Sep 17, 2019

SmartSeq2 for HTP Generation of Bulk RNA Libraries- with Pipetting plan V.1

Forked from SmartSeq2 for HTP Generation of Bulk RNA Libraries

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In Development dx.doi.org/10.17504/protocols.io.7eahjae

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ABSTRACT

[insert abstract from MACA_Bulk paper]

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

Ensure access to necessary automated liquid handlers or suitable equivalents to facilitate the high throughput processing of samples that this protocol was adapted for.

RNA Plating and Normalization

1 Extracted bulk RNA samples were plated in 96 well hard-shell PCR plates (Bio-Rad HSP9601) by hand and normalized to 25 ng/µL in RNAse free water and Recombinant RNase Inhibitor. Two µL aliquots of normalized RNA was transferred to a new 96-well plate and mixed with 2 µL of RNA Buffer Master Mix. These sample plates were incubated at 72 ˚C for 3 mins and slowly cooled to facilitate oligo-dT annealing. Plates were stored at -80˚C until processing.

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RNA Buffer Master Mix

2 U/µL Recombinant RNase Inhibitor (Takara Bio, 2313B)
5 mM dNTP mix (Thermo Fisher, R0193)
5 µM Oligo-dT30VN (Integrated DNA Technologies, 5'AAGCAGTGGTATCAACGCAGAGTACT30VN-3')

cDNA Synthesis

2 First Strand Synthesis

cDNA synthesis was performed using the Smart-seq2 protocol[1]. Briefly, 96-well plates containing bulk RNA were thawed on ice followed by first-strand synthesis. 6 µl of RT Master Mix was added to each well and mixed by hand. Reverse transcription was carried out by incubating wells on a Biorad S1000 thermal-cycler (BioRad) 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).

3 2nd Strand Synthesis and cDNA Amplification

Subsequently, 15 µl of PCR mix was added to each well and mixed by hand, and second- strand synthesis was performed on a Biorad S1000 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) 6 cycles :
   98°C for 20s
   67°C for 15s
   72°C for 4min
4)72°C for 5min.

4 cDNA Cleaning, Reformatting, and Quantification

The amplified products were purified once with 0.7x AMPure beads (Fisher, A63881) by hand and reformatted from 96-well plates to new 384-well hard-shell PCR plates (Bio- Rad HSP3901) by hand for quanitification and further processing. 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). Samples were normalized by two rounds of dilutions to 1.5 ng/µl and then 0.5 ng/µl in 384-well Echo 384-well Polypropylene Microtiter plates (Labcyte, PP-0200). For every sample, 0.8 µl of normalized cDNA was transfered to a new 384-well plate using a Viaflow 384 Multichannel Pipette (Integra).

Library Preparation

5 **Homebrew Library Preparation**

Illumina sequencing libraries were prepared using a homebrew library preprartion protocol modified from previously reported tagmentation-based protocols[2,3] and a Mosquito liquid handler (TTP Labtech). 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 at (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 (Thermo Fisher) using the following program:

1) 72 °C for 3min
2) 95 °C for 30 s
3) 12 cycles :
   98°C for 10s
   67°C for 30s
   72°C for 1min
4)72°C for 5min.

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-dimethyformamide (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)

2. 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).

3. 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

6 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). 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). Pooled libraries were normalized to 2 nM and volumes were mixed together based on number of libraries in the pool to make the sequencing sample pool. A PhiX control library was spiked in at 0.2% before sequencing.

7 To ensure relatively even sequencing depth across all libraries, an initial sequencing run was performed on the iSeq 100 Sequencing System (Illumina) using 2 x 100-bp paired end reads and 2 x 12-bp index reads with iSeq 100 i1 300-cycle single kit (Illumina, 20021533). The pooled volume of individual bulk libraries was adjusted according to the sequencing depth observed from this initial run, and libraries were subsequently purified and QC-ed as described in Step 7.

8 Pooled libraries were split and sequenced on the NovaSeq 6000 Sequencing System (Illumina) using 2 × 100-bp paired-end reads and 2 × 12-bp index reads with 2 x S4 300-cycle kit (Illumina, 20012861 or 20012860).

Data Processing

9 Sequences from the NovaSeq were de-multiplexed using bcl2fastq version 2.20. Reads were aligned to the GRCm39.p6 (mus musculus) genome with Gencode v.M19 annotations using using STAR version 2.5.2b with parameters TK. Gene counts were produced using HTSEQ version 0.6.1p1 with default parameters, except “stranded” was set to “false”, and “mode” was set to “intersection-nonempty”.