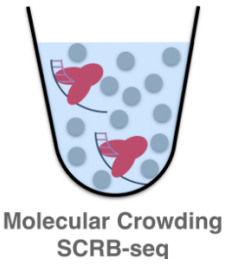
May 22, 2018 Version 2

mcSCRB-seq protocol V.2



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Human Cell Atlas Metho...

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OPEN ACCESS

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Protocol status: Working
We use this protocol and it's working

Created: May 21, 2018

Last Modified: July 26, 2018

Protocol Integer ID: 12300

Keywords: molecular crowding, scRNA-seq, SCRB-seq

Abstract

Single-cell RNA sequencing (scRNA-seq) has emerged as a central genome-wide method to characterize cellular identities and processes. Consequently, improving its sensitivity, flexibility and cost-efficiency can advance many research questions. Among the flexible plate-based methods, “Single-Cell RNA-Barcoding and Sequencing” (SCRB-seq) is one of the most sensitive and efficient ones. Here, we systematically evaluated experimental conditions of this protocol and find that adding polyethylene glycol considerably increases sensitivity by enhancing cDNA synthesis. Furthermore, using Terra polymerase increases efficiency due to a more even cDNA amplification that requires less sequencing of libraries. We combined these and other improvements to a new scRNA-seq library protocol we call “molecular crowding SCRB-seq” (mcSCRB-seq), which we show to be the most sensitive and one of the most efficient and flexible scRNA-seq methods to date.

Attachments

TXT

mcSCRBseq_oligodT.tx..

44KB

Guidelines

- For troubleshooting help, feel free to join our **mcSCRB-seq Slack channel**, leave your question in the comments section, or message us directly.
- The complete list of reagents and plastic ware with order numbers can be found in the 'Materials' section.
- Make sure all steps involving single-cell lysate and RNA before reverse transcription are carried out swiftly.
- Size selection of libraries is optional, but has in our experience improved reliability in cluster densities when sequencing.
- All primer sequences are listed below:

	Oligo	Vendor	Purification	Concentration	Sequence
	barcoded oligo-dT (E3V6NEXT)	IDT	TruGrade	2 µM	Biotin-ACACTCTTTCCCTACACGACGCTCTTCCGATCT[BC6][UMI10][T30]VN
	TSO unblocked (E5V6NEXT)	IDT	HPLC	100 µM	ACACTCTTTCCCTACACGACGCrGrGrG
	PreAmp (SINGV6)	IDT	Desalted	10 µM	Biotin-ACACTCTTTCCCTACACGACGC
	3' enrichment primer (P5NEXTPT5)	IDT	HPLC	5 µM	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG*A*T*C*T
	i7 Index Primer (N7XX)	IDT	TruGrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG

Find the cell barcode sequences in the attached text file (Abstract tab).

Materials

MATERIALS

- EDTA 0.5M **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**E7889
- Ethanol, absolute **Carl Roth** **Catalog #**9065.4
- Exonuclease I (20 U/µl) **Thermo Fisher Scientific** **Catalog #**EN0582
- Exonuclease I Reaction Buffer (10x) **Thermo Fisher Scientific** **Catalog #**EN0582
- IGEPAL CA-630 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I8896
- Maxima H- Reverse Transcriptase (200 U/µl) **Thermo Fisher Scientific** **Catalog #**EP0753
- Maxima RT Buffer (5x) **Thermo Fisher Scientific** **Catalog #**EP0753
- Polyethylene glycol 8000 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**89510
- PBS 7.4 **Gibco - Thermo Fisher Scientific** **Catalog #**10010-23
- Phusion HF Buffer **New England Biolabs** **Catalog #**B0518
- Proteinase K **Takara Bio Inc.** **Catalog #**9034
- Sera-Mag Speed Beads **Thermo Fisher Scientific** **Catalog #**65152105050250
- Terra PCR Direct Polymerase Mix **Takara Bio Inc.** **Catalog #**639271
- UltraPure DNase/RNase-Free Distilled Water **Invitrogen - Thermo Fisher** **Catalog #**10977-049
- Sodium Azide 99.5% **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-100G
- Sodium Chloride 5M **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S5150-1L
- Trizma hydrochloride solution 1M pH 8.0 **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2694
- Bioanalyzer High Sensitivity DNA Analysis Kits **Agilent Technologies** **Catalog #**5067-4626
- MinElute Gel Extraction Kit **Qiagen** **Catalog #**28606
- Nextera XT DNA Library Preparation Kit **Illumina, Inc.** **Catalog #**FC-131-1096
- Quant-iT PicoGreen dsDNA Assay Kit **Invitrogen - Thermo Fisher** **Catalog #**P7589
- dNTPs (25 mM each) **Thermo Fisher Scientific** **Catalog #**R0182
- Aluminium seals for cold storage **Catalog #**391-1275
- Adhesive PCR film seals **Thermo Fisher Scientific** **Catalog #**AB0558
- twin.tec 96-well DNA LoBind Plates **Eppendorf** **Catalog #**0030129504
- twin.tec 384-well DNA LoBind Plates **Eppendorf** **Catalog #**0030129547
- 0.5 ml PCRclean tube DNA LoBind **Eppendorf** **Catalog #**0030108035
- 1.5 ml PCRclean tube DNA LoBind **Eppendorf** **Catalog #**0030108051
- 5.0 ml PCRclean tube DNA LoBind **Eppendorf** **Catalog #**0030108310

15 ml PCRclean tube DNA LoBind **Eppendorf Catalog #**0030122208

E-Gel EX Agarose Gels, 2% **Invitrogen - Thermo Fisher Catalog #**G402002

Before start

Wipe bench surfaces with RNAse Away and keep working environment clean.

Preparation of lysis plates

1 Prepare **Lysis Buffer** according to the number of plates to be filled.

	Reagent	96-well plate	384-well plate
	NEB HF Phusion buffer (5x)	1.1 µL	4.4 µL
	Proteinase K (20 mg/mL)	27.5 µL	110 µL
	UltraPure Water	411.4 µL	1645.6 µL
	Total	**440 µL**	**1760 µL**

2 Prepare 96/384 well plate(s) containing 4 µL **Lysis Buffer** per well.

Add 1 µL barcoded oligo-dT primer [2 µM] (E3V6NEXT adapter) to each well (12-/64-channel pipette).

Note

Lysis plates with barcode primers can be prepared ahead of time and stored at -20 °C

4 µL Lysis Buffer

1 µL barcoded oligo-dT primer [2 µM]

Sample Collection

3 Sort 1 cell to each well of a 96/384 well plate containing 5 µL **Lysis Buffer** and **barcoded oligo-dT primer.**

4 Immediately seal the plate with an aluminium cold storage seal.

5 In a cooled centrifuge, spin down the plate for 30 sec @ max. speed and place immediately on dry ice.

-80 °C Store plates containing single-cell lysates in a -80 °C freezer for up to 6 months.

Proteinase K Digest

6 Thaw plates briefly (up to 1 min) at room temperature

7 Spin down (30 sec @ 1000 rcf) in a centrifuge pre-cooled to 4 °C.

4 °C

8 Replace aluminum foil seal with PCR plate seal to avoid excessive stickiness of the glue.

9 In a thermocycler with heated lid, incubate as follows:

50 °C 10 min (Proteinase K digest)

80 °C 10 min (Heat inactivation)

8 °C ∞ (Store)

Note

During incubation, proceed with preparation of **Reverse Transcription Mix**.

Reverse Transcription

10 Prepare **Reverse Transcription Mix** as follows:

Reagent	96-well plate	384-well plate
UltraPure Water	88 µL	352 µL
PEG 8000 (50 % solution)	165 µL	660 µL
Maxima RT Buffer (5x)	220 µL	880 µL
dNTPs (25 mM each)	44 µL	176 µL
TSO E5V6NEXT unblocked (100 µM)	22 µL	88 µL
Maxima H Minus RT (200 U/µl)	11 µL	44 µL

Total	**550 µL**	**2200 µL**

Note

Caution: Reverse Transcription Mix with PEG needs to be mixed carefully!

4 °C Keep Reverse Transcription Mix on ice

11 Add 5 µL Reverse Transcription Mix to each well.

5 µL Reverse Transcription Mix

Note

If a robot (eg. Formulatrix Mantis) is used, make sure to calibrate correctly to the viscous solution.

12 Seal plate with a PCR seal, vortex briefly and spin down (30 sec @ 1000 rcf) in a centrifuge pre-cooled to 4 °C.

13 In a thermocycler with heated lid, incubate:

42 °C 90 min

8 °C ∞

cDNA Pooling & Purification

14 Prepare **Pooling Beads:**

Reagent	Amount
PEG 8000	15 g
NaCl, 5M	20 mL
Tris-HCL, 1M, pH 8.0	500 µL
EDTA, 0.5M	100 µL
IGEPAL, 10% solution	50 µL

	Sodium Azide, 10% solution	250 µL
	UltraPure Water	up to 50 mL
	Total	**50 mL**

Add all ingredients into a 50 mL falcon tube, but do not add the total amount of water until after PEG is completely solubilized
Incubate at 40°C and vortex regularly until PEG is completely dissolved
Resuspend bead stock carefully (Sera-Mag Speed Beads)
Pipette 100 µL of bead suspension into a 96-well plate well
Place on magnet stand
Remove supernatant
Add 100 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads
Place on magnet stand
Remove supernatant
Repeat wash one more time
Add 90 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads
Add to PEG solution above and mix well.

Note

Beads can be prepared ahead of time and stored at 4 °C or room temperature.

Note

Caution: Beads tend to pellet and need to be carefully resuspended before use.

15 **For 96-well plates**: Pool all wells of one plate into a 2 mL tube and add 960 µL (ratio 1/1) **30% PEG Pooling Beads**
For 384-well plates: Pool all wells of one plate into a 15 mL falcon tube and add 3840 µL (ratio 1/1) **30% PEG Pooling Beads**

960 µL Pooling Beads (96-well plates)

3840 µL Pooling Beads (384-well plates)

16

00:05:00 binding of the cDNA onto the beads

20 °C Room temperature

17 Place on magnet stand until clear
For 384-well plates: Remove supernatant, leaving about 1 mL in the tube. Resuspend the beads in the leftover supernatant and transfer to a 1.5 mL tube for easier handling.

18 Discard supernatant

19 Wash twice with 1 mL 80% ethanol (while on magnet) and discard supernatant
1 mL 80% ethanol (freshly prepared)

20 00:05:00 air dry beads

21 Elute cDNA in 17 µL UltraPure Water & transfer to new tube
17 µL UltraPure Water

Exonuclease I Treatment

22 To the 17 µl cDNA, add:

2 µL Exonuclease I Buffer (10x)
1 µL Exonuclease I (20 U/µl)

23 In a thermocycler with heated lid, incubate:
37 °C 20 min (ExoI digest)
80 °C 10 min (Heat inactivation)
8 °C ∞ (Store)

Full length cDNA amplification

24 Prepare **PreAmplification Mix** as follows:

	Reagent	1x
	Terra direct Buffer (2x)	25 µL
	SINGV6 Primer (10 µM)	1 µL
	Terra polymerase (1.25 U/µL)	1 µL
	UltraPure Water	3 µL
	Total	**30 µL**

25 Add 30 µL of **PreAmplification Mix** directly to the Exonuclease I digested sample.

30 µL PreAmplification Mix

26 In a thermocycler with heated lead, incubate as follows:

Step	Temperature	Time	Cycles
Initial Denaturation	98 °C	3 min	1x
Denaturation	98 °C	15 sec	13-21x
Annealing	65 °C	30 sec	
Elongation	68 °C	4 min	
Final Elongation	72 °C	10 min	1x
Store	8 °C	∞	

Note

Cylce number highly depends on the input amount and should be optimized depending on the specific celltype used in the experiment. For ES cells, 13-15 cycles are sufficient.

cDNA purification & quantification

27 Prepare **Clean-up Beads:**

Reagent	Amount
PEG 8000	11 g
NaCl, 5M	10 mL
Tris-HCL, 1M, pH 8.0	500 µL
EDTA, 0.5M	100 µL
IGEPAL, 10% solution	50 µL
Sodium Azide, 10% solution	250 µL
UltraPure Water	up to 49 mL
Total	**49 mL**

Add all ingredients into a 50 mL falcon tube, but do not add the total amount of water until after PEG is completely solubilized
Incubate at 40°C and vortex regularly until PEG is completely dissolved
Resuspend bead stock carefully (Sera-Mag Speed Beads)
Pipette 1000 µL of bead suspension into a 1.5 mL tube
Place on magnet stand
Remove supernatant
Add 1000 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads
Place on magnet stand
Remove supernatant
Repeat wash one more time
Add 900 µL 10 mM Tris-HCl, pH 8.0, 1 mM EDTA (TE), and resuspend beads
Add to PEG solution above and mix well.

Note

Beads can be prepared ahead of time and stored at 4 °C or room temperature.

28 Mix PreAmplification PCR with 40 µL **Clean-up Beads** (1/0.8 ratio)

40 µL Clean-up Beads

29 00:05:00 binding of the cDNA onto the beads

30 Place on magnet until clear and discard supernatant

31 Wash twice with 150 µL 80% ethanol (while on magnet) and discard supernatant
150 µL 80% ethanol (freshly prepared)

32 00:05:00 air dry beads

33 Elute cDNA in 15 µL UltraPure Water & transfer to new tube
15 µL UltraPure Water

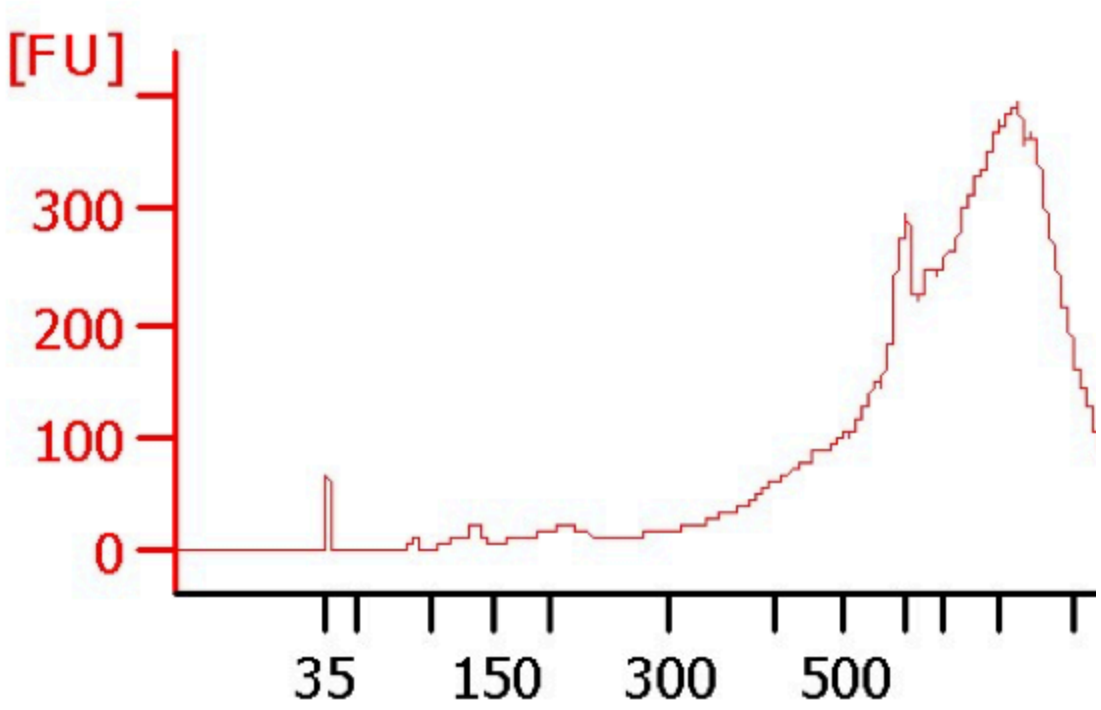
34 Quantify the cDNA using the Quant-iT PicoGreen dsDNA assay kit or equivalent Qubit following the manufacturer's protocol. Use 1 µl of clean cDNA for quantification.

Expected result

cDNA concentration should be > 1 ng/µl, depending on cell type and cycle number

35 Optional: Quality check the cDNA using the Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits.

Expected result



Tagmentation, Library PCR & Indexing

36 Prepare **Tagmentation Mix** and dispense 19 µL to a new 96-well plate.

	Reagent	1x
	Tagment DNA Buffer (2x)	10 µL
	Amplicon Tagment Mix (Tn5)	5 µL
	UltraPure Water	4 µL
	Total	**19 µL**

19 µL Tagmentation Mix

37 Dilute cDNA to 0.8 ng/µL and add 1 µL to each reaction.

1 µL cDNA (0.8 ng/µL)

38 In a thermocycler with heated lid, incubate as follows:

55 °C Tagmentation

00:10:00 Tagmentation

39 To stop the reaction, add 5 µL NT buffer to each reaction and mix by pipetting up and down.

5 µL NT Buffer

00:05:00 Incubation at room temperature

40 Prepare **3' Enrichment PCR Mix** as follows and add 24.5 µL to each tagmentation reaction.

	Reagent	1x
	NPM PCR Mix	15 µL
	P5NEXTPT5 (5 µM)	0.5 µL
	UltraPure Water	9 µL
	Total	**24.5 µL**

24.5 µL 3' Enrichment PCR Mix

41 Add 0.5 µL of i7 index primer (5 µM)

0.5 µL i7 index primer (5 µM)

42 In a thermocycler with heated lead, incubate as follows:

Step	Temperature	Time	Cycles
Gap-fill	72 °C	3 min	1x
Initial Denaturation	95 °C	30 sec	
Denaturation	95 °C	10 sec	13x
Annealing	55 °C	30 sec	
Elongation	72 °C	1 min	
Final Elongation	72 °C	5 min	1x
Store	8 °C	∞	

43 Mix Index PCR with 50 µL **Clean-up Beads** (1/1 ratio)

50 µL Clean-up Beads

44 00:05:00 binding of DNA onto the beads

45 Place on magnet until clear and discard supernatant

46 Wash twice with 150 µL 80% ethanol (while on magnet) and discard supernatant

150 µL 80% ethanol (freshly prepared)

47 00:05:00 air dry beads

48 Elute cDNA in 20 µL UltraPure Water & transfer to new tube

20 µL UltraPure Water

Size selection

49 Load complete library onto an 2% Agarose E-Gel EX and run for 10 minutes.

00:10:00

50 As soon as the Gel run has finished open the Gel framing using the Gel opening tool

51 Excise the Library from 300bp to 900bp using a clean scalpel

52 Gel purify the slice using the Qiagen MinElute Kit following manufacturer's guidelines:

- Add 450 µL Buffer QG
- Dissolve the gel slice in QG for 10 min @ 42 °C
- Add 150 µL Isopropanol to the sample and mix by inverting
- Transfer sample to spin column and centrifuge at 16 000 x g for 1 min
- Discard flow through and add 500 µL Buffer QG
- Centrifuge at 16 000 x g for 1 min and discard flow through
- Add 700 µL Buffer PE
- Centrifuge at 16 000 x g for 1 min and discard flow through
- Centrifuge again at 16 000 x g for 1 min to remove residual ethanol
- Transfer column to a new 1.5 mL microcentrifuge tube
- Add 20 µL H2O to column and incubate for 1 min
- Centrifuge at 16 000 x g for 1 min to elute and discard the spin column

Library Quantification

53 Quantify and quality control the library using the Agilent 2100 Bioanalyzer with High Sensitivity DNA Analysis Kits.

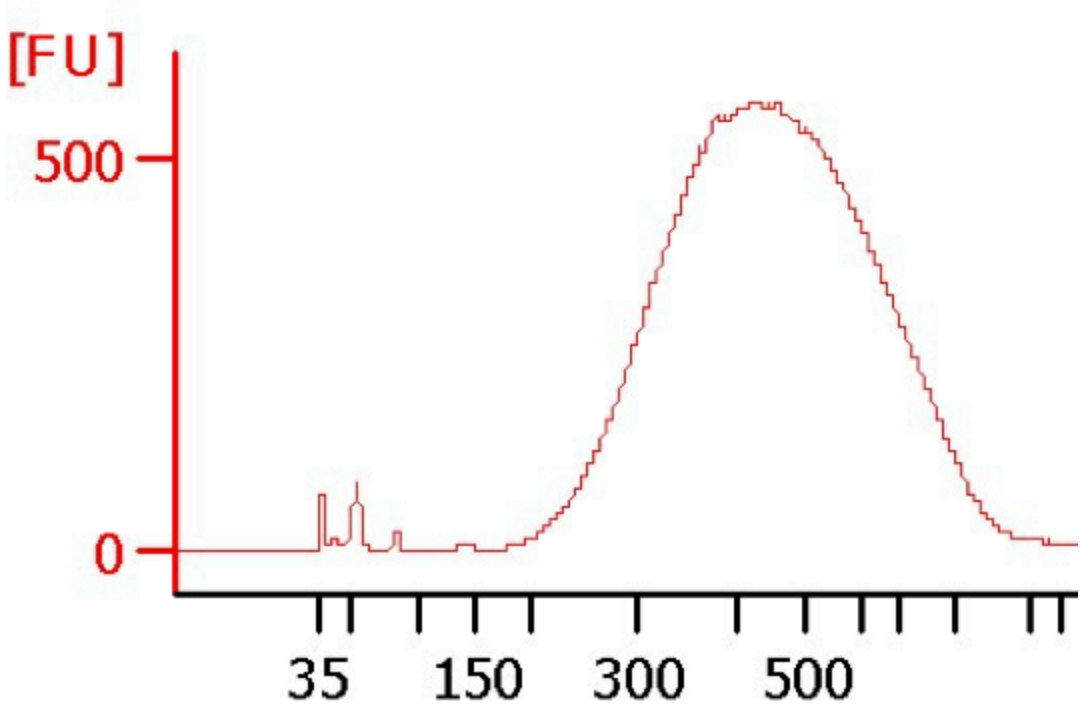
Load both the library and a 1/10 dilution on two different lanes of the chip.

Note

If cDNA was not quality controlled previously, it is strongly suggested to run cDNA on the same chip.

Expected result

Successful libraries will typically exceed 3-5 ng/µl concentration.



54 If pooling several libraries, combine equal molar amounts.

Sequencing

55 Sequence your library on any compatible Illumina sequencer.
Dilute libraries to recommended molarity according to Illumina's recommendations (eg. 2 nM).
Select the following paired-end read-length settings:

	Read	Cycles	Content
	Read 1	16	Cell barcode & UMI
	Index 1	8	i7 Index
	Index 2	0	
	Read 2	50	cDNA fragment

Primary data processing using zUMIs

56 Download and install zUMIs including all dependencies.

Software

NAME	OS	SOURCE LINK
zUMIs	Linux	https://github.com/sdparekh/zUMIs

57 Copy the sequencing data from the sequencer and run bcl2fastq without demultiplexing.

Command

```
bcl2fastq --use-bases-mask Y16,I8,Y50 --create-fastq-for-index-reads
```

58 Run zUMIs with the following parameters. Replace Read names and paths to reference genome and annotation with actual files of your instance.

Command

```
zUMIs-master.sh -f lane1.R1.fastq.gz -c 1-6 -m 7-16 -T
lane1.I1.fastq.gz -U 1-8 -r lane1.R2.fastq.gz -l 50 -n mcSCRBseqrun -
p 16 -a /path_to/gene_annotation.gtf -g
/path_to/reference_genome_index
```