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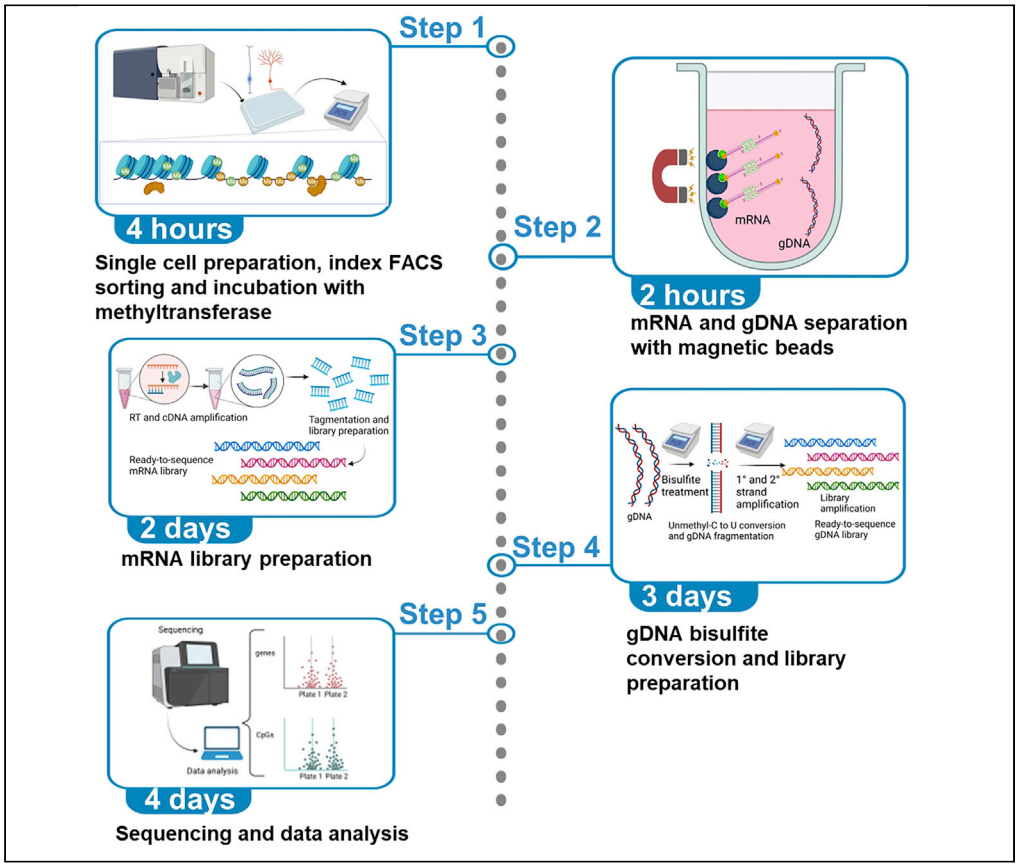
High-throughput scNMT protocol for multiomics profiling of single cells from mouse brain and pancreatic organoids



Single-cell nucleosome, methylome, and transcriptome (scNMT) sequencing is a recently developed method that allows multiomics profiling of single cells. In this scNMT protocol, we describe profiling of cells from mouse brain and pancreatic organoids, using liquid handling platforms to increase throughput from 96-well to 384-well plate format. Our approach miniaturizes reaction volumes and incorporates the latest Smart-seq3 protocol to obtain higher numbers of detected genes and genomic DNA (gDNA) CpGs per cell. We outline normalization steps to optimally distribute per-cell sequencing depth.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Miniaturization of scNMT protocol to increase cost-effectiveness

Throughput increase from 96-well plate to 384-well plate format

Incorporation of Smart-seq3 pipeline, obtaining higher number of genes per cell

Normalization steps to optimally distribute per-cell sequencing depth

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High-throughput scNMT protocol for multiomics profiling of single cells from mouse brain and pancreatic organoids

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SUMMARY

Single-cell nucleosome, methylome, and transcriptome (scNMT) sequencing is a recently developed method that allows multiomics profiling of single cells. In this scNMT protocol, we describe profiling of cells from mouse brain and pancreatic organoids, using liquid handling platforms to increase throughput from 96-well to 384-well plate format. Our approach miniaturizes reaction volumes and incorporates the latest Smart-seq3 protocol to obtain higher numbers of detected genes and genomic DNA (gDNA) CpGs per cell. We outline normalization steps to optimally distribute per-cell sequencing depth.

For complete details on the use and execution of this protocol, please refer to Clark (2019), Clark et al. (2018), and Hagemann-Jensen et al. (2020a, 2020b).

BEFORE YOU BEGIN

The profiling of mRNA species, chromatin accessibility and epigenetic information in single cells is key to understanding the molecular mechanisms governing how fate choices are executed within a cell. In this regard, single cell sequencing methods underwent an exciting development in the last years, with the incorporation of multiomics profiling, which has the advantage of capturing multiple molecular layers from the same cell (Dimitriu et al., 2022). Recently, the scNMT protocol was used to capture the transcriptomic and epigenetic landscape of mouse gastrulation (Clark et al., 2018; Argelaguet et al., 2019). However, the protocol is cost-intensive, uses Smart-seq2 as the RNA sequencing pipeline and works on a 96-well plate format.

Due to tissue heterogeneity and the stochasticity of states present even within the very same cell type, it is important to profile a significant number of cells to have statistically relevant readouts and address the biology of a tissue.

This would allow a comprehensive understanding of the regulatory mechanisms underlying transcriptional commitment and cell fate decisions, together with the discovery of key molecular drivers and mechanisms underpinning cellular states in homeostasis and disease. Ultimately, precise tuning of epigenetic states may permit greater control of engineered cell type and activity for cell therapies.

Here, we improve the scNMT protocol by incorporating the Smart-seq3 pipeline for the transcriptomic profiling, which uses unique molecular identifiers (UMIs) to pre-label each unique molecule,

addressing potential bias introduced during amplification and enabling the quantitative measurement of RNA molecules. Moreover, the implementation of Smart-seq3 allows capturing a much higher number of genes per cell, including transcription factors, which are very important to regulate chromatin and are generally lowly expressed. In addition, we miniaturize the reaction volume and increase the throughput to a 384-well plate format, with the possibility of profiling up to 768 cells per sequencing run (at the mRNA level). To improve the number of cells that reach the lower threshold of genes/cell, we also incorporate normalization steps in order to optimally distribute sequencing depth among all the cells.

For an optimal protocol, we recommend the use of automatic pipetting machines, such as Viaflo 384™ (Integra), Mosquito™ HV (SPT Labtech) and Mantis® (FORMULATRIX). In the absence of these machines, a multichannel pipette can be utilized, which would increase the plate-processing time.

The protocol below describes the specific steps for profiling mouse primary neural stem cells (NSCs), astrocytes, neuroblasts, oligodendrocytes and neurons, as well as cells from pancreatic acinar organoids.

Institutional permissions

All animal experiments were performed in accordance with the institutional guidelines of the DKFZ and were approved by the "Regierungspräsidium Karlsruhe" (Germany).

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Buffer RLT Plus	QIAGEN	Cat#1053393
Dynabeads MyOne Streptavidin C1	Invitrogen	Cat#65001
10 mM dNTP mix	Thermo Scientific	Cat#R0192
1 M DTT	Sigma-Aldrich	Cat#43816-10 mL
KAPA HiFi HotStart Ready mix (2×)	Roche Diagnostics	Cat#KK2602/07958935001
KAPA HiFI Hotstart PCR mix	Roche Diagnostics	Cat#KK2502/07958897001
CT Conversion Reagent	Zymo Research	Cat#D5003-1
M-Solubilization buffer	Zymo Research	Cat#D5021-7
M-dilution buffer	Zymo Research	Cat#D5002-2
M-Reaction buffer	Zymo Research	Cat#D5021-8
MagBinding Beads	Zymo Research	Cat#D4100-2-8
M-binding buffer	Zymo Research	Cat#D5002-3
M-Desulphonation buffer	Zymo Research	Cat#D5001-5
Klenow (3′-5′ exo-)	Biozym	Cat#280310
Exonuclease I	NEB	Cat#M0293L
Recombinant RNAse inhibitor	Takara Bio	Cat#2313A
Agencourt AMPure XP beads	Beckman Coulter	Cat#A63881
Maxima H Minus	Thermo Scientific	Cat#EP0752
Nextera XT DNA sample preparation kit	Illumina	Cat#FC-131-1096
IGEPAL CA-630	Sigma-Aldrich	Cat#I8896-50mL
1 M Tris-HCl buffer pH 8.0	Thermo Fisher Scientific	Cat#15568025
0.5 M EDTA solution	Thermo Fisher Scientific	Cat#15575020
Tween 20	Sigma-Aldrich	Cat#P1379-1L
Sytox Blue dead cell stain	Thermo Scientific	Cat#S34857
TrypLE™ Express Enzyme (1×), no phenol red	Thermo Scientific	Cat#12604013
PEG 8000	Sigma-Aldrich	Cat#P2139
Critical commercial assays		
Bioanalyzer DNA High sensitivity chip	Agilent	Cat#5067-4626
Qubit assay tubes	Thermo Fisher Scientific	Cat#Q32856

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Qubit dsDNA HS Assay kit	Thermo Fisher Scientific	Cat#Q32854
Quant-iT™ PicoGreen™ dsDNA Assay-Kits	Thermo Fisher Scientific	Cat#P7589
Neural Tissue Dissociation Kit (NTDK)	Miltenyi Biotec	Cat#130-093-231
Experimental models: Cell lines		
Primary cells from mouse brain	JANVIER LABS	C57BL/6NRj
Mouse pancreatic organoids	JANVIER LABS	C57BL/6NRj
Oligonucleotides		
Biotynilated Oligo-dT_SS3 /5BiotinTEG/	This paper	AC GAG CAT CAG CAG CAT ACG ATT TTT TTT TTT TTT TTT TTT TTT TTT TVN
Template-switching oligo (TSO) /5Me-isodC// iisodG//iMe-isodC/	This paper	AGA GAC AGA TTG CGC AAT GNN NNN NNN NNN NrGrG+G
mRNA index primers - combinatorial indexing - 5er and 7er	Nextera index primers. For the original design see (Buenrostro et al., 2015), in the references of the manuscript.	On Table S1
Fwd_PCR_primer	(Hagemann-Jensen et al., 2020a, 2020b), see references on manuscript	TCGTCGGCAGCGTCAGATGTGTA TAAGAGACAGATTGCGCAA*T*G
Rev_PCR_primer	(Hagemann-Jensen et al., 2020a, 2020b), see references on manuscript	ACGAGCATCAGCAGCATAC*G*A
Preamp oligo	(Clark et al., 2018) see references on manuscript	CTACACGACGCTCTTCCGATCTNNNNNN
Adapter 2 oligo	(Clark et al., 2018) see references on manuscript	TGCTGAACCGCTCTTCCGATCTNNNNNN
gDNA index primers - Unique dual indexing	This paper	On Table S2
iTAG sequencing primer	(Clark et al., 2018) see references on manuscript	AAGAGCGGTTCAGCAGGAATG CCGAGACCGATCTC
Software and algorithms		
zUMIs	(Parekh et al., 2018) see references on manuscript.	https://github.com/sdparekh/zUMIs
FASTQC	(Andrews et al., 2010) see references on manuscript.	https://www.bioinformatics.babraham.ac.uk/projects/fastqc/
STAR mapper 2.7.3a	(Dobin et al., 2013) see references on manuscript.	https://github.com/alexdobin/STAR
Trim Galore	(Krueger, 2015) see references on manuscript.	https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/
Bismark	(Krueger and Andrews, 2011) see references on manuscript.	https://www.bioinformatics.babraham.ac.uk/projects/bismark/
Biorender	Science Suite Inc.	https://biorender.com/
Inkscape	The Inkscape Project	https://inkscape.org/
Other		
Twin.tec PCR plate 384 loBind	Eppendorf	Cat#00301129547
Hard-Shell® 384-Well PCR Plates, thin wall, skirted, clear/clear	Bio-Rad	Cat#HSP3801
5 XYZ Racks of 384 tips	Integra	Cat#6465
384 well plate magnetic block	SPT Labtech	Cat#3268-02008
neoVortex® shaker, with fixed rotation 2500 rpm	neoLab	Cat#D-6013
Spool of 18500 Gamma Ray Sterilized Pipettes 4.5 mm Pitch HV	SPT Labtech	Cat#4150-03033
Viaflo 384™ base unit	INTEGRA	Cat#6031
384-Channel Pipetting Head 2–50 µL	INTEGRA	Cat#6136
16 Channel VIAFLO Lightweight Electronic Pipette	INTEGRA	Cat#4646
Mantis V3.3 with integrated RFID	FORMULATRIX	Cat#MANTV3.3_RFID
Mantis Chip - Silicone, LV (0.1 ul & 0.5 ul), RFID	FORMULATRIX	Cat#233649
Mantis Chip - Silicone, HV (1 ul & 5 ul), RFID	FORMULATRIX	Cat#233648
Mantis Chip – Silicone, Continuous Flow (CF), RFID	FORMULATRIX	Cat#232724
Mosquito HV Liquid handling system	SPT Labtech	Cat#3097-01057

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
NextSeq 2000 Sequencing System	Illumina	Cat#20038897
NextSeq 550 Sequencing System	Illumina	Cat#SY-415-1002
NextSeq 1000/2000 P2 Reagents (200 Cycles) v3	Illumina	Cat#20046812
NextSeq 500/550 Mid Output KT v2.5 (150 CYS)	Illumina	Cat#20024904
NextSeq 500/550 Hi Output KT v2.5 (150 CYS)	Illumina	Cat#20024907

*phosphorothioate.

MATERIALS AND EQUIPMENT

As described before, we recommend liquid handler platforms to obtain reproducible results. Viaflo 384™ (INTEGRA) can be used for the nucleic acid purification steps and gDNA and mRNA separation. Mantis® (FORMULATRIX) is used for adding low amounts of cost-intensive reagents with little dead volume. Viaflo 16-channel electronic pipette (INTEGRA) is used to prepare pre-aliquoted plates with master mixtures or reagents.

For dispensing low volumes (less than 3 µL) we usually use Mantis® (FORMULATRIX) and Mosquito™ HV (SPT Labtech), while for dispensing larger volumes we pre-aliquot the specific solution into a fresh 384-deep well plate and then use Viaflo 384™ (INTEGRA) to transfer it. For aspirating lower volumes, we use Mosquito™ HV (SPT Labtech) and for higher volumes we use Viaflo 384™ (INTEGRA). When proceeding with the protocol, it is up to the user to decide how to dispense and aspirate solutions.

Of note, the 384-well plates should be DNA low-bind and PCR-clean. We opted for twin.tec® PCR Plates LoBind® (Eppendorf, see key resources table, KRT), but Hard-Shell 384-well PCR Plates (Bio-Rad) worked equally well based on our experience.

Below we describe the solutions that need to be prepared in order to perform the protocol.

Transcriptomic part

Oligo dT bead preparation

Timing: 20 min

Dynabead solution A		
Reagent	Final concentration	Amount
NaOH (10 M)	0.1 M	100 µL
NaCl (5 M)	0.05 M	100 µL
ddH_2O	n/a	9.8 mL
Total	n/a	**10 mL**

Can be stored at 4°C for 1 month.

Dynabead solution B		
Reagent	Final concentration	Amount
NaCl (5 M)	0.1 M	200 µL
ddH_2O	n/a	9.8 mL
Total	n/a	**10 mL**

Can be stored at 4°C for 1 month.

Dynabead 2× "Bind and wash" buffer (B&W)		
Reagent	Final concentration	Amount
Tris-HCl pH 7.5 (1 M)	0.01 M	100 μL
EDTA (0.5 M)	1 mM	20 μL
NaCl (5 M)	2 M	4 mL
ddH_2O	n/a	5.88 mL
Total	n/a	**10 mL**
Can be stored at 4°C for 1 month.		

Genome and Transcriptome wash buffer (G&T)		
Reagent	Final concentration	Amount
Tris-HCl pH 8.3 (1 M)	0.05 M	500 μL
KCl (3 M)	0.075 M	250 μL
$MgCl_2$ (1 M)	3 mM	30 μL
DTT (0.1 M)	0.01 M	1 mL
Tween-20 (100%)	0.5%	50 μL
ddH_2O	n/a	5.88 mL
Total	n/a	**10 mL**
Can be stored at 4°C for 1 month.		

Genomic part

Conversion reagent

Timing: 20 min

Reagent	Final concentration	Amount
CT Conversion Reagent (powder)	n/a	1 bottle
M-solubilization buffer	n/a	7.9 mL
M-Dilution buffer	n/a	3 mL
M-Reaction buffer	n/a	1.6 mL
Total	n/a	**12.5 mL**

- Add 7.9 mL of M-solubilization Buffer + 3 mL of M-Dilution Buffer into CT Conversion Reagent (Powder bottle). Vortex 10 min.
- Add 1.6 mL of M-Reaction Buffer. Vortex 3 min. Aliquot into dark 1.5 mL microcentrifuge tubes and store for up to 1 month a −20°C. 1 bottle of conversion reagent can be used to process 1 $^1/_2$ plates.

PEG buffer 18%

Timing: 20 min

Reagent	Final concentration	Amount
PEG 8000	18% w/v	9 g
NaCl	2.5 M	14.61 g
Tris-HC pH 8 (1 M)	10 mM	500 μL
EDTA (0.5 M)	1 mM	100 μL
100% Tween-20	0.05%	25 μL
ddH_2O	n/a	Add until 10 mL
Total	n/a	**10 mL**

Sterilize through a 0.2 μm filter and treat the buffer with UV irradiation for 1 h (a laminar flow hood with UV light can be used to sterilize the solution). It can be stored for 1 month at 4°C.

STEP-BY-STEP METHOD DETAILS

Tissue processing for the preparation of single cell suspension

Timing: 2–3 h

Here a solution of single cells will be prepared starting from the tissue or cell system of origin.

The time for obtaining a single cell preparation depends on the starting tissue. For isolating neural stem cells, neuroblasts and neurons, the times per mouse are explained below. Subventricular zone and olfactory bulb dissociation: 15 min. Tissue processing and single cell preparation with the Neural Tissue Dissociation Kit (NTDK, Miltenyi, see key resources table): 40 min (follow the manufacturer protocol: https://www.miltenyibiotec.com/upload/assets/IM0001320.PDF). Antibody labeling and final wash: 30 min. For more information on the sorting strategy see the following paper (Kremer et al., 2021).

For the isolation of single cells from pancreatic organoids the times are explained below. Incubation of organoid with TrypLE Express: 30 min. Washing and single cell resuspension: 30 min. For more information on the generation of pancreatic organoids, see the following papers (Wollny et al., 2016; Krieger et al., 2021).

Single cell collection and methyltransferase reaction

Timing: 1 h

Through this step, cells will be sorted into 384-well plates and will undergo a reaction with a methyltransferase to obtain information about DNA accessibility.

Prepare a single cell suspension of your cell type of interest. Then proceed to cell collection. Collect cells on individual wells of a low binding 384-well plate. For sorting live cells, we use Sytox ™ blue dead cell stain (see key resources table) on our sorting strategy. The use of FACS is preferred. Alternatively, cell printing devices like cellenONE® (Cellenion) can also be used. The collection solution is composed of a mild lysis buffer (IGEPAL CA-630), used to permeabilize the nuclear membrane and a GpC methyltransferase (M.CviPI), which adds methyl groups to cytosines in GpC islands located in non-nucleosome bound DNA. The latter allows the assessment of chromatin accessibility throughout the genome.

1. Prepare GpC methyltransferase reaction buffer. Prepare fresh and keep on ice.

GpC methylase master mix.

Reagent	Final concentration	Amount for 1 reaction	Amount for 1 plate (384 + 10%)
ddH_2O		0.57 μL	240.79 μL
GpC methyltransferase buffer (10×)	1×	0.1 μL	42.24 μL
SAM (32 mM)	0.16 mM	0.005 μL	2.11 μL
IGEPAL CA-630 (1%)	0.1%	0.1 μL	42.24 μL
RNAse-in (40 U/μL)	1 U/μL	0.025 μL	10.56 μL
M.CviPI (4 U/μL)	0.8 U/μL	0.2 μL	84.48 μL
Total		1 μL	422.42 μL

2. Add 1 μL of the mix into every well of a 384-well plate. Index sort single cells on the plate. Index sorting is a mode of the FACS instrument that allows the isolation of single cells while recording the coordinates of all fluorescence and scatter parameters for each individual event. It is advisable to keep the temperature of the plate holder at 4°C during sorting.

   Optional: Sort minibulks of 25–100 cells per cell type into single wells as positive control and to obtain a deeper coverage from both the transcriptome and the genome of specific cell populations. For minibulks, the volume per well and the incubation time can remain the same as with single cells.

   ⚠ CRITICAL: The FACS sorter or cell printing device (e.g FACS Aria II) should have a 4°C cooling block, to keep the methyltransferase reaction inhibited until all the cells are sorted.

3. After sorting, centrifuge the plate at 1,000 × *g* for 10 s.
4. Incubate on a thermocycler for 15 min at 37°C with the lid at 50°C. Add 2 μL of RLT Plus buffer and centrifuge at 1,000 × *g* for 20 s.

   Pause point: Store plate at −80°C or proceed directly with the separation of the mRNA and gDNA.

Separation of mRNA from gDNA

Oligo dT bead preparation

Timing: 40 min

Here the binding of the dynabeads to the oligo-dT takes place through the strong interaction between Streptavidin and Biotin. To that end, we prepare and wash the dynabeads with a series of buffers.

5. Add 225 μL Dynabeads (MyOne Streptavidin C1) to a 1.5 mL microcentrifuge tube. Place on a magnet, wait 30 s and remove supernatant (SN).
6. Wash the beads with Solutions A, B and B&W buffer.
   a. Resuspend beads in 400 μL of Solution A by pipetting outside the magnet. Place on magnet, wait 30 s and remove the SN.
   b. Repeat step a one more time.
   c. Resuspend beads in 200 μL of Solution B by pipetting outside the magnet. Place on magnet, wait 30 s and remove the SN.
   d. Resuspend the beads in 225 μL of 2× B&W buffer and add 225 μL of Oligo-dT-SS3 (100 μM).
   e. Incubate by rotation for 15 min at 18°C–25°C.

   Pause point: This solution can be stored overnight at 4°C.

7. Prepare the bead resuspension solution, G&T wash buffer + RNAse inhibitor and the reverse transcription (RT) Master Mix.

Bead Resuspension Solution

Reagent	Final concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
ddH_2O		3.1 μL	1368.96 μL
Buffer RT (5 ×) from Maxima enzyme	1 ×	0.8 μL	353.28 μL
RNAse Inhibitor (40 U/μL)	1 U/μL	0.1 μL	44.16 μL
Total		4 μL	1766.4 μL

Store on ice until use. Once used to resuspend the Oligo-dT beads, aliquot solution into a fresh 384-well plate.

G&T Wash buffer with RNAse Inhibitor			
Reagent	Final concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
G&T Wash buffer		14.85 µL	6557.76 µL
RNAse Inhibitor (40 U/µL)	0.4 U/µL	0.15 µL	66.24 µL
Total		15 µL	6624 µL
Store on ice until use.			

Optional: Aliquot the G&T wash buffer + RNAse Inhibitor in a new plate to increase the efficiency of the separation of mRNA and gDNA. By aliquoting the G&T wash buffer in a different plate, we reduced the time for transferring the wash buffer to the sample plate, thereby decreasing the time that the beads are drying out. Over-drying beads might prevent an efficient elution of the mRNA in the RT mix.

RT master mix (mind the order)				
Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH_2O		1.185 µL	523.30 µL	
dNTP mix	10 mM	0.2 µL	88.32 µL	1 mM
Tris-HCl pH 8.3	1 M	0.040 µL	17.66 µL	25 mM
NaCl	1 M	0.060 µL	26.5 µL	30 mM
MgCl2	100 mM	0.050 µL	22.08 µL	2.5 mM
GTP	100 mM	0.020 µL	8.83 µL	1 mM
TSO (Template switching oligo)	100 µM	0.040 µL	17.66 µL	2 µM
RNAse Inhibitor	40 U/µL	0.025 µL	11.04 µL	0. 5 U/µL
PEG	50%	0.2 µL	88.32 µL	5%
DTT (add second to last)	100 mM	0.160 µL	70.66 µL	8 mM
Maxima H-minus RT (add last)	200 U/µL	0.020 µL	8.83 µL	2 U/µL
Total		2 µL	883.2 µL	
Store on ice until use.				

Bead resuspension and separation of genomic and transcriptomic fractions

Timing: 30 min

Through these steps the polyadenylated RNAs will bind to the oligo-dT-bound dynabeads which will be kept on the well through the use of a magnetic block. On a second step, the gDNA will be separated from the RNA faction by a series of washes.

8. Resuspend the beads in 400 µL of B&W buffer 1× by pipetting outside the magnet. Place on magnet, wait 30 s and remove the SN. Repeat this step 3 times more to reach a total of 4 washes.
9. Resuspend beads with the prepared Bead Resuspension solution. Mix by vortexing thoroughly 15 s (we apply 2500 rpm for vortexing).

⚠ CRITICAL: After resuspending the beads with the bead resuspension solution, please use the solution within the next 30 min, to keep the RNAse inhibitor within its optimal activity time window.

10. Transfer 4 µL of bead resuspension solution to the mRNA plate. Vortex 30 s at maximum speed, spin down the plate and incubate 3 min at 18°C–25°C. Repeat twice more to reach a total incubation time of approx. 10 min. After the incubation centrifuge at 1,000 × *g* for 1 min at room temperature (RT, 18°C–25°C).

11. Separation of gDNA from polyadenylated RNAs.
    a. Place the plate on magnet and incubate 2 min until the suspension is clear.
    b. Aspirate 7 µL from the mRNA plate still on the magnet and transfer to the gDNA plate.
    c. Add 5 µL of G&T wash buffer to the mRNA plate.
    d. Vortex mRNA plate for 30 s, spin down on a benchtop centrifuge for 10 s and incubate 1 min.
    e. Repeat step d two more times to achieve an incubation time of 5 min. Centrifuge the mRNA plate for 1 min at 1,000 × *g*.
    f. Put mRNA plate on magnet, incubate 2 min and transfer SN to gDNA plate.
    g. Repeat steps c-f twice more. Now you have 22 µL of gDNA eluate on the gDNA plate. Store the gDNA plate at −20°C until proceeding with the gDNA library preparation at step 38.
12. Immediately add 2 µL of the 18°C–25°C mix to the mRNA plate on ice.

Transcriptomic part

Reverse transcription, cDNA amplification and cDNA purification

Timing: 6 h

Here the polyadenylated RNA will be retrotranscribed, and then subsequently amplified. At the end the cDNA will be purified with magnetic beads.

Reverse transcription and cDNA amplification.

Through these steps the polyA mRNA will be reverse-transcribed and amplified. Afterwards, the obtained cDNA will be purified before proceeding with tagmentation and library amplification.

The first PCR is a standard reverse transcription reaction with no amplification step. In the following PCR amplification step, generating a double stranded cDNA from the single stranted cDNA template needs to be optimized per cell type to ensure amplification of just sufficient cDNA with minimal PCR amplification bias. For testing this, we advise users to prepare a so-called validation plate per cell type to run tests for determining RNA quality and optimal PCR cycle before proceeding with a sample plate.

13. Incubate plate on a thermocycler as follows.

Reverse transcription. PCR cycling conditions

Temperature	Time	Cycles
42°C	90 min	1
50°C	2 min	10
42°C	2 min	
85°C	5 min	1
4°C	Hold	

Optional: A pre-wash of unbound TSO before proceeding to the cDNA amplification step might help reducing TSO dimers (peak between 80 and 150 bp in the Bioanalyzer traces). To avoid this, perform a TSO wash after the reverse transcription. We therefore advise to perform a test run with few samples to see whether TSO dimers represent a problem for the tested samples. If so, proceed with TSO wash.

a. After reverse transcription, place mRNA plate on magnet and incubate 3 min.
b. Discard SN. Add 20 µL of 1× RT buffer.

c. Vortex 30 s and spin down. Incubate on magnet 3 min. Discard SN and add TSO-wash cDNA amplification master mix (5 µL).

TSO-wash cDNA amplification master mix (when doing TSO-wash)

Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH_2O		3.075 µL	1357.92 µL	
HiFi HotStart (KAPA HiFI HotStart Polymerase)	1 U/µL	0.1 µL	44.16 µL	0.02 U/µL
5× HiFi Fid. With Mg (KAPA High Fidelity Buffer 5×)	5 ×	1 µL	441.6 µL	1 ×
MgCl2	100 mM	0.075 µL	33.12 µL	0.5 mM
dNTP mix	10 mM	0.15 µL	66.24 µL	0.5 mM
Forward Primer	10 µM	0.25 µL	110.4 µL	0.5 µM
Reverse Primer	10 µM	0.05 µL	22.08 µL	0.1 µM
PEG	50%	0.2 µL	88.32 µL	5%
Tris-HCl pH 8.3	1 M	0.040 µL	17.66 µL	25 mM
NaCl	1 M	0.060 µL	26.5 µL	30 mM
Total		5 µL	2208 µL	

14. If no TSO wash is performed, prepare cDNA amplification master mix (MM) and add 3 µL of the MM to the plate. Vortex gently and spin down on benchtop centrifuge.

cDNA amplification master mix

Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH_2O		1.425 µL	629.28 µL	
HiFi HotStart (KAPA HiFI HotStart Polymerase)	1 U/µL	0.1 µL	44.16 µL	0.02 U/µL
5× HiFi Fid. With Mg (KAPA High Fidelity Buffer 5×)	5 ×	1 µL	441.6 µL	1 ×
$MgCl_2$	100 mM	0.025 µL	11.04 µL	0.5 mM
dNTP mix	10 mM	0.15 µL	66.24 µL	0.5 mM
Forward Primer	10 µM	0.25 µL	110.4 µL	0.5 µM
Reverse Primer	10 µM	0.05 µL	22.08 µL	0.1 µM
Total		3 µL	1324.8 µL	

a. Incubate plate on thermocycler as follows:

cDNA Amplification. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	20 s	18–25
Annealing	65°C	30 s	
Extension	72°C	4 min	
Final extension	72°C	5 min	1
Hold	4°C		

Note: Cycle number depends strongly on the cell type used. In our case we used 24 cycles for primary NSCs, astrocytes, oligodentrocytes, neuroblasts and neurons. For pancreatic organoid cells we used 20 cycles. It is advisable to test different cycle numbers between 18 and

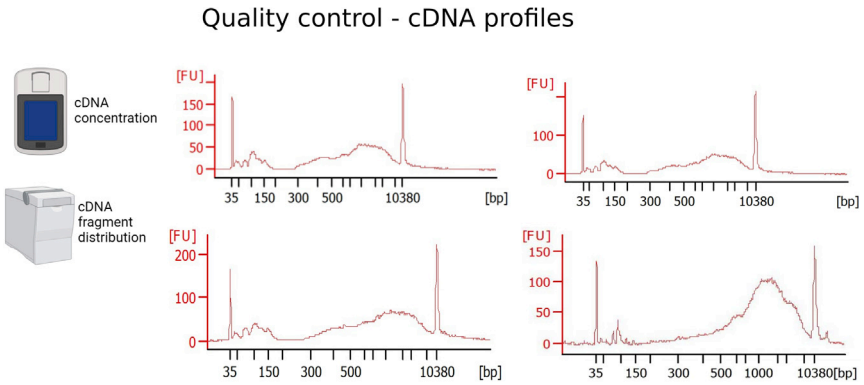

Figure 1. Expected outcomes after cDNA amplification

Measure the concentration of the cDNA with a Qubit™ Fluorometer (Thermo Fisher Scientific) and the fragment distribution with Bioanalyzer or Tape Station (Agilent). cDNA profile of 4 random samples. Note that the proportion of primer dimer (50–150 bp) is lower than the one of the cDNA fragments (400–10000 bp). The proportion of primer dimers should not exceed the one showed here. Parts of this figure are created with BioRender.com.

25 before proceeding with the full protocol. A concentration of more than 0.3 ng/μL and the lack of fragments smaller than 500 bp are characteristics of a good cDNA profile.

Pause point: Store at 4°C up to 16 h or proceed directly with the purification of the cDNA.

cDNA purification: Bead clean-up.

15. Equilibrate AMPure XP beads at 18°C–25°C for 30 min. Add 3 μL of beads to the mRNA plate (reaching 0.6× ratio of beads to sample volume).
16. Vortex for 30 s and spin down. Incubate 3 min. Repeat twice more to reach a total of 10 min of incubation at RT. Centrifuge the plate at 1,000 × *g* for 1 min.
    a. During the incubation time prepare 18 mL of EtOH 80% and aliquot 41 μL into a new 384-well plate.
17. Incubate the mRNA plate 2 min on magnet. Discard the SN and perform the following step twice (EtOH wash):
    a. Add 20 μL of EtOH 80% to the mRNA plate (on magnet). Wait 30 s and discard SN. Let the beads from the mRNA plate dry for 2 min, until no EtOH 80% is seen on the wells.
18. Remove mRNA plate from magnet. Add 8 μL of nuclease-free water. Vortex 30 s, spin down and incubate 1 min at RT. Repeat the vortexing and incubation two times more, to reach a total of 5 min incubation.
19. Place the plate on magnet for 2 min and transfer SN to a new plate (cDNA plate).
20. Perform quality control:
    a. Measure the concentration of 10 random samples (wells) with Qubit™ dsDNA HS Assay Kit as described by the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2FQubit_dsDNA_HS_Assay_UG.pdf):
       i. Mix 1 μL of sample + 199 μL of a mix consisting of 199 μL of Qubit HS buffer + 1 μL of Qubit reagent. Vortex and measure on Qubit™ Fluorometer (Thermo Fisher Scientific).
    b. Load 1 μL of the 10 samples onto a Bioanalyzer High Sensitivity DNA chip (or TapeStation, Agilent) (concentration should not exceed 5 ng/μL, otherwise dilute in nuclease-free water before loading). Follow the manufacturer's instructions (https://www.agilent.com/cs/library/usermanuals/public/G2938-90322_HighSensitivityDNAKit_QSG.pdf).
    c. Check the quality of the cDNA (see Figure 1 that displays good quality cDNA profiles, and troubleshooting 1).

Optional: To get a better overview of the quality of the whole plate, the concentration of all the samples (384) can be measured using a fluorescent microplate reader (Biotek Synergy LX, Agilent) together with the Quant-IT™ PicoGreen™ dsDNA Assay-Kit. This also allows an optimal normalization of the cDNA before proceeding to the tagmentation.

For more information follow the manufacturer's instructions (https://www.thermofisher.com/document-connect/document-connect.html?url=https://assets.thermofisher.com/TFS-Assets%2FLSG%2Fmanuals%2Fmp07581.pdf).

Tagmentation-based library preparation

Timing: 2 h

Through these steps, the cDNA will be tagmented with sequencing adaptors and indexed. This allows the final multiplexing of the 384 or 768 single cells, depending on whether you plan to sequence 1 or 2 plates on the same transcriptomics sequencing run. For more information about the amount of input and cycle number required for the tagmentation process, please refer to the Smart-seq3 protocol.

Tagmentation.

21. Normalize the cDNA to a concentration of 1 ng/μL. If the overall concentration of the plate is lower, normalize to 0.7 ng/μL and adjust appropriately the reaction volumes.
    a. If a microplate reader is not available, average the concentration measurements from 10–15 random cells and then dilute the cDNA to the final concentration of 1 ng/μL with nuclease-free water.
    b. If a microplate reader is available, determine the cDNA concentrations and add the proper volumes of water per well in order to normalize the concentration of each well to 0.4–2.5 ng/μL. This can be done with Mantis.
22. Prepare the tagmentation master mix and aliquot 1.2 μL on a new plate (tagmentation plate).

Tagmentation master mix

Reagent	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
Tagment DNA buffer	0.8 μL	353.28 μL
Amplicon Tagment mix	0.4 μL	176.64 μL
Total	1.2 μL	529.92 μL

23. Add 0.4 μL of normalized cDNA (100–1000 pg of input) to the tagmentation plate. Vortex 30 s, and spin down. Repeat vortex and spin two times more. Incubate on thermocycler.

Note: Input amounts of cDNA can vary. For a specific table describing the input amount for tagmentation please refer to the Smart-seq2 (Picelli et al., 2014) and Smart-seq3 (Hagemann-Jensen et al., 2020a, 2020b) papers. In addition, if having remaining TSO dimers from the cDNA amplification part, increase the input amount to compensate for their presence. The same procedure can be applied in case of obtaining suboptimal profiles after tagmentation (see troubleshooting).

cDNA tagmentation. PCR cycling conditions

Steps	Temperature	Time	Cycles
Tagmentation	55°C	10 min	1
Hold	4°C		

24. Add 0.4 μL of 0.2% SDS to the tagmentation plate. Vortex for 30 s and spin down. Incubate 5 min at 18°C–25°C.

Optional: Instead of 0.2% SDS, NT buffer (Nextera XT DNA sample preparation kit) can be used.

SDS 0.2%		
Reagent	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
SDS 0.2%	0.4 μL	70.66 μL

Library amplification.

25. Add 1.2 μL of Nextera PCR master mix (NPM) to the tagmentation plate.

Optional: Alternatively, KAPA HiFi ready mix (2×) can be used instead of the Nextera PCR master mix. In this case use 3.2 μL instead of 1.2 μL for the final mix. Our experience shows that the results are similar by comparing the use of the 2 polymerases. If using the KAPA mix, make sure to keep the same proportion of beads to the sample volume in the further clean-up steps.

Nextera PCR Master mix		
Reagent	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
Nextera PCR Mastermix	1.2 μL	529.92 μL

26. Add 0.8 μL of Nextera Primer index mix (i5 + i7 indexes combined at a concentration of 12.5 μM, see enclosed table with the index sequences: Table S1: mRNA index primers). Vortex 30 s, spin down and incubate on thermocycler.

Note: Example layout of indexes for multiplexing 768 cells for the same sequencing run (Figure 2). This strategy uses combinatorial indexing and the numbers represent the indexes used. We provide the files with the index sequences in a format that can be easily used for ordering the oligos (5er and 7er indexes). The original design of the primers can be found here (Buenrostro et al., 2015).

Library amplification. PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Extension	72°C	3 min	1
Initial Denaturation	98°C	3 min	1
Denaturation	98°C	10 s	6–16
Annealing	55°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	5 min	1
Hold	4°C		

⚠ CRITICAL: Cycle number can be adjusted according to the input of cDNA. For primary NSCs, astrocytes, oligodendrocytes, neuroblasts and neurons we have used 13 cycles for an input of 500 pg. For pancreatic organoids we have used 12 cycles for an input of 400 pg. However, the number of cycles and the amount of input depend on the cell type. When using KAPA instead of Nextera PCR Mastermix, the PCR conditions are

Index for cDNA library preparation

	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724
501	501-701	501-702	501-703	501-704	501-705	501-706	501-707	501-708	501-709	501-710	501-711	501-712	501-713	501-714	501-715	501-716	501-717	501-718	501-719	501-720	501-721	501-722	501-723	501-724
502	502-701	502-702	502-703	502-704	502-705	502-706	502-707	502-708	502-709	502-710	502-711	502-712	502-713	502-714	502-715	502-716	502-717	502-718	502-719	502-720	502-721	502-722	502-723	502-724
503	503-701	503-702	503-703	503-704	503-705	503-706	503-707	503-708	503-709	503-710	503-711	503-712	503-713	503-714	503-715	503-716	503-717	503-718	503-719	503-720	503-721	503-722	503-723	503-724
504	504-701	504-702	504-703	504-704	504-705	504-706	504-707	504-708	504-709	504-710	504-711	504-712	504-713	504-714	504-715	504-716	504-717	504-718	504-719	504-720	504-721	504-722	504-723	504-724
505	505-701	505-702	505-703	505-704	505-705	505-706	505-707	505-708	505-709	505-710	505-711	505-712	505-713	505-714	505-715	505-716	505-717	505-718	505-719	505-720	505-721	505-722	505-723	505-724
506	506-701	506-702	506-703	506-704	506-705	506-706	506-707	506-708	506-709	506-710	506-711	506-712	506-713	506-714	506-715	506-716	506-717	506-718	506-719	506-720	506-721	506-722	506-723	506-724
507	507-701	507-702	507-703	507-704	507-705	507-706	507-707	507-708	507-709	507-710	507-711	507-712	507-713	507-714	507-715	507-716	507-717	507-718	507-719	507-720	507-721	507-722	507-723	507-724
508	508-701	508-702	508-703	508-704	508-705	508-706	508-707	508-708	508-709	508-710	508-711	508-712	508-713	508-714	508-715	508-716	508-717	508-718	508-719	508-720	508-721	508-722	508-723	508-724
509	509-701	509-702	509-703	509-704	509-705	509-706	509-707	509-708	509-709	509-710	509-711	509-712	509-713	509-714	509-715	509-716	509-717	509-718	509-719	509-720	509-721	509-722	509-723	509-724
510	510-701	510-702	510-703	510-704	510-705	510-706	510-707	510-708	510-709	510-710	510-711	510-712	510-713	510-714	510-715	510-716	510-717	510-718	510-719	510-720	510-721	510-722	510-723	510-724
511	511-701	511-702	511-703	511-704	511-705	511-706	511-707	511-708	511-709	511-710	511-711	511-712	511-713	511-714	511-715	511-716	511-717	511-718	511-719	511-720	511-721	511-722	511-723	511-724
512	512-701	512-702	512-703	512-704	512-705	512-706	512-707	512-708	512-709	512-710	512-711	512-712	512-713	512-714	512-715	512-716	512-717	512-718	512-719	512-720	512-721	512-722	512-723	512-724
513	513-701	513-702	513-703	513-704	513-705	513-706	513-707	513-708	513-709	513-710	513-711	513-712	513-713	513-714	513-715	513-716	513-717	513-718	513-719	513-720	513-721	513-722	513-723	513-724
514	514-701	514-702	514-703	514-704	514-705	514-706	514-707	514-708	514-709	514-710	514-711	514-712	514-713	514-714	514-715	514-716	514-717	514-718	514-719	514-720	514-721	514-722	514-723	514-724
515	515-701	515-702	515-703	515-704	515-705	515-706	515-707	515-708	515-709	515-710	515-711	515-712	515-713	515-714	515-715	515-716	515-717	515-718	515-719	515-720	515-721	515-722	515-723	515-724
516	516-701	516-702	516-703	516-704	516-705	516-706	516-707	516-708	516-709	516-710	516-711	516-712	516-713	516-714	516-715	516-716	516-717	516-718	516-719	516-720	516-721	516-722	516-723	516-724

	701	702	703	704	705	706	707	708	709	710	711	712	713	714	715	716	717	718	719	720	721	722	723	724
517	517-701	517-702	517-703	517-704	517-705	517-706	517-707	517-708	517-709	517-710	517-711	517-712	517-713	517-714	517-715	517-716	517-717	517-718	517-719	517-720	517-721	517-722	517-723	517-724
518	518-701	518-702	518-703	518-704	518-705	518-706	518-707	518-708	518-709	518-710	518-711	518-712	518-713	518-714	518-715	518-716	518-717	518-718	518-719	518-720	518-721	518-722	518-723	518-724
519	519-701	519-702	519-703	519-704	519-705	519-706	519-707	519-708	519-709	519-710	519-711	519-712	519-713	519-714	519-715	519-716	519-717	519-718	519-719	519-720	519-721	519-722	519-723	519-724
520	520-701	520-702	520-703	520-704	520-705	520-706	520-707	520-708	520-709	520-710	520-711	520-712	520-713	520-714	520-715	520-716	520-717	520-718	520-719	520-720	520-721	520-722	520-723	520-724
521	521-701	521-702	521-703	521-704	521-705	521-706	521-707	521-708	521-709	521-710	521-711	521-712	521-713	521-714	521-715	521-716	521-717	521-718	521-719	521-720	521-721	521-722	521-723	521-724
522	522-701	522-702	522-703	522-704	522-705	522-706	522-707	522-708	522-709	522-710	522-711	522-712	522-713	522-714	522-715	522-716	522-717	522-718	522-719	522-720	522-721	522-722	522-723	522-724
523	523-701	523-702	523-703	523-704	523-705	523-706	523-707	523-708	523-709	523-710	523-711	523-712	523-713	523-714	523-715	523-716	523-717	523-718	523-719	523-720	523-721	523-722	523-723	523-724
524	524-701	524-702	524-703	524-704	524-705	524-706	524-707	524-708	524-709	524-710	524-711	524-712	524-713	524-714	524-715	524-716	524-717	524-718	524-719	524-720	524-721	524-722	524-723	524-724
525	525-701	525-702	525-703	525-704	525-705	525-706	525-707	525-708	525-709	525-710	525-711	525-712	525-713	525-714	525-715	525-716	525-717	525-718	525-719	525-720	525-721	525-722	525-723	525-724
526	526-701	526-702	526-703	526-704	526-705	526-706	526-707	526-708	526-709	526-710	526-711	526-712	526-713	526-714	526-715	526-716	526-717	526-718	526-719	526-720	526-721	526-722	526-723	526-724
527	527-701	527-702	527-703	527-704	527-705	527-706	527-707	527-708	527-709	527-710	527-711	527-712	527-713	527-714	527-715	527-716	527-717	527-718	527-719	527-720	527-721	527-722	527-723	527-724
528	528-701	528-702	528-703	528-704	528-705	528-706	528-707	528-708	528-709	528-710	528-711	528-712	528-713	528-714	528-715	528-716	528-717	528-718	528-719	528-720	528-721	528-722	528-723	528-724
529	529-701	529-702	529-703	529-704	529-705	529-706	529-707	529-708	529-709	529-710	529-711	529-712	529-713	529-714	529-715	529-716	529-717	529-718	529-719	529-720	529-721	529-722	529-723	529-724
530	530-701	530-702	530-703	530-704	530-705	530-706	530-707	530-708	530-709	530-710	530-711	530-712	530-713	530-714	530-715	530-716	530-717	530-718	530-719	530-720	530-721	530-722	530-723	530-724
531	531-701	531-702	531-703	531-704	531-705	531-706	531-707	531-708	531-709	531-710	531-711	531-712	531-713	531-714	531-715	531-716	531-717	531-718	531-719	531-720	531-721	531-722	531-723	531-724
532	532-701	532-702	532-703	532-704	532-705	532-706	532-707	532-708	532-709	532-710	532-711	532-712	532-713	532-714	532-715	532-716	532-717	532-718	532-719	532-720	532-721	532-722	532-723	532-724

Figure 2. mRNA library amplification indexes
The figure shows the possible combinations of i5 and i7 indexes in order to multiplex 768 cells on a single sequencing run.

different for certain steps, as follows. Denaturation: 98°C for 20, Annealing: 63°C for 15 s. For more information on input and cycle number please refer to the Smart-seq2 and Smart-seq3 protocols.

cDNA library purification, quality control and sequencing

Timing: 4 h

Through these steps the cDNA library will be purified and checked for quality control.

Purification by bead clean-up.

27. Equilibrate AMPure XP beads at RT for 30 min. Add 3 μL of beads to the tagmentation plate, reaching a 0.75× proportion.
28. Vortex for 30 s and spin down. Incubate 3 min. Repeat twice more to reach a total of 10 min of incubation at RT. Centrifuge the plate at 1,000 × *g* for 1 min.
    a. During the incubation time prepare 18 mL of EtOH 80% and aliquot 41 μL into a new 384-well plate.
29. Incubate the tagmentation plate 2 min on Magnet. Discard the SN perform the following step twice (EtOH wash):
    a. Add 20 μL of EtOH 80% to the tagmentation plate. Wait 30 s and discard SN. Let the beads from the tagmentation plate dry for 2 min, until no EtOH 80% is seen on the wells.
30. Remove the tagmentation plate from the magnet. Add 7 μL of nuclease-free water. Vortex 30 s, spin down and incubate 1 min. Repeat the vortexing and incubation two times more, to reach a total of 5 min incubation.

31. Place plate on magnet for 2 min and transfer SN to a new plate (purified mRNA library plate).
32. Repeat the purification by adding 4.5 μL of AMPure beads (0.65×). Elute in 7 μL of nuclease-free water.
33. Perform Quality control similarly to the quality control of the cDNA (step 20).

Pooling of final library.

34. Normalize tagmented cDNA to a concentration of 0.5 ng/μL and pool 5 μL of every well into a final tube (for concentration measurement and normalization please use a similar workflow as step 21). Pooling procedure:
    a. Use Mosquito™ HV (SPT Labtech) to aspirate 5 μL from every column of the source plate (sample plate) and transfer to one column of a deep-well plate (called pool plate). Always change tips between transfers, to avoid contamination between the samples.
    b. After pooling into a single column, use a normal 1000 μL pipette to transfer the contents into a 2 mL microcentrifuge tube. The ideal amount should be 384*5=1920 μL. Mix the solution by pipetting up and down 10 times. The volume might be sometimes less than 1920 μL.
    c. Transfer 960 μL into a new 2 μL microcentrifuge tube. Now the pool should be divided on 2 tubes.
35. Perform a 1× bead cleanup.
    a. Measure exactly the volume of pool in every tube and add the same amount of AMPure XP beads in order to reach a 1× proportion. Mix by pipetting.
    b. Incubate 10 min at RT. Place the tube 3 min on magnet and discard SN.
    c. Wash two times with EtOH 80% and resuspend in 35 μL of nuclease-free water.

⚠ CRITICAL: When adding the water, resuspend first the beads with water on one tube, and then transfer the resuspended beads to the second tube (which has the dry beads). With this procedure, the two tubes are now fused into one, and the pool is resuspended in 35 μL of water containing the pooled cDNA library.

36. Measure the DNA concentration of the final pool using Qubit™ dsDNA HS Assay Kit as described before. Analyze the fragment distribution with Bioanalyzer or TapeStation (Agilent).

Sequencing

37. The pool can be sequenced on an Illumina NextSeq 550 sequencer (or another non-patterned flow cell Sequencer: HiSeq2000, NextSeq500). Because of the combinatorial indexing that is used on this part of the protocol, it is recommended to sequence on a non-patterned flow cell, since sequencing on a patterned flow cell might lead to index hopping (more information at: https://www.illumina.com/techniques/sequencing/ngs-library-prep/multiplexing/index-hopping.html). For an 1-plate run (384 cells) it is advisable to sequence it on a NexSeq 550 75 bp Paired End Mid output (MO) (104 million reads), in order to reach 300,000 reads per cell. For a 2-plate run (786 cells) the NS550 75 bp Paired End High output (HO) (320 Million reads) is recommended. Runs with 50 bp should also work. To reach more depth per cell, a single plate can also be sequenced on a NS550 HO, reaching 0.8 million reads per cell. Note that if the library is unique dual-indexed, a patterned flow cell sequencer such as NextSeq2000 and NovaSeq6000 can be used. It is always recommended to spike in PhiX to the sample before sequencing. PhiX is an adapter-ligated library used as a control for Illumina sequencing runs. It offers benefits for sequencing and alignment. We have used 1% as a final concentration of PhiX in our sequencing runs for the mRNA part.

Genomic part

gDNA purification and bisulfite conversion

Timing: 4 h

Through these steps the gDNA will be purified and bisulfite-converted. In this process, the gDNA will be fragmented and non-methylated cytosines will be chemically modified into uracils. In the conversion step, unmethylated cytosines will be deaminated. In order to save time, this step can be performed on the same day as the final part of the cDNA library preparation.

gDNA purification.

38. Equilibrate AMPure XP beads at RT for 30 min. Add 15 μL of beads to the gDNA plate (reaching 0.68× proportion) which was frozen and stored at −20°C on step 11.
39. Vortex for 30 s and spin down. Incubate 10 min at RT. Repeat twice more to reach a total of 30 min of incubation. Centrifuge the plate at 1,000 × *g* for 1 min.
    a. During the incubation time, prepare 18 mL of EtOH 80% and aliquot 41 μL into a new 384-well plate.
40. Incubate 2 min on magnet. Discard SN and perform the following step twice (EtOH wash):
    a. Add 20 μL of EtOH 80% to the gDNA plate. Wait 30 s and transfer SN to waste plate. Let the beads dry for 2 min, until no EtOH 80% is seen on the wells.
41. Remove gDNA plate from magnet. Add 2.5 μL of nuclease-free water. Vortex 30 s, spin down and incubate 1 min. Repeat the vortexing and incubation twice more, to reach a total of 5 min incubation.

⚠ CRITICAL: Do not aspirate the water from the gDNA plate.

Bisulfite conversion.

42. Place gDNA plate on magnet. Add 16 μL of pre-warmed (37°C) CT conversion reagent. Do not vortex (in order to avoid the beads mixing with the conversion reagent). Incubate on thermocycler as follows:

Bisulfite Conversion. PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	98°C	8 min	1
Conversion	64°C	180 min	1
Hold	4°C		

Pause point: gDNA plate can be left 16–24 h at 4°. Do not exceed 24 h of incubation at 4°C.

Desulfonation, 1° strand synthesis and exonuclease treatment.

Timing: 8 h

Through these steps the converted gDNA will be purified and the desulphonation process will take place, whereby the sulfite moiety from the cytosines will be removed to generate uracil bases. At the end the process of DNA amplification will start, with the 1° strand synthesis and the remaining free primers will be excised by exonuclease treatment.

gDNA clean-up with MagBinding beads.

43. Prepare Zymo MagBinding bead mix by mixing beads with M-Binding buffer in the following proportion:

MagBinding bead mix		
Reagent	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, X441.6)
MagBinding beads	5 µL	2208 µL
M-Binding buffer	70 µL	30912 µL
Total	75 µL	33120 µL

44. Aliquot 75 µL of bead mix into a 384-deep well plate. Also prepare an EtOH plate with 41 µL per well.
45. Perform the binding of the Bead mix with the gDNA.
    a. Transfer 25 µL of bead mix from the deep-well plate to the gDNA plate, perform a pipette mix and transfer 45 µL back to the deep-well plate.
    b. Add again 25 µL of bead mix to the gDNA plate, mix and transfer 25 µL back to the deep-well plate.
    c. Rinse the gDNA plate with 20 µL of bead mix and transfer all the remaining liquid to the deep-well plate. Vortex the deep-well plate 30 s and incubate 5 min at 18°C–25°C.
46. Procedure for discarding of SN and washing of the gDNA:
    a. Transfer 33.3 µL of bead mix from the deep-well plate to the gDNA plate. Spin down the gDNA plate at 1,000 × *g* for 1 min and then put plate on the magnet. Incubate 2 min. Discard SN.
    b. Repeat step a twice more and make sure that there are no beads remaining on the deep-well plate.
    c. Perform the following step **once** (EtOH wash):
        i. Add 20 µL of EtOH 80% to the gDNA plate. Wait 30 s and transfer SN to waste plate. Let the beads air dry at 18°C–25°C for 2 min.

Desulphonation and 1° strand synthesis.

47. Add 25 µL of M-Desulphonation to the gDNA plate. Vortex 30 s and spin the samples shortly. Incubate 15 min at 18°C–25°C. Meanwhile, prepare the 1° strand synthesis mix:

1° strand synthesis master mix				
Reagent	Initial c oncentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH_2O		7.9 µL	3488.64 µL	
Blue buffer (Part of the Klenow enzyme kit, see key resources table)	10 ×	1 µL	441.6 µL	1×
dNTP mix	10 mM	0.4 µL	176.64 µL	0.4 mM
Preamplification Oligo (1° Strand oligo)	10 µM	0.4 µL	176.64 µL	0.4 µM
Total		9.7 µL	4283.52 µL	

48. Centrifuge the gDNA plate at 1,000 × *g* for 1 min and place on magnet for 2 min. Perform two EtOH washes (as explained on step 46.c.I).
49. To dry the beads, put the gDNA plate **without foil** on a thermocycler with the lid open for 8 min at 55°C.
50. Add 9.7 µL of First Strand synthesis mix and seal the plate. Vortex 30 s, spin down briefly and incubate 1 min. Repeat the vortexing step twice more to reach a total of 5 min incubation.
51. Incubate the sealed plate 5 min at 55°C on a thermocycler with the lid closed. Centrifuge plate at 1,000 × *g* for 1 min. Put on magnet for 2 min and transfer SN to a new, fresh plate. Incubate 3 min at 65°C on a thermocycler. Immediately cool on ice.
52. Add 0.3 µL of Klenow (50 U/µL). Vortex 10 s and spin down. Incubate on thermocycler as follows:

1° strand synthesis – short PCR. PCR cycling conditions - 1 part			
Steps	Temperature	Time	Cycles
Initial Incubation	4°C	5 min	1
Slow ramping	4°C–37°C at a rate of 4 °C/min (0.06 °C/s)	8.25 min	1
Extension	37°C	30 min	1
Hold	4°C		

53. Incubate at 95°C for 45 s and afterward cool to 4°C on an ice block.
54. Add 0.6 μL of "first strand extra cycles" mix.

1° strand synthesis "extra cycles" master mix			
Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
ddH_2O		0.156 μL	68.89 μL
Blue Buffer	10×	0.06 μL	26.50 μL
dNTP mix	10 mM	0.024 μL	10.60 μL
Preamplification Oligo (1° Strand oligo)	10 μM	0.24 μL	105.98 μL
Klenow exo-	50 U/μL	0.12 μL	52.99 μL
Total		0.6 μL	264.96 μL

55. Vortex 10 s, spin down and incubate as follows:

1° Strand synthesis – short PCR. PCR cycling conditions - 2 Part			
Steps	Temperature	Time	Cycles
Initial incubation	4°C	5 min	1
Slow ramping	4°C–37°C at a rate of 4 °C/min (0.06 °C/s)	8.25 min	1
Extension	37°C	30 min	1
Hold	4°C		

56. Go to step "53" and repeat steps 53–55 three more times. For the last repetition, use the following program for the thermocycler:

1° strand synthesis – long PCR. PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Incubation	4°C	5 min	1
Slow ramping	4°C–37°C at a rate of 4 °C/min (0.06 °C/s)	8.25 min	1
Extension	37°C	90 min	1
Hold	4°C		

57. In total you have done 5 PCRs.

Pause point: You can incubate the plate at 4°C between 16–24 h or proceed to the next step.

58. Add 15 μL of exonuclease mix. Vortex 30 s and spin down. Incubate on thermocycler at 37°C for 60 min with the lid at 50°C. The total volume in plate is now 27.4 μL.

Pause point: The plate can be stored up to 1 month at −20°C.

Exonuclease mix			
Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)
ddH2O		14.5 μL	6403.2 μL
Exonuclease I	20 U/μL	0.5 μL	220.8 μL
Total		15 μL	6624 μL

1° strand product purification, 2° strand synthesis and purification, and library amplification.

Timing: 4 h

Through these steps the 1° strand product will be purified and further amplified using a second primer (Adapter 2 oligo). This product will be purified at the end. On the final step, the library will be amplified by using indexed primers (Unique dual index, UDI) and the KAPA enzyme.

1° strand purification.

59. Equilibrate AMPure XP beads at RT for 30 min. Add 17 μL of beads to the gDNA plate (reaching 0.62× bead proportion).
60. Vortex for 30 s and spin down. Incubate 3 min. Repeat twice more to reach a total of 10 min of incubation. Centrifuge the plate at 1,000 × *g* for 1 min.
    a. During the incubation time prepare 18 mL of EtOH 80% and aliquot 41 μL into a new 384-well plate.
    b. During the incubation time also prepare the 2° strand synthesis master mix.

2° strand synthesis master mix				
Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH2O		9.8 μL	4327.68 μL	
Blue Buffer	10 ×	1.22 μL	538.75 μL	1 ×
dNTP mix	10 mM	0.49 μL	216.38 μL	0.4 mM
Adaptor 2 Oligo (2° Strand oligo)	10 μM	0.49 μL	216.38 μL	0.4 μM
Total		12 μL	5299.2 μL	

61. Incubate 2 min on Magnet. Transfer the SN to the waste plate and perform the following step twice (EtOH wash).
    a. Add 20 μL of EtOH 80% to the mRNA plate. Wait 30 s and transfer SN to waste plate. Let the beads from the gDNA plate dry for 2 min, until no EtOH 80% is seen on the wells.

2° strand amplification.

62. Remove gDNA plate from magnet. Add 12 μL of 2° strand synthesis master mix. Vortex 30 s, spin down and incubate 1 min. Repeat the vortexing and incubation two times more, to reach a total of 5 min incubation. Transfer the content of every well to a new plate (without magnet).
63. Incubate at 95°C for 45 s and afterward cool to 4°C on an ice block.
64. Add 0.3 μL of Klenow-exo. Vortex 10 s and spin down. The total volume is 12.3 μL. Incubate on thermocycler as follows:

2° strand synthesis – long PCR. PCR cycling conditions.			
Steps	Temperature	Time	Cycles
Initial Incubation	4°C	5 min	1
Slow ramping	4°C–37°C at a rate of 4 °C/min (0.06 °C/s)	8.25 min	1
Extension	37°C	90 min	1
Hold	4°C		

2° strand purification.

65. Equilibrate PEG buffer at RT for 30 min. Add 12 µL of nuclease-free water and mix. Add 18 µL of PEG buffer and mix again (the bead proportion is now 0.74×).
66. Vortex for 30 s and spin down. Incubate 3 min. Repeat twice more to reach a total of 10 min of incubation. Centrifuge the plate at 1,000 × *g* for 1 min.
    a. During the incubation time prepare 18 mL of EtOH 80% and aliquot 41 µL into a new 384-well plate.
    b. During the incubation time also prepare the library amplification master mix (KAPA mix).

Library amplification master mix				
Reagent	Initial concentration	Amount for 1 reaction (1×)	Amount for 1 plate (384 + 15%, i.e., 441.6×)	Final concentration
ddH_2O		5.75 µL	2539.2 µL	
KAPA HiFi ready mix	2×	6.25 µL	2760 µL	1×
Total		12 µL	5299.2 µL	

67. Incubate 2 min on magnet. Transfer the SN to the waste plate and perform the following step twice (EtOH wash).
    a. Add 20 µL of EtOH 80% to the mRNA plate. Wait 30 s and transfer SN to waste plate. Let the beads from the gDNA plate dry for 2 min, until no EtOH 80% is seen on the wells.

Library amplification and purification

68. Add 12 µL of KAPA mix, vortex 30 s, and spin down. Incubate 10 min at RT to elute the DNA.
69. Add 0.5 µL of Index mix (UDI primer mix for gDNA, see key resources table and enclosed file: Table S2: gDNA index primers). Vortex 30 s and spin down. Incubate on thermocycler as follows.

gDNA library amplification. PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial denaturation	95°C	2 min	1
Denaturation	94°C	80 s	14
Annealing	65°C	30 s	
Extension	72°C	30 s	
Final extension	72°C	3 min	1
Hold	4°C		

70. Equilibrate PEG buffer at RT for 30 min. Add 12 µL of nuclease-free water and mix. Add 18 µL of PEG and mix again (the bead proportion is now 0.73×).
71. Vortex for 30 s and spin down. Incubate 3 min. Repeat twice more to reach a total of 10 min of incubation. Centrifuge the plate at 1,000 × *g* for 1 min.

72. Incubate 2 min on magnet. Transfer the SN to the waste plate and perform the following step twice (EtOH wash).
    a. Add 20 μL of EtOH 80% to the mRNA plate. Wait 30 s and transfer SN to waste plate. Let the beads from the gDNA plate dry for 2 min, until no EtOH 80% is seen on the wells.
73. Add 7 μL of nuclease-free water. Vortex 30 s, spin down and incubate 1 min. Repeat the vortexing and incubation twice more, to reach a total of 5 min incubation.
74. Place plate on magnet for 2 min and transfer SN to a new plate (final gDNA plate).
75. Perform quality control similarly to the quality control of the cDNA in step 20.

Pooling of final gDNA library.

76. Normalize the gDNA library to a concentration of 0.5 ng/μL and pool 3μL of every well into a final tube (for concentration measurement and normalization, please use a similar workflow as in step 21; for pooling, use a similar workflow as in step 34).
77. Perform a 1× bead clean-up as described for the tagmented cDNA in step 27.
78. Measure the DNA concentration of the final pool with Qubit dsDNA HS™ Assay Kit as described before. Analyze the fragment distribution with Bioanalyzer.

Sequencing

79. The pool can be sequenced on an Illumina NextSeq 1000–2000 sequencer (or any other patterned-flow cell sequencer: HiSeq X, HiSeq 3000/4000, NovaSeq 6000). For 96 cells per sequencing run, it is advisable to aim for 4–6 million reads per cell, in order to reach the minimum of 50.000 CpGs per cell. It should always be sequenced paired-end and with a read length of at least 75 bp. Note that the gDNA libraries can also be sequenced on non-patterned flow cell sequencers (NextSeq550), since the samples have UDI. However, we prefer to use patterned-flow cell sequencer, since the output of sequencing data is more cost-effective. We have used 15% as a final concentration of PhiX in our sequencing runs for the gDNA part. Note that this high concentration is due to the low diversity of bisulfite treated gDNA libraries.

Alignment and quantification of transcriptomic data

Timing: 4 h

Through these steps the transcriptomic data will be mapped to the reference genome. This allows the quantification of the gene expression profile of single cells.

Follow the steps recommended by (Hagemann-Jensen et al., 2020a, 2020b) in the SmartSeq3 protocol. For convenience, we briefly recapitulate these methods.

80. Convert Binary Base Call (BCL) files to FASTQ format with the bcl2fastq software provided by Illumina.

```
bcl2fastq -use-bases-mask Y150N,I8,I8,Y150N -no-lane-splitting -create-fastq-for-index-reads -R /run_directory/
```

where `/run_directory/` is the directory containing the BCL files.

a. Inspect the quality of several FASTQ files with FASTQC (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/).
b. All FASTQ files may then be demultiplexed, mapped, and quantified with the software zUMIs (https://github.com/sdparekh/zUMIs). To configure your zUMIs run, you will need to prepare

a YAML file that denotes all parameters and paths (Hagemann-Jensen et al., 2020a, 2020b). provide the following template for a typical Smart-seq3 run:

```
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
```

Continued

```
    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
```

81. After substituting the appropriate paths in this template, your zUMIs run may be started:

```
zUMIs.sh -c -y Smartseq3_config.yaml
```

Alignment and quantification of epigenomic data

Timing: 3 days

Through these steps the epigenomic data will be mapped to the reference genome. This allows the quantification of the methylation and chromatin accessibility profile of single cells.

The total time will depend on the power of the computer cluster used for processing the data. On average we find that 3 days are enough to map 96 cells. This part of the protocol is based on recommendations described in the original scNMT-seq paper (Clark et al., 2018). We recommend Bismark (https://www.bioinformatics.babraham.ac.uk/projects/bismark/) (10.1093/bioinformatics/btr167) for mapping and quantification of genomic reads, since Bismark can quantify both CpG-methylation

and GpC-methylation. Since mapping of bisulfite-converted whole-genome reads is computationally expensive, you should consider performing this analysis on a large compute cluster or a similar system. The following steps are required for every cell, so it's advisable to use a workflow manager like Snakemake or Nextflow to process multiple cells in parallel. It is not advisable to process FASTQ-files with extremely low read number (e.g., below 10.000 reads) since these cells will not pass later quality checks anyways, and they might cause crashes in later processing steps.

82. Follow the steps recommended by Illumina to demultiplex your sequencing data and to convert them to FASTQ format.
83. Prepare the genome for methylation-aware alignment with Bismark. This step is only required once.

```
bismark_genome_preparation –path_to_aligner /usr/bin/bowtie2/ –verbose /genomes/homo_sapiens/GRCh37/
```

84. Trim sequencing adapters with trim_galore (https://www.bioinformatics.babraham.ac.uk/projects/trim_galore/) as follows:

```
trim_galore –output_dir trimmed/ –paired cell1_read1.fastq.gz cell1_read2.fastq.gz
```

85. Map sequencing reads to the genome in single-end non-directional mode:

```
bismark –non_directional –genome /genomes/homo_sapiens/GRCh37/ cell1_read1.fastq.gz –output_dir mapped/

bismark –non_directional –genome /genomes/homo_sapiens/GRCh37/ cell1_read2.fastq.gz –output_dir mapped/
```

86. Remove PCR duplicates:

```
deduplicate_bismark mapped/cell1_read1_bismark_bt2.bam –single –bam –output_dir mapped/ –outfile read1_deduplicated.bam

deduplicate_bismark mapped/cell1_read2_bismark_bt2.bam –single –bam –output_dir mapped/ –outfile read2_deduplicated.bam
```

87. Merge mates:

```
samtools merge merged/cell1_merged.bam cell1_read1_deduplicated.bam cell1_read2_deduplicated.bam
```

88. Quantify DNA methylation at all cytosines.

```
bismark_methylation_extractor –gzip –CX –output methylation-calls/ merged/cell1_merged.bam
```

89. Use Bismark's NOMe-seq option to distinguish between CpG and GpC methylation:

```
coverage2cytosine -nome-seq -dir methylation-calls/ -genome_folder /genomes/homo_sapiens/GRCh37/ -output cell1 methylation-calls/cell1_merged.bismark.cov.gz
```

EXPECTED OUTCOMES

cDNA amplification

cDNA profile after amplification: The profiles should look as indicated in Figure 1. The presence of TSO dimers at around 80–150 bp in high proportion might hinder the downstream processing of the sample, in particular the tagmentation process. In this case, perform the TSO-wash as described previously. In addition, to obtain a good profile, it is advisable to reduce the time that the beads are without liquid (in particular in the mRNA and gDNA separation steps) and to pre-aliquot reagents in fresh 384-well plates in order to increase the speed of reagent transfer (see troubleshooting for more information).

cDNA library preparation

cDNA library profile: The profiles should look as shown in Figure 3. An average fragment size of 500–900 bp is expected. If primer dimers are still present, perform another 0.8× purification.

cDNA library pool

Pooled cDNA library profile: The profile should look as shown in Figure 4. An average fragment size of 400–900 bp is expected. If primer dimers are still present, repeat again a 1× purification. On the final pool there should not be free adaptors (fragments of size less than 200 bp), as they will interfere with the sequencing.

gDNA library

gRNA library profile: The profiles should look as shown in Figure 5. An average fragment size of 500–900 bp is expected. We had some runs with an average fragment size of 500 bp and some other runs with an average fragment size of 800–900 bp. For both neural cells and pancreatic organoid cells, we have used 14 cycles. Presence of fragment at ~200–300 bp indicates the presence of primer concatemers, which might reduce the quality of the data.

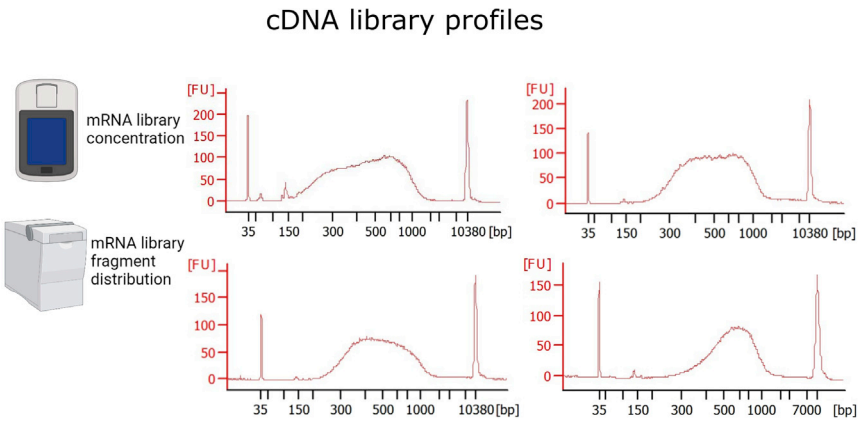

Figure 3. cDNA library profile of 4 random samples

Measure the concentration with a Qubit™ Fluorometer (Thermo Fisher Scientific) and the fragment distribution with Bioanalyzer or Tape Station (Agilent). The average fragment size should vary between 400–900 bp. If primer dimers are still present, perform one 0.8× purification. Parts of the figure are created with BioRender.com.

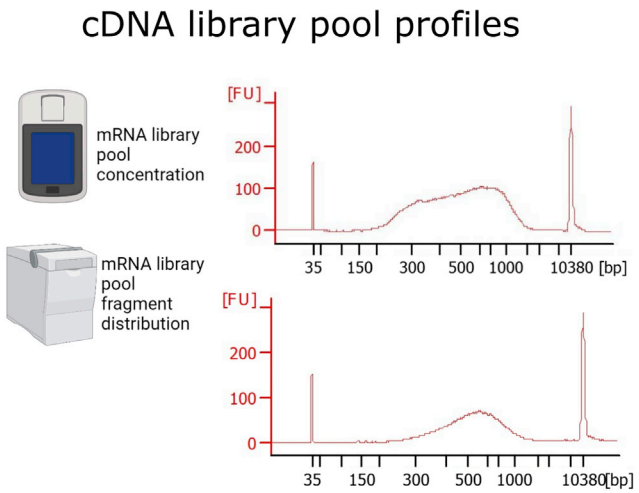

Figure 4. Pooled cDNA libraries

Measure the concentration with a Qubit™ Fluorometer (Thermo Fisher Scientific) and the fragment distribution with Bioanalyzer or Tape Station (Agilent). Here two random mRNA library pools are shown. The average fragment size should be between 400–900 bp free of fragment smaller than 200 bp. Parts of the figure are created with BioRender.com.

gDNA library pool

gRNA library pool profile: The profiles should look as shown in Figure 6. An average fragment size of 500–900 bp is normal.

Alignment of transcriptomic data

zUMIs will create a directory `zUMIs_output/expression` that contains the single-cell RNA sequencing (scRNA-seq) count matrix in .loom and .rds formats for standard scRNA-seq analysis in R or Python. As is common practice, we recommend to filter cells with an unusually low number of observed genes. Note that the gene number depends not just on RNA quality and sequencing depth, but also on the overall transcriptional activity of the sample (Figure 7).

Alignment of epigenomic data

This workflow will produce two output files per cell, `methylation-calls/cell1.NOMe.CpG.cov` and `methylation-calls/cell1.NOMe.GpC.cov`, which list the methylation level at all CpG sites and GpC sites. Downstream analysis of these raw data may be performed with R or Python.

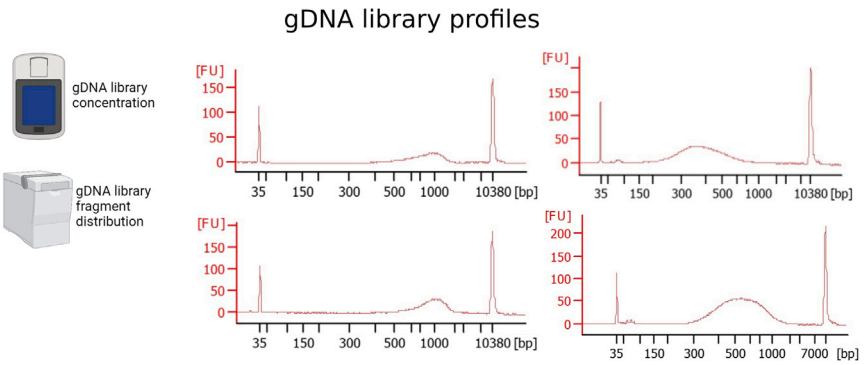

Figure 5. gDNA library profile of 3 random samples

Measure the concentration of the gDNA with a Qubit™ Fluorometer (Thermo Fisher Scientific) and the fragment distribution with Bioanalyzer or Tape Station (Agilent). The average fragment size should vary between 500 and 900 bp. Parts of the figure are created with BioRender.com.

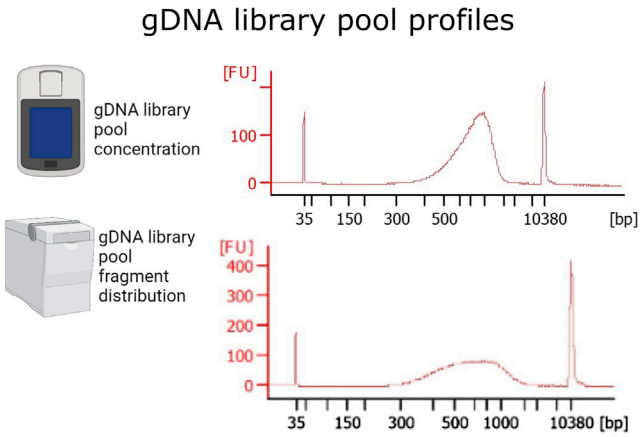

Figure 6. Pooled gDNA library profiles of two random samples

Measure the concentration of the gDNA with a Qubit™ Fluorometer (Thermo Fisher Scientific) and the fragment distribution with Bioanalyzer or Tape Station (Agilent). The average fragment size should be between 500–900 bp. Parts of the figure are created with BioRender.com.

For quality filtering, we recommend to discard cells with less than 50.000 observed CpG sites, and possibly cells with extremely high or low global DNA methylation. Depending on DNA quality, sequencing depth, and the proportion of PCR duplicate reads, the number of observed CpG sites may vary between experiments (Figure 8).

It may also be useful to inspect DNA methylation patterns and chromatin accessibility patterns at transcription start sites, to judge the quality of the data. In mammalian cells, the average transcription start site should be less methylated and more accessible than neighboring regions (Figure 9).

LIMITATIONS

This protocol allows the processing of 384 cell per protocol run, but the profiling of 96 cells at the gDNA level per sequencing run. To increase the number of cells that can be sequenced per run, it is advisable to design new 384 UDI primers, by considering the color complexity of the index sequences regarding the sequencer to be used. Our design is based on 96 UDI. The protocol is also laborious and might take to up to 1 week. To reduce time, the use of the liquid handlers is recommended. As explained before, the gDNA purification and conversion step can be done on the last day of the mRNA library part, in order to save one day of work.

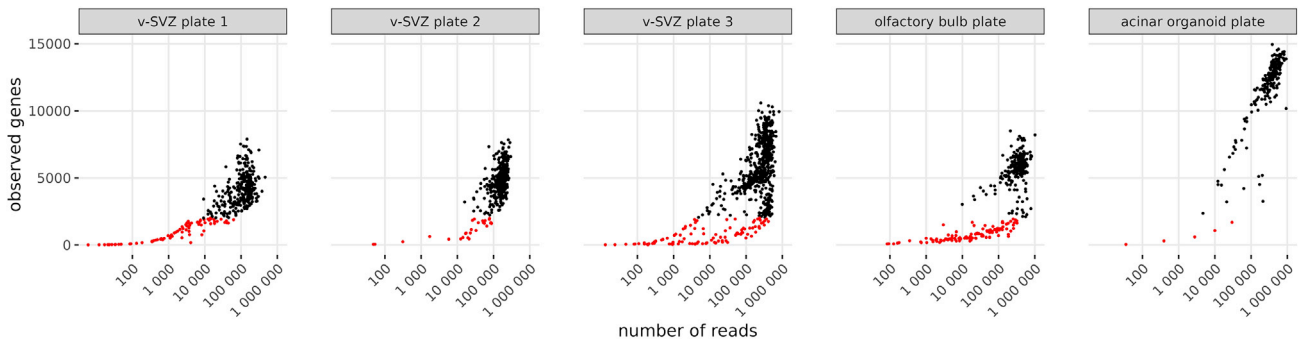

Figure 7. Quality metrics of single cell transcriptomes from five different plates

Cells with less than 2000 observed genes are highlighted in red. These cells are candidates for quality filtering. Note that the *in vitro* acinar organoid cells appear to express more genes than murine *in vivo* cells since this difference cannot be explained by read number alone.

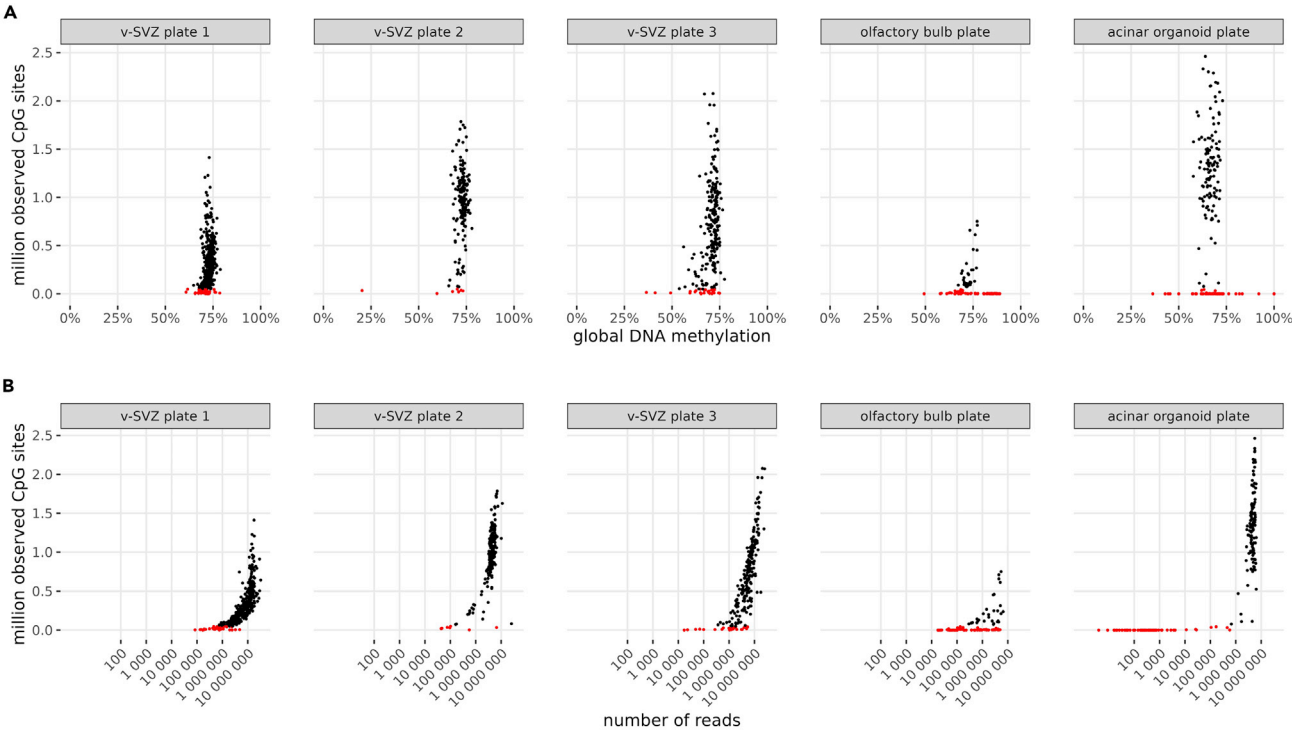

Figure 8. Quality metrics of single cell methylomes from five different plates

(A) Observed CpG sites per cell versus global DNA methylation. Cells with less than 50,000 observed CpG sites are highlighted in red. These cells are candidates for quality filtering.

(B) Observed CpG sites per cell versus number of reads. This shows that with at least 3–4 million reads per cell, the number of CpGs pass the lower threshold of 50,0000 CpGs.

TROUBLESHOOTING

Problem 1

High proportion of primer or TSO dimers after cDNA amplification (steps 13 and 14).

Potential solution

- Perform TSO-wash (See step 13).

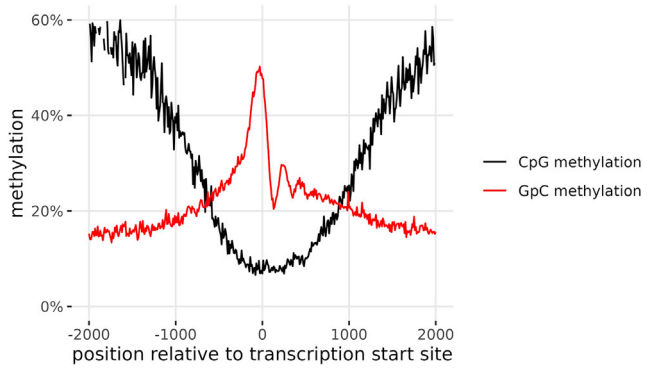

Figure 9. Average methylation of all transcription start sites one a single high-quality cell

Genomic position is binned in bins of 10 bp width.

- Prealiquot the G&T wash buffer in a fresh plate. That enables an efficient separation of the gDNA and the mRNA and also reduces the time that the OligodT beads are exposed to air.
- Increase the number of PCR cycles in order to amplify more the cDNA.

Problem 2

Primer dimer presence after cDNA tagmentation or suboptimal profiles (steps 21–33).

Potential solution

- Perform another 0.6× bead cleanup in order to eliminate the presence of primer dimers.
- Increase the input of cDNA for tagmentation.

Problem 3

Small average fragment size on gDNA library (200–300 bp, steps 68–75).

Potential solution

- The initial material (gDNA) is in low concentration. Increase the incubation time with water from 30 min to 45 min on the gDNA purification step (step 39).
- Decrease the proportion of beads in the purification steps of the gDNA library to 0.6×.

Problem 4

Low amount of gDNA library (less than 0.2 ng/μL, steps 68–75).

Potential solution

Repeat library amplification with 2–4 cycles.

Problem 5

High average fragment size on gDNA library (1200–1400 bp, steps 68–75).

Potential solution

The conversion was not efficient. However, the library is still sequence-ready. For the next trial use freshly prepared conversion reagent and make sure that you don't see crystals on the solution before aliquoting into a new plate.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Ana Martin-Villalba (a.martin-villalba@dkfz-heidelberg.de).

Materials availability

This study did not generate new unique reagents.

Data and code availability

All sequencing data are available at the NCBI Gene Expression Omnibus (GEO) under the SuperSeries accession GSE210806. The four brain data sets depicted in Figure 7 correspond to plates pD, pH, pI, and pE. The code used to process scNMT-seq data is provided as part of the protocol.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101555.

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AUTHOR CONTRIBUTIONS

Conceptualization: A.M.V., S.C., and O.K. Writing, Review & Editing: S.C., L.K., O.K., and A.M.V. Methodology: Performed the scNMT protocol with different cell types: S.C., T.E., A.S., J.S., and A.K. Development of the miniaturized protocol: S.C., O.K., and T.E. Design and implementation of troubleshooting alternatives: A.S.M. and A.S. Establishment of pancreatic organoid culture: J.B. and A.S. Formal analysis: Data processing and statistical analysis: L.K. Funding acquisition: A.M.V.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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