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

Created: Apr 04, 2023

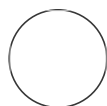
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Bulk FLASH-seq

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Rebecca A Siwicki

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

REAGENTS - CELL LYSIS MIX

PROTOCOL integer ID:
79989

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



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 Thermo Fisher Scientific Catalog
##EP0741



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 -80°C 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/AAGCAGTGGTATCAACGCAGAGTACTTTTTT TTTTTTTTTTTTTTTTTTTTTTTTTVN | 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_N 714 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTCATGAGCGTCTCG TGGGCTCG*G |
| Nextera_v2_N 715 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCCTGAGATGTCTCG TGGGCTCG*G |
| Nextera_v2_N 716 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTAGCGAGTGTCTCG TGGGCTCG*G |
| Nextera_v2_N 718 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGTAGCTCCGTCTCG TGGGCTCG*G |
| Nextera_v2_N 719 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTACTACGCGTCTCG TGGGCTCG*G |
| Nextera_v2_N 720 | /5Biosg/CAAGCAGAAGACGGCATACGAGATAGGCTCCGGTCTCG TGGGCTCG*G |
| Nextera_v2_N 721 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGCAGCGTAGTCTCG TGGGCTCG*G |
| Nextera_v2_N 722 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCTGCGCATGTCTCG TGGGCTCG*G |
| Nextera_v2_N 723 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGAGCGCTAGTCTCG TGGGCTCG*G |
| Nextera_v2_N 724 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCGCTCAGTGTCTCG TGGGCTCG*G |
| Nextera_v2_N 726 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGTCTTAGGGTCTCG TGGGCTCG*G |
| Nextera_v2_N 727 | /5Biosg/CAAGCAGAAGACGGCATACGAGATACTGATCGGTCTCG TGGGCTCG*G |
| Nextera_v2_N 728 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTAGCTGCAGTCTCG TGGGCTCG*G |
| Nextera_v2_N 729 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGACGTCGAGTCTCG TGGGCTCG*G |
| Nextera_v2_S5 02 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCTCTCTATT CGTCGGCAGCGT*C |
| Nextera_v2_S5 13 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTCGACTAGT CGTCGGCAGCGT*C |
| Nextera_v2_S5 03 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTATCCTCTT CGTCGGCAGCGT*C |
| Nextera_v2_S5 15 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTTCTAGCTT CGTCGGCAGCGT*C |
| Nextera_v2_S5 05 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTAAGGAGT CGTCGGCAGCGT*C |
| Nextera_v2_S5 16 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCCTAGAGTT CGTCGGCAGCGT*C |
| Nextera_v2_S5 06 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACTGCATAT CGTCGGCAGCGT*C |
| Nextera_v2_S5 17 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGCGTAAGAT CGTCGGCAGCGT*C |

| A | B |
|---------------------|--|
| Nextera_v2_S5 07 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACAAGGAGTAT CGTCGGCAGCGT*C |
| Nextera_v2_S5 18 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCTATTAAGT CGTCGGCAGCGT*C |
| Nextera_v2_S5 08 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCTAAGCCTT CGTCGGCAGCGT*C |
| Nextera_v2_S5 20 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACAAGGCTATT CGTCGGCAGCGT*C |
| Nextera_v2_S5 10 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCGTCTAATT CGTCGGCAGCGT*C |
| Nextera_v2_S5 21 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGAGCCTTAT CGTCGGCAGCGT*C |
| Nextera_v2_S5 11 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTCTCTCCGT CGTCGGCAGCGT*C |
| Nextera_v2_S5 22 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTTATGCGAT CGTCGGCAGCGT*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/CAAGCAGAAGACGGCATACGAGATGCCTATCAGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_2 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCTTGGATGGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_3 | /5Biosg/CAAGCAGAAGACGGCATACGAGATAGTCTCACGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_4 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCTCATCAGGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_5 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTGTACCGTGTCTCG TGGGCTCG*G |
| Nextera_extra_ i7_6 | /5Biosg/CAAGCAGAAGACGGCATACGAGATAAGTCGAGGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_7 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCACGTTGTGTCTCG TGGGCTCG*G |
| Nextera_extra_ i7_8 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTACAGCAGTCTC GTGGGCTCG*G |
| Nextera_extra_ i7_9 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCTACTTGGGTCTCG TGGGCTCG*G |
| Nextera_extra_ i7_10 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCCTCAGTTGTCTCG TGGGCTCG*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/CAAGCAGAAGACGGCATACGAGATGAAGGTTCTGTCTCGTGGGCTCG*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/CAAGCAGAAGACGGCATACGAGATTCAACTGGGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_36 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCTGCACTTGTCTCG TGGGCTCG*G |
| Nextera_extra_i7_37 | /5Biosg/CAAGCAGAAGACGGCATACGAGATACACGGTTGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_38 | /5Biosg/CAAGCAGAAGACGGCATACGAGATAATACGCGGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_39 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTGCGAACTGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_40 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGCTGACTAGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_41 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGTGGTGTGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_42 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGTGCTTACGTCTCG TGGGCTCG*G |
| Nextera_extra_i7_43 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTCAAGGACGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_44 | /5Biosg/CAAGCAGAAGACGGCATACGAGATTGAACCTGGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_45 | /5Biosg/CAAGCAGAAGACGGCATACGAGATAGTGTGGGTCTC GTGGGCTCG*G |
| Nextera_extra_i7_46 | /5Biosg/CAAGCAGAAGACGGCATACGAGATGTACTCTCGTCTCG TGGGCTCG*G |
| Nextera_extra_i7_47 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCCGTATCTGTCTCG TGGGCTCG*G |
| Nextera_extra_i7_48 | /5Biosg/CAAGCAGAAGACGGCATACGAGATCGAAGAACGTCTC GTGGGCTCG*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/AATGATACGGCGACCACCGAGATCTACACCGACCATT TCGTCTCGGCAGCGT*C |
| Nextera_extra_i5_2 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGATAGCGA TCGTCTCGGCAGCGT*C |
| Nextera_extra_i5_3 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACAATGGACG TCGTCTCGGCAGCGT*C |
| Nextera_extra_i5_4 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCGCTAGTA TCGTCTCGGCAGCGT*C |

| A | B |
|-------------------------|---|
| Nextera_extra_i 5_5 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTCTCTAGG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_6 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACATTGCG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_7 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTGAGGTGT TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_8 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACAATGCCTC TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_9 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCTGGAGTA TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_10 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTATGCTG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_11 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTGGAGAGT TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_12 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCGATAGAG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_13 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCTCATTGCT CGTCGGCAGCGT*C |
| Nextera_extra_i 5_14 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACCAGCTT TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_15 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGAATCGTG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_16 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACAGGCTTCT TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_17 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCAGTTCTG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_18 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTTGGTGAG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_19 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCATTCCGGT TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_20 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTGTGAAGC TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_21 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTAAGTGGC TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_22 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACGTGATG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_23 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTAGAGCA TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_24 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTCAGTTG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_25 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACATTCGAGG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_26 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGATACTGG TCGTCGGCAGCGT*C |
| Nextera_extra_i 5_27 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGCCTTGTTT CGTCGGCAGCGT*C |
| Nextera_extra_i 5_28 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACTTGGTCTCT CGTCGGCAGCGT*C |

| A | B |
|-------------------------|--|
| Nextera_extra_i 5_29 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACCCGACTAT TCGTCTCGGCAGCGT*C |
| Nextera_extra_i 5_30 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTCCTAAG TCGTCTCGGCAGCGT*C |
| Nextera_extra_i 5_31 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACCAATGC TCGTCTCGGCAGCGT*C |
| Nextera_extra_i 5_32 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGATGCACT TCGTCTCGGCAGCGT*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 sticking to 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.

3m 30s

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 |
| MgCl ₂ (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 |

| A | B | C | D | E |
|-----|----------------------|------|--------|--------|
| 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 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 a 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.

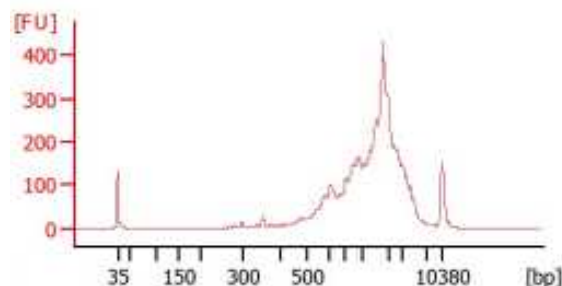
Safe stopping point. Amplified and clean cDNA can be stored for several months at -20 C. We recommend storing in LoBind plates to avoid material loss during long-term storage.

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 |


| A | B | C | D |
|---|-----------------------------------|---------------|--------|
| 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 |
| 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 be 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 |
|---|---|---|---|




| 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 MgCl ₂ | 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 ( 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 |

| A | B | C | D |
|---------------------|------------|---|-----------------|
| 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 |
| 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

- Take an aliquot from each sample well (i.e. 5 µl) and transfer it to a 1.5 mL Eppendorf tube for the final 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.

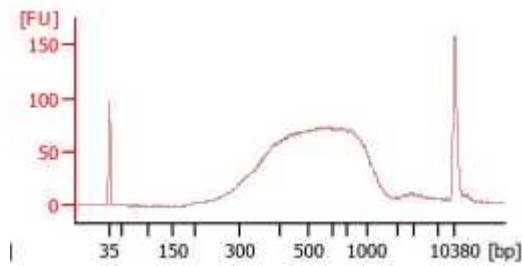
Remove  49 μL of the supernatant and transfer it to a new 1.5-ml LoBind tube. Store the cDNA at  -20 °C long-term or until ready for sequencing.

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.