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Working

gmcSCRB-seq protocol

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ABSTRACT

gmcSCRB-seq is an alternative lysis protocol to mcSCRB-seq (Publication and Protocol). gmcSCRB-seq uses Guanidine Hydrochloride in the lysis buffer and requires an additional cean up step. We recommend using this alternative lysis protocol in cases where cells are difficult to lyse or where the DNA appears degraded following the pre-amplification step. However, we have observed reduced sensitivity with gmcSCRB-seq compared to mcSCRB-seq and would therefore only recommend using it in cases where the latter does not produce high quality libraries.

mcSCRBseq_oligodT.txt

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 TEXT

NAME	CATALOG #	VENDOR
EDTA 0.5M	E7889	Sigma Aldrich
Ethanol, absolute	9065.4	Carl Roth

Exonuclease I (20 U/µl)	EN0582	Thermo Fisher Scientific
Exonuclease I Reaction Buffer (10x)	EN0582	Thermo Fisher Scientific
IGEPAL CA-630	I8896	Sigma Aldrich
Maxima H- Reverse Transcriptase (200 U/µl)	EP0753	Thermo Fisher Scientific
Maxima RT Buffer (5x)	EP0753	Thermo Fisher Scientific
Polyethylene glycol 8000	89510	Sigma Aldrich
PBS 7.4	10010-23	Gibco - Thermo Fischer
Phusion HF Buffer	B0518	New England Biolabs
Proteinase K	9034	Takara
Sera-Mag Speed Beads	65152105050250	Thermo Fisher Scientific
Terra PCR Direct Polymerase Mix	639271	Takara
UltraPure DNase/RNase-Free Distilled Water	10977-049	Invitrogen - Thermo Fisher
Sodium Azide 99.5%	S2002-100G	Sigma Aldrich
Sodium Chloride 5M	S5150-1L	Sigma Aldrich
Trizma hydrochloride solution 1M pH 8.0	T2694	Sigma Aldrich
Bioanalyzer High Sensitivity DNA Analysis Kits	5067-4626	Agilent Technologies
MinElute Gel Extraction Kit	28606	Qiagen
Nextera XT DNA Library Preparation Kit	FC-131-1096	illumina
Quant-iT PicoGreen dsDNA Assay Kit	P7589	Invitrogen - Thermo Fisher
dNTPs (25 mM each)	R0182	Thermo Fisher Scientific
Aluminium seals for cold storage	391-1275	
Adhesive PCR film seals	AB0558	Thermo Fisher Scientific
twin.tec 96-well DNA LoBind Plates	0030129504	Eppendorf
twin.tec 384-well DNA LoBind Plates	0030129547	Eppendorf
0.5 ml PCRclean tube DNA LoBind	0030108035	Eppendorf
1.5 ml PCRclean tube DNA LoBind	0030108051	Eppendorf
5.0 ml PCRclean tube DNA LoBind	0030108310	Eppendorf
15 ml PCRclean tube DNA LoBind	0030122208	Eppendorf
E-Gel EX Agarose Gels, 2%	G402002	Invitrogen - Thermo Fisher

BEFORE STARTING

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

Preparation of lysis plates

1 Prepare **Lysis Buffer** for the number of plates needed.

Note that 96-well plates are the preferred setup for gmcSCRB-seq, as the well cleanup (Step 9-15) would be more difficult with 384-well plates.

Reagent	**96-well plate**
8M Guanadine Hydrochloride	343.75
2-mercaptoethanol	5.5
NEB HF Phusion buffer (5x)	1.1
H2O	199.65

Total	550

2 Prepare 96 well plate(s) containing 5 µL **Lysis Buffer** per well.

Lysis plates should be prepared shortly before use, but can be stored for up to 1 week at room temperature. Prior to use, double check to make sure salts have not fallen out of solution or that the lysis buffer has not evaporated.

5 µl Lysis Buffer

Sample Collection

3 Sort 1 cell to each well of a 96 well plate containing 5 µL **Lysis Buffer.**

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 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.

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

9 Add 10 uL of **Clean-up Beads** to each well (1:2 ratio).

10 µl Clean-up Beads

10 Seal the plate and vortex to mix the bead-lysate mixture. Briefly spin down the plate (<200 rcf, <10 sec) so all of the mixture is at the bottom of the well but the beads are still in solution.

11 Incubate for 5 minutes at RT.

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

20 °C Room temperature

12 Place on magnet stand until clear

13 Discard supernatant

14 Wash twice with 50 uL 80% ethanol (while on magnet) and discard supernatant

50 µl 80% ethanol (freshly prepared)

15 **00:05:00 air dry beads**

Depending on lab temperature and humidity, drying times can vary.

Reverse Transcription

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

Reagent	96-well plate
UltraPure Water	88 µL
PEG 8000 (50 % solution)	165 µL
Maxima RT Buffer (5x)	220 µL
dNTPs (25 mM each)	44 µL
TSO E5V6NEXT unblocked (100 µM)	22 µL
Maxima H Minus RT (200 U/µl)	11 µL
Total	**550 µL**

- RT MM can be prepared while plate is incubating or while drying on magnet.
- If ERCCs will be used, decrease the amount of H2O and add appropriate amount of ERCCs.
- Caution: Reverse Transcription Mix with PEG needs to be mixed carefully!

4 °C **Keep Reverse Transcription Mix on ice**

17 Once drying is complete, add 4 µL H2O to each well.

4 µl H2O

18 Add 5 µL Reverse Transcription Mix to each well.

5 µl Reverse Transcription Mix

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

19 Add 1 µL of barcoded oligo-dT primer [2 µM] (E3V6NEXT adapter) to each well.

1 µl barcoded oligo-dT primer [2 µM] (E3V6NEXT adapter)

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

21 In a thermocycler with heated lid, incubate:

42 °C 90 min

8 °C ∞

cDNA Pooling & Purification

22 Vortex to mix the bead-cDNA mixture. Briefly spin down the plate (<200 rcf, <10 sec) so all of the mixture is at the bottom of the well but the beads are still in solution.

23 Prepare **Bead Binding Buffer**

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 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

24 Pool all wells (including beads) of one plate into a 2 mL tube and add 960 µL (ratio 1/1) **Bead Binding Buffer**

960 µl Bead Binding Buffer

Vortex to mix the sample and bead binding buffer.

25 00:05:00 **binding of the cDNA onto the beads**

20 °C **Room temperature**

26 Place on magnet stand until clear

27 Discard supernatant

28 Wash twice with 2 mL 80% ethanol (while on magnet) and discard supernatant

2 ml **80% ethanol (freshly prepared)**

29 00:05:00 **air dry beads**

Exonuclease I Treatment

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

17 µl **UltraPure Water**

Avoid transfering beads as these can inhibit the downstream PCR.

31 To the 17 µl cDNA, add:

2 µl **Exonuclease I Buffer (10x)**

1 µl **Exonuclease I (20 U/µl)**

Full length cDNA amplification

32 In a thermocycler with heated lid, incubate:

37 °C **20 min (ExoI digest)**

80 °C **10 min (Heat inactivation)**

8 °C **∞ (Store)**

33 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**

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

30 µl **PreAmplification Mix**

cDNA purification & quantification

35 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	∞	

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.

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

40 µl Clean-up Beads

37 **00:05:00 binding of the cDNA onto the beads**

38 Place on magnet until clear and discard supernatant

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

150 µl 80% ethanol (freshly prepared)

40 **00:05:00 air dry beads**

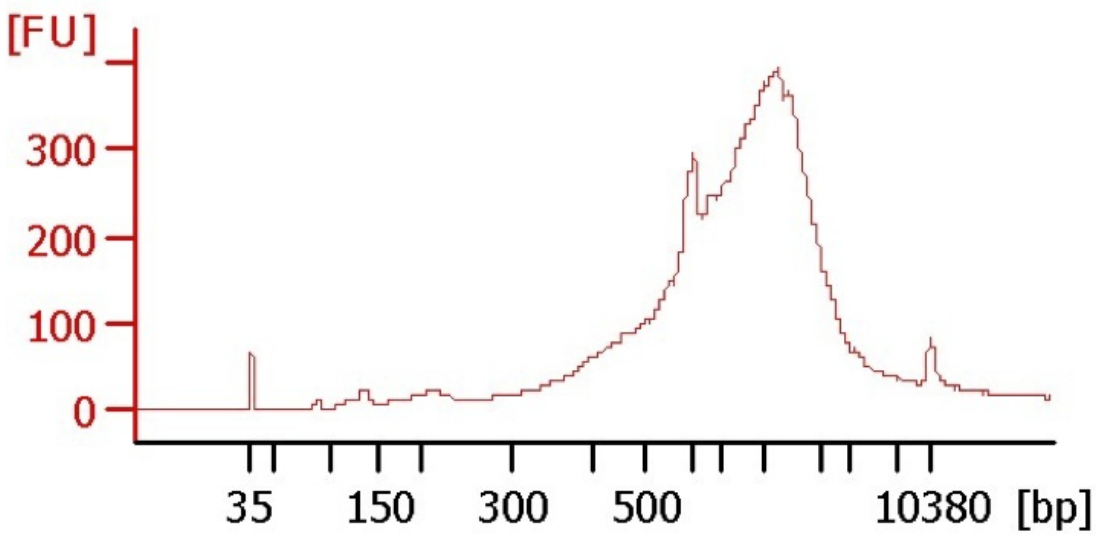
41 Elute cDNA in 15 µL UltraPure Water & transfer to new tube

15 µl UltraPure Water

42 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.

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

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



Tagmentation, Library PCR & Indexing

44 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

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

1 µl cDNA (0.8 ng/µL)

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

55 °C Tagmentation

00:10:00 Tagmentation

47 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

48 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

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

0.5 µl i7 index primer (5 µM)

50 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	∞	

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

50 µl Clean-up Beads

52 **00:05:00 binding of DNA onto the beads**

53 Place on magnet until clear and discard supernatant

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

150 µl 80% ethanol (freshly prepared)

55 **00:05:00 air dry beads**

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

20 µl UltraPure Water

Size selection

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

00:10:00

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

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

60 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

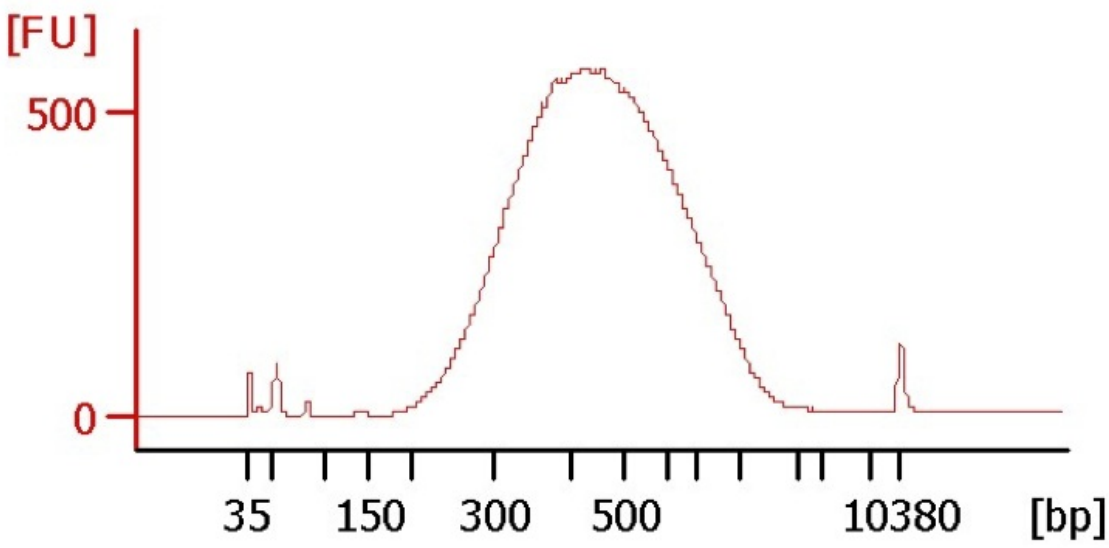
The Monarch DNA Gel Extraction Kit (NEB T1020L) can also be used.

Library Quantification

61 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.

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

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



62 If pooling several libraries, combine equal molar amounts.

Sequencing

63 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

64 Download and install zUMIs including all dependencies.

zUMIs
Linux
source

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

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

66 Run zUMIs by specifying your parameters in the associated yaml file or using the YAML config Rshiny application. Below is an example yaml file for a typical gmcSCRB-seq run.

```
project: Example_project_name
sequence_files:
  file1:
    name: /data/R2.fq.gz
    base_definition: BC(1-8)
  file2:
    name: /data/R1.fq.gz
    base_definition:
    - BC(1-6)
    - UMI(7-16)
  file3:
    name: /data/R3.fq.gz
    base_definition: cDNA(1-50)
reference:
  STAR_index: /data/STAR5idx
  GTF_file: /data/Species.gtf
  additional_STAR_params: ''
  additional_files: /data/ERCC92.fa
out_dir: /data/zUMIs
num_threads: 15
mem_limit: 0
filter_cutoffs:
  BC_filter:
    num_bases: 1
    phred: 20
  UMI_filter:
    num_bases: 1
    phred: 20
barcodes:
  barcode_num: ~
  barcode_file: /data/bc.txt
  automatic: no
  BarcodeBinning: 0
  nReadsperCell: 100
counting_opts:
  introns: yes
  downsampling: '0'
  strand: 0
  Ham_Dist: 0
  velocyto: no
  primaryHit: yes
  twoPass: yes
make_stats: yes
which_Stage: Filtering
Rscript_exec: Rscript
STAR_exec: STAR
pigz_exec: pigz
samtools_exec: samtools
zUMIs_directory: /data/zUMIs2/zUMIs
read_layout: SE
```

Example zUMIs yaml