Oct 28, 2020

In-house automated Smart-Seq2

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1 Works for me dx.doi.org/10.17504/protocols.io.bnzsmf6e

Human Cell Atlas Method Development Community

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ABSTRACT

This SOP describes the procedure for the in-house automated Smart-Seq2 workflow with off-the-shelf reagents. This set up utilises the benchtop robots and significantly reduces the experimental cost, hands-on time, as well as increase the reproducibility and workflow efficiency, while delivering a throughput of thousands of cells per day. Following library construction, samples are pooled in equivolume and quantified, prior to sequencing on the Illumina platform.

DOI

dx.doi.org/10.17504/protocols.io.bnzsmf6e

PROTOCOL CITATION

Lira Mamanova 2020. In-house automated Smart-Seq2. **protocols.io** https://dx.doi.org/10.17504/protocols.io.bnzsmf6e

KEYWORDS

null, RNA-Seq, Automation, SmartSeq2, Zephyr, Mantis

CREATED

Oct 26, 2020

LAST MODIFIED

Oct 28, 2020

PROTOCOL INTEGER ID

43794

MATERIALS TEXT

Reagents:
Triton™ X-100 (Sigma, cat. no. t9284)
ERCC RNA Spike-In Mix (Ambion, cat. no. 4456740)
RNase free water (not-depc treated) (Ambion, cat. no. AM9937)
RNaseZap (Ambion, cat. no. AM9780)
CD45 MicroBeads, mouse (Miltenyi Biotec, cat. no. 130-052-301)
dNTP Mix (ThermoFisher UK Ltd, cat. no. 11853933)
Recombinant Ribonuclease Inhibitor (Takara Bio, cat. no. 2313B)
Betaine solution, 5 M (Sigma-Aldrich Co. Ltd, cat. no. B0300-5VL)
SmartScribe RT (Takara Bio, cat. no. 639538)

Maxima H Minus Reverse Transcriptase (Thermo Scientific™, EPO752)
MgCl 2 (1 M) (Life Technologies Ltd, cat. no. AM9530G)
KAPA Hifi HS Mix (Kapa Biosystems, cat. no. KK2603)
Oligo-dT(30)VN (5'–AAGCAGTGGTATCAACGCAGAGTACTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN-3'; IDT, PAGE purification)
IS PCR Primers (5'-AAGCAGTGGTATCAACGCAGAGT-3'; IDT, Standard Desalt)
Template-switching oligo (TSO) (5'-AAGCAGTGGTATCAACGCAGAGTACATrGrG+G-3'; EXIQON, HPLC, RNAse-free)
80% Ethanol Freshly prepared Ethanol 95–97% (vol/vol) AnalaR Normapur analytical reagent (VWR, cat. no. 20823.327) ! CAUTION Ethanol is flammable, and it should be stored carefully and handled with appropriate safety equipment.
DTT (Invitrogen, cat. no. 18064-014) ! CAUTION DTT is toxic when ingested. Avoid inhaling fumes or contact with the skin. Handle it using appropriate safety equipment.
RNAClean XP, 40 mL (Beckman Coulter Genomics, cat. no. A63987) ! CAUTION Agencourt AMPure XP Beads contains Sodium Azide, which is Toxic and should be handled with appropriate care.
Buffer RLT (Qiagen, cat. no. 79216) ! CAUTION Buffer RLT contains guanidine thiocyanate, which is harmful and should be handled with appropriate safety equipment.
Agencourt AMPure XP Beads (Beckman Coulter, cat. no. A63882) ! CAUTION Agencourt AMPure XP Beads contains Sodium Azide, which is Toxic and should be handled with appropriate care.
Bleach (VWR International Ltd, cat. no. 1053393)! CAUTION Bleach can cause an irritation in the eyes, mouth, lungs and on skin.
Agilent High-Sensitivity DNA Kit (Agilent Technologies, cat. no. 5067-4626)
Agilent 1000 DNA Kit (Agilent Technologies, cat. no. 5067-1505)
Nextera XT DNA Sample Preparation Kit, 96 samples (Illumina, cat. no. FC-131-1096)
Nextera XT 96-Index Kit, 384 samples (Illumina, cat. no. FC-131-1002)

Equipment:
Seal - Adhesive PCR Film Kbioscience, cat. no. 4TI-0500)
50 Framestar 96-well PCR Plate (4titude, cat. no. 4TI-0960)
Eppendorf twin.tec 96-well PCR Plate (Eppendorf, cat. no. 0030128648)
Thermo Scientific Nunc 96-well deepwell storage plates (Thermofisher, cat. no 278743)
2ml 96 well V-Bot Assay (Corning, cat. no. 3961)
Dna Lobind Tubes, 1,5ml, PCR Clean, (Eppendorf, cat. no. 0030 108.051)
Falcon® Conical Tubes, 50 mL (Stem Cell Technologies SARL, cat. no. 38010)
Falcon® Conical Tubes, 15 mL (Stem Cell Technologies SARL, cat. no. 38009)
Eppendorf Combitips advanced® 25 mL. Pack of 100. Red (Eppendorf, cat. no. 0030089472)
Tips LTS 20UL Filter RT-RT-L10FLR (Anachem, cat. no. 17007957)
Tips GP-LTS-A-10µL-/F-960/10 (Anachem, cat. no. 30389274)
Pipette Tips LQR LTS 20µL FL 960/10 (Anachem, cat. no.17014400)
Tips 5-200ul Filter Greenpak LTS Pk960Tip (Anachem, cat. no. 30389276)
Tips 50-1000ul filter Greenpak LTS Pk/768 (Anachem, cat. no. 30389272)
80 uL - Barrier Sterile 96 Rack Tips (Perkin Elmer, cat. no. 111624)
150 uL - Barrier Sterile 96 Rack Tips - (Perkin Elmer, cat. no. 111426)
Pipet-Lite Multi Pipette L8-20XLS+(2-20 µL) (Anachem, cat. no. 17014392)
Pipet-Lite Multi Pipette L8-200XLS+(20-200 µL) (Anachem, cat. no. 17014391)
Pipet-Lite LTS Pipette L-1000XLS+ (Anachem, cat. no. 17014382)
Pipet-Lite Multi Pipette L8-10XLS+(0.5-10 µL) (Anachem, cat. no. 17014388)
Multi-dispenser (Multipette Eppendorf, E3f), Multipette Xstream®, with charging adapter, 100-240V/50-60Hz Eppendorf UK Ltd, cat. no. 4986000025)
Zephyr® G3 NGS Workstation (Perkin Elmer, cat. no.133750)
Plate-Magnetic, 96 ring, bead separator (Perkin Elmer, cat. no.128316)
Spacer, Magnetic plate, Sciclone NGS (Perkin Elmer, cat. no.133514)
96S Super Magnet Plate (Alpaqua, cat. no. A001322)
MANTIS® Liquid Handler (Formulatrix, cat. no. MANTV3.1)
Low Volume Silicone based Mantis Chip (Formulatrix, cat. no. MCLS12)
High Volume Silicone based Mantis Chip (Formulatrix, cat. no. MCHS12)
Alpha Cycler 4 (LabTech, cat. no.AC496)
VWR® PCR Workstation (VWR, cat. no. 732-2542)
2100 Bioanalyzer (Agilent Technologies LDA UK Limited, cat. no.G2938C)
DynaMag Spin Magnet (Life Technologies, cat. no. 12320D)
Vortex mixer (e.g., Grant Instruments, model no. PV-1)
Microcentrifuge (e.g., Starlab, model no. N2631-0007)

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Fluorescence-activated cell sorter compatible with single-cell deposition into 96-well plates (e.g., BD Influx, Sorter CL2-BD INFLUX or Beckman Coulter MoFlo)
Refrigerated centrifuge and adaptors for 96-well plates (e.g., Eppendorf, model no. 5810 R)
An Illumina next-generation sequencing platform (HiSeq platform)

ABSTRACT

This SOP describes the procedure for the in-house automated Smart-Seq2 workflow with off-the-shelf reagents. This set up utilises the benchtop robots and significantly reduces the experimental cost, hands-on time, as well as increase the reproducibility and workflow efficiency, while delivering a throughput of thousands of cells per day. Following library construction, samples are pooled in equivolume and quantified, prior to sequencing on the Illumina platform.

Preparation of lysis buffer plates and single-cell samples

1

A single-cell contains a very low amount of RNA. Therefore, extra effort should be taken to avoid sample degradation and reagent contamination. All work surfaces and equipment should be decontaminated with DNA and RNAZap before and after the experiment. RNA related work should be carried out in a specially designated cleanroom or in a laminar flow hood equipped with UV light for sterilization. When working with a few batches of samples, we would advise pre-aliquotting reagents, especially if the reagent supplied in a large volume.

For reagent dispensing steps, we use the Formulatrix Mantis microfluidic liquid handler, and for all purification steps, we use a Zephyr automated liquid-handling robot.

Steps 1-12 should be performed in specially designated RNAse free areas, keeping reagents and cells chilled at all times.

The Formulatrix Mantis microfluidic liquid handler is positioned in a laminar flow hood. The lysis buffer dispensing step can be performed on alternative liquid handling platforms or manually. The reagents were dispensed from a 0.2 ml or 1 ml tip inserted into the High Volume (HV) Silicone based Mantis chip. We recommend having an individual set of chips for various reagent dispensing. Clean the dispensing chips prior to use by dispensing 50-100 µl of solutions into the waste plate in the following order: Water => 10% Bleach (incubate for **00:05:00**) => Water=> 80% EtOH=>Water. Repeat these procedures after finishing the reagent dispensing.

For each well, prepare the Triton buffer by adding **0.2 µl** of RNAse inhibitor and **0.02 µl** of diluted (1/500,000) ERCCs to the **1.8 µl** of **0.2 %** Triton X-100. Prepare an RLT lysis buffer by using **9.8 µl** of RLT buffer (Qiagen); **0.2 µl** of DTT (2M) and **0.02 µl** of diluted (1/500,000) ERCCs. Dispense **2 µl** of Triton lysis buffer or **10 µl** of RLT lysis buffer into each well of a 96-well plate.

We recommend to seal dispensed plates and centrifuge them immediately (**1000 rpm** for **00:01:00** at **4 °C**) to spin down the lysis buffer to the bottom of the well to prevent a sample degradation.

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Sealed Lysis buffer plates can be stored at **-80 °C** prior to cell sorting for < 6 months.

2 FACS sort single cells into the plate. Immediately centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) plates containing cells and keep them chilled on ice or at **-80 °C** until further processing. 1m

When FACS sorting, take care of plate calibration/priming prior to single-cell deposition. If many plates are deposited at the same time, repeat the calibration/priming at least every 8 plates.We recommend defrost lysis buffer plates on ice prior to cell sorting, centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) and keep chilled on ice.

Samples can be stored at **-80 °C** for at least 6months.

Preparation of RNA Elution buffer

3 Prepare the RNA elution buffer fresh for each experiment, as described below. We would recommend noting the lot numbers for reagents and plastics for every run, as this can be useful in troubleshooting. Keep the RNA elution buffer chilled until use.

Reagent	Per well (µl)	Final concentration
100 µM oligo dT	0.15	2.5 µM
10 mM dNTP	1.5	2.5 mM
RNase Inhibitor (40U/µl)	0.15	10 U/µl
Nuclease-free water	4.2	
Total	6	

4 Depending on the lysis buffer used, continue with option RLT buffer treated cells **(A)** or Triton buffer treated cells **(B)**. We recommend introducing the non-template and multicell (10-50 cells) controls and locate them in the middle of the plate. 27m 30s

RLT buffer should be removed prior to subsequent reverse transcription.

A. RLT buffer treated cells: Single-cell RNA cleaning with RNAClean XP beads

i. Allow RNAClean XP beads to warm up to room temperature for **00:15:00** before use, gently mixing to ensure that

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the beads are evenly resuspended.

ii. Dispense **25 µl** of RNAClean XP beads into a clean 96-well Bead Plate with Eppendorf Multi-dispenser.

iii. Seal the plate and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**) to remove air bubbles in the wells.

Keep bead plate covered with a plate seal on a shaker between cleaning procedures if there are many plates to be processed.

iv. Thaw and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) plate containing lysed cells from Step 2. Add additional RNAse-free water to the samples if required to a final volume of **10 µl** .

We perform all purification steps using a Zephyr automated liquid-handling robot. Expose Zephyr deck to UV light for at least **00:20:00** . For the csRNA cleaning procedures use Zephyr 150 µl Barrier Sterile tips.

v . Add **22 µl** of RNAClean XP beads to each well of the working plate from step iv at room temperature, and mix thoroughly by pipetting up and down on the Zephyr instrument. Allow the mixture to stand for **00:05:00** at room temperature.

vi. Move the plate to a low-elution magnet, and allow the beads to settle for **00:02:00** or until the solution is clear.

vii. Once the RNAClean XP beads have settled, carefully remove and discard the supernatant without disturbing the beads. Keeping the 96-well plate on the magnet, wash the RNAClean XP beads with **100 µl** of freshly prepared **80 %** (vol/vol) ethanol for **00:00:30** , then remove and discard the ethanol wash. Do not disturb the beads.

viii. Repeat Step vii once more.

ix.Dispense **6 µl** of RNA elution buffer from step 3 to each well of a new clean 96-well plate and place it on a chilled position of the robot deck. Add **5 µl** of RNA elution buffer to the RNAClean XP beads with purified single-cell RNA using the Zephyr.

CRITICAL STEP Take the plate that contains beads with RNA and elution buffer under the hood and manually resuspend the beads in the RNA elution buffer with low-bind 10 µl tips with a multichannel pipette to facilitate the resuspension. Make sure that all beads are removed from the tips.

x. Seal the plate and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**).

xi. Proceed directly to the denaturation step 5.

B. Triton Buffer treated cells

i. Thaw and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) plate containing lysed single cells from Step2.

ii. Dispense **5 µl** of freshly prepared RNA elution buffer from step 3 to each well of 96-well plate from step i. Seal the plate and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**).

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Proceed directly to the denaturation step 5.

RNA denaturation

5 Perform RNA denaturation on the thermal cycler with the heated lid set to 100 °C.

Temperature	Time	Cycles
72 °C	3 min	1
4 °C	forever	

6 When the temperature reaches **4 °C**, immediately transfer plate with denatured single-cell RNA onto ice and proceed to the reverse transcription immediately.

Reverse Transcription

7 Prepare the Reverse Transcription (RT) Master mix, as described below. We used this protocol for two reverse transcriptases, SmartScribe (SMT) and Maxima -H (Maxima), with enzymes corresponding to Master Mix compositions.

RT Master mix for Maxima enzyme

Reagent	Per well (µl)	Final concentration
Maxima buffer (5x)	2	2.5x
Betaine (5 M)	2	1.5 M
MgCl2 (100 mM)	0.9	13 mM
TSO (10 µM)	1.0	1.5 µM
RNase inhibitor (40 U/µl)	0.25	10 U/µl
Maxima RNaseH-minus RT (200 U/µl)	0.1	20 U/µl
H2O	0.75	
Total	7.0	

RT Master mix for SMT enzyme

Reagent	Per well (µl)	Final concentration
Smartscribe buffer(5x)	2	2.5x
DTT (100mM)	0.5	8.5 mM
Betaine (5M)	2	1.5 M
MgCl2 (1M)	0.09	0.013 M
TSO (100 µM)	0.1	1.8 µM

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RNase inhibitor (40U/ µl)	0.25	1.76 U/µl
SmartScribe (100U/ µl)	0.5	10 U/µl
H2O	0.56	
Total	5.7	

8 Dispense **7 µl** of Maxima RT master mix or **5.7 µl** of SMT RT master mix to the plate with denatured RNA from step 6. Seal the plate, vortex on a medium speed and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**). 1m

9 Reverse transcription will convert polyA (+) mRNA to cDNA. Place the plate in the thermal cycler and perform the following steps on a thermal cycler:

Reverse Transcription conditions for Maxima RT

Temperature	Time	Cycles
50 ºC	90 min	1
85 ºC	5 min	1
4 ºC	Hold	1

Reverse Transcription conditions for SMT RT

Temperature	Time	Cycles
42 ºC	90 min	1
50 ºC	2 min	10
42 ºC	2 min	
70 ºC	15 min	1
4 ºC	hold	1

10 Collect the plate from the thermal cycler, centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**) and keep chilled on ice. 1m

PCR Amplification

11 Prepare the PCR amplification Master mix, as described below.

Identify the number of PCR cycles for each investigated cell type to avoid under- or over-amplification.

PCR amplification master mix for Maxima enzyme

Reagent	Per well (µl)	Final concentration
ISPCR primer (10 µM)	0.5	0.4 µM

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KAPA HiFi Hot Start Ready Mix (2x)	12.5	2x
H2O	1.0	
Total	14	

PCR amplification master mix for SMT enzyme

Reagent	Per well (µl)	Final concentration
ISPCR primer (10 µM)	0.25	0.3 µM
KAPA HiFi Hot Start Ready Mix (2x)	12.5	2x
H2O	2.25	
Total	15	

12 Dispense **14 µl** of PCR master mix for Maxima RT or **15 µl** of PCR master mix for SMT RT to each sample on the 96-well plate from step 10 using a Mantis. Seal the plate, vortex on a medium speed and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**). Place the plate in the thermal cycler and perform the following steps: 1m

PCR amplification protocol for Max RT

Temperature	Time	Cycles
98 °C	3 min	1
98 °C	15 sec	20 / 22 / 25 cycles depending on the cell type
67 °C	20 sec	
72 °C	6 min	
72 °C	6 min	1
4 °C	hold	

PCR amplification protocol for SMT RT

Temperature	Time	Cycles
98 °C	3 min	1
98 °C	20 sec	20 / 22 / 25 cycles depending on the cell type
67 °C	15 sec	
72 °C	6 min	
72 °C	5 min	1
4 °C	hold	

Amplified cDNA samples can be stored in **-20 °C** for several months before purification.

13 Proceed with the Agencourt AMPure beads for cleaning on the Zephyr instrument, or keep the plate at **4 °C** if

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continuing the next day, or -20 °C for long term storage.

Purification of the amplified cDNA 15m

14 15m

Steps 14–25should be performed in a dedicated post-amplification room.

Allow Agencourt AMPure XP beads to warm up to room temperature for 00:15:00 before use, gently mixing to ensure that the beads are evenly resuspended. Dispense 25 µl of Agencourt AMPure XP beads into the clean 96-well Bead Plate with Eppendorf Multi-dispenser.

15 1m

Centrifuge (1000 rpm for 00:01:00 at 4 °C) the plate containing the amplified cDNA from Step13.

16 5m

Add 25 µl of the Agencourt AMPure XP beads to each well of the 96-well plate containing the PCR-amplified cDNA molecules at room temperature, and mix thoroughly by pipetting up and down. Allow the mixture to stand for 00:05:00 at room temperature.

17 2m

Move the plate to a magnet, and allow the beads to settle for 00:02:00 or until the solution is clear.

18

Carefully remove and discard the supernatant without disturbing the beads, once the beads have settled.

19 30s

Keeping the 96-well plate on the magnet, wash the beads with 100 µl of freshly prepared 80 % (vol/vol) ethanol for 00:00:30 , and then remove and discard the ethanol wash. Do not disturb the beads.

20

Repeat Step 19 once more.

21 5m

Remove and discard any remaining ethanol solution from the well without disturbing the beads, and then allow the beads to dry for ~ 00:05:00 .

22

Add 25 µl of nuclease-free water to the beads, remove the plate from the magnet and resuspend by pipetting up and down at room temperature.

23 2m

Incubate this 96-well plate for 00:02:00 off the magnet at room temperature.

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24 Return the 96-well plate to the magnet, and allow the beads to settle for **00:05:00** or until the solution is clear. 5m

25 Transfer the supernatant, which contains the purified cDNA to a new 96-well plate at room temperature without disturbing the beads.

The amplified cDNA can be stored at **-20 °C** for >6 months before library preparation.

Quality Control of Amplified cDNA and input normalization

26 We use an Agilent Bioanalyzer for a cDNA QC on a high-sensitivity chip. Run **1 µl** of the eluted sample on a high-sensitivity chip on the Agilent. Dilute the library to 1 in 10 if cDNA is highly concentrated. It is important to quantify cDNA concentration accurately. Overestimation can affect the normalization of the cDNA input for the Nextera library preparation and lead to skewed Nextera libraries profiles.

Sample normalization

27

Steps 27–29 should be performed in a dedicated post-amplification room. Illumina libraries are prepared using the tagmentation approach with Nextera XT DNA Library Prep Kit (Illumina). We recommend following the manufacturer's instructions for the applied kit.

For cDNA input normalization, we check 11 randomized wells throughout a plate (A6; B4; B11; C3; D4; D12; F2; F9; G3; G8; H5). Measure cDNA concentration between 200-8000bp in all 11 samples, and find an average concentration that will be applied to the whole plate. Remove the outliers for accurate template normalization. The final average concentration of cDNA should be normalized to 0.3 ng/µl for optimal tagmentation. Normalize multicell controls separately.

28 Sample normalization is performed on the Zephyr instrument. A fixed amount of cDNA will be used for the dilution. Take **5 µl** of cDNA from the individual wells and dilute with water to achieve a final concentration of 0.3 ng/ul.

29 Seal the normalised plate and centrifuge it (**1000 rpm** for **00:01:00** at **4 °C**), vortex on medium speed for **00:01:00** and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) again. 3m

Normalised cDNAcan be stored at **+4 °C** for continuation the next day, or at **-20 °C** for a week.

Library construction using the Nextera XT Library Preparation Kit

30 Transfer **1.2 µl** of normalised samples from step 29 using the Zephyr instrument to the working Nextera Plate. Keep plates chilled and continue with the Nextera protocol.

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31 Prepare the Nextera master mix, as described below and dispense with the Mantis using a Low Volume (LV) chip.

Reagent	Per well (µl)
Tagmentation DNA buffer	2.5
Amplification Tagment Mix	1.25
Total	3.75

32 Seal the plate and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**), vortex on medium speedfor **00:01:00** and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) again. 3m

33 Place the Nextera plate in a thermal cycler and run the following program:

Temperature	Time
55 ^{0}C	10min
4 ^{0}C	Hold

34 Once the temperature reaches **4 °C** , remove from the thermal cycler and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**) the plate. 1m

35 Wash Mantis LV chip with water by dispensing 50-100 µl of water into the waste plate.

36 Dispense **1.2 µl** NT buffer into each well to neutralize the reaction.

Nextera Index preparation 1m

37 2m

Prepare the Indexes set mix (A-B-C-D) in 96-well Index Plate according to the manufacturer's instructions. Defrost and spin down Index 1(i7) and Index 2 (i5). Arrange the index primers in the Index Plate Fixture as follows: Index 1 (i7) adapters in columns 1-12 in ascending or descending order. Index 2 (i7) adapters in columns A-H in ascending or descending order(Figure S3).
Using a multichannel pipette, add **10 µl** of i7 adapters down each row. Replace the vial cap with a new cap. Using a multichannel pipette, add **10 µl** of i5 adapters down each column. Replace the vial cap with the new cap.

Seal the plate and centrifuge (**1000 rpm** for **00:01:00** at **4 °C**), vortex on medium speedfor 1min and

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centrifuge (1000 rpm for 00:01:00 at 4 °C) again.

38 Keep premade plates with index barcodes chilled until use or in the freezer at -20 °C for long term storage.

39 Dispense 2.5 µl of Index Adapters on the Zephyr instrument to the working Nextera plate from step 36. Seal the plate and centrifuge (1000 rpm for 00:01:00 at 4 °C). 1m

Nextera Library amplification

40 40.Wash Mantis LV chip by dispensing 50-100 µl of water into the waste plate.

41 Dispense 3.7 µl of NPM buffer by the Mantis dispenser to each well of the working Nextera plate.

42 Seal the plate and centrifuge (1000 rpm for 00:01:00 at 4 °C), vortex on medium speed for 1min and centrifuge (1000 rpm for 00:01:00 at 4 °C) again. 2m

43 Place the library prep plate in a thermal cycler and perform the following program:

Temperature	Time	Cycles
72 ºC	3 min	1
95 ºC	30 sec	1
95 ºC	10 sec	12
55 ºC	30 sec	
72 ºC	30 sec	
72 ºC	5 min	1
4 ºC	Hold	1

44 Once the temperature reaches 4 °C , remove from the thermal cycler. Centrifuge the plate (1000 rpm for 00:01:00 at 4 °C), vortex on medium speed for 1min and centrifuge (1000 rpm for 00:01:00 at 4 °C) again. 2m

Nextera library pooling and purification

45 Collect 5 µl of each sample (except the Control wells) into 8-well PCR strip tubes using a multichannel pipette. Pool control wells if these samples are used for the analysis.

46 Then pool amplified libraries from PCR strips into a single 1.5 ml vial (≈ 595 µl). Briefly spin down the strips and collect the rest of the samples.

47 Measure the sample volume with the pipette and add Agencourt AMPure XP beads at 1:1 ratio. Mix well by pipetting. 10m

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Incubate for **00:10:00** at room temperature.

48 Place the vial with the beads/sample mix on the magnet for **00:05:00** or wait till the solution is clear. 5m

49 Carefully remove and discard the supernatant without disturbing the beads, once the beads have settled.

50 Keeping the vial on the magnet, wash the beads with **100 µl** of freshly prepared **80 %** (vol/vol) ethanol for **00:00:30** , and then remove and discard the ethanol wash. Do not disturb the beads. 30s

51 Repeat Step 50 once more.

52 Remove and discard any remaining ethanol solution from the well without disturbing the beads, and then allow the beads to dry for ~ **00:05:00** . Leave the vial cap open to air-dry the beads. 5m

53 Remove the vial from the magnet, add **100 µl** of nuclease-free water to beads and resuspend by pipetting up and down at room temperature.

54 Incubate the vial for **00:02:00** off the magnet at room temperature. 2m

55 Return the vial to the magnet, and allow the beads to settle for **00:05:00** or until the solution is clear. 5m

56 Transfer the supernatant, which contains the purified Nextera libraries to a new vial without disturbing the beads.

57 Keep the samples at **4 °C** if continued the next day or **-20 °C** for long term storage.

Quality Control of Nextera sequencing libraries and normalization for sequencing

58 Run **1 µl** of the eluted sample from step 57 in triplicate on an Agilent Bioanalyser using a DNA 1000 assay chip or 1 in 10 diluted sample on a high-sensitivity chip.

59 Note the molarity of each reading and take an average of the 3 triplicates discarding any outliers.

Citation: Lira Mamanova (10/28/2020). In-house automated Smart-Seq2. https://dx.doi.org/10.17504/protocols.io.bnzsmf6e

60 Taking the molarity reading from step 59. In a fresh 1.5 ml tube combine the calculated volume of the amplified library pool with nuclease-free water to produce a final sequencing library pool of 2.8 nM.

61 In a fresh 1.5 ml tube equimolar pool the 2.8 nM sequencing library from step 60 with 3 additional sequencing library pools which have been processed through this workflow. To produce a final sequencing pool containing 384 samples.

Library sequencing

62 Libraries were submitted for sequencing on an Illumina HiSeq 4000 chemistry (paired-end, 75-bp reads). Perform single-end (SE) or paired-end sequencing (PE) of the Single-cell derived libraries according to the manufacturer's protocol. Alternative sequencing options to achieve comparable sequencing depth are; the NextSeq 550 with high output flow cell or NovaSeq 6000 SP flow cell on one lane of an XP workflow.

Citation: Lira Mamanova (10/28/2020). In-house automated Smart-Seq2. https://dx.doi.org/10.17504/protocols.io.bnzsmf6e