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mcSCRB-seq2 protocol

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

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Disclaimer

Abstract

mcSCRB-seq2 is the improved version of our published protocol molecular crowding SCRB-seq (**mcSCRB-seq**) (**https://www.nature.com/articles/s41467-018-05347-6/**).

Following changes were introduced:

- re-evaluted the Lysis Buffer formulation, making it more robust against RNAses
- added additional dCTP in the Reverse Transcription leading to higher yield by enhanced Template switching
- updated the oligo dT primers to be compatible with 10x's three prime gene expression layout (see **prime-seq** for more information)
- changed to KapaHifi for PreAmplification as it produces less chimeric rerads compared to Tera Polymerase
- increased Primer concentration in PreAmp (SingV6)
- included an option for cheaper library prep with NEBNext

Guidelines

- All reagents and plasticware can be found in the 'Materials' section.
- Use only RNase free supplies and clean all surfaces and tools with RNase Away prior to working
- Make sure all steps before reverse transcription are carried out swiftly and on ice.
- All primer sequences are listed below:

	A	B	C	D	E
	Oligo	**Vendor**	**Purification**	**Concentration**	**Sequence**
	barcoded oligo-dT (E3V7NEXT)	Sigma	Cartridge	2 µM	ACACTCTTTCCCTACACGACGCTCTTCCGATCT[BC12][UMI16][T30]VN
	TSO unblocked (E5V7NEXT)	Sigma	RNase-Free HPLC	100 µM	Biotin-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 (Nextera)	IDT	TruGrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG
	i5 Index Primer (TruSeq)	IDT	Trugrade	5µM	AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT

Specific barcoded oligodT (E3V7NEXT) sequences:

E3V7_Set1.txt

E3V7_Set2.txt

Materials

General:

- RNase AWAY™ Surface Decontaminant **Carl Roth Catalog #**A998.4
- Ethanol (100%, Molecular Biology Grade) **Fisher Scientific Catalog #**BP2818500
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Quant-iT™ PicoGreen® dsDNA Assay Kit **Life Technologies Catalog #**P11496 or
- QuantiFluor(R) dsDNA System **Promega Catalog #**E2670

Homemade Beads:

- Sera-Mag SpeedBeads Carboxylate-Modified Magnetic Particles **GE Healthcare Catalog #**44152105050350
- Sodium Chloride **Fisher Scientific Catalog #**S271
- Tris HCl Buffer 1M Solution, Sterile pH 8.0 **Bio Basic Inc. Catalog #**SD8127.SIZE.450ml
- EDTA (0.5 M), pH 8.0 **Life Technologies Catalog #**AM9260G
- IGEPAL-CA630 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**I3021 SIGMA-ALDRICH
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2002-100G

Lysis:

- Phusion HF Buffer Pack - 6.0 ml **New England Biolabs Catalog #**B0518S
- NxGen RNAse Inhibitor **Lucigen Catalog #**30281-2

Reverse Transcription:

- PEG 8000 **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**81268
- Maxima H Minus Reverse Transcriptase **Thermo Fisher Scientific Catalog ##**EP0741
- dNTP Set 100 mM Solutions **Thermo Fisher Scientific Catalog #**R0181
- dCTP Solution (100 mM) **Thermo Fisher Scientific Catalog #**R0151
- ERCC RNA Spike-In Mix **Thermo Fisher Catalog #**4456740 (optional)
- Exonuclease I (E.coli) - 15,000 units **New England Biolabs Catalog #**M0293L

PreAmp:

- Kapa HiFi Hotstart ReadyMix (2x) **Kapa Biosystems Catalog #**KK2612

Library:

Bioanalyzer chips and reagents (DNA High Sensitivity kit) **Agilent Technologies**

NEBNext Ultra II DNA Library Prep with Sample Purification Beads - 96 rxns **New England Biolabs Catalog #**E7103L

NEBNext Q5 Hot Start HiFi PCR Master Mix - 250 rxns **New England Biolabs Catalog #**M0543L

SPRIselect reagent **GE Healthcare Catalog #**B23317

Before start

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

Sample Collection

1 Prepare Lysis Buffer for the number of plates you intend to use.

	A	B	C
	Reagent	**per well**	**per plate (96 well)**
	Buffer Phusion HF 5x	0.008	0.88
	NxGen RNAi (40U/ul)	0.125	13.75
	H2O	3.867	425.37
	Total	**4**	**440**

Lysis Buffer PPi (**P**rimer, **P**husion and **i**nhibitor)

2 Distribute 4 µL **PPi Lysis Buffer** per well using a repeater pipette (stepper) or similar.

Add 1 µL barcoded oligo-dT primer [2 µM] (E3V7) to each well using a multichannel pipette.

Note

When preparing many plates at once we usually make one **master plate including the primers** and then distribute 5 µL to each well of the Lysis plates.
Lysis plates with barcode primers can be prepared ahead of time and stored at -20 °C for short term (< 1 week).

3 Deposit one cell per well in the Lysis plate using e.g. FACS

Note

Keep plate cooled whenever possible, briefly spin down before use to make sure lysis buffer is at the bottom of the well.

4 Immediately after sorting close plate tightly with an aluminum seal.

5 In a cooled centrifuge, spin down the plate 30s
1000 x g, 4°C, 00:00:30 , max speed if 1000 x g are not possible and place immediately on dry ice.
Store plates at -70 °C or lower for up to 6 month before processing.

Preparation

6 Before starting clean all surfaces and pipettes with RNase Away.
Apply RNase Away for 3-5 minutes and wipe away with a clean tissue.

7 When running the protocol for the **first time**, prepare **Cleanup Beads** as described in Appendix 1.

Note

The **Cleanup Beads** can be stored at 4 °C for up to six months and do not have to be prepared fresh every time.

8 When running the protocol for the **first time**, prepare **Pooling Beads** as described in Appendix 2.

Note

The **Pooling Beads** can be stored at 4 °C for up to six months and do not have to be prepared fresh every time. **Pooling Beads** contain a 5 times lower amount of Bead particles and can alternatively be prepared by diluting **Cleanup Beads** 1/5 in Bead Binding Buffer (see Table in the corresponding section, add H2O to 50 ml)

Reverse Transcription

9

Note

When processing several plates for an experiment, try to balance the different conditions to be able to correct for potential batch effects. In the ideal case process all plates in one go. However, for the first steps until pooling of the cDNA you will need one Thermal Cycler per plate, so depending on the number of available Thermo Cyclers, batches can be bigger or smaller.

10 Prepare **Reverse Transcription Mix**

	A	B	C
	Reagent	**Well**	**Plate**
	UltraPure Water	0.7 µL	77 µL
	PEG 8000 (50% solution)	1.5 µL	165 µL
	Maxima RT Buffer (5x)	2 µL	220 µL
	dNTPs (25 mM)	0.4 µL	44 µL
	dCTPs(100 mM)	0.2 µL	22
	TSO E5V7NEXT (100 µM)	0.1 µL	11 µL
	Maxima H Minus RT (200 U/µL)	0.1 µL	11 µL
	Total	5 µL	550 µL

If you wish to add spike-in molecules like ERCCs or molecular spikes add them to the RT Mix and decrease the water accordingly

Note

Caution: Reverse Transcription Mix with PEG needs to be mixed carefully! RT Mix should be kept on ice until use but not longer than 00:15:00 minutes

11 Gently thaw plates on ice for 00:01:00 at most. 1m

12 Spin down in a pre-cooled centrifuge 1000 rcf, 4°C, 00:00:30 . 30s

13 Add 5 µL **Reverse Transcription Mix** to each well using a repeater pipette or a liquid handling robot like the Mantis.

14 Incubate for 01:30:00 at 42 °C in a Thermal Cycler with heated lid 105 °C .

cDNA Pooling & Purification

15 Mix each well (10 µL per well) with 10 µL of **Pooling Beads** for a 1:1 ratio. Pooling Beads can be added using a repeater pipette.

> Note
>
> The EDTA in the **Pooling Beads** will inactivate the RT and make pooling easier due to the color.

16 Pool all wells of one plate into a 2 mL tube or 15 mL tube when pooling up to 384 cells.

> Note
>
> We usually transfer all wells of a 96 well plate into the first column using a 20 µL 8-channel pipette and then transfer everything to a bigger tube using a 200 µL pipette.

17 Incubate for 00:05:00 at Room temperature to allow binding of the first strand cDNA onto beads.

18 Place the tube on the magnet stand until clear, approximately 00:05:00 and discard supernatant. 5m

19 Wash with 1 mL of **80% EtOH** while the tube is on the magnet. Discard the supernatant.

> Note
>
> Volume of EtOH should be adjusted depending on the number of cells/plates in a pool. More samples will require more EtOH to cover the beads completely and wash them efficiently.

20 Repeat wash step once more.

21 Air dry beads for 00:05:00

> Note
>
> Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

22 Resuspend the beads in 17 µL of **UltraPure Water** and remove from Magnet.

23 Incubate for 00:05:00 at RT and place on magnet to transfer supernatant to a new well of a 96 well PCR plate. 5m

Exonuclease I Treatment

24 Add 2 µL of **Exonuclease I Buffer (10x)** and 1 µL of **Exonuclease I** per pool.

> Note
>
> The **Exonuclease I** step **is important** to remove remaining single stranded oligo dT primers. **When not removed** completely **oligo dT primers** will prime in the subsequent PCR and thus **lead to extremly high levels of cross contamination and inflation of UMI counts!**

25 Incubate as follows in a Thermal Cycler with heated Lid 105 °C :

	Step	Temperature	Time
	Incubation	37 C	20 min
	Heat Inactivation	80 C	10 min
	Storage	4 C	∞

Exonuclease I digest

26 Mix sample with 20 µL **Clean Up Beads (22% PEG)** for a ratio of 1:1

Note

This Clean up is necessary because the Exonuclease Reaction inhibits the Kapa HiFi polymerase. As this clean up is done on the pooled cDNA the loss of molecules is negligible.

27 Incubate for 00:05:00 at 20 °C (Room Temp)

28 Place the plate on the magnet stand until clear (~ 00:05:00) and discard supernatant. 5m

29 Wash with 100 µL of **80% EtOH** while the plate is on the magnet.

30 Discard the supernatant and keep plate on the magnet.

31 Repeat wash step once more. go to step #29

32 Air dry beads for 00:05:00

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

33 Remove the plate from the magnet and resuspend the beads in 20 µL of **UltraPure Water.**

34 Incubate for 00:05:00 at RT and place on magnet to transfer supernatant to a new well.

Full length cDNA Amplification

35 Prepare **Pre Amplification Mix.** Adjust to the number of pools.

	Reagent	1x
	KAPA HiFi 2x RM	25 µL
	SINGV6 Primer (10 uM)	3 µL
	UltraPure Water	2 µL
	Total	**30 µL**

Pre Amplification PCR Master mix for one pool.

36 Add 30 µL **Pre Amplification Mix** to each pool.

37 Incubate the Pre Amplification PCR as follows:

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98 C	3 min	1 cycle
	Denaturation	98 C	15 sec	15 cycles*
	Annealing	65 C	30 sec	
	Elongation	72 C	4 min	
	Final Elongation	72 C	10 min	1 cycle
	Storage	4 C	∞	

Note

Adjust the number of cycles based on input (cell number and expected amount of RNA per cell).
15 cycles is a good starting point for one full 96 well plate containing big cells like iPSCs.
For immune cells increase cycle number to 17-19 cycles.

cDNA Bead Purification

38 Mix sample with 40 µL **Clean Up Beads (22% PEG)** for a ratio of 1:0.8

39 Incubate for 00:05:00 at 20 °C (Room Temp)

40 Place the plate on the magnet rack until clear (~ 00:05:00) . 5m

41 Discard supernatant and keep plate on the magnet.

42 Wash with 100 µL of **80% EtOH** while the plate is on the magnet.

43 Discard supernatant and keep plate on the magnet.

44 Repeat wash step once more. go to step #42

45 Air dry beads for 00:05:00

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

46 Remove from Magnet and resuspend the beads in 10 µL of **UltraPure Water**.

47 Incubate for 00:05:00 at RT and place on magnet to transfer supernatant to a new well.

Note

Safe Stoping Point! The Pre-Amplified cDNA can be stored for up to one week at -20 °C . However usually we want to know if the experiment worked first and continue to do the concentration measurement and Bioanalyzer QC.

cDNA Quantification and Quality Check

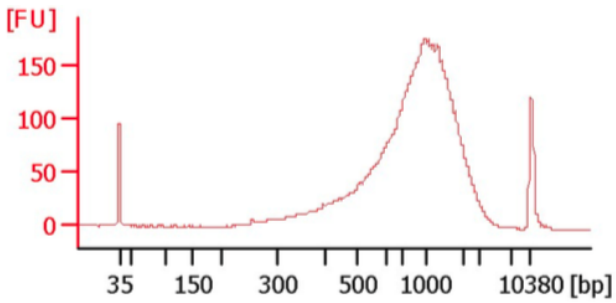
48 Quantify the cDNA using the **Quant-iT PicoGreen dsDNA assay kit** or equivalent following the manufacturer's protocol. Use 1 µl of clean cDNA for quantification.

49 Quality check the cDNA using the Agilent 2100 Bioanalyzer with **High Sensitivity DNA Analysis Kits**. Other instruments like the Tape Station or the Fragment Analyzer can be used as well but make sure to not waste more than 4 µL of your cDNA.

Note

Passing the cDNA quality check does not guarantee that the data will be of high quality, however, if the cDNA fails the quality check it will usually not yield good libraries and will therefore generate lower quality data.

Expected result



Exemplary cDNA trace with a **peak around 1000 to 1500 bp** and **few small fragments below 300 bp.**

50

Note

In previous versions of SCRB-seq/mcSCRB-seq we used the Nextera XT Kit for Library Preparation, but have switched to using NEB Next Ultra II recently. While the data quality is similar, the downscaled NEB Next Ultra II Kit as we use it is a fraction of the cost per library and allows for using more of the cDNA as input. However both methods can be used depending on availability of the kits in the lab.

Library Preparation

51 Use one fourth of your cDNA 2.5 µL but not more than 20 ng in total . If your cDNA concentration is higher than 8 ng/µL, dilute accordingly.

Note

As the volumes in the scaled down NEBNext Ultra II kit are very low we recommend always preparing a 2x master mix for all subsequent steps.

Library Preparation - Fragmentation

52 Start by setting up the Thermo Cycler to be able to immediately proceed to the incubation after adding the Fragmentation Mix to the cDNA.

Note

Set heated lid to 75° C. Make sure the lid is at the correct temperature before you start the reaction.
Skip the first incubation step once you have added your samples.

	A	B	C
	Step	**Temperature**	**Time**
	Pre-Cool	4 °C	infinite
	Fragmentation	37 °C	5 min
	A Tailing and Phosphorylation	65 °C	30 min
	Storage	4 °C	infinite

53 Prepare **Fragmentation Mix**

	A	B
	Reagent	**2x**
	Ultra II FS Reaction Buffer	2.8 µL
	Ultra II FS Enzyme Mix	0.8 µL
	TE	3.4 µL
	Total	7 µL

Note

Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up and down several times to break it up, and quickly vortex to mix. Place on ice until use.

Vortex the Ultra II FS Enzyme Mix for 5-8 seconds prior to use for optimal performance.

54 Add 2.5 µL of cDNA (between 0.5 & 8 ng/µl) to a new well of a 96 well plate and add 3.5 µL of Fragmentation Mix.

55 Vortex the Fragmentation Mix for 00:00:05 and immediately proceed to next step. 5s

Safety information

Proceed to next step immediately to avoid over-fragmentation.

56 Place plate containing the samples into Thermal Cycler and start incubation by skiping the initial 4 °C hold.

Library Preparation - Adapter Ligation

57 Prepare Adapter Ligation Mix:

	A	B
	Reagent	**1 x**
	Ultra II Ligation Master Mix	6 µL
	Ultra II Ligation Enhancer	0.2 µL

	A	B
	prime Adapter (1.5 µM)	0.5 µL
	Total	**6.7 µL**

Adapter Ligation mix for one Library.

58 Add 6.7 µL Adapter Ligation Mix to each replicate.

59 Incubate for 00:15:00 at 20 °C 15m

Note

Turn off heated lid or run cycler with open lid.

Library Preparation - Double size selection

60 Add 37.3 µL Buffer EB to Samples for a total of 50 µL

61 Mix Index PCR with 25 µL SPRI select beads (ratio of 0.5x).

Note

We use SPRI Select Beads here instead of our home made 22% Clean Up beads for their guaranteed QCed size selection properties.

62 Incubate for 00:05:00 at room temperature. 5m

63 Place the plate on the magnet stand until clear and transfer 75 µL supernatant to a clean well.

Safety information

Be careful not to discard the supernatant! This is your library!

64 Mix supernatant with 10 µL SPRI select beads (ratio of 1:0.7)

65 Incubate for 00:05:00 at Room temperature 5m

66 Place the plate on the magnet stand until clear.

67 Discard supernatant and keep plate on magnet.

68 Wash with 150 µL of 80% EtOH while the plate is on the magnet.

69 Discard the supernatant and keep plate on magnet.

70 Repeat wash step once more. go to step #68

71 Air dry beads for 00:05:00 5m

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

72 Take plate off the magnet and resuspend samples in 10.5 µL 0.1X TE (dilute 1X TE Buffer 1:10 in water).

73 Elute for 00:05:00 minutes. 5m

74 Place plate on magnet and transfer samples to clean wells.

Library Preparation - Library PCR and Indexing

75

Note

For Illumina sequencers with patterned flow cells (e.g. Nova-seq or Next-seq Series) it is recommended to use unique dual indexing, meaning both the i5 and i7 indices are used for only one library. Library replicates may have the same indices.

76 Add 1 µL of Nextera i7 Index Primer ([M] 5 micromolar (µM)) to each well.

77 Add 1 µL of TruSeq i5 Index Primer ([M] 5 micromolar (µM)) to each well.

78 Add 12.5 µL of 2x Q5 Master Mix (NEBNext Ultra II) to each well.

Note

Although scaled down, there will not be sufficient Q5 Master Mix (M0544L) in the kit. This item will have to be ordered separately.

79 Incubate Library PCR reaction as follows with the heated Lid set to 105 °C :

	A	B	C	D
	Step	**Temperature**	**Time**	**Cycles**
	Initial Denaturation	98 °C	30 sec	
	Denaturation	98 °C	10 sec	10*
	Annealing/Elongation	65 °C	1 min	10*
	Final Elongation	65 °C	5 min	
	Storage	8 °C	∞	

Library Amplification PCR

80 Adjust the number of cycles based on total cDNA input.
As a general guide we recommend:

	A	B
	Input (ng)	Cycles
	20	10
	10	11
	5	12
	2.5	13

Library Preparation - Final Double Size Selection and Clean Up

81 Add 25 µL Buffer EB to Index PCR.

82 Mix Index PCR with 25 µL SPRI select beads (ratio of 1:0.5)

83 Incubate for 00:05:00 at Room temperature 5m

84 Place the plate on the magnet stand until clear and transfer 75 µL supernatant to a clean well.

Safety information

Be careful not to discard! This is your library.

85 Mix supernatant with 10 µL SPRI select beads (ratio of 1:0.7)

86 Incubate for 00:05:00 at Room temperature . 5m

87 Place the plate on the magnet stand until clear.

88 Discard supernatant and keep plate on the magnet.

89 Wash with 150 µL of 80% EtOH while the plate is on the magnet.

90 Discard supernatant and keep plate on the magnet.

91 Repeat wash step once more. go to step #89

92 Air dry beads for 00:05:00 . 5m

Note

Depending on temperature and humidity, the beads may dry faster. Therefore it is important to regularly check the beads and avoid over-drying.

93 Elute in 15 µl UltraPure Wate r. 107 Incubate for 00:05:00 and then place on magnet until clear. Transfer eluted library to new well. Stopping point. The libraries can be safely stored at -20 °C until they will be QCed and sequenced. Library QC 45m

94 Take plate off the magnet and resuspend samples in 15 µL **UltraPure Water.**

95 Elute for 00:05:00 minutes.

96 Transfer 15 µL clean sequencing Library to a 0.5 mL tube for storage.

Note

Safe Stopping Point! The final library can be stored at -20 °C until sequencing ideally not longer than one month.

Library Preparation - Quantification and QC

97 Quantify the cDNA using the **Quant-iT PicoGreen dsDNA assay kit** or equivalent following the manufacturer's protocol. Use 1 µL of the final Library for quantification.

Note

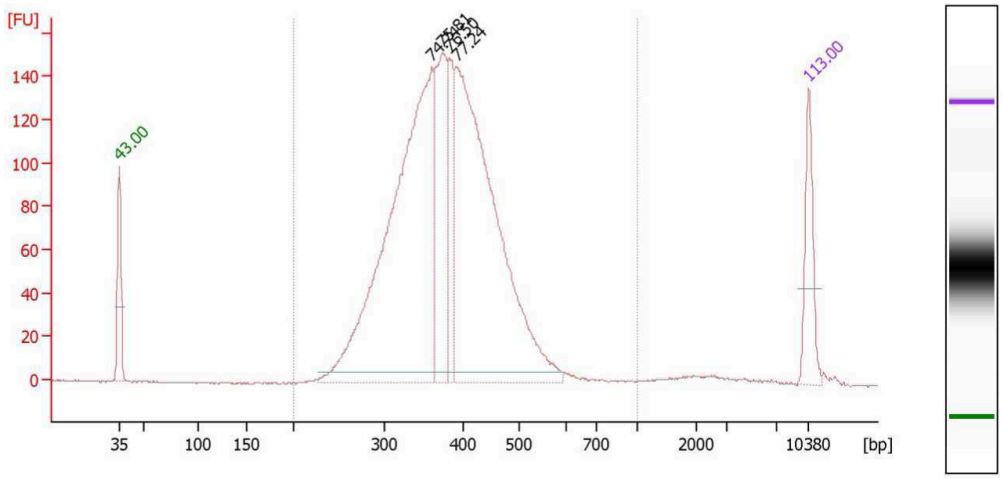
Expected Library concentrations are between 1 and 10 ng/µl. Very low yield can be an indicator of low quality cDNA. For Yields between 0.1 and 1 ng/µl consider Amplifying the Library for 5 to 10 more Cycles using the Q5 Master Mix with universal Illumina Fwd and Rev. primers. (see Materials)

98 Quality control and Quantify the Library using the Agilent 2100 Bioanalyzer with **High Sensitivity DNA Analysis Kits**. Other instruments like the Tape Station or the Fragment Analyzer can be used as well.

Note

Use Library concentrations between 1 and 2 ng/µL for optimal quantification results. If Library concentration is above 2 ng/µl dilute accordingly.

Expected result



Optimal Bioanalyzer trace of an NEB Next Library. Narrow distribution with a peak between 300 and 400 bp. If the Fluorescence intensity maximum is markedly higher than the Marker peaks (35bp and ~10000 bp) the quantification of the Bioanalyzer will be inaccurate. The trace in the example is still in a quantifiable range.

99 Quantify the Library Molarity using the Bioanalyzer2000 (or else) software. Make sure to set the Region from 200 bp to 1000 bp. The Bioanalyzer will calculate the Molarity based on fragment size and Fluorescence intensity.
We usually aim for a Library concentration of 10nM (10000pM) to submit for sequencing. Lower or higher molarities might be required depending on the sequencing provider.

Sequencing

100 Samples should be submitted according to your Sequencing Facility specifications. prime-seq is compatible with Illumina Sequencing.

At least 8 cycles are required for the Index Reads (i7+i5) and 28 cycles for the Read 1 (BC+UMI). Read 2 (DNA) should be adjusted based on the quality of the genome being mapped to, but for human and mouse 50 cycles are sufficient.

Some potential sequencing options:

	A	B	C	D	E	F
	Sequencer	**Read1**	**Read2**	**Index Read (i7)**	**Index Read (i5)**	**Kit**
	NovaSeq 6000	28	94	8	8	SP v1.5 100 cycle
	NovaSeq 6000	150	150	10	10	S4 v1.5 300 cycle
	NextSeq 500/550	28	63	8	8	NextSeq 500/550 HiOut v3 75 cycle
	NextSeq 1000/2000	28	88	8	8	NextSeq 1000/2000 P2 100 cycle
	NextSeq 2000	28	46	8	0	NextSeq 2000 P3 50 cycle

NextSeq 2000 P3 50 Cycle is only possible when not pooling with other libraries as no index read is included.

101 Sequencing Depth should be adjusted to the scientific question, for example for broad cell type classification few read per cell between 10 k and 25 k are usually sufficient. For in depth transcriptome anaylsis between 100k - 500k reads per cell are adequate. Please note that these are just general remarks and library complexity may differ considerably depending on cell type, state and quality of the input.

	A	B	C
	Number of cells	Mio. reads shallow seq. (25k per Cell)	Mio. reads deep seq. (500k per Cell)
	96	2.4	48

	A	B	C
	384	9.6	192
	1536	38.4	768

Exemplary sequencing depth calculations.

Appendix: Prepare Clean Up Beads (SPRI 22% PEG)

102 Prepare **PEG Solution (22%)** by adding all ingredients to a 50 mL falcon tube

	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**

Bead Binding Buffer

Note

Do not add the total amount of water until after PEG is completely solubilized

103 Incubate at 40 °C and vortex regularly until PEG is completely dissolved

104 Resuspend **Sera-Mag Speed Beads** carefully and pipette 1000 µL of bead suspension into a 1.5 mL tube

105 Place on magnet stand and remove storage buffer

106 Add 1000 µL of **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads

107 Place on magnet stand and remove supernatant

108 Repeat wash step one more time

109 Add 900 µL **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads

110 Add the washed **Sera-Mag Speed Beads** to the **PEG Solution (22%)** and mix well

Note

The final **Cleanup Beads** can be aliquoted and stored at 4 °C for up to six months

Appendix: Prepare Pooling Beads (SPRI 22% PEG 5 times less Beads)

111 Prepare **PEG Solution (22%)** by adding all ingredients to a 50 mL falcon tube

	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**

Note

Do not add the total amount of water until after PEG is completely solubilized

112 Incubate at 40 °C and vortex regularly until PEG is completely dissolved

113 Resuspend **Sera-Mag Speed Beads** carefully and pipette 200 µL of bead suspension into a 1.5 mL tube

114 Place on magnet stand and remove storage buffer

115 Add 1000 µL of **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads

116 Place on magnet stand and remove supernatant

117 Repeat wash step one more time

118 Add 900 µL **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads

Appendix: Prepare Pooling Beads (SPRI 22% PEG 5 times less Beads)

119 Add the washed **Sera-Mag Speed Beads** to the **PEG Solution (22%)** and mix well

Note

The final **Cleanup Beads** can be aliquoted and stored at 4 °C for up to six months