2019

Working

SmartSeq2 for HTP Generation of FACS Sorted Single Cell Libraries

Tabula Muris Consortium[1], Shayan Hosseinzadeh[1]

[1]Chan Zuckerberg Biohub

dx.doi.org/10.17504/protocols.io.2uwgexe

Chan Zuckerberg Biohub

Shayan Hosseinzadeh
Chan Zuckerberg Biohub

MATERIALS TEXT

All required reagents for SmartSeq2 are described in the body of the protocol for each step of the workflow.

SAFETY WARNINGS

Library preparation protcols involve handling of 100% N,N- dimethylformamide which is a carcinogen and irritant. This chemical should be handled with care wearig proper PPE in a chemical fume hood until diluted to a concentration <10% and all waste that contacts this reagent should be disposed of in a separate chemical waste.

BEFORE STARTING

Prepare lysis plates for cell sorting before dissecting tissue or preparing single cell suspensions for FACS sorting. Ensure access to necessary automated liquid handlers or suitable equivalents to facilitate the high throughput processing of samples that this protocol was adapted for.

Lysis Plate Preparation

1 Lysis plates were created by dispensing 0.4 µl lysis buffer into 384-well hard-shell PCR plates (Bio- Rad HSP3901) using a Tempest liquid handler (Formulatrix). 96-well lysis plates were also prepared with 4 µl lysis buffer. All plates were sealed with AlumaSeal CS Films (Sigma-Aldrich Z722634) and spun down (3,220*g*, 1 min) and snap-frozen on dry ice. Plates were stored at −80 °C until sorting.

Lysis Buffer Master Mix
0.5 U Recombinant RNase Inhibitor (Takara Bio, 2313B)
0.0625% TritonTM X-100 (Sigma, 93443-100ML)
3.125 mM dNTP mix (Thermo Fisher, R0193)
3.125 µM Oligo-dT30VN (Integrated DNA Technologies, 5'AAGCAGTGGTATCAACGCAGAGTACT30VN-3')
1:600,000 ERCC RNA spike-in mix (Thermo Fisher, 4456740)

FACS Sorting

2 ***Is this step necessary to include?***

After dissociation, single cells from each organ and tissue were isolated into 384- or 96-well plates via FACS. Most organs were sorted into 384-well plates using SH800S (Sony) sorters. Heart and liver were sorted into 96-well plates and cardiomyocytes were hand-picked into 96-well plates. Limb muscle and dia- phragm were sorted into 384-well plates on an Aria III (Becton Dickinson) sorter. The last two columns of each 384 well plate were intentionally left as blanks. For most organs, single cells were selected with forward scatter, and dead cells and common cell types were excluded with a single colour channel. Combinations of fluorescent antibodies were used for most organs to enrich for rare cell popula- tions (see Supplementary Information), but some were stained only for viable cells. Colour compensation was used whenever necessary. On the SH800, the highest purity setting ('Single cell') was used for all but the rarest cell types, for which the 'Ultrapure' setting was used. Sorters were calibrated using FACS buffer every day before collecting any cells, and also after every eight sorted plates. For a typical sort, 1–3 ml of pre-stained cell suspension was filtered, vortexed gently, and loaded onto the FACS machine. A small number of cells were flowed at low pressure to check cell and debris concentrations. The pressure was then adjusted, flow paused, the first

destination plate unsealed and loaded, and sorting started. If a cell suspension was too concentrated, it was diluted using FACS buffer or 1X PBS. For some cell types, such as hepatocytes, 96-well plates were used because it was not possible to sort individual cells accurately into 384-well plates. Immediately after sorting, plates were sealed with a pre-labelled aluminium seal, centrifuged, and flash frozen on dry ice. On average, each 384-well plate took 8 min to sort.

cDNA Synthesis

3 First Strand Synthesis

cDNA synthesis was performed using the Smart-seq2 protocol[1]. In brief, 384-well plates containing single- cell lysates were thawed on ice followed by first-strand synthesis. 0.6 µl of reaction mix was added to each well using a Tempest liquid handler or Mosquito liquid handler (TTP Labtech). Reverse transcription was carried out by incubating wells on a ProFlex 2 × 384 thermal-cycler (Thermo Fisher) at 42 °C for 90 min, and stopped by heating at 70 °C for 5 min.

RT Master Mix
16.7 U/µl SMARTScribe Reverse Transcriptase (Takara Bio, 639538)
1.67 U/µl Recombinant RNase Inhibitor (Takara Bio, 2313B)
1.67X First-Strand Buffer (Takara Bio, 639538),
1.67 µM TSO (Exiqon, 5'-AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG-3')
8.33 mM dithiothreitol (Bioworld, 40420001-1)
1.67 M Betaine (Sigma, B0300-5VL)
10 mM MgCl2 (Sigma, M1028-10X1ML)

1. Picelli, S. *et al.* Smart-seq2 for sensitive full-length transcriptome profiling in single cells. *Nat Methods* **10,** nmeth.2639 (2013).

4 2nd Strand Synthesis and cDNA Amplification

Subsequently, 1.5 µl of PCR mix was added to each well with a Mantis liquid handler (Formulatrix) or Mosquito liquid handler, and second-strand synthesis was performed on a ProFlex 2x384 thermal-cycler by using the following program:

cDNA PCR Master Mix
1.67X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, KK2602)
0.17 µM IS PCR primer (IDT, 5'-AAGCAGTGGTATCAACGCAGAGT-3')
0.038 U/µl Lambda Exonuclease (NEB, M0262L))

PCR Conditions:
1) 37 °C for 30 min
2) 95 °C for 3 min
3) 23cycles :
   98°Cfor20s
   67°Cfor15s
   72°Cfor4min
4) 72°Cfor5min.

5 cDNA Quantification and QC

The amplified product was diluted with a ratio of 1 part cDNA to 10 parts 10 mM Tris-HCl (Thermo Fisher, 15568025)

For the sample plates from mice aged 3 months, concentrations were measured with a dye-fluorescence assay (Quant-iT dsDNA High Sensitivity kit; Thermo Fisher, Q33120) on a SpectraMax i3x microplate reader (Molecular Devices). Sample plates were selected for downstream processing if the mean concentration of blanks (ERCC-containing, non-cell wells) was greater than 0 ng/µl, and, after linear regression of the values obtained from the Quant-iT dsDNA standard curve, the R^2 value was greater than 0.98. Sample wells were then selected if their cDNA concentrations were at least one standard deviation greater than the mean concentration of the blanks. These wells were reformatted to a new 384-well plate at a concentration of 0.3 ng/µL and a final volume of 0.4 µl using an Echo 550 acoustic liquid dispenser (Labcyte).

For the sample plates from mice aged 18, 21, and 24 months, sample plates were not quantified, cherrypicked, or normalized. For every sample plate, 0.8 µl of cDNA was transfered to a new 384-well plate using a Viaflow 384 Multichannel Pipette (Integra).

Library Preparation

6 Nextera Library Preparation

For the sample plates from mice aged 3 months, Illumina sequencing libraries were prepared as described previously[2]. In brief, tagmentation was carried out on double-stranded cDNA using the Nextera XT Library Sample Preparation kit (Illumina, FC-131-1096) and a Mantis liquid handler. Each well was mixed with 0.8 µl Nextera tagmentation DNA buffer (Illumina) and 0.4 µl Tn5 enzyme (Illumina), then incubated at 55 °C for 10 min. The reaction was stopped by adding 0.4 µl Neutralize Tagment Buffer (Illumina) and centrifuging at room temperature at 3,220*g* for 5 min. Indexing PCR reactions were performed by adding 0.4 µl of 5 µM i5 indexing primer, 0.4 µl of 5 µM i7 indexing primer, and 1.2 µl of Nextera NPM mix (Illumina). PCR amplification was carried out on a ProFlex 2x384 thermal cycler using the following program:

1) 72 °C for 3 min
2) 95 °C for 30 s
3) 12 cycles :
   95°Cfor10s
   55°Cfor30s
   72°Cfor1min
4) 72°Cfor5min.

2. Darmanis, S. et al. A survey of human brain transcriptome diversity at the single cell level. *Proc. Natl Acad. Sci.* USA **112**, 7285–7290 (2015)

7 Homebrew Library Preparation

For the sample plates from mice aged 18, 21, and 24 months, Illumina sequencing libraries were prepared using a homebrew library preprartion protocol modified from previously reported tagmentation-based protocols[3,4] and a Mosquito liquid handler. Tagmentation was carried out by mixing each well with 1 uL of 1.6x Homebrew Tn5 Tagmentation Buffer and 0.2 uL of Tn5 enzyme (UC Berkeley Macrolab), then incubated at 55 °C for 3 min. The reaction was stopped by adding 0.4 µl 0.1% sodium dodecyle sulfate(Fisher Scientific, BP166-500) and centrifuging at room temperature at 3,220*g* for 5 min. Indexing PCR reactions were performed by adding 0.4 µl of 5 µM i5 indexing primer, 0.4 µl of 5 µM i7 indexing primer, and 1.2 µl of KAPA HiFi Non-Hot Start Master Mix (Kapa Biosystems). PCR amplification was carried out on a ProFlex 2x384 thermal cycler using the following program:

1) 72 °C for 3 min
2) 95 °C for 30 s
3) 12 cycles :
   98°Cfor10s
   67°Cfor30s
   72°Cfor1min
4) 72°Cfor5min.

1.6x Homebrew Tn5 Tagmentation Buffer
16 mM Tris-HCl, pH 7.4 (Lonza, 51247)
16 mM $MgCl_2$ (Invitrogen, AM9530G)
8% v/v N,N-dimethyformami

de (Sigma-Aldrich, 227056-1L)

KAPA HiFi Non-Hot Start Master Mix
0.067 U/µL KAPA HiFi Standard Polymerase (Kapa Biosystems, KK2102)
3.33x KAPA HiFi Fidelity Buffer(Kapa Biosystems, KK2102)
1 µM dNTP Mix (Kapa Biosystems, KK2102)

3.Hennig, B. P. *et al.* Large-Scale Low-Cost NGS Library Preparation Using a Robust Tn5 Purification and Tagmentation Protocol. *G3 Genes Genomes Genetics* **8,** 79–89 (2018).

4. Picelli, S. *et al.* Tn5 transposase and tagmentation procedures for massively scaled sequencing projects. *Genome Res* **24,** 2033–2040 (2014).

Library Pooling, QC, and Sequencing

8

After library preparation, wells of each library plate were pooled using a Mosquito liquid handler. Pooling was followed by two purifications using 0.7x AMPure beads (Fisher, A63881) or 0.9x Homebrew SPRI beads. Library quality was assessed using capillary electrophoresis on a

Fragment Analyzer (AATI) or Tapestation (Agilent), and libraries were quantified by qPCR (Kapa Biosystems, KK4923) on a CFX96 Touch Real-Time PCR Detection System (Biorad). Plate pools were normalized to 2 nM and equal volumes from 10 or 20 plates were mixed together to make the sequencing sample pool. A PhiX control library was spiked in at 0.2% before sequencing.

Homebrew SPRI Beads
1M NaCl (Teknova, S0250)
10 µM Tris-HCl, pH 8.0 (Lonza, 51238)
5 µM EDTA, pH 8.0 (Invitrogen, 15575-038)
18 % w/v PEG8000 (Sigma-Aldrich, P1458-50ML)
0.055% v/v Tween-20 (Acros Organics, 233362500)
1 mg/mL SeraMag Carboxylated Speedbeads, (GE Healthcare 65152105050250)

Sequencing 384-well and 96-well Libraries

9 Libraries were sequenced on the NovaSeq 6000 Sequencing System (Illumina) using 2 × 100-bp paired-end reads and 2 × 8-bp or 2 × 12-bp index reads with either a 200- or 300-cycle kit (Illumina, 20012861 or 20012860).