mcSCRB-seq protocol

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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.

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Guidelines

- 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).

Before start

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

Materials

EDTA 0.5M E7889 by Sigma Aldrich

Ethanol, absolute 9065.4 by Carl Roth

Exonuclease I (20 U/µl) EN0582 by Thermo Fisher Scientific

Exonuclease I Reaction Buffer (10x) EN0582 by Thermo Fisher Scientific

IGEPAL CA-630 I8896 by Sigma Aldrich

Maxima H- Reverse Transcriptase (200 U/µl) EP0753 by Thermo Fisher Scientific

Maxima RT Buffer (5x) EP0753 by Thermo Fisher Scientific

Polyethylene glycol 8000 89510 by Sigma Aldrich

PBS 7.4 10010-23 by Gibco - Thermo Fischer

Phusion HF Buffer B0518 by New England Biolabs

Proteinase K 9034 by Takara

Sera-Mag Speed Beads 65152105050250 by Thermo Fisher Scientific

Terra PCR Direct Polymerase Mix 639271 by Takara

UltraPure DNase/RNase-Free Distilled Water 10977-049 by Invitrogen - Thermo Fisher

Sodium Azide 99.5% S2002-100G by Sigma Aldrich

Sodium Chloride 5M S5150-1L by Sigma Aldrich

Trizma hydrochloride solution 1M pH 8.0 T2694 by Sigma Aldrich

Bioanalyzer High Sensitivity DNA Analysis Kits 5067-4626 by Agilent Technologies

MinElute Gel Extraction Kit 28606 by Qiagen

Nextera XT DNA Library Preparation Kit FC-131-1096 by illumina

Quant-iT PicoGreen dsDNA Assay Kit P7589 by Invitrogen - Thermo Fisher

dNTPs (25 mM each) R0182 by Thermo Fisher Scientific

Aluminium seals for cold storage 391-1275 by Contributed by users

Adhesive PCR film seals AB0558 by Thermo Fisher Scientific

twin.tec 96-well DNA LoBind Plates 0030129504 by Eppendorf

twin.tec 384-well DNA LoBind Plates 0030129547 by Eppendorf

0.5 ml PCRclean tube DNA LoBind 0030108035 by Eppendorf

1.5 ml PCRclean tube DNA LoBind 0030108051 by Eppendorf

5.0 ml PCRclean tube DNA LoBind 0030108310 by Eppendorf

15 ml PCRclean tube DNA LoBind 0030122208 by Eppendorf

E-Gel EX Agarose Gels, 2% G402002 by Invitrogen - Thermo Fisher

Protocol

Preparation of lysis plates

Step 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**

Preparation of lysis plates

Step 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).

AMOUNT

4 µl Additional info: Lysis Buffer

AMOUNT

1 µl Additional info: barcoded oligo-dT primer [2 µM]

NOTES

Christoph Ziegenhain 09 Mar 2018

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

ANNOTATIONS

Brian Muegge 18 Apr 2018

Thanks for sharing this outstanding resource. I was wondering if you tested the importance of the biotin modification of the oligo-dT primer? Just as you found that unblocked TSO's weren't superior to blocked TSO, I'm wondering if the biotin modification is really necessary to prevent concatemeters. The major upfront cost is the barcoded primer, so anything that reduces the costs of these primers would make the protocol even more cost effective.

Sample Collection

Step 3.

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

Sample Collection

Step 4.

Immediately seal the plate with an aluminium cold storage seal.

Sample Collection

Step 5.

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

TEMPERATURE

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

Proteinase K Digest

Step 6.

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

Proteinase K Digest

Step 7.

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

TEMPERATURE

4 °C Additional info:

Proteinase K Digest

Step 8.

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

Proteinase K Digest

Step 9.

In a thermocycler with heated lid, incubate as follows:

TEMPERATURE

50 °C Additional info: 10 min (Proteinase K digest)

TEMPERATURE

80 °C Additional info: 10 min (Heat inactivation)

TEMPERATURE

8 °C Additional info: ∞ (Store)

NOTES

Christoph Ziegenhain 09 Mar 2018

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

Reverse Transcription

Step 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**

TEMPERATURE

4 °C Additional info: Keep Reverse Transcription Mix on ice

NOTES

Christoph Ziegenhain 09 Mar 2018

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

Reverse Transcription

Step 11.

Add 5 µL Reverse Transcription Mix to each well.

AMOUNT

5 µl Additional info: Reverse Transcription Mix

NOTES

Christoph Ziegenhain 09 Mar 2018

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

solution.

Reverse Transcription

Step 12.

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

Reverse Transcription

Step 13.

In a thermocycler with heated lid, incubate:

🌡 TEMPERATURE

42 °C Additional info: 90 min

🌡 TEMPERATURE

8 °C Additional info: ∞

cDNA Pooling & Purification

Step 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.

NOTES
Christoph Ziegenhain 09 Mar 2018

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

Christoph Ziegenhain 09 Mar 2018

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

ANNOTATIONS
Alexander Chamessian 05 Apr 2018

For 384, is this literally aspirating well by well, one at a time? Or do you all have a way to automate? I can envision aspirating into a trough, but there would likely be loss.

cDNA Pooling & Purification
Step 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**

AMOUNT
960 µl Additional info: Pooling Beads (96-well plates)
AMOUNT
3840 µl Additional info: Pooling Beads (384-well plates)

cDNA Pooling & Purification
Step 16.

TEMPERATURE
20 °C Additional info: Room temperature

cDNA Pooling & Purification
Step 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.

cDNA Pooling & Purification
Step 18.
Discard supernatant

cDNA Pooling & Purification

Step 19.

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

AMOUNT
1 ml Additional info: 80% ethanol (freshly prepared)

cDNA Pooling & Purification

Step 20.

cDNA Pooling & Purification

Step 21.

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

AMOUNT
17 µl Additional info: UltraPure Water

Exonuclease I Treatment

Step 22.

To the 17 µl cDNA, add:

AMOUNT
2 µl Additional info: Exonuclease I Buffer (10x)
AMOUNT
1 µl Additional info: Exonuclease I (20 U/µl)

Exonuclease I Treatment

Step 23.

In a thermocycler with heated lid, incubate:

TEMPERATURE
80 °C Additional info: 10 min (Heat inactivation)
TEMPERATURE
37 °C Additional info: 20 min (ExoI digest)
TEMPERATURE
8 °C Additional info: ∞ (Store)

Full length cDNA amplification

Step 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**

Full length cDNA amplification

Step 25.

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

AMOUNT

30 µl Additional info: PreAmplification Mix

Full length cDNA amplification

Step 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	∞	

NOTES

Christoph Ziegenhain 09 Mar 2018

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

Step 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.

NOTES

Christoph Ziegenhain 09 Mar 2018

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

cDNA purification & quantification

Step 28.

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

AMOUNT

40 µl Additional info: Clean-up Beads

cDNA purification & quantification

Step 29.

cDNA purification & quantification

Step 30.

Place on magnet until clear and discard supernatant

cDNA purification & quantification

Step 31.

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

AMOUNT

150 µl Additional info: 80% ethanol (freshly prepared)

cDNA purification & quantification

Step 32.

cDNA purification & quantification

Step 33.

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

AMOUNT

15 µl Additional info: UltraPure Water

cDNA purification & quantification

Step 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 RESULTS

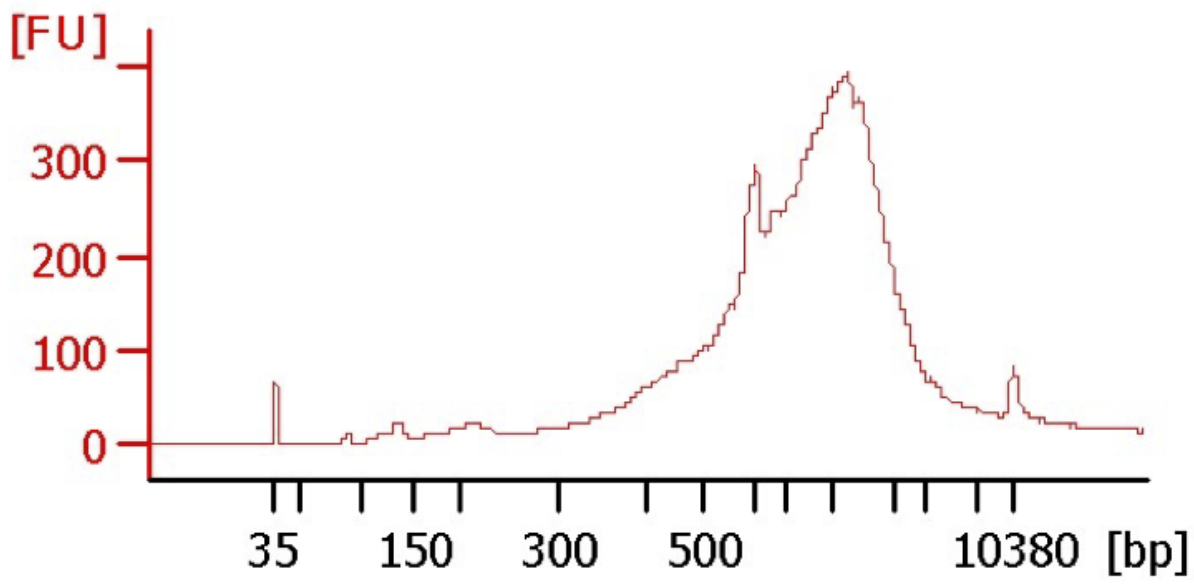
- cDNA concentration should be > 1 ng/µl, depending on cell type and cycle number

cDNA purification & quantification

Step 35.

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

EXPECTED RESULTS



Tagmentation, Library PCR & Indexing

Step 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**

AMOUNT

19 µl Additional info: Tagmentation Mix

Tagmentation, Library PCR & Indexing

Step 37.

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

AMOUNT

1 µl Additional info: cDNA (0.8 ng/µL)

Tagmentation, Library PCR & Indexing

Step 38.

In a thermocycler with heated lid, incubate as follows:

TEMPERATURE

55 °C Additional info: Tagmentation

Tagmentation, Library PCR & Indexing

Step 39.

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

AMOUNT

5 µl Additional info: NT Buffer

Tagmentation, Library PCR & Indexing

Step 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**

AMOUNT

24.5 µl Additional info: 3' Enrichment PCR Mix

Tagmentation, Library PCR & Indexing

Step 41.

Add 0.5 µL of i7 index primer (5 µM)

AMOUNT

0.5 µl Additional info: i7 index primer (5 µM)

Tagmentation, Library PCR & Indexing

Step 42.

In a thermocycler with heated lead, incubate as follows:

Step	**Temperature**	**Time**	**Cycles**

Gap-fill	72 °C	3 min	
Initial Denaturation	95 °C	30 sec	1x
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	∞	

Tagmentation, Library PCR & Indexing

Step 43.

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

AMOUNT

50 µl Additional info: Clean-up Beads

Tagmentation, Library PCR & Indexing

Step 44.

Tagmentation, Library PCR & Indexing

Step 45.

Place on magnet until clear and discard supernatant

Tagmentation, Library PCR & Indexing

Step 46.

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

AMOUNT

150 µl Additional info: 80% ethanol (freshly prepared)

Tagmentation, Library PCR & Indexing

Step 47.

Tagmentation, Library PCR & Indexing

Step 48.

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

AMOUNT

20 µl Additional info: UltraPure Water

Size selection

Step 49.

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

Size selection

Step 50.

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

Size selection

Step 51.

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

Size selection

Step 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

ANNOTATIONS

Alexander Chamessian 06 Apr 2018

Any reason why you used gel purification for size selection vs. double-sided bead clean up? Would the latter not work?

Library Quantification

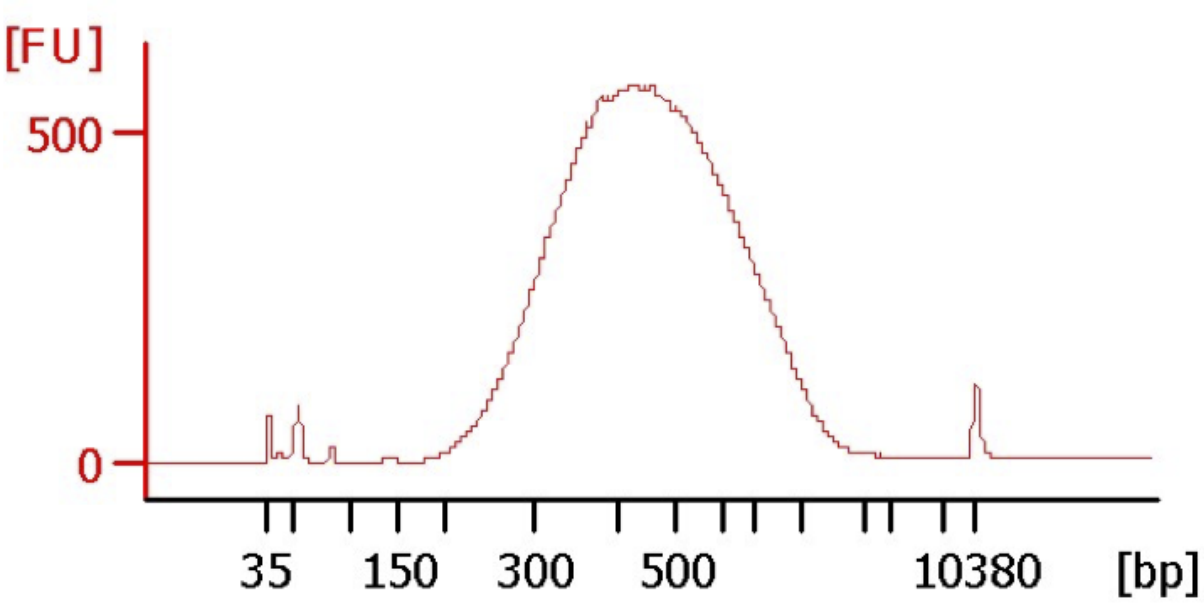
Step 53.

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.

EXPECTED RESULTS

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



NOTES

Christoph Ziegenhain 09 Mar 2018

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

Library Quantification

Step 54.

If pooling several libraries, combine equal molar amounts.

Sequencing

Step 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

Step 56.

Download and install zUMIs including all dependencies.

SOFTWARE PACKAGE (Linux)
zUMIs
https://github.com/sdparekh/zUMIs

Primary data processing using zUMIs

Step 57.

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

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

Primary data processing using zUMIs

Step 58.

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

cmd 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
```