Sep 07, 2023

High-Throughput Generation of Single Cell Libraries using SmartSeq2 Plate Assay

Forked from SmartSeq2 for HTP Generation of FACS Sorted Single Cell Libraries

Science Advances

DOI
dx.doi.org/10.17504/protocols.io.8epv5xx54g1b/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.8epv5xx54g1b/v1

External link: https://doi.org/10.1016/j.cell.2020.07.017

Protocol Citation: Michael Borja, Tabula Muris Consortium, Shayan Hosseinzadeh 2023. High-Throughput Generation of Single Cell Libraries using SmartSeq2 Plate Assay. **protocols.io** https://dx.doi.org/10.17504/protocols.io.8epv5xx54g1b/v1

Manuscript citation:
Maynard A, McCoach CE, Rotow JK, Harris L, Haderk F, Kerr DL, Yu EA, Schenk EL, Tan W, Zee A, Tan M, Gui P, Lea T, Wu W, Urisman A, Jones K, Sit R, Kolli PK, Seeley E, Gesthalter Y, Le DD, Yamauchi KA, Naeger DM, Bandyopadhyay S, Shah K, Cech L, Thomas NJ, Gupta A, Gonzalez M, Do H, Tan L, Bacaltos B, Gomez-Sjoberg R, Gubens M, Jahan T, Kratz JR, Jablons D, Neff N, Doebele RC, Weissman J, Blakely CM, Darmanis S, Bivona TG, Therapy-Induced Evolution of Human Lung Cancer Revealed by Single-Cell RNA Sequencing. Cell 182(5). doi: 10.1016/j.cell.2020.07.017

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Protocol status: Working
We use this protocol and it's working

Created: August 23, 2023

Last Modified: September 07, 2023

Protocol Integer ID: 86829

Keywords: Tabula Muris, MACA, SmartSeq2, NGS, Tn5, Tagmentation, Library Prepration, cDNA, Single Cell, RNA-Seq, optimized smartseq2 assay, aging cell atlas, compendium of cell atlas data, cell atlas data, cell atlas, generation of single cell library, throughput generation of single cell library, smartseq2 assay with the assistance, genomics platform, single cell library, larger efforts of quantitative cell science, single cell plate, quantitative cell science, single cell, smartseq, cell, plate assay as part, sequencing, plate assay, rna

Abstract

As part of the larger efforts of Quantitative Cell Science at the Chan Zuckerberg Biohub, single cell plate-based protocols such as the SmartSeq 2 assay has been frequently utilized to generate compendium of cell atlas data. With the help of previous works of MACA (mouse aging cell atlas), the Genomics platform has optimized Smartseq2 assay with the assistance of high-throughput liquid dispensers from SPT LabTech and ViaFlo. Here, we list the latest protocol detailing the generation of single cell libraries for most projects that utilize plate-based RNA sequencing.

Materials

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

Troubleshooting

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 start

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 master mix into 384-well hard-shell PCR plates (Bio- Rad HSP3901) using the SPT LabTech DragonFly. 96-well lysis plates were also prepared with 4 µl lysis buffer. All plates were sealed with Microseal Seal F foil seal and spun down (1000 rcf, 1 min) and snap-frozen on dry ice. Plates were stored at −80 °C until sorting.

Lysis Buffer Master Mix
Recombinant RNase Inhibitor (Takara Bio, 2313B)
10% TritonTM X-100 (Sigma, 93443-100ML)
10 mM dNTP mix (Thermo Fisher, R0193)
100 µM Oligo-dT30VN (Integrated DNA Technologies, 5′AAGCAGTGGTATCAACGCAGAGTACT30VN-3′)
1:600,000 ERCC RNA spike-in mix (Thermo Fisher, 4456740)

FACS Sorting

2 After dissociation, single cells are isolated into 384- or 96-well plates via either using SH800S (Sony) sorter or equivalent sorters. Under the guidance of appropriate FACS manual and protocols, flow cytometry was performed onto prepared lysis plates from step 1. When completed, plates were stored in −80 °C until first strand synthesis.

cDNA Synthesis

3 **First Strand Synthesis**

cDNA synthesis was performed using the Smart-seq2 protocol[1]. In brief, 384-well or 96-well plates containing single- cell lysates from previous step were thawed on ice followed by first-strand synthesis. 0.6 µl of reaction mix was added for 384-well plates and 6 µl of reaction mix to each well using the SPT LabTech DragonFly. All plates were sealed with Microseal Seal B adhesive seal and spun down (1000 rcf, 1 min). Reverse transcription was carried out by incubating wells on a ProFlex 2 × 384 thermal-cycler (Thermo Fisher) at 42 °C for 90 min, stopped by heating at 70 °C for 5 min and cooled to 4 °C hold. When completed, plates were either stored in −20 °C or immediately processed for 2nd strand synthesis and cDNA amplification.

RT Master Mix
SMARTScribe Reverse Transcriptase (Takara Bio, 639538)
Recombinant RNase Inhibitor (Takara Bio, 2313B)
5X First-Strand Buffer (Takara Bio, 639538),

100 µM TSO (Exiqon, 5′-AAGCAGTGGTATCAACGCAGAGTGAATrGrGrG-3′)
100 mM dithiothreitol (Bioworld, 40420001-1)
5 M Betaine (Sigma, B0300-5VL)
1 M 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 for 384-well plates or 15 µl for 96-well plates of cDNA amp mix was added to each well using the SPT LabTech DragonFly. All plates were sealed with Microseal Seal B adhesive seal and spun down (1000 rcf, 1 min). 2nd strand synthesis and cDNA amplification were performed on a ProFlex 2×384 thermal-cycler by incubating at 37 °C for 30 min, 95 °C for 3 min, 23 cycles of the following steps: 98°C for 20 s; 67°C for 15 s; 72°C for 4 min, and finally 72°C for 5 min. After completion, all plates were spun down (1000 rcf, 1 min) and stored in −20 °C until ready for plate quantification and dilution.

cDNA Amp Mix
2X KAPA HiFi HotStart ReadyMix (Kapa Biosystems, KK2602)
10 µM IS PCR primer (IDT, 5′-AAGCAGTGGTATCAACGCAGAGT-3′)
Lambda Exonuclease (NEB, M0262L))

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). Measured average fragment size and concentration of diluted cDNA plates using either a Fragment Analyzer or a Quant-iT dsDNA High Sensitivity kit on a SpectraMax i3x microplate reader. For every sample plate, 0.4 µl of cDNA was transfered to a new 384-well plate using a Viaflow 384 Multichannel Pipette (Integra). All plates were sealed with Microseal Seal F foil seal, spun down (1000 rcf, 1 min) and stored in −20 °C until ready for Nextera Library Preparation.

Library Preparation

6 **Nextera Library Preparation**

In brief, tagmentation was carried out on diluted cDNA plates from previous step using the Nextera XT Library Sample Preparation kit (Illumina, FC-131-1096) using the SPT LabTech FireFly or Mosquito LV. Each well was mixed with 0.8 µl Nextera tagmentation DNA buffer (Illumina) and 0.4 µl Tn5 enzyme (Illumina). All plates were then sealed with Microseal Seal B adhesive seal, spun down (1000 rcf, 1 min) then incubated at 55 °C for

10 min. The reaction was stopped by adding 0.4 µl Neutralize Tagment Buffer (Illumina), sealed with Microseal Seal F foil seal, and centrifuging at room temperature at 1000 rcf 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). All plates were then sealed with Microseal Seal B adhesive seal and spun down (1000 rcf, 1 min). PCR amplification was carried out on a ProFlex 2×384 thermal cycler using the following program: 72 °C for 3 min; 95 °C for 30 s; 12 cycles of 95 °C for 10 s, 55 °C for 30 s, 72 °C for 1 min; and 72 °C for 5 min. After Library Preparation, plates were stored in −20 °C until ready for Library Pooling and QC.

Library Pooling and QC

7 After library preparation, wells of each library plate were pooled using either the SPT LabTech FireFly or Mosquito liquid handlers. Pooling was followed by two purifications using 0.7x AMPure beads (Fisher, A63881). Library quality was assessed using the 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.