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Smart-seq3 Protocol V.2

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3 *Works for me* dx.doi.org/10.17504/protocols.io.bbpyimpw

Michael Hagemann-Jensen

GUIDELINES

• If you are pro, and want to change the volumes indicated in the protocol, make sure that the reaction concentrations stay similar. Also ensure that the spillover concentrations into the next reaction also stay similar. Failing to do so can lead to decreased performance of the protocol. In terms of the tagmentation reaction make sure to keep the ratio of cDNA input to Nextera TN5 amount, if you contemplate to miniaturize this reaction.

• The current protocols is based on the TN5 from Nextera Xt kit. However Illumina TDE1, works as well.

• For determining PCR cycles, a good general guideline is to add 1-2 cycles more than using Smart-seq2. However as always, this is good to empirically test first, before running important samples.

List of oligos:

Oligo	Vendor	Purification	Working concentration	Sequence	
Smartseq3_OligodT30VN	IDT	HPLC	100uM	/5Biosg/ACGAGCATCAGCAGCATACGATTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	
Smartseq3_N8_TSO	IDT	RNase-Free HPLC	100uM	/5Biosg/AGAGACAGATTGCGCAATGNNNNNNNNrGrGrG	
Fwd_PCR_primer	IDT	HPLC	100uM	TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGATTGCGCAA*T*G	
Rev_PCR_primer	IDT	HPLC	100uM	ACGAGCATCAGCAGCATAC*G*A	

* phosphorothioate bonds

• It is absolute fine to use standard desalting instead of HPLC, both works fine in our hands, same goes for hand-mixed vs machine mixed degenerate bases. Using the regular DNA oligos service at IDT should provide based on their QC full length oligos.

• We use custom Nextera Indexes primers (standard 25 nmol oligo preps from IDT, delivered at 200 nM concentration in IDTE buffer) and we typically get performance that is indistinguishable from Illumina's primers.

For making your own primers, we recommend using the "DNABarcodes" R package. using the following settings:
Barcode length: 10 bp (or 8bp like Illumina primers, depending on the amount of cells you need indexed and sequenced at the same time)
Minimal levenshtein distance: 3
Filter out homopolymers >= 3
Filter for uneven GC content

Additionally, there seems to be an artifact on the NovaSeq platform for i5 index primers starting with the bases "AC", so we recommend to avoid those too!
(see supplementary information in this paper: https://bmcgenomics.biomedcentral.com/articles/10.1186/s12864-018-4703-0#Sec13)

• For troubleshooting: feel free to leave comments or message directly.

MATERIALS

NAME	CATALOG #	VENDOR
Triton X-100	T8787-50ML	Sigma Aldrich

NAME	CATALOG #	VENDOR
Agilent High Sensitivity DNA Kit	5067-4626	Agilent Technologies
Nextera XT DNA Library Preparation Kit	FC-131-1096	illumina
QIAquick Gel Extraction Kit	28704	Qiagen
DNA LoBind Tubes, 1.5 mL	0030108051	Eppendorf
Recombinant RNAse Inhibitor	2313A	Takarabio
Dithiothreitol (DTT)	707265ML	Thermo Fisher Scientific
dNTP Set 100 mM Solutions	R0182	Thermo Fisher Scientific
UltraPure™ DNase/RNase-Free Distilled Water	10977035	Thermo Fisher
EDTA (0.5 M), pH 8.0, RNase-free	AM9260G	Thermo Fisher
SDS, 10% Solution, RNase-free	AM9822	Thermo Fisher
Maxima H Minus Reverse Transcriptase (200 U/µL)	EP0751	Thermo Fisher
Poly Ethylene Glycol (PEG) 8000	89510-250G-F	Sigma Aldrich
Sodium Chloride (5M)	AM9760G	Invitrogen - Thermo Fisher
Magnesium Chloride (1M Solution)	AM9530G	Invitrogen - Thermo Fisher
GTP (Tris buffered solution 100mM)	R1461	Thermo Scientific
Trizma-base	T6791-100G	Sigma Aldrich
KAPA HiFi Hotstart PCR kit	KK2502	Roche
Phusion High-Fidelity DNA Polymerase (2 U/µL)	F530L	Thermo Scientific
Sera-Mag Speed Beads	65152105050250	Ge Healthcare
Sodium Azide	S2002-100G	Sigma Aldrich
IGEPAL® CA-630	I8896	Sigma Aldrich
Armadillo PCR Plate 96-well clear semi-skirted white wells	AB3596	Thermo Scientific
Armadillo PCR Plate 384-well	AB2384B	Thermo Scientific
QuantiFluor® dsDNA System	E2670	Promega
E-Gel™ EX Agarose Gels 2%	G402002	Thermo Scientific
2-propanol	I9516	Sigma Aldrich
NN-Dimethylformamide	D4551	Sigma Aldrich

Before starting

1 This protocol should be carried out in a clean environment. Use ethanol, RNAseZAP, DNA-OFF, or similar to prepare work bench before start.

Work quickly and preferably on **On ice** .

Prepare master-mixes right before use.

Use multichannel pipettes, liquid dispensers etc. to dispense the master-mixes. Avoid pipetting up and down, to minimize the loss of material.

• Take a look at the Guidelines section for more info about Oligos etc. used in this protocol.

Prepare lysis plates

2 Prepare **lysis buffer mix**

Reagent	Reaction conc.	uL per. reaction	96 well plate	384 well plate
Guanidine Hydrochloride (8000mM ; Optional)	0mM - 50mM	0.00	-	-
Poly-ethylene Glycol 8000 (50% solution)	5%	0.40	44	164
Triton X-100 (10% solution)	0.1%	0.03	3.3	12.3
ERCC spike-ins (Optional)	-	-	-	-
RNAse Inhibitor (40u/uL)	0.5u/uL	0.04	4.1	15.4
OligodT30VN (100uM)	0.5uM	0.02	2.2	8.2
dNTPs (25mM/each)	0.5mM/each	0.08	8.8	32.8
Nuclease Free Water		2.43	267.6	997.3
Total		**3 uL**	**330 uL**	**1230 uL**

Reaction concentrations for PEG8000, OligodT30VN and dNTPs, are adjusted to and reflect their concentration in the reverse transcription reaction (4uL)

The lysis master-mix contains PEG! Ensure that PEG is fully mixed into solution, by either pipetting up and down until the liquid is clear, or start with vortexing the required master-mix volume of water and PEG together before adding the remaining reagents.

Add **3 µl** lysis buffer to each well of a 96/384 well plate.

Quick centrifugation to collect the lysis buffer before storage until use.

Sample collection

3 Sort single cells into **3 µl** lysis in either 96 or 384 wells.

Seal with appropriate seals (-80 C to >100 C) and centrifuge the finished sorted plate immediately after, and transfer it to a **-80 °C** freezer or dry-ice.

Cell lysis

4 Remove the plate of sorted cells from the -80 freezer and incubate in a thermocycler with heated lid at **72 °C** for **00:10:00** , followed by a **4 °C** hold (keeping the storage seal sheet on the plate, unless damaged or loose).

Reverse Transcription

5 While the plate is incubating as per **step 4**, prepare the following **Reverse transcription master-mix**.

Reagent	Reaction conc.	uL per. reaction	96 well plate	384 well plate
Tris-HCl pH 8.3 (1M)	25mM	0.1	11	41
NaCl (1M)	30mM	0.12	13.2	49.2
MgCl2 (100mM)	2.5mM	0.1	11	41
GTP (100mM)	1mM	0.04	4.4	16.4
DTT (100mM)	8mM	0.32	35.2	131.2
RNase Inhibitor (40u/uL)	0.5u/uL	0.05	5.5	20.5
TSO (100uM)	2uM	0.08	8.8	32.8
Maxima H-minus RT enzyme (200U/uL)	2u/uL	0.04	4.4	16.4
Nuclease Free Water		0.15	16.5	61.5
Total		**1uL**	**110uL**	**410uL**

Add **1 µl** RT mix to each well of a 96/384 well plate.

Replace the storage seal with a PCR seal. Ensure that the plate is properly sealed, to avoid evaporation.

Do a quick centrifugation to collect reaction at the bottom, before incubating the plate in a thermocycler at;

42 °C	90 min	1x
50 °C	2 min	10x
42 °C	2 min	
85 °C	5 min	1x

Preamplification PCR

6

Start preparing the **PCR mix,** when the incubation of the reverse transcription reaction is near completion, by combining the following components.

! Note that the KAPA DNA polymerase has a 3-5' exonuclease activity that is not HotStart. Therefore add polymerase just before using the master-mix.

Reagent	Reaction conc.	uL per. reaction	96 well plate	384 well plate
Kapa HiFi HotStart buffer (5X)	1X	2.0	220	820
dNTPs (25mM/each)	0.3mM/each	0.12	13.2	49.2
MgCl2 (100mM)	0.5mM	0.05	5.5	20.5
Fwd Primer (100uM)	0.1uM	0.01	1.1	4.1
Rev Primer (100uM)	0.1uM	0.01	1.1	4.1
Polymerase (1U/uL)	0.02U/uL	0.2	22	82
Nuclease Free Water		3.61	379.1	1480.1
Total		**6uL**	**660uL**	**2460uL**

Add **6 µl** PCR mix to each well of a 96/384 well plate.

Quick centrifugation to collect reaction at the bottom, before running the following PCR program in a thermocycler.

Step	Temperature	Time	Cycles
Initial denaturation	98 °C	3 min	1x
Denaturation	98 °C	20 sec	18-25x
Annealing	65°C	30 sec	
Elongation	72 °C	4 min	
Final Elongation	72 °C	5 min	1x
Hold	4 °C	Hold	

The PCR cycle number depends on the input, and is very cell-type specific. See the Guidelines & warnings for help determining PCR cycles needed.

cDNA purification (preferable but optional)

7 Before purification prepare **22% PEG Clean-up Beads** used for cleaning up the preamplified cDNA. These beads perform similar to Ampure XP beads. Beads are prepared as per mcSCRB-seq protocol

Johannes Bagnoli, Christoph Ziegenhain, Aleksandar Janjic, Lucas Esteban Wange, Beate Vieth, Swati Parekh, Johanna Geuder, Ines Hellmann, Wolfgang Enard. mcSCRB-seq protocol. Nature Communications.
http://dx.doi.org/10.17504/protocols.io.p9kdr4w

Reagent	Amount
PEG 8000	11 g
NaCl, 5M	10 mL
Tris-HCL, 1M, pH 8.0	500 µL
EDTA, 0.5M	100 µL
IGEPAL, 10% solution	50 µL
Sodium Azide, 10% solution	250 µL
UltraPure Water	up to 49 mL
Total	**49 mL**

Add all ingredients into a **50 ml** falcon tube, but do not add the total amount of water until after PEG is completely solubilized.

Incubate at **40 °C** and **vortex regularly until PEG is completely dissolved.**

Resuspend bead stock carefully (Sera-Mag Speed Beads).

Pipette **1000 µl** of bead suspension into a 1.5 mL tube.

Place on magnet stand Remove supernatant.

Add **1000 µl** 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads. Place on magnet stand.

Remove supernatant. Repeat wash one more time.

Add **900 µl** 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads.

Add to PEG solution above and mix well.

8 1. To purify cDNA add **0.6:1** ratio of 22% PEG beads to sample, and mix by gently pipetting up and down.

2. Incubate at **Room temperature** for **00:08:00** .

3. Place on magnet and allow beads to settle. Roughly **00:05:00** .

4. Discard supernatant, and wash once with **20 µl** / **100 µl** of **freshly prepared 80% Ethanol** for 384 / 96 well plates respectively.

5. Remove Ethanol and let the beads air dry for **00:02:00** - **00:05:00**

6. Elute cDNA in **12 µl** UltraPure Water, resuspend beads and incubate for **00:05:00** .

DNA concentration measurement and normalization (Optional, but recommended)

9 1. Prepare 1X TE buffer by either diluting the 20X TE buffer from the QuantiFluor® dsDNA kit or by preparing a solution of 10mM Tris-HCl, 0.1mM EDTA, pH 8.

2. Dilute the QuantiFluor® dsDNA Dye 1:400 in 1X TE buffer and mix.

3. Prepare dsDNA standards for plate read-out, according to manufacturers protocol.

4. Dispense **49 µl** / **99 µl** per well of the ready Quantiflour dye mix into black, flat-bottom 384/96 well plates, respectively.

5. Add Standards to a separate plate.

6. Add **1 µl** of cDNA to each well. Incubate asssay for **00:05:00** at **Room temperature**

7. Use a plate reader, to measure fluorescence (504nM Excitation/ 531nM Emission)

8. Calculate cDNA concentration.

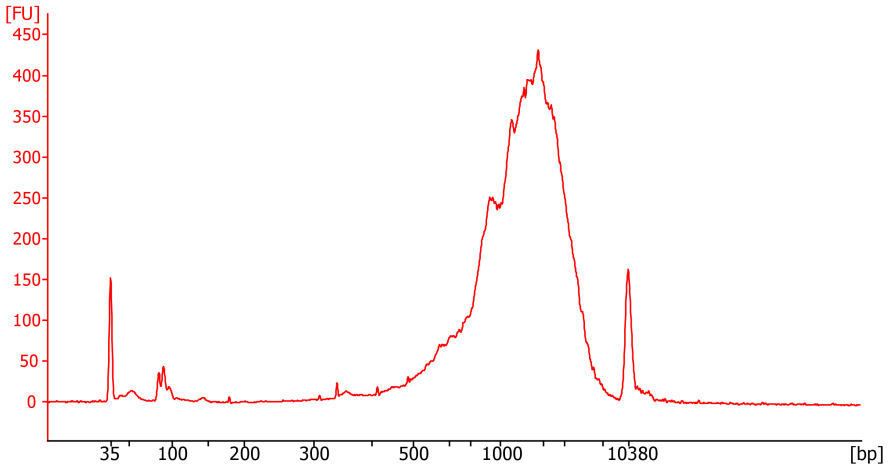
9. Calculate water needed to dilute **1 µl** cDNA to **100 pg/uL** .

10 1. Prepare **normalization plate** by adding the calculated water volumes to each well.

2. Add **1 µl** of preamplified cDNA to each well.

Quality Control check (Optional)

11 Check the cDNA preamplification library content and quality on a Agilent Bioanalzyer, using High Sensitivity DNA Analysis chips.



Example of one HEK cell.

Tagmentation

12

1. Prepare 4x Tagmentation buffer as following. Aliquots of 4xTD buffer can be stored for later use. The TD buffer (2x) from Nextera Kits can also be used, however with the current small amount of ATM used, the Illumina TD buffer will at some point run out.

Dimethylformamide (DMF) should be handled in a fume hood and according to local safety regulations.

Reagent	Amount (uL)	Concentration in 4X	
Tris-HCl pH 7.5 (1M)	40	40mM	
MgCl2 (100mM)	200	20mM	
Dimethylformamide (DMF)	200	20%	
UltraPure Water	560		
Total	**1000 uL**		

2. Prepare **Tagmentation mix.**

• Please note that the ATM amount is a suggested starting point for 100pg/uL input, and some optimization might be necessary to reach a desired UMI-read to Internal-read ratio, based on input and celltype..

Reagent	Reaction conc.	uL per. reaction	96 well plate	384 well plate
Tagmentation buffer (4x)	1X	0.5	55	205
Amplicon Tagmentation Mix (Tn5)		0.08	8.8	32.8
UltraPure water		0.42	46.2	172.2
Total		**1uL**	**110uL**	**410uL**

3. Dispense **1 µl** of Tagmentation mix to a new 96 or 384 well plate.

4. Add **1 µl** of **normalized 100pg/uL cDNA (step 10)** to the plate containing tagmentation mix.

5. Apply a quick spin-down of the plate before incubation in a thermocycler at **55 °C** for **00:10:00** .

6. To strip off the Tn5 from the DNA, add **0.5 µl** of 0.2% SDS to each well. Centrifuge quickly and incubate for **00:05:00** .

7. **Concerning Nextera Index primers: We highly suggest to design or order custom Nextera Index primers. This ensures higher flexibility while also being much cheaper in the long run! The following protocol is designed as such. If using Nextera index primers purchased from Illumina, dilute all primers 5x with UltraPure water, and proceed to use similar volume as follows.**

8. Add **1.5 µl** **Nextera Index primers t**o each well as follows

Reagent	Reaction conc.	uL per. reaction		
Custom S50X index primer (0.5uM)	0.1uM	0.75		
Custom N70X index primer (0.5uM)	0.1uM	0.75		

9. Prepare **Tagmentation PCR mix**.

Reagent	Reaction conc.	uL per. reaction	96 well plate	384 well plate
Phusion HF buffer (5X)	1X	1.4	154	574
dNTPs (25mM/each)	0.2mM/each	0.06	6.2	23
Phusion HF (2U/uL)	0.01U/uL	0.04	3.9	14.4
H2O		1.51	166	618.7
Total		**3uL**	**330uL**	**1230uL**

10. Add **3 µl** of **Tagmentation PCR mix** to each well, centrifuge quickly and incubate in a thermocycler using the following PCR program.

Step	Temperature	Time	Cycles
Gap-filling	72 °C	3 min	1x
Initial denaturation	98 °C	3 min	1x
Denaturation	98 °C	10 sec	12x
Annealing	55 °C	30 sec	
Elongation	72 °C	30 sec	
Final Elongation	72 °C	5 min	1x
Hold	4 °C	Hold	

Library clean-up

13 For the final library clean-up, pool all the Tagmented cDNA (step 12) sample wells in a 1.5mL or 5mL eppendorf tube.

1. Add **0.6:1 22% PEG beads to final volume of the pooled tagmentation cDNA**. Mix gently by pipetting and incubate for **00:08:00** at **Room temperature**

2. Place on magnet and allow beads to settle. Roughly **00:05:00** .

4. Discard supernatant, and wash twice with >= **1000 µl** **freshly prepared 80% Ethanol.**

5. Remove Ethanol and let the beads air dry for at least **00:05:00**

6. Elute cDNA in **40 µl** UltraPure Water, resuspend beads and incubate for **00:05:00** .

(Optional) Size selection via Gel-cutting and extraction

14 To further select for longer tagmented fragments, an optional step including a size selection step can be included.

1. Load **20 µl** of the eluted tagmented library from step 13 into a **2% Agarose E-Gel EX** together with 50bp DNA ladder.

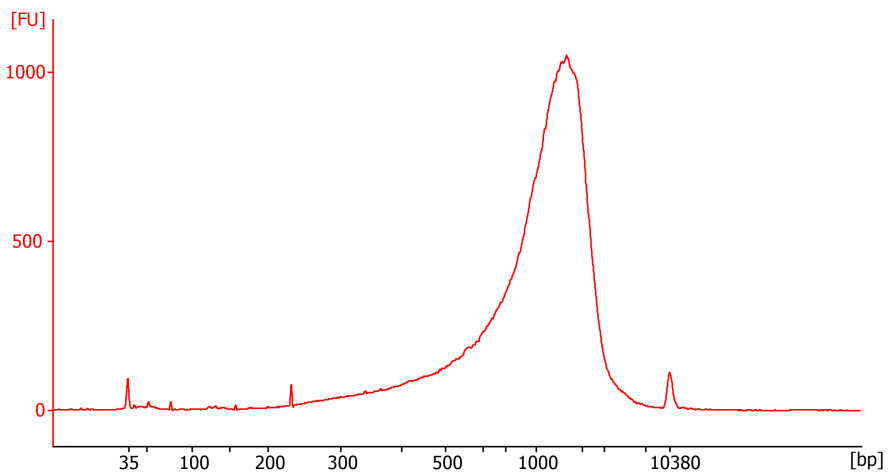
2. Run gel for **00:12:00**

3. After finished run, open the gel casing and cut the gel between **550bp - 2kb** using a clean scalpel or blade.

4. Purify the excised gel slice using Qiagen QIAquick Gel extraction kit according to manufacturers protocol.

Final Library Quantification

15 Run the final library on a Agilent Bioanalyzer (High Sensitivity DNA), to inspect the quality and median base-pair length of your library.



Suggestive example of a finshed HEK library. A bit on the large size. Can still be sequenced.

Use Qubit fluorometer or similar to quantify the library.

Calcutate the final library concentration, using above metrics.

Sequencing

16 The sequencing ready library should be sequenced on any Illumina compatible sequencer, either Single-end or Paired-end, depending on the question and need.

For final library whether gel cut/size selected or not, the expected median base-pair should be around or above 1kb. In our experience NovaSeq/HiSeq sequncers are more tolerant towards wider or longer size fragment distributions, than the NextSeq. Because of this consider increasing the loading concentration a bit to ensure proper cluster density. However "your milage may vary". Empircal investigation or a pilot run is always adviced, if possible.

Primary Data processing

17 After sequencing has completed successfully, binary base-call files (BCL) need to be converted to fastq.
For this, bcl2fastq should be used in the latest version (bcl2fastq v2.20).

bcl2fastq 2.20
by Illumina

At this stage, demultiplexing into per-cell fastq files is not necessary - a sample sheet is thus not needed.
Be sure to adjust the base mask to represent your sequencing layout and the length of your barcode reads.
Remove the option --no-lane-splitting if the same cell barcodes have been reused for different libraries on different lanes of the flow cell.
You may restrict the number of cores used with the following options:
--loading-threads
--processing-threads
--writing-threads

bcl2fastq: 2x150bp dual-index

```
bcl2fastq --use-bases-mask Y150N,I8,I8,Y150N --no-lane-splitting --create-fastq-for-index-reads -R /mnt/storage1/NextSeqNAS/191011_NB502120_0154_AHVG7JBGXB
```

-R denotes the runfolder and you may redirect the fastq output to a different folder with the -o option.

18 After generating fastq files, the zUMIs pipeline should be used to process Smart-seq3 data to ensure correct handling of UMI reads and internal reads.

Parekh S, Ziegenhain C, Vieth B, Enard W, Hellmann I (2018). zUMIs - A fast and flexible pipeline to process RNA sequencing data with UMIs.. GigaScience.
https://doi.org/10.1093/gigascience/giy059

zUMIs
Linux
source

We recommend the newest version v2.5.6 at the time of this protocol.

All options are set in a configuration file following the YAML format. Here is a best practice example:

Smartseq3.yaml

```
project: Smartseq3
sequence_files:
  file1:
    name: /smartseq3/fastq/Undetermined_S0_R1_001.fastq.gz
    base_definition:
      - cDNA(23-150)
      - UMI(12-19)
    find_pattern: ATTGCGCAATG
```

```
  file2:
    name: /smartseq3/fastq/Undetermined_S0_R2_001.fastq.gz
    base_definition:
      - cDNA(1-150)
  file3:
    name: /smartseq3/fastq/Undetermined_S0_I1_001.fastq.gz
    base_definition:
      - BC(1-8)
  file4:
    name: /smartseq3/fastq/Undetermined_S0_I2_001.fastq.gz
    base_definition:
      - BC(1-8)
reference:
  STAR_index: /resources/genomes/Mouse/STAR5idx_noGTF/
  GTF_file: /resources/genomes/Mouse/Mus_musculus.GRCm38.91.gtf
  additional_STAR_params: '--clip3pAdapterSeq CTGTCTCTTATACACATCT'
  additional_files:
    - /resources/genomes/spikes/ERCC92.fa
out_dir: /smartseq3/zUMIs/
num_threads: 20
mem_limit: 50
filter_cutoffs:
  BC_filter:
    num_bases: 3
    phred: 20
  UMI_filter:
    num_bases: 2
    phred: 20
barcodes:
  barcode_num: ~
  barcode_file: /smartseq3/expected_barcodes.txt
  automatic: no
  BarcodeBinning: 1
  nReadsperCell: 100
  demultiplex: no
counting_opts:
  introns: yes
  downsampling: '0'
  strand: 0
  Ham_Dist: 1
  write_ham: no
  velocyto: no
  primaryHit: yes
  twoPass: no
make_stats: yes
which_Stage: Filtering
samtools_exec: samtools
pigz_exec: pigz
STAR_exec: STAR
Rscript_exec: Rscript
```

Be sure to use full paths to all files and folders. For further descriptions of the individual options visit the zUMIs GitHub repository wiki

Now, simply start zUMIs with the following command:

Invoke zUMIs

zUMIs-master.sh -y Smartseq3.yaml

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