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# FLASH-seq with SEQURNA RNAse Inhibitor

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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

This protocol is a modification of our FLASH-seq method (Protocols.io: https://dx.doi.org/10.17504/protocols.io.kxygxzkrwv8j/v4; PubMed: **https://pubmed.ncbi.nlm.nih.gov/35637419/**).
In this version we used the novel RNAse inhibitor form SEQURNA, with gains in performance (i.e. number of genes detected per cell) and reduced costs compared to our usual RRI (70% cost saving).
Moreover, we found out that DTT is not required when using the SEQURNA inhibitor and it rather leads to inferior results. Therefore, no DTT has been used for the lysis as well as the RT-PCR steps.

## Materials

Reagents are grouped by the step in which they are needed.
Reagents used in more than one reaction are reported only once, in the first reaction where they are required.

### General consumables

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | Item | Company | Pack size | Cat. # | Comments |
| | RNase AWAY‰ Surface Decontaminant | Thermo Fisher | 6 bottles | 7002 | Alternatively a 0.5% solution of sodium hypochlorite can be used instead |
| | Adhesive PCR Plate Seals | Thermo Fisher | 100 pcs | AB-0558 | To use to seal the plate in ALL STEPS EXCEPT after cell sorting |
| | Aluminium foil seals for -80ºC storage | VWR | 100 pcs | 391-1281 | To use to seal the plate ONLY after cell sorting |
| | Twin.Tec PCR plates 384 (LoBind, colourless) | Eppendorf | 25 pcs | EP0030129547 | |
| | UltraPure DNase/RNase-Free Distilled Water | Thermo Fisher | 10 × 500 ml | 10977-049 | |
| | DNA LoBind 1.5 mL, PCR clean, colourless | Eppendorf | 5 × 50 pcs | 30108051 | |
| | Ethanol, for molecular biology | Sigma Aldrich | 500 ml | 51976-500ML-F | |

### Cell Lysis Mix

| | A | B | C | D |
|---|---|---|---|---|
| | Item | Company | Pack size | Cat. # |
| | SEQURNA Thermostable RNase Inhibitor | Genovis | 3 × 150 µl | SQ-RIT-045 |

| | A | B | C | D |
|---|---|---|---|---|
| | dNTP-Set 1 (100 mM each) | Roth | 5 × 4 × 250 µl | K039.2 |
| | Triton X-100 | Sigma Aldrich | 100 ml | X100-100ML |
| | Betaine (5 M solution) | Sigma Aldrich | 5 × 1.5 ml | B0300-5VL |

**RT-PCR Mix**

| | A | B | C | D |
|---|---|---|---|---|
| | Item | Company | Pack size | Cat. # |
| | KAPA HiFi HotStart ReadyMix (2x) | Roche | 6.25 ml | KK2602 |
| | Maxima H Minus Reverse Transcriptase | Thermo Fisher | 4 × 10,000U | EP0753 |
| | Magnesium chloride (1 M solution) | Thermo Fisher | 100 ml | AM9530G |
| | dCTP (100 mM solution) | Thermo Fisher | 250 µl | 10217016 |

**Magnetic bead preparation**

Any commercial bead solution, such as AMPure XP beads (Beckman-Coulter), can be used instead.

| | A | B | C | D |
|---|---|---|---|---|
| | Item | Company | Pack size | Cat. # |
| | Polyethylenglycol (MW = 8000) | Sigma Aldrich | 1 Kg | 89510-1KG-F |
| | Sodium chloride (5 M solution) | Sigma Aldrich | 1000 ml | 59222C-1000ML |

| | A | B | C | D |
|---|---|---|---|---|
| | Sera-Mag SpeedBead Carboxylate-Modified Magnetic Particles (Hydrophylic) | GE Healthcare | 15 ml | GE24152105050250 |
| | Sodium azide | Sigma Aldrich | 25 gr | S2002-25G |
| | EDTA (0.5 M solution, pH 8.0) | Thermo Fisher | 100 ml | AM9260G |
| | Tris-HCl, pH 8.0 (1 M solution) | Thermo Fisher | 1000 ml | 15568025 |
| | Tween-20 (100% solution) | Sigma Aldrich | 100 ml | P9416-100ML |

**Sample QC**

| | A | B | C | D |
|---|---|---|---|---|
| | Item | Company | Pack size | Cat. # |
| | Quant-iT PicoGreen dsDNA Assay Kit | Thermo Fisher | 10 × 100 ul | P11496 |
| | Nunc F96 MicroWell Polystyrene Plate, black | Thermo Fisher | 50 pcs | 237105 |
| | Qubit Assay Tubes | Thermo Fisher | 500 pcs | Q32856 |
| | Qubit 1X dsDNA HS Assay Kit | Thermo Fisher | 500 assays | Q33231 |
| | Agilent High Sensitivity DNA Kit | Agilent Technologies | 10 chips + reagents | 5067-4626 |
| | AMPure XP kit* | Beckman-Coulter | 60 ml | A63881 |

*Required only if choosing a commercial solution for sample cleanup.

**Tagmentation**

| | Item | Company | Pack size | Cat. # |
|---|---|---|---|---|
| | KAPA HiFi plus dNTPs and 5x buffer | Roche | 250U | KK2102 |
| | N,N-Dimethylformamide (100% solution) | Sigma Aldrich | 250 ml | D4551-250ML |
| | UltraPure‰ SDS (10% solution) | Thermo Fisher | 1000 ml | 24730020 |
| | TAPS | Sigma Aldrich | 100 gr | T9659-100G |
| | Sodium Hydroxide (pellet, purity 98%)* | Sigma Aldrich | 1 Kg | 71690-1KG |

*Sodium hydroxide is required to titrate the TAPS and adjust the solution to the required pH.

**Oligonucleotides**

**Standard FLASH-Seq RT-PCR**

| | Oligo ID | Sequence (5′ → 3′) | Purification / synthesis scale |
|---|---|---|---|
| | Smart dT30VN | /5Biosg/AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTVN | desalted or HPLC |
| | FS TSO | /5Biosg/AAGCAGTGGTATCAACGCAGAGTACrGrGrG | desalted or HPLC |

**Tn5 transposase loading**

| | A | B | C |
|---|---|---|---|
| | Oligo ID | Sequence (5′ → 3′) | Comments |
| | TN5MErev | /5Phos/ CTGTCTCT TATACACAT CT | 2 uM scale - desalted* |

| | A | B | C |
|---|---|---|---|
| | TN5ME-A | TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG | 1 uM scale - desalted* |
| | TN5ME-B | GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAG | 1 uM 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.

**Nextera index adaptors**

One can order the 4 Nextera XT Index Kit v2 (set A, B, C, D) sets 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. 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.

| | 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 |
| | 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 |

**Additional index adaptors**

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

| | 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 |
| | 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 |
| | 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 |

| | | |
|---|---|---|
| | 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 |
| | 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 |
| | Nextera_extra_i5_30 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGTCCTAAGTCGTCGGCAGCGT*C |

| | | |
|---|---|---|
| | Nextera_extra_i5_31 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACACCAATGCTCGTCGGCAGCGT*C |
| | Nextera_extra_i5_32 | /5Biosg/AATGATACGGCGACCACCGAGATCTACACGATGCACTTCGTCGGCAGCGT*C |

## Safety warnings

- **IMPORTANT!** Note that NO DTT is used in this version of FLASH-seq. Including DTT will decrease performance of the protocol.
- **IMPORTANT!** Note that NO additional RNase Inhibitor should be included in the RT mix. Adding additional RNase inhibitor in this step will decrease performance of the protocol.
- **IMPORTANT!** Dimethylformamide (DMF) is toxic and should be handled under the hood according to local safety regulations.

## Before start

- 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. There is no need to use mineral oil to prevent evaporation. However, the FLASH-seq protocol has been shown to perform well when cells are sorted in 200 nl lysis buffer + 800 nl RT-PCR mix using 1-2 µl mineral oil.
- The protocol below uses 1 U/ul of SEQURNA inhibitor. This represents the final concentration in the RT-PCR mix and it has been shown to be the most suitable one for the FLASH-seq. However, the optimal concentration may differ for other protocols, even if using the same SMART-seq chemistry.
- 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 (in Lysis Mix)** | **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 |
| | SEQURNA inhibitor (50 mass units/µl) | 5 U/µl | 0.100 | 42.240 |
| | Betaine (5 M) | 0.2 M | 0.200 | 84.480 |
| | Nuclease-free water | - | 0.422 | 178.253 |
| | **Total volume (µl)** | | **1.000** | **422.400** |

**IMPORTANT!**
**NO** DTT is used in this version of FLASH-seq. Including DTT will decrease performance of the protocol.

2 Add 1 µl Lysis Mix to each well of a 384-well plate.

3 Seal the plate with a PCR seal and quickly spin it down to collect the Lysis Mix to the bottom.

4 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.

**SAFE STOPPING POINT** - Plates containing lysis buffer can be stored for >6 months at -20ºC.

## Sample collection

10m

5 Sort single cells into 384-well plates containing 1 µl of Lysis Mix.

6 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.

7 Even if proceeding with the protocol immediately after sorting, it is advisable to put the plate on dry ice for 5 minutes, followed by heat denaturation, as freeze-thawing facilitates cell lysis.

**SAFE STOPPING POINT** - Sorted cells in lysis buffer can be stored for >6 months at -80ºC. Longer storage might lead to lower yield or increased presence of shorter cDNA fragments, indicating RNA degradation.

## Cell Lysis

3m

8 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.

9 Place the plate in a thermocycler with a heated lid and incubate for 3 minutes at 72°C, followed by a 4°C hold step.

10 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

3h 30m

11 While the plate is in the thermocycler, prepare the following RT-PCR Mix:

| | A | B | C | D |
|---|---|---|---|---|
| | **Reagent** | **Reaction concentration (final)** | **Volume (µl)** | **384-well plate** |
| | MgCl2 (1 M) | 9.2 mM | 0.046 | 19.430 |
| | Betaine (5 M) | 1 M | 0.800 | 337.920 |
| | Nuclease-free water | - | 0.422 | 178.253 |
| | dCTP (100 mM) | 1.8 mM | 0.090 | 38.016 |
| | Maxima H- RT (200 U/µl) | 2 U/ul | 0.050 | 21.120 |
| | KAPA HiFi HotStart ReadyMix (2x) | 1x | 2.500 | 1056.000 |

| | A | B | C | D |
|---|---|---|---|---|
| | FS TSO (100 µM) | 1.84 µM | 0.092 | 38.861 |
| | **Total volume (µl)** | | **4.000** | **1689.600** |

**IMPORTANT!**

**NO** additional RNase Inhibitor should be included in the RT mix. Adding additional RNase inhibitor in this step will decrease performance of the protocol.

**NO** DTT is used in this version of FLASH-seq. Including DTT will decrease performance of the protocol.

12 Add 4 µl of the RT-PCR Mix into each well of the 384-well plate.

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

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

| | A | B | C | D | E | F |
|---|---|---|---|---|---|---|
| | **Step** | | **Temperature** | **Time** | **Cycles** | |
| | RT | | 50ºC | 60 min | 1x | |
| | PCR | Initial denaturation | 98ºC | 3 min | 1x | |
| | | Denaturation | 98ºC | 20 sec | 18-21x* | |
| | | Annealing | 67ºC | 20 sec | | |
| | | Elongation | 72ºC | 5 min | | |
| | | | 15ºC | Hold | | |

*Adjust the number of cycles according to the cell type used. We recommend 18-19 cycles for HEK 293T cells and 21 cycles for hPBMC.

**SAFE STOPPING POINT** - Amplified cDNA before purification can be stored for several months at -20ºC.

## cDNA purification

30m

15 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: **Picelli S, Methods Mol Biol . 2019:1979:25-44**

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

17 We recommend adding extra nuclease-free water to each sample, to increase the volume, simplify the handling and improve recovery rate. We generally add 10 µl of nuclease-free water to 5 µl of amplified cDNA.

18 Add a 0.8x volume ratio of Sera-Mag SpeedBeads™ working solution to each well (i.e., 12 µl beads for each 15 µl cDNA). Mix thoroughly by pipetting or vortexing.

19 Incubate the plate off the magnetic stand for 5 min at room temperature.

20 Place the plate on the magnetic stand and leave it for 5 min or until the solution appears clear.

21 Remove the supernatant without disturbing the beads.

22 Performing an ethanol wash is not necessary. We do not recommend it when working in 384-well plates and with liquid handling robots, to avoid cDNA losses.

23 Remove the plate from the magnetic stand, add 15 µl of nuclease-free water and mix well by pipetting or vortexing to resuspend the beads. Do not let the bead pellet dry, as it will decrease the final cDNA yield!

24 Incubate 2 min off the magnetic stand.

25 Place the plate back on the magnetic stand and incubate for 2 min or until the solution appears clear.

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

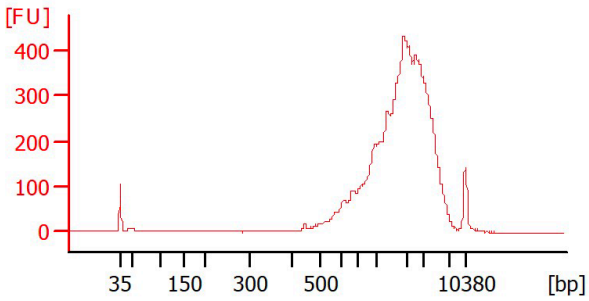
**SAFE STOPPING POINT** - Amplified and purified cDNA can be stored for several months at -20°C. We recommend using LoBind plates to avoid material losses upon long-term storage.

## Quality control check

45m

27 Check the cDNA quality on Agilent Bioanalyzer High Sensitivity DNA chip. Follow the instructions as described in the user manual. A good sample is characterized by a low proportion of fragments <500 bp, absence of residual primers (ca. 100 bp) and an average cDNA size of 2.0–2.5 Kb.

Expected result




Example of amplified cDNA from a single HEK293 cell (19 cycles)

## cDNA quantification

15m

28 Allow the Quant-iT PicoGreen reagent to warm to room temperature before opening the vial. PicoGreen is light sensitive; while thawing, wrap in aluminum foil.

29 Prepare a 1x working solution of TE using 20x TE (supplied) and nuclease-free water.

30 Prepare a 1:800 dilution of PicoGreen solution and always use a plastic vessel (tubes, Falcon, etc.). Do not use glass as PicoGreen may adsorb to glass.

31 Prepare the standard curve using Lambda DNA standard (supplied at a concentration of 100 ng/µl, with the PicoGreen 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.

32 Vortex well and spin down the DNA standards before every use. Not vortexing thoroughly the standards is going to negatively affect the standard curve and your readings! Serial dilutions should be prepared as shown in the table below.

| | A | B | C | D |
|---|---|---|---|---|
| | **Tube no.** | **Contents** | **Concentration** | **Final 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 |
| | 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 | - |

33 Prepare the PicoGreen solution by pipetting 0.25 µl of PicoGreen dye + 99.5 µl of 1X TE for each sample. Vortex to mix.

34 Pipette 1 µl of each of the 7 standards + 1 Blank into a black, flat-bottom Nunc™ F96 MicroWell™ Plate. Place the standards on one column.

35 Pipet 1 µl of your samples into the center of each well of the Nunc™ F96 MicroWell™ Polystyrene Plate.

36 Add 99 µl of PicoGreen + TE mix into every well. There is no need to mix.

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

38 Allow 2 minutes 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.

39 Use a plate reader to measure fluorescence (Excitation: 485 nm; Emission: 530 nm; Read from top; Endpoint reading).

## Plate normalization

10m

40 Prepare a normalization plate by adding 1 µl of purified cDNA and nuclease-free water to a final concentration of 200 pg/µl.

**SAFE STOPPING POINT** - Normalized cDNA can be stored for several months at -20°C. LoBind plates must be used to avoid material losses upon long-term storage.

## Tagmentation and indexing PCR

1h

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

42 Prepare the Tagmentation Mix as described below:

| | A | B | C |
|---|---|---|---|
| | **Reagent** | **Final Concentration** | **Volume (µl)** |
| | TAPS-Mg Buffer, pH=7.3 (5x) | 10 mM TAPS, 5 mM MgCl2 | 0.800 |
| | Dimethylformamide (DMF) (100%) | 20% | 0.800 |
| | Tn5 transposase (2 µM working dil.) | 62.5 nmol | 0.125 |
| | Nuclease-free water | - | 2.275 |
| | **Total volume (µl)** | | **3.000** |

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

43 Dispense 3 µl of Tagmentation Mix in a new 384-well plate.

44 Add 1 µl of normalized cDNA (200 pg/µl) to each well containing the Tagmentation Mix.

45 Seal the plate, vortex, spin down, and carry out the tagmentation reaction: 55°C for 8 min, 4°C hold. Upon completion proceed immediately to the next step.

46 Add 1 µl of 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.

47 Add 2 µl of prediluted N7xx + S5xx Index Adaptors (5 µM each).

48 Add 3 µl of Enrichment PCR Mix to each well:

| | A | B | C |
|---|---|---|---|
| | **Reagent** | **Final Concentration** | **Volume (µl)** |
| | KAPA HiFi enzyme (1 U/µl) | 0.02 U/µl | 0.200 |
| | KAPA HiFi Buffer (5x) | 1x | 2.000 |
| | dNTPs (10 mM) | 300 mM | 0.300 |
| | Nuclease-free water | - | 0.500 |
| | **Total volume (µl)** | | **3.000** |

49 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.

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | **Step** | | **Temperature** | **Time** | **Cycles** |
| | Gap filling | | 72ºC | 3 min | 1x |
| | Enrichment PCR | Initial denaturation | 98ºC | 30 sec | 1x |
| | | Denaturation | 98ºC | 10 sec | 12x |

| | A | B | C | D | E |
|---|---|---|---|---|---|
| | | Annealing | 55ºC | 30 sec | |
| | | Elongation | 72ºC | 30 sec | |
| | | | 15ºC | hold | |

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

## Library cleanup and quantification

45m

50 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.

51 Remove the Sera-Mag SpeedBeads™ working solution (alternatively: AMPure XP beads or SPRI beads) from the +4°C storage and equilibrate it at room temperature for 15 min.

52 Add Sera-Mag SpeedBeads™ working solution to a final ratio of 0.8x and mix well to homogenization. Use a different ratio if the goal is to recover longer (i.e., lower ratio) or shorter (i.e., higher ratio) fragments.

53 Incubate the tube off the magnetic stand for 5 min at room temperature.

54 Place the tube on the magnetic stand and leave it for 5 min or until the solution appears clear.

55 Remove the supernatant without disturbing the beads.

56 Recommended: wash the pellet with 1 ml of 80% v/v ethanol. Incubate 30 sec without removing the tube from the magnetic stand.

57 Remove any trace of ethanol and let the bead pellet dry for 2 min. Do not cap the tube or remove it from the magnetic stand during this time. Do not completely air-dry the beads.

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

59 Incubate 2 min off the magnetic stand.

60 Place the tube back on the magnetic stand and incubate for 2 min or until the solution appears clear.

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

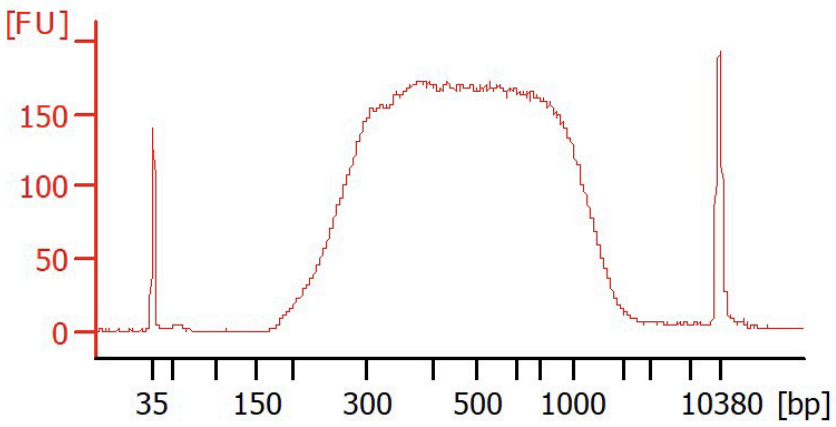
62 Use Qubit fluorometer or a similar fluorimetric assay to quantify the library. Library yield can vary from c1 to e100 ng/µl depending on the number of cells being pooled as well as PCR cycles used.

63 Check the final library size on the Agilent Bioanalyzer. Follow the instructions as described in the High Sensitivity DNA chip user manual.

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

65

Expected result




Example of sequencing-ready library (pool of 384 HEK293 cells).

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

## Pooling and sequencing

15m

66 The purified library can be sequenced on any Illumina or Element Bio sequencer. Follow the specifications reported for each instrument.

## Protocol references

"SEQURNA enhances FLASH-seq gene detection while eliminating DTT dependence"
Irina Khven, Mariana Ribeiro, Sara Crausaz, Simone Picelli
bioRxiv 2025.12.12.693841; doi: https://doi.org/10.64898/2025.12.12.693841