Barcoded Plate-Based Single Cell RNA-seq

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Abstract

This protocol presents a simple modification of the SMART-seq2 (Picelle et al, 2014) and SCRB-seq (Soumillon et al, 2014) protocols, with the goal of combining the sensitivity of SMART-Seq2 with the improved cost and throughput of 3' single cell protocols. Reverse transcription (RT) and PCR proceed as in SMART-Seq2, but using 3' barcoded oligos from SCRB-seq. While most barcoded methods (including SCRB-seq) pool cells together after RT, we perform PCR individually in each well, as this negates the need for an additional exonuclease and purification step, and therefore maintains the same molecular sensitivity as SMART-Seq2. However, we pool amplified cDNA from single wells immediately after PCR, and generate a single tagmentation-based 3' cDNA library. While this protocol therefore has advantages in both sensitivity and throughput, the absence of an exonuclease step prior to PCR disables the use of unique molecular identifiers for quantification.

Citation: Satija Lab Barcoded Plate-Based Single Cell RNA-seq. **protocols.io**
dx.doi.org/10.17504/protocols.io.nkgdctw
Published: 23 Mar 2018

Materials

- Deoxynucleotide (dNTP) Solution Mix N0447S by New England Biolabs
- NxGen RNAse Inhibitor 30281-2 by Lucigen
- Maxima RT 5X Buffer Provided with EP0752 by Thermo Fisher Scientific
- Betaine solution (5M PCR Reagent) B0300 by Sigma – Aldrich
- Magnesium chloride solution for molecular biology (1.00 M) M1028 by Sigma – Aldrich
- Maxima H Minus Reverse Transcriptase (200 U/uL) EP0752 by Thermo Fisher Scientific
- Superscript II Reverse Transcriptase 18064071 by Thermo Fisher Scientific
- KAPA HiFi HotStart ReadyMixPCR Kit KK2602 by Kapa Biosystems
- Agencourt Ampure XP A63AA0 by Beckman Coulter
- Qubit dsDNA HS Assay Kit Q32851 by Thermo Fisher Scientific
- Agilent DNA 12000 Kit 5067-1508 by Agilent Technologies
- Nextera XT DNA Library Preparation Kit FC-131-1096 by illumina

Protocol

Pre-FACS

Step 1.

Prepare the following master mix (Mix 1).

Reagent	uL/ sample
dNTP Mix (10mM)	0.5
NxGen RNAse Inhibitor (40U/uL)	0.1
Maxima RT Buffer	1.0
Molecular Grade Water	1.3
Total per well	2.9

Pre-FACS

Step 2.

Add 2.9uL of Mix1 to each well of 96 well plates.

Pre-FACS

Step 3.

Transfer 0.5uL of 3'_UMI_primers from primer plate (10mM) to each well. Be sure to note which primer goes in each well of the working plate.

Pre-FACS

Step 4.

Seal plates, spin down and freeze at -20°C until ready to sort cells.

FACS

Step 5.

Once cells are sorted into Mix 1 in 96 well plates seal and store at -80°C.

Post-FACS: RT PCR

Step 6.

Preheat thermal cycler to 72°C.

Post-FACS: RT PCR

Step 7.

Spin plate with samples and Mix1 at 2500rmp for 1 minute.

Post-FACS: RT PCR

Step 8.

Incubate plate at 72°C for 3 minutes and cool to 4°C.

Post-FACS: RT PCR

Step 9.

Prepare the following master mix (Mix 2). **This is different is you are using Maxima H Minus Reverse Transcriptase or SuperScript II Reverse Transcriptase. We find that SuperScript II Reverse Transcriptase tends to work best on immune cells.**

Maxima Master Mix

Reagent	uL/ well
Drops TSO Primer (10uM)	0.5
Betaine (5M)	0.925
Magnesium Chloride (100mM)	0.4
NxGen RNAse Inhibitor (40U/uL)	0.125
Maxima H Minus RT	0.05
Total per well	2

*Add enzyme last to master mix

OR

SuperScript II Master Mix

Reagent	uL/ well
Drops TSO Primer (10uM)	0.5
Betaine (5M)	1
Magnesium Chloride (1M)	0.045
NxGen RNAse Inhibitor (40U/uL)	0.25
SuperScript II RT	0.25
Total per well	2.045

*Add enzyme last to master mix

Post-FACS: RT PCR

Step 10.

Add 2uL of Mix 2 to each well. Seal plate and mix on Thermomixer for 1 minute at 2000rpm. Spin down.

Post-FACS: RT PCR

Step 11.

Run Reverse Transcription program on thermal cycler.

Temperature	Time
42°C	90 minutes
50°C	2 minutes
42°C	2 minutes

70°C	15 minutes	** heat inactivation
4°C	forever	

Post-FACS: RT PCR

Step 12.

Make the following master mix (Mix 3).

Reagent	uL/ well
SS2 PCR Primer (10mM)	0.25
KAPA HiFi ReadyMix	6.25
Molecular Grade Water	0.5
Total per well	7.0

Post-FACS: RT PCR

Step 13.

Add 7uL of Mix 3 to each well. Seal, shake on Thermomixer for 1 min at 2000rpm. Spin down.

Post-FACS: RT PCR

Step 14.

Run PCR program on thermal cycler.

Note: prefer to run this program overnight than storing plate after reverse transcription.

Temperature	Time	Cycles
98°C	3 minutes	1 cycle
98°C	15 seconds	18 cycles
67°C	20 seconds	
72°C	6 minutes	
72°C	5 minutes	
4°C	forever	1 cycle

*Number of cycles in second step depends on input sample.

Can store plate at -20°C before clean-up.

cDNA Purification

Step 15.

Using a 12-multichannel pipette pool all columns of one plate into 12 strip tubes. Spin down and pool all of these tubes into a 1.5mL DNA LoBind eppindorf tube. Split this volume into two tubes noting the volume in each 1.5mL tube.

cDNA Purification

Step 16.

Do 0.6X AmPure XP bead clean up on sample making sure to warm beads to room temperature.

cDNA Purification

Step 17.

Add 0.6X beads to each tube mixing gently by pipetting up and down and incubate at room temperature for 5 minutes.

cDNA Purification

Step 18.

Place on magnet and wait for beads to separate.

cDNA Purification

Step 19.

Remove supernatant, being sure to not draw up any beads, and discard.

cDNA Purification

Step 20.

Wash beads 2X with 900uL of freshly made 70% molecular grade ethanol.

cDNA Purification

Step 21.

Allow beads to dry (but not crack) on magnet.

cDNA Purification

Step 22.

Elute with 15uL of molecular grade water.

cDNA Purification

Step 23.

Let sit off magnet for 3 minutes.

cDNA Purification

Step 24.

Put back on magnet and collect sample.

cDNA Quality Checks

Step 25.

Using manufacturer directions, quantify cDNA concentrations with Qubit Flourometer and dsDNA HS

(high sensitivity) Assay Kit.

cDNA Quality Checks

Step 26.

Using manufacturer directions, assess the quality of the cDNA trace with BioAnalyzer and Agilent DNA 12000 Kit.

Nextera Library Preparation

Step 27.

Make a dilution of cDNA with molecular grade water to 0.20 ng/uL based of Qubit values in a final volume of 10uL. May have to make serial dilution to be accurate. If so, rerun first dilution on Qubit to confirm concentration before proceeding.

Nextera Library Preparation

Step 28.

Combine the following tagmentation reaction in PCR tube strips. Mix by pipetting and spin down. Keep tubes on ice.

Reagent	uL/well
Sample (diluted to 0.20ng/uL)	5
Tagment DNA Buffer	10
Amplicon Tagment Mix	5

Nextera Library Preparation

Step 29.

Incubate at 55°C for 5 minutes on thermal cycler. Wait until temperature reaches 55°C before putting tubes in thermal cycler and wait until it cools down to 4°C to take tubes out and place on ice.

Nextera Library Preparation

Step 30.

Add 5uL of Neutralization Buffer to each tube. Mix by pipetting and spin down.

Nextera Library Preparation

Step 31.

Incubate tubes at room temperature for 5 minutes. Place tubes back on ice.

Nextera Library Preparation

Step 32.

Add the following to each cDNA dilution

Reagent	uL/well
NPM PCR Master Mix	15

P5 TSO Hybrid (10uM)	1	*use in all reactions
P7 primer (10uM)	1	*unique for each sample-keep track
Molecular Grade Water	8	

Nextera Library Preparation

Step 33.

Run Nextera PCR Program on thermal cycler

Temperature	Time	Cycles
95°C	30 seconds	1 cycle
95°C	10 seconds	13 cycles
55°C	30 seconds	
72°C	30 seconds	
72°C	5 minutes	
10°C	forever	1 cycle

Nextera Library Purification

Step 34.

Do 0.7X AmPure XP bead clean up followed by a 0.6X on sample making sure to warm beads to room temperature.

Nextera Library Purification

Step 35.

Add 0.7X beads to each tube mixing gently by pipetting up and down and incubate at room temperature for 5 minutes.

Nextera Library Purification

Step 36.

Place on magnet and wait for beads to separate.

Nextera Library Purification

Step 37.

Remove supernatant, being sure to not draw up any beads, and discard.

Nextera Library Purification

Step 38.

Elute beads with 50uL of molecular grade water.

Nextera Library Purification

Step 39.

Add 30mL of beads to eluted beads (0.6X of 50uL).

Nextera Library Purification

Step 40.

Put on magnet and remove supernatant.

Nextera Library Purification

Step 41.

Wash beads 2X with 400uL of freshly made 70% molecular grade ethanol.

Nextera Library Purification

Step 42.

Allow beads to dry (but not crack) on magnet.

Nextera Library Purification

Step 43.

Elute with 17uL of molecular grade water.

Nextera Library Purification

Step 44.

Let sit off magnet for 3 minutes.

Nextera Library Purification

Step 45.

Put back on magnet and collect sample.

Nextera Library Quality Checks

Step 46.

Using manufacturer directions, quantify Nextera library concentrations with Qubit Flourometer and dsDNA HS (high sensitivity) Assay Kit.

Nextera Library Quality Checks

Step 47.

Using manufacturer directions, assess the quality of the Nextera library trace with BioAnalyzer and Agilent DNA 12000 Kit.

Nextera Library Quality Checks

Step 48.

If the quality of the libraries is satisfactory, pool together and proceed with sequencing.