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High-throughput Smart-seq3

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We use this protocol and it's working

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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

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32854	In 2 steps
BioAnalyzer High Sensitivity Chip **Agilent Technologies Catalog #**5067-4626	Step 17
Ampure XP beads **Beckman Coulter Catalog #**A63881	In 2 steps
Nextera XT DNA Library Preparation Kit **Illumina, Inc. Catalog #**FC-131-1096	Step 15

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 µL **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. 2m

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 .

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**

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 Hot Start 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
	MgCl2	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 Technologies (IDT)	100uM	0.1uM	0.01
	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 6 µL **PCR Master Mix** into each well of the 384-well plate using **Mantis**. 2m

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 [M] 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 ([M] 100 pg/ul) 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 °C for 00:05:00

Hold at 4 °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

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