



Oct 02, 2024

High-throughput Smart-seq3

 [BMC Genomics](#)

DOI

<https://dx.doi.org/10.17504/protocols.io.ewov196j0lr2/v1>

Kai-Hui Sun¹, Hsiu-Chun Chuang¹

¹Gilead



Kai-Hui Sun

Gilead, Gilead

Create & collaborate more with a free account

Edit and publish protocols, collaborate in communities, share insights through comments, and track progress with run records.

Create free account

OPEN  ACCESS



DOI: <https://dx.doi.org/10.17504/protocols.io.ewov196j0lr2/v1>

Protocol Citation: Kai-Hui Sun, Hsiu-Chun Chuang 2024. High-throughput Smart-seq3. [protocols.io](#)
<https://dx.doi.org/10.17504/protocols.io.ewov196j0lr2/v1>

License: This is an open access protocol distributed under the terms of the [Creative Commons Attribution License](#), which permits unrestricted use, distribution, and reproduction in any medium, provided the original author and source are credited

Protocol status: Working

We use this protocol and it's working



Created: September 25, 2024

Last Modified: October 02, 2024

Protocol Integer ID: 108293

Keywords: single-cell RNA sequencing, single-cell TCR sequencing, smart-seq3, high-throughput smart-seq3, seq3 protocol, seq3, throughput smart, optimized protocol, automated workflow, workflow, ht smart, protocol, throughput, smart

Abstract

We built upon the Smart-seq3 protocol to develop the high-throughput Smart-seq3 (HT Smart-seq3) workflow, an automated workflow with a detailed and optimized protocol.

Materials

- List of oligos: refer to the published Smart-seq3 protocol V3 (<https://www.protocols.io/view/smart-seq3-protocol-bcq4ivyw.pdf>)
- Details of reagents were listed in each key step.

Protocol materials

⊗ Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096**

⊗ Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

⊗ BioAnalyzer High Sensitivity Chip **Agilent Technologies Catalog #5067-4626**

⊗ Ampure XP beads **Beckman Coulter Catalog #A63881**

Single Cell Collection via FACS

- 1 Prepare **Cell Lysis Buffer Master Mix** on the same day as single cell collection via FACS, and keep it  On ice .

A	B	C	D	E
Master mix component	Cat # (Vendor)	Stock conc.	Reaction conc.	Volume (ul) per well
Poly-ethylene glycol 8000	89510-250G-F (Sigma)	50%	5%	0.4
Triton X-100	T8787-50ML (Sigma)	10%	0.1%	0.04
Recombinant RNase inhibitor	2313A (Takara Bio)	40U/ul	0.5U/ul	0.05
Smart-seq3 Oligo-dT-30VN	Integrated Dna Technologies (IDT)	100uM	0.5uM	0.02
dNTPs	R0182 (Thermo Fisher Scientific)	25mM/each	0.5mM/each	0.08
Nuclease-free water	AM9930 (Invitrogen)			3.41
Total				4

- 2 Dispense  4 μ L **Cell Lysis Buffer Master Mix** into each well of the 96-well plates using **Mantis**. Keep the plates sealed and  On ice prior to FACS.
- 3 Before loading the 96-well plate onto FACS, perform a quick spin-down and then sort single cells directly into wells containing **Cell Lysis Buffer**.
- 4 After completion of single cell collection, seal the 96-well plate, perform a quick spin-down, and place it on dry ice before transferring to storage at  -80 °C .

2m

Cell Lysis

10m

5 Remove the 96-well plates from the -80 °C freezer and incubate them in a thermocycler at 72 °C for 00:10:00 , followed by a hold at 4 °C .

10m

Reverse Transcription

2h 30m

6 Prepare **Reverse Transcription (RT) Master Mix**, and dispense 1 µL **RT Master Mix** into each well of the 384-well plates using **Mantis**.

	A	B	C	D	E
	Master mix component	Cat # (Vendor)	Stock conc.	Reaction conc.	Volume (ul) per well
	Tris-HCl, pH 8.3	T1083 (Teknova)	1M	25mM	0.1
	NaCl	AM9759 (Invitrogen)	1M	30mM	0.12
	MgCl2	AM9530G (Invitrogen)	100mM	2.5mM	0.1
	GTP	R1461 (Thermo Fisher)	100mM	1mM	0.04
	Dithiothreitol (DTT)	707265ML (Thermo Fisher Scientific)	100mM	8mM	0.32
	Recombinant RNase inhibitor	2313A (Takara Bio)	40U/ul	0.5U/ul	0.05
	Smart-seq3 TSO	Integrated Dna Technologies (IDT)	100uM	2uM	0.08
	Maxima H-minus RT enzyme	EP0751 (Thermo Fisher)	200U/ul	2U/ul	0.04
	Nuclease-free water	AM9930 (Invitrogen)			0.15
	Total				1

Reverse Transcription

2h 30m

- 7 After completion of cell lysis at step 5, use **Integra VIAFLO** to transfer the lysates from four 96-well plates into one 384-well plate pre-filled with **RT Master Mix** at step 6, and then mix well. 1m
- 8 Seal the plate, perform a quick spin-down, and then incubate it in a thermocycler as follows: 2h 15m

🌡️ 42 °C for ⌚ 01:30:00

10 cycles of:

▪ 🌡️ 50 °C for ⌚ 00:02:00

▪ 🌡️ 42 °C for ⌚ 00:02:00

🌡️ 85 °C for ⌚ 00:05:00

Hold at 🌡️ 4 °C

cDNA Amplification

2h 30m

- 9 Prepare **PCR Master Mix**, and keep it 🌡️ On ice .

	A	B	C	D	E
	Master mix component	Vendor	Stock conc.	Reaction conc.	Volume (ul)
	Kapa HiFi HotStart buffer	KK2502 (Roche)	5X	1X	2
	DNA polymerase	KK2502 (Roche)	1U/ul	0.02U/ul	0.2
	dNTPs	R0182 (Thermo Fisher Scientific)	25mM/each	0.3mM/each	0.12
	MgCl ₂	AM9530G (Invitrogen)	100mM	0.5mM	0.05
	Smart-seq3 forward primer	Integrated Dna Technologies (IDT)	100uM	0.5uM	0.05
	Smart-seq3 reverse primer	Integrated Dna	100uM	0.1uM	0.01

	A	B	C	D	E
		Technologies (IDT)			
	Nuclease-free water	AM9930 (Invitrogen)			3.57
	Total				6

10 After completion of RT at step 8, perform a quick spin-down, and then dispense

2m

 6 µL **PCR Master Mix** into each well of the 384-well plate using **Mantis**.

11 Seal the plate, perform a quick spin-down, and then incubate it in a thermocycler as follows:

2h 15m

 98 °C for  00:03:00

18-25 cycles of:

▪  98 °C for  00:00:20

▪  65 °C for  00:00:30

▪  72 °C for  00:04:00

 72 °C for  00:05:00

Hold at  4 °C

cDNA Purification

30m

12 Program **Integra VIAFLO** to perform cDNA purification as follows:

30m

1. Add  6 µL  Ampure XP beads **Beckman Coulter Catalog #A63881** (0.6X) to each well of the 384-well plate and mix by pipetting up and down.
2. Incubate  00:05:00 at  Room temperature .
3. Place on magnet until the solution clears.
4. Remove the supernatant.
5. Wash twice with  30 µL freshly prepared 80% ethanol.
6. Remove the ethanol, and air dry for  00:02:00 .
7. Add  11 µL nuclease-free water and mix by pipetting up and down.
8. Incubate  00:05:00 at  Room temperature .
9. Place on magnet until the solution clears.
10. Transfer  10 µL purified sample to a new 384-well plate.



11. Store at -20 °C .

cDNA Quantification

10m

13 Use Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** with our modified protocol to perform cDNA quantification as followed:

10m

1. Prepare the **Qubit® Working Solution**: Dilute the Qubit® dsDNA HS Reagent at a ratio of 1:200 in Qubit® dsDNA HS Buffer

2. Prepare Standards: Create a series of standards at concentrations of 0, 1.25, 2.5, and 5 ng/μL by performing serial dilutions of Qubit™ 1X dsDNA HS Standard #2, which has a concentration of 10 ng/μL. This will allow for accurate quantification during the assay.

3. Dispense Working Solution: Using an **Integra VIAFLO**, dispense 34 μL of the prepared **Qubit® working solution** into each well of the black, flat-bottom 384-well plates (Corning, Catalog#3820)

4. Add **Standards**: In a separate plate, add 1 μL of each prepared **standard** to the corresponding wells. This will enable the creation of a standard curve for quantifying the cDNA concentration.

5. Add cDNA Samples: Add 1 μL of cDNA sample to each well of the plate containing the **Qubit® working solution**. Ensure that samples are added to the correct wells to maintain accurate results.

6. Measure Fluorescence: Use a **SpectraMax microplate reader** to measure the fluorescence of the samples. Set the excitation wavelength to 485 nm and the emission wavelength to 525 nm.
Record the fluorescence intensity for each well.

7. Calculate cDNA Concentration: Analyze the fluorescence data to calculate the cDNA concentration in the samples using the standard curve generated from the known standards

cDNA Normalization

10m

14 Calculate and normalize each sample to 100 pg/ul using nuclease-free water as follows:

10m



1. Prepare a new 384-well plate for cDNA normalization by dispensing calculated volumes of nuclease-free water (diluent) into each well using **Mantis**.
2. Transfer  1 μL purified cDNA into this new 384-well plate prepared above.

Library Generation

5m

- 15 Use  Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #FC-131-1096** with our modified protocol to perform library generation.

- 15.1 Prepare **Tagmentation Master Mix**, and dispense  3 μL into a new 384-well plate using **Mantis**.

2m

A	B
Mater mix component (Kit)	Volume (ul)
Tagment DNA buffer (TD)	2
Amplicon tagment mix (ATM)	1
Total	3

- 15.2 Add  1 μL normalized cDNA ( 100 pg/ μL) to **Tagmentation Master Mix**, and mix by pipetting up and down using **Integra VIAFLO**.

1m

- 15.3 Seal the plate, perform a quick spin-down, and then incubate it in a thermocycler as follows:

5m

 55 $^{\circ}\text{C}$ for  00:05:00

Hold at  4 $^{\circ}\text{C}$

- 15.4 Add  1 μL **Neutralize tagment buffer (NT)** using **Mantis**, mix and perform a quick spin-down, followed by incubation for  00:05:00 at  Room temperature .

5m



15.5 Dispense  3 μL **Nextera PCR Master Mix (NPM)** using **Mantis**, followed by adding  2 μL UDIs (1:1 dilution) and mixing using **Integra VIAFLO**.

3m

15.6 Seal the plate, perform a quick spin-down, and then incubate it in a thermocycler as follows:

9m 40s

 72 °C for  00:03:00

 95 °C for  00:00:30

14 cycles of:

▪  95 °C for  00:00:10

▪  55 °C for  00:00:30

▪  72 °C for  00:00:30

 72 °C for  00:05:00

Hold at  4 °C

Pooled Library Purification

15m

16 Pool libraries with different UDIs to perform library purification as follows:

15m

1. Take  2 μL from each library with a different UDI from the plates in step 15, and pool them together for purification.
2. Add 0.6:1  Ampure XP beads **Beckman Coulter Catalog #A63881** to final volume of the pooled libraries, mix by pipetting up and down.
3. Incubate  00:05:00 at  Room temperature .
4. Place on magnet until the solution clears.
5. Remove the supernatant.
6. Wash twice with  1000 μL freshly prepared 80% ethanol.
7. Remove the ethanol, and air dry for  00:05:00 .
8. Add  26 μL nuclease-free water and mix by pipetting up and down.
9. Incubate  00:05:00 at  Room temperature .
10. Place on magnet until the solution clears.
11. Transfer  25 μL purified sample to a new Eppendorf tube.
12. Store at  -20 °C .

Library Quality Control



- 17 Measure the concentration of pooled library using  Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** , and determine the size using  BioAnalyzer High Sensitivity Chip **Agilent Technologies Catalog #5067-4626** .

Sequencing

- 18 Sequence the pooled library on an Illumina sequencer (Illumina, San Diego, CA, USA) using paired-end (PE) 150 bp reads, with a target of 1-2 million reads per sample.

Protocol references

1. Hagemann-Jensen M, Ziegenhain C, Chen P, Ramsköld D, Hendriks GJ, Larsson AJM, Faridani OR, Sandberg R: Single-cell RNA counting at allele and isoform resolution using Smart-seq3. *Nat Biotechnol* 2020, 38(6):708-714.
2. Michael Hagemann-Jensen CZ, Ping Chen, Daniel Ramsköld, Gert-Jan Hendriks, Anton J.M Larsson, Omid R. Faridani, Rickard Sandberg: Smart-seq3 Protocol V.3. In. protocols.io; 2020.