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SMART-Seq

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This protocol details the cell lysis / Oligot-dT priming, reverse transcription, PCR preamplification and quality Check cDNA and tagmentation reaction.

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SmartSeq

Cell lysis, Oligot-dT priming, Reverse transcription, PCR preamplification, Quality Check cDNA, Tagmentation reaction, ASAPCRN

protocol ,

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Part of collection

SmartSeq

REAGENTS:

- PBS (phosphate buffered saline) with no Ca/Mg **Thermo Fisher Scientific Catalog #Invitrogen 14190-144**

TrypLE™ Express Enzyme (1X), no phenol red **Thermo**

- **Fisher Catalog #12604021**
- RNaseZap **Ambion Catalog #AM9780**

DNA-OFF™ **Takara Bio**

- **Inc. Catalog #9036**

Triton™ X-100 **Sigma**

- **Aldrich Catalog #T9284**

dNTP Mix (dATP, dCTP, dGTP, and dTTP, each at 10mM) **Thermo Fisher**

- **Scientific Catalog #R0192**
- **First-strand buffer (5×; 250 mM Tris-HCl, pH 8.3, at room temperature (25 °C); 375 mM KCl; 15 mM MgCl2; Invitrogen, cat. no. 18064-014)**

Superscript II **Invitrogen - Thermo**

- **Fisher Catalog #18064-014**
- **DTT (Invitrogen, cat. no. 18064-014)**

Recombinant RNAse

- Inhibitor **Takarabio Catalog #2313A**

Betaine BioUltra ≥99.0% (NT) **Sigma**

- **Aldrich Catalog #61962**

1 M Magnesium Chloride (MgCl2) **Sigma**

- **Aldrich Catalog #M8266**

Kapa HiFi Hotstart Readymix **Kapa**

- **Biosystems Catalog #KK2601**

Critical: A HotStart DNA polymerase is necessary to minimize the background amplification when working with single cells and is more practical when working with automated liquid-handling platforms.

Agencourt AMPure XP beads **Beckman**

- **Coulter Catalog #A63881**
- **Ethanol 99.5% (vol/vol); Kemethyl, cat. no. SN366915-06)**

Caution: It is flammable; handle it using appropriate safety equipment.

Elution Buffer

- (EB) **Qiagen Catalog #19086**

TruSeq Dual Index Sequencing Primer Box **Illumina,**

- **Inc. Catalog #FC-121-1003**

Nextera XT DNA Sample Preparation Kit, 96

- samples **illumina Catalog #FC-131-1096**

Nextera XT Index Kit (24 indexes 96 samples) **Illumina,**

- **Inc. Catalog #FC-131-1001**

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Cell Lysis / Oligot-dT Priming 6m 20s

1

Timing: ~15 min (for eight-strip tubes)

Dilute the oligo-dT30VN primer to **10 Micromolar (µM)** by adding **10 µL** of **100 Micromolar (µM)** oligo-dT primers and **90 µL** of nuclease-free water to a tube and mix well.

2

Prepare cell lysis buffer by adding **1 µL** of RNase inhibitor to **19 µL** of a 0.2% (vol/vol) Triton X-100 solution.

If you are working with purified RNA, this step can be omitted and a corresponding volume of water can be used instead.

3

Isolate single cells in the lowest possible volume (preferably ≤ **0.5 µL** , possibly **0.3 µL**) or pipet the appropriate amount of RNA into a **0.2 mL** thin-walled PCR tube. Single cells can be obtained either by using a micro capillary pipette or via FACS.

4

Place each single cell into a **0.2 mL** thin-walled PCR tube containing **2 µL** of cell lysis buffer, **1 µL** of oligo-dT primer and **1 µL** of dNTP mix.

5 10s

Quickly vortex the tube to mix, and then spin down the solution (**700 g** for **00:00:10** at **Room temperature**) and immediately place it **On ice** .

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6 3m

Incubate the samples at 72 °C for 00:03:00 and immediately put the tube back On ice .

7 3m 10s

Spin down the samples (700 g for 00:00:10 at Room temperature) to collect the liquid at the bottom of the tubes, and then put them immediately back On ice .

The oligo-dT primer is now hybridized to the poly(A) tail of all the mRNA molecules.

Purified RNA:

Xul	RNA up to 2.5ul
1ul	oligo-dT Primer (10uM)
1ul	dNTP (10mM)
xul	H2O
4.5ul	Total

72 °C for 00:03:00 , snap cool.

Reverse Transcription

8

Prepare the RT mix for all reactions plus one additional reaction by combining and mixing the reagents listed in the table below.

A	B	C
Component	**Volume (ul)**	**Final Conc**
Superscipt II	0.50	100 U
RNAse inhibitor (40 U/ul)	0.25	10 U
Superscript II FS buffer (5X)	2.00	1X
DTT (100 mM)	0.50	5 mM
Betaine (5 M)	2.00	1 M
MgCl2 (1 M)	0.06	6 mM
TSO (100uM)	0.10	1 uM
H2O	0.29	-
Total	5.70	-

9

Add 5.7 µL of RT mix to Samples for a total of 10 µL .

10

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Spin and incubate as follows:

A	B	C	D
Cycle	**Temperature (°C)**	**Time**	**Purpose**
1	42	90 min	RT and template-switching
2–11	50	2 min	Unfolding of RNA secondary structures
	42	2 min	Completion/continuation of RT and template-switching
12	70	15 min	Enzyme inactivation
13	4	Hold	Safe storage

PCR preamplification

11

Prepare the PCR mix for all reactions plus one additional reaction by combining and mixing the following components:

A	B	C
Component	**Volume (µl)**	**Final concentration**
First-strand reaction	10	–
KAPA HiFi HotStart ReadyMix (2×)	12.50	1×
IS PCR primers (10 µM)	0.25	0.1 µM
Nuclease-free water	2.25	–
Total volume	25	–

12

Add **15 µL** of PCR mix to each tube from Step 12, which contains the first-strand reaction and perform the PCR in a thermal cycler by using the following program:

A	B	C	D	E
Cycle	Denature	Anneal	Extend	Hold
1	98 °C, 3 min	–	–	–
2–19 (see below)	98 °C, 20 s	67 °C, 15 s	72 °C, 6 min	–
20	–	–	72 °C, 5 min	–
21	–	–	–	4 °C

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A	B	C
Input Amount Total RNA	**Input Amount, Cells**	**Typical No. of PCR Cycles**
10 ng	1,000 cells	12
1 ng	100 cells	12
500 pg	50 cells	13
100 pg	10 cells	15
10 pg	1 cell	18

Ampure Cleanup

13 Perform a typical Ampure cleanup using 1:1 ratio of Ampure:cDNA.

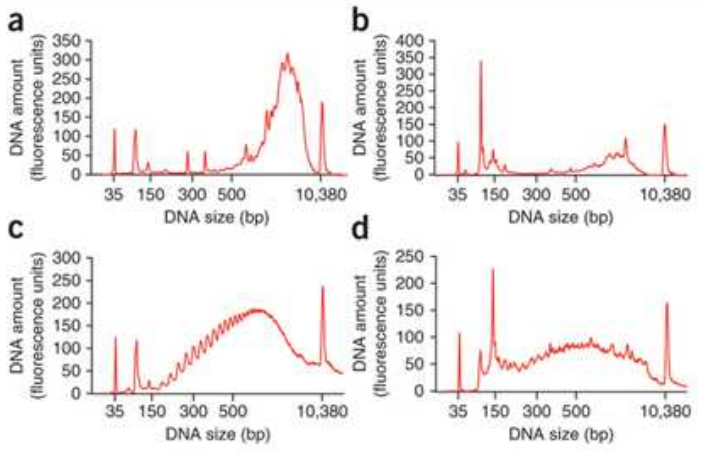
14 

Elute using **17.5 µL** EB solution and pipette **15 µL** to transfer to a new tube.

Quality Check cDNA

15 Run a High Sensitivity Bioanalyzer Chip to check for quality of cDNA.

16 A good library should be free of short (<500 bp) fragments and should show a peak at 1.5–2 kb.



(a) Representative example of the cDNA size distribution obtained from the successful cDNA preamplification from a single MEF cell (mouse). The profile has a peak ~1.5–2 kb with a small number of fragments below 500 bp and a small amount of primer dimers. (b) Representative example of the primer dimer–dominated cDNA profile from a single HEK293T cell (human). Amplification of full-length cDNA is visible but a large peak of primer dimers that were not removed by bead purification dominates the profile. (c) Data from a single T cell (human) with a 'hedgehog' pattern, most probably due to the formation of TSO concatamers. (d) Data from a single interneuron (mouse), with a wide distribution of fragments indicative of RNA degradation before library preparation.

Tagmentation Reaction 10m

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17 Setup the tagmentation RXN as follows:

A	B	C
Component	Volume (µl)	Final concentration
Tagment DNA buffer (TD, 2×)	10	1×
Amplicon tagment mix	5	–
DNA from PCR	Variable	–
Nuclease-free water	Variable	–
Total volume	20	–

18 5m

Incubate in a thermal cycler at **55 °C** for **00:05:00** and bring to **4 °C** HOLD.

19

Add **5 µL** of NT buffer to the **20 µL** RXN and mix.

20 5m

Incubate at **Room temperature** for **00:05:00** .

Enrichment of Tagmented cDNA

21 Prepare the following PCR RXN as follows:

A	B
Component	**Volume (µl)**
DNA	25
Nextera PCR master mix	15
Index 1 primers (N7xx)	5
Index 2 primers (N5xx)	5
Total volume	50

22 Run the PCR RXN on a thermal cycler with the following conditions:

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A	B	C	D	E
Cycle	Denature	Anneal	Extend	Hold
1	–	–	72 °C, 3 min	–
2	95 °C, 30 s	–	–	–
3–14*	95 °C, 10 s	55 °C, 30 s	72 °C, 30 s	–
15	–	–	72 °C, 5 min	–
16	–	–	–	4 °C

*for 1ng, 8-12 cycles could be used

Expected results:

23

Using Single Cell (thus, purified RNA should yield better stats)

Sequence reads from each individual cell are normally in the range of 1–20 million, depending on the level of multiplexing in the sequencing. When sequencing 50-bp single-end reads, we find that normally 60% of reads map uniquely to the genome (20% multimapping and 20% with no match); of the uniquely mapping reads, >60% of the reads map to annotated RefSeq exons, 20% intronic and 20% intergenic, but these values depend on the completeness of the gene annotations. The read coverage across transcripts should be even.

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