequencing is not required.

GUIDELINES

Bulk FS has been optimised for 1 ng RNA input. Any other input in the range 10 pg-1 ng will not require any change to the protocol we describe here. Any input <5 ng RNA might also be fine with the 5ul reaction volume. However, inputs >10 ng will almost certainly require a reagent titration, in particular dNTPs, oligodT, TSO and perhaps, RT. The reaction can also be scaled to 96-well plates but reaction volume will need to be increased from 5uL. The user should be aware of this and perform the necessary titrations. Bulk FS is, similarly to scFS, a polyA-only, non-stranded protocol and therefore unsuitable when preserving strand information is crucial.

MATERIALS

Created: Nov 16, 2023

Last Modified: Nov 20, 2023

PROTOCOL integer ID: 91033

Keywords: RNA-seq, FLASH-seq, bulk RNA

REAGENTS - CELL LYSIS MIX

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

SAMPLE NORMALISATION & ADDITION

- Qubit RNA HS (High Sensitivity) assay **Thermo Fisher Scientific Catalog #Q32852**

REAGENTS - RT-PCR MIX

- KAPA HiFi HotStart ReadyMix (2x) **Roche Catalog #KK2602**
- Superscript IV **Thermo Fisher Scientific Catalog #18090050**
- Maxima H Minus Reverse Transcriptase (200 U/uL) **Thermo Fisher Scientific Catalog #EP0753**
- 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 Solution 5 M **Merck MilliporeSigma (Sigma-Aldrich) Catalog #59222C**
- Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #44152105050350**
- Sodium azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #S2002**
- 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 - SAMPLE & LIBRARY QC

Quant-iT™ PicoGreen® dsDNA Assay Kit **Life Technologies Catalog #P11496**

384 Well Black Plate Non-Treated Surface Non-Sterile Pack of 25 **Thermo Scientific Catalog #262260**

Nunc™ F96 MicroWell™ Polystyrene Plate black **Thermo Fisher Scientific Catalog #237105**

Qubit™ 1X dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #Q33231**

Qubit assay tubes **Thermo Fisher Scientific Catalog #Q32856**

Agilent High Sensitivity DNA Kit **Agilent Technologies Catalog #5067-4626**

REAGENTS - TAGMENTATION WITH NEXTERA XT KIT

Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**

Nextera XT Index Kit v2 (set A B C D) **Illumina, Inc. Catalog #FC-131-2001; FC-131-2002; FC-131**

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 Catalog #391-1281**

twin.tec PCR Plate 384 **Eppendorf Catalog #951020729**

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/AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTVN	desalted or HPLC
	FS TSO	/5Biosg/AAGCAGTGGTATCAACGCAGAGTACrGrGrG	desalted or HPLC

/5Biosg/ = C6-linker biotin

OLIGONUCLEOTIDES - IN-HOUSE Tn5 PROTOCOL ONLY

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

* 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 - ALL TAGMENTATION PROTOCOLS (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
	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

	A	B
	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 - ALL TAGMENTATION PROTOCOLS (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/CAAGCAGAAGACGGCATACGAGATGCCTATCAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_2	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTTGGATGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_3	/5Biosg/CAAGCAGAAGACGGCATACGAGATAGTCTCACGTCTCGTGGGCTCG*G
	Nextera_extra_i7_4	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTCATCAGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_5	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGTACCGTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_6	/5Biosg/CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_7	/5Biosg/CAAGCAGAAGACGGCATACGAGATCACGTTGTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_8	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCACAGCAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_9	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTACTTGGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_10	/5Biosg/CAAGCAGAAGACGGCATACGAGATCCTCAGTTGTCTCGTGGGCTCG*G

	A	B
	Nextera_extra_i7_11	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCCTACCTGTCTCGTGGGCTCG*G
	Nextera_extra_i7_12	/5Biosg/CAAGCAGAAGACGGCATACGAGATATGGCGAAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_13	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTTACCTGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_14	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTCGATACGTCTCGTGGGCTCG*G
	Nextera_extra_i7_15	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCCGTGAAGTCTCGTGGGCTCG*G
	Nextera_extra_i7_16	/5Biosg/CAAGCAGAAGACGGCATACGAGATTAGAGCTCGTCTCGTGGGCTCG*G
	Nextera_extra_i7_17	/5Biosg/CAAGCAGAAGACGGCATACGAGATTGACTGACGTCTCGTGGGCTCG*G
	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

	A	B
	Nextera_extra_i7_35	/5Biosg/CAAGCAGAAGACGGCATACGAGATTCAACTGGGTCTCGTGGGCTCG*G
	Nextera_extra_i7_36	/5Biosg/CAAGCAGAAGACGGCATACGAGATCTGCACTTGTCTCGTGGGCTCG*G
	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
	Nextera_extra_i5_4	/5Biosg/AATGATACGGCGACCACCGAGATCTACACCGCTAGTATCGTCGGCAGCGT*C

	A	B
	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
	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

	A	B
	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.

SAFETY WARNINGS

Sodium azide and Dimethylformamide (DMF) are both toxic and should be handled with caution under a fume hood.

BEFORE START INSTRUCTIONS

The protocol should be carried out in a clean environment, ideally on a dedicated PRE-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. For 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.

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)**	**Volume 384-well plate (µl)**
	Triton-X100 (10% v/v)	0.2%	0.020	9.216
	dNTP mix (25 mM each)	6 mM	0.240	110.592
	SMART dT30VN (100 µM)	1.8 µM	0.018	8.294
	RNase Inhibitor (40 U/µl)	1.2 U/µl	0.030	13.824
	DTT (100 mM)	1.2 mM	0.012	5.530
	dCTP	9 mM	0.090	41.472
	Betaine	1 M	0.200	92.160
	Nuclease Free Water	NA	0.068	31.334
	Total volume		**0.678**	**312.422**

Add 0.68 µL lysis mix to each well of a 384-well plate.

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

Process immediately to the next step or store plate long term at -20 °C. Plates that will be used the same day can be stored in the fridge 4 °C or on ice.

Safe stopping point. Plates containing lysis buffer can be stored for 6+ months at -20 °C

Sample Normalisation & Addition

30m

2 Quantify input RNA using a spectrophotometry and fluorometry assay (ex: NanoDrop™ or Qubit™ RNA High Sensitivity).

Normalise input RNA to 2 ng/µL with Nuclease-free water.

Add 0.5 µL of normalised RNA into corresponding 384-well plate containing lysis mix.

> **Note**
>
> We successfully tested this protocol with a wide-range of RNA inputs, ranging from single-cell levels (see Hahaut *et al*) to 1 ng. Going above this threshold may require an increase in reagents, especially the oligonucleotides. When using lower RNA inputs, the number of PCR cycles must be adapted.

Seal the plate with an aluminium seal, quickly spin down. If processing more than one plate at once, keep each plate on dry ice until ready to transfer all to -80 °C for long term storage. Plates containing RNA should ideally be processed within 6 months.

Cell Lysis

3m

3 Remove plates from -80 °C and check that aluminum seal is still intact. If damaged or not stic 3m 30s the plate, wait a few minutes for the plate to partially thaw, remove damaged foil and replace with new one.

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

> **Note**
>
> During incubation prepare RT-PCR mix.

Spin down any condensation droplets (00:00:30 , 750 x g) that may have formed during incubation and return the plate to a cold block. Process quickly to the next step. If not ready with RT-PCR mix, keep the plate on the cold block at all times.

RT-PCR Reaction

15m

4 Prepare RT-PCR mix: 30s

A	B	C
Reagent	**Volume (µl)**	**Volume 384-well plate (µl)**
DTT	0.238	109.670
MgCl2 (1M)	0.046	21.197
Betaine (5M)	0.800	368.640
Takara RNAse inhibitor (40 U/µl)	0.096	44.237
Maxima RT or Superscript IV (200U/µl)	0.050	23.040
KAPA HiFi HotStart Ready Mix (2X)	2.500	1152.000
FS TSO	0.092	42.394
Total volume	**3.822**	**1792.512**

Add 3.822 µL RT-PCR mix into each well of the 384-well plate.

Seal the plate with a PCR seal, gently vortex and spin down (00:00:30 , 750 x g) to collect the liquid at the bottom of the well.

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

A	B	C	D	E
Step		**Temperature**	**Time**	**Cycles**
RT		50°C	60 min	1
PCR	initial denaturation	98°C	3 min	1
	denaturation	98°C	20 sec	12-14x
	annealing	67°C	20 sec	
	elongation	72°C	6 min	
		4°C	hold	

*Adjust the number of cycles according to the amount of RNA used.

Safe Stopping Point. Amplified cDNA before purification can be stored for several months at -20 °C .

Magnetic bead working solution preparation

1h

5 You can either use AMPure XP beads, SPRI beads or prepare your own solution of SeraMag beads containing 18% w/v PEG to reduce costs. A detailed protocol for making your own magnetic bead solution is described in:

CITATION

Picelli S (2019). Full-Length Single-Cell RNA Sequencing with Smart-seq2..
LINK
https://doi.org/10.1007/978-1-4939-9240-9_3

Below is a short description of how to prepare 50 ml of working solution:

	A	B	C
	Reagent	**Final concentration**	**Amount to add**
	Sodium chloride	1 M	2.92 gr
	Tris-HCl, pH = 8.0 (1 M)	10 mM	500 µl
	EDTA (500 mM)	1 mM	100 µl
	PEG (MW=8000)	18% w/v	9.5 gr
	Nuclease-free water	-	to a final volume of 50 ml

Combine the sodium chloride, Tris-HCl, EDTA and PEG in a a 50-ml Falcon tube. Add 25 mL water.

Solubilise the PEG by stirring and heating the solution at 37 °C. If needed, progressively add more water.

While the PEG is dissolving, prepare the Sera-Mag SpeedBeads™. Vortex thoroughly to ensure complete resuspension. Withdraw 1 mL Sera-Mag SpeedBeads™ stock solution and transfer it into a new 1.5-ml tube.

Pellet the beads by placing the tube on a magnetic stand. Wait until the solution clears and then discard the supernatant.

Add 1 mL TE buffer (10 mM Tris–HCl pH 8.0 + 1 mM EDTA) and resuspend the beads off the magnet.

Pellet the beads again, wait until the solution is clear, discard the supernatant and resuspend off the magnet with 0.9 mL TE buffer.

Once the PEG solution is clear, add the resuspended beads prepared in the previous step.

Add 50 µL Tween-20 (10% v/v) and 250 µL sodium azide (NaN3, 10% w/v) and adjust the volume to 50 ml with nuclease-free water.

Safety information

Sodium azide is extremely toxic and should be handled under a fume hood.

Note

Tween-20 is added at the end to prevent foaming during PEG resuspension

Store at 4 °C. Do not freeze.

Note

Confirm that the beads have been properly prepared by cleaning up a control sample and running a High Sensitivity DNA chip on the Agilent Bioanalyzer. Batch-to-batch variations in PREG concentrations will influence size-cutoffs.

cDNA purification

25m

6 Remove the Sera-Mag SpeedBeads™ working solution (AMPure XP beads or SPRI beads when using commercial solution) from the 4 °C storage and equilibrate it at room temperature for 00:15:00 . 29m

Note

We recommend adding nuclease-free water to each sample to increase volume, simplify handling, and improve recovery rate. For instance, adding 10 µL nuclease free water to 5 ul amplified cDNA.

Add 0.8X volume ratio Sera-Mag SpeedBeads™ working solution to each well. Mix thoroughly by pipetting or vortexing.

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

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

Remove the supernatant without disturbing the beads. Do not let the bead pellet dry completely as it can decrease the final cDNA yield.

Remove the plate from the magnetic stand, add 15 µL nuclease free water and mix well by pipetting or vortexing to resuspend the beads.

Incubate for 00:02:00 off the magnetic stand.

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

Remove 14 µL of the supernatant and transfer it to a new plate.

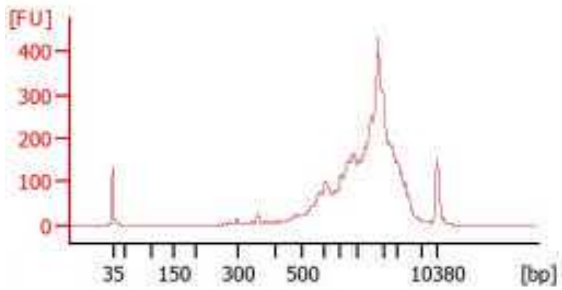
Safe stopping point. Amplified and clean cDNA can be stored for several months at -20 C. We recommend storing in LoBind plates to avoid material loss during long-term storage.

Quality control check (highly recommended)

45m

7 Check the cDNA quality on the Agilent Bioanalyzer High Sensitivity DNA chip. A good sample is characterised by a low proportion of fragments <400 bp, absence of residual primers (~100bp) and an average cDNA size of 1.8-2.2 Kb.

Expected result



cDNA quantification (optional but recommended)

15m

8 Allow the Quant-iT™ PicoGreen™ dsDNA Assay reagents to come to room temperature before opening the vial. PicoGreen™ dye is light sensitive, it should be thawed in dark drawer or wrapped in foil. 2m

This step can be performed in either 384-well or 96-well plates. Volume of PicoGreen + TE solution should be adjusted depending on plate type.

Prepare a 1X TE solution using TE 20X (supplied) and nuclease-free water.

Prepare the standard curve using Lambda DNA standard (supplied at concentration of 100 ng/µl, with the PicoGreen™ dsDNA Assay kit) and 1X TE in 8 tubes as below. The stock tubes can be used multiple times, keep any leftover in the fridge at 4 C between experiments.

Vortex well and spin down the DNA standard before every use. Not vortexing the standards thoroughly will negatively affect the standard curve and concentration readings. Serial dilutions should be prepared as shown in the table below and stored at 4 °C :

	A	B	C	D
	Tube	**Content**	**Concentration**	**Volume**
	1	90 µl TE + 10 µl Lambda DNA stock	10 ng/µl	100 µl
	2	50 µl from Tube 1 + 50 µl TE	5 ng/µl	100 µl
	3	50 µl from Tube 2 + 50 µl TE	2.5 ng/µl	100 µl
	4	50 µl from Tube 3 + 50 µl TE	1.25 ng/µl	100 µl

	A	B	C	D
	5	50 µl from Tube 4 + 50 µl TE	0.625 ng/µl	100 µl
	6	50 µl from Tube 5 + 50 µl TE	0.3125 ng/µl	100 µl
	7	50 µl from Tube 6 + 50 µl TE	0.15625 ng/µl	100 µl
	8	TE only	blank	

For 384-well plates, prepare a 1:400 solution of PicoGreen™ dsDNA Assay in 1X TE buffer (39 µL per sample). Vortex to mix.

Note

When using 96-well plates, prepare a 1:200 solution of PicoGreen™ dsDNA Assay in 1X TE buffer (99 µL per sample).

Pipette 1 µL each of the 7 standards + 1 Blank into a black, flat-bottom 384 MicroWell™ plate. Place the standards on one column.

Pipette 1 µL of your samples into the center of each well of the Nunc™ F384 MicroWell™ polystyrene plate.

Add 39 µL PicoGreen + TE mix into every well. There is no need to mix.

Cover the plate with the provided plastic (transparent) lid to prevent possible contaminations.

Allow 00:02:00 for the dye to bind the DNA. Protect from light but keep at room temperature. For optimal results, the plate should be read within the next hour.

Use a plate reader to measure fluorescence (excitation: 485 nm; emission: 530 nm; read from top; endpoint reading).

Plate normalisation

10m

9 Prepare a normalisation plate by adding 1 µL purified cDNA and nuclease-free water to a final concentration of 200 pg/µL.

Tagmentation and enrichment PCR

1h

10 This step can be carried out either by using the commercially available **Nextera XT kit** or an **in-house Tn5 transposase**, as described below. Indexing primers can be purchased from Illumina (Nextera XT index kit v2) or ordered from your local oligonucleotide manufacturer. Indexing primer sequences can found in the "Materials" section.

Note

Tagmentation instructions with the **Nextera XT Kit** information can be found in the single cell FS protocol.

CITATION

Hahaut V. & Picelli S. (2022). FLASH-seq . protocol.io.
LINK
https://protocols.io/view/flash-seq-protocol-cmnnu5de

10.1 Tagmentation with in-house Tn5 transposase

13m 30s

Note

Please note that the Tn5 transposase amount indicated below is a suggested starting point for tagmenting 200 pg/µl cDNA. Optimisation might be necessary, depending on the specific activity of each batch of Tn5.

Prepare the tagmentation mix:

	A	B	C	D
	Reagent	**Volume (µl)**	**Final Concentration**	**Volume 384-well plate (µl)**
	Dimethylformamide (DMF) (100%)	0.8	20%	368.64
	TAPS-Mg buffer (5x) pH 7.3	0.8	10 mM TAPS, 5 mM MgCl2	368.64
	Tn5 transposase (2 µM working dil.)	0.1	62.5 nmol	46.08
	Nuclease-free water	1.3	-	599.04
	Total volume	**3.0**		**1,382.4**

Safety information

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

Dispense 3 µL tagmentation mix in a new 384-well plate.

Add 1 µL normalised cDNA (200 pg/µl) 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 1 µL 0.2% SDS to each well. Seal the plate, vortex, spin down and incubate 00:05:00 at Room temperature . Do not put the plate back on ice.

Add 2 µL N7xx + S5xx index adaptors ([M] 5 micromolar (µM)) each.

Add 3 µL enrichment PCR mix to each well.

	A	B	C	D
	Reagent	**Per sample (µl)**	**Final Concentration**	**Volume 384-well plate (µl)**
	KAPA HiFi enzyme (1 U/µl)	0.2	0.02 U/µl	92.16
	dNTPs (10 mM)	0.3	300 nM	138.24
	KAPA HiFi Buffer (5x)	2.0	1X	921.60
	Nuclease-free water	0.5		230.40
	Total volume	**3.0**		**1,382.40**

Seal the plate, vortex, spin down (00:00:30 , 750 x g), 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.

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Gap-filling	72°C	3 min	1
	Initial denaturation	98°C	30s	1

	A	B	C	D
	Denaturation	98°C	10s	14-16x
	Annealing	55°C	30 sec	
	Elongation	72°C	30 sec	
	Final Elongation	72°C	5 min	1
	Hold	4°C	Hold	

Library clean-up and quantification

30m

11 Take an aliquot from each sample well (i.e. 5 µl) and transfer it to a 1.5 mL Eppendorf tube for the library pool clean-up. The plate containing the rest of the libraries can be stored long-term at -20 °C 31m 30s

Remove the Sera-Mag SpeedBeads™ working solution (AMPure XP beads or SPRI beads when using a commercial solution) from the 4 °C storage and equilibrate it at room temperature for 00:15:00 .

Add 0.8X volume ratio Sera-Mag SpeedBeads™ working solution to the pool. Mix thoroughly by pipetting or vortexing.

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.

Note

You can resuspend the final pool in higher volume than 50 µL depending on volume of beads used.

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.

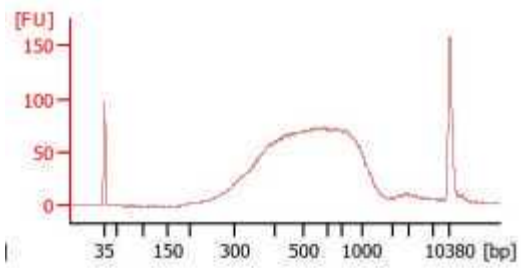
Remove 49 µL of the supernatant and transfer it to a new 1.5-ml LoBind tube. Store the cDNA at -20 °C long-term or until ready for sequencing.

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



Sequencing

12 The clean library pool can be sequenced on any Illumina sequencer. Follow the specifications reported for each individual instrument. Single-End 75 bp is generally sufficient but longer read modes or paired-end sequenced are also options depending on the biological question.

Analysis

13 Data generated with FLASH-seq bulk can be analysed with standard unstranded bulk RNA-sequencing pipelines. We suggest the following tools:

1. (optional) Trim left-over adapters / TSO / oligo-dT with trimmomatic or bbduk.
2. Map reads with STAR.
3. Select properly mapped reads with samtools (-F 260).
4. Visualise the alignment with IGV.
5. Explore the mapping statistics with rseqc (gene body coverage, intron/exon/intergenic mapping).
6. Assign reads to features with featureCounts.
7. Explore isoforms with RSEM or BRIE.
8. Calculate differential expression with DESeq2, EdgeR or limma.