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We use this protocol and it's working

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Abstract

prime-seq is a simple and open RNA-seq method, which can be easily established in most research labs or facilities. Based on two of the most sensitive single cell RNA-seq methods available, namely smart-seq2 and mcSCRB-seq **(Picelli et al. 2013; Bagnoli et al. 2018)**, prime-seq is an adaptable, affordable, robust, and high-throughput option. Additionally, prime-seq can be used on a wide range of model organisms, from apes to zebrafish, and many in between.

Guidelines

- All reagents and plastic-ware 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 involving cell lysate and RNA before reverse transcription are carried out swiftly and on ice.
- All primer sequences are listed below:

	Oligo	Vendor	Purification	Working Conc.	Sequence	Notes
	Barcoded Oligo-dT (E3V7NEXT)	Sigma	Cartridge	10 µM	ACACTCTTTCCCTACACGACGCTCTTCCGATCT[12 bp BC]NNNNNNNNNNNNNNNVTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	
	Template Switching Oligo (TSO) (E5V7NEXT)	Sigma	RNase-Free HPLC	100 µM	Biotin-ACACTCTTTCCCTACACGACGCrGrGrG	
	Preamp Primer (SINGV6)	Sigma	Standard Desalting	10 µM	Biotin-ACACTCTTTCCCTACACGACGC	
	3' enrichment primer (P5NEXTPT5)	Sigma	Standard Desalting	5 µM	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCT	
	i7 Index Primer (N7XX)	IDT	Trugrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG	
	prime-seq Adapter AntiSense	IDT	Standard Desalting	1.5 µM	/5Phos/CTGTCTCTTATACACATCT	Duplexed DNA
	prime-seq Adapter Sense	IDT	Standard Desalting	1.5 µM	GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGT	Duplexed DNA

Specific barcoded oligodT (E3V7NEXT) sequences:

E3V7_Set1.txt

E3V7_Set2.txt

Materials

MATERIALS

- DNase I Reaction Buffer - 6.0 ml **New England Biolabs** **Catalog #**B0303S
- DNase I (RNase-free) - 1,000 units **New England Biolabs** **Catalog #**M0303S
- Deoxynucleotide Solution Mix - 40 umol of each **New England Biolabs** **Catalog #**N0447L
- Exonuclease I (E.coli) - 3,000 units **New England Biolabs** **Catalog #**M0293S
- Quant-it™ PicoGreen® dsDNA Assay Kit **Life Technologies** **Catalog #**P7589
- ß -mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**M3148
- QuantiFluor(R) RNA System **Promega** **Catalog #**E3310
- Proteinase K solution, 20 mg ml – 1 **Ambion** **Catalog #**AM2546
- 5 M Sodium chloride (NaCl) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S5150-1L
- Agilent High Sensitivity DNA Kit **Agilent Technologies** **Catalog #**5067-4626
- Buffer RLT Plus **Qiagen** **Catalog #**1053393
- Maxima H Minus Reverse Transcriptase (200 U/uL) **Thermo Fisher Scientific** **Catalog #**EP0752
- NEBNext Ultra II FS DNA Library Prep with Sample Purification Beads - 24 rxns **New England Biolabs** **Catalog #**E6177S
- EDTA **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**E7889
- Ethanol absolute **Carl Roth** **Catalog #**9065.4
- Igepal **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**I8896
- KAPA HiFi 2x RM **Kapa Biosystems** **Catalog #**KR0370
- Poly(ethylene glycol) **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**89510
- UltraPure DNase/RNase Free Distilled Water **Catalog #**10977-049
- Trizma hydrochloride solution **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**T2694
- Aluminium seals for cold storage **Catalog #**391-1275
- Filter tips 96 low retention 10 uL **Catalog #**771265
- PCR Seals **Thermo Scientific** **Catalog #**AB0558
- twin.tec 96-well DNA LoBind Plates **Eppendorf** **Catalog #**0030129504
- Sera-Mag Speed Beads **GE Healthcare** **Catalog #**65152105050250
- Sodium Azide **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2002-100G
- NEBNext Ultra II FS DNA Library Prep Kit for Illumina **New England Biolabs** **Catalog #**E7805S

Troubleshooting

Safety warnings

Please follow all Manufacturer safety warnings and recommendations.

Before start

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

Preparation

12m

1 Clean all surfaces and pipettes with RNase Away. 5m

2 Thaw frozen buffers and primers on ice. 10m

3 Prepare 80% EtOH (approximately 45 mL for 96 samples) 2m

Prepare Cleanup Beads (22% PEG)

10m

4 Prepare **PEG Solution (22%)** by adding all ingredients to a 50 mL falcon tube 10m

	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

5 Incubate at 40 °C and vortex regularly until PEG is completely dissolved 10m

6 Resuspend **Sera-Mag Speed Beads** carefully and pipette 1000 µL of bead suspension into a 1.5 mL tube 1m

7 Place on magnet stand and remove storage buffer 1m

8 Add 1000 µL of **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads 30s

9 Place on magnet stand and remove supernatant 30s

10 Repeat wash step one more time 1m

11 Add 900 µL **TE Buffer** (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and resuspend beads 30s

12 Add the washed **Sera-Mag Speed Beads** to the **PEG Solution (22%)** and mix well 1m 30s

Note

The final **Cleanup Beads (22% PEG)** can be aliquoted and stored at 4 °C for up to six months

13 prime-seq can be used on lysate or extracted RNA. It is essential, however, that the samples either have the same input or that they are normalized after the RNA is extracted, otherwise sequencing depth per sample will be impacted. Based on your starting material, please follow one of the following cases:

STEP CASE

Lysate (similar input) 96 steps

Follow this case if you are testing samples that have **similar input** (i.e. the expected RNA amount is the same between samples). The steps here will guide you in digesting residual proteins in your samples, extracting the RNA, digesting DNA,

preparing RNA-seq libraries, and finally sequencing.

Example: investigating the genotype effect on transcription in 5,000 neurons

Prepare Bead Binding Buffer

10m

14 Prepare **Bead Binding Buffer (2x)** 10m

	Reagent	
	PEG 8000	1.1 g
	NaCl (5 M)	1 mL
	Tris-HCl (1 M, pH 8.0)	50 µL
	Igepal (10% solution)	5 µL
	Sodium Azide (10% solution)	25 µL
	H2O	to 5 mL
	Total	**5 ml**

Note

The **Bead Binding Buffer (2x)** can be stored at Room temperature for up to six months.

Sample Collection

2m

15 Prepare **Lysis Buffer** according to the number of samples. 2m

	Reagent	**Well**	**Plate**
	RLT Plus Buffer	99 µL	10.89 mL
	β-mercaptoethanol	1 µL	110 µL

	Total	**100 µL**	**11 mL**

Note

If sample volume exceeds 25 % of total lysate, use 2x TCL buffer (Qiagen, #1070498) + 1 % β-mercaptoethanol

16 Add 100 µL of **Lysis Buffer** to each well of a semi-skirted 96-well PCR plate 1m

17 Add cells or tissue to wells

Note

Cells

Minimum: 100 cells, Optimum: 10,000 cells

Make sure that the same number of cells are used for each sample. Large differences between cells will impact distribution of sequencing reads and can potentially affect normalization.

Note

Tissue

If samples are difficult to lyse they should be homogenized using a tissue homogenizer.

Tissue should be a relatively small and not exceed more than 1000 ng of RNA. Tissue samples should be normalized by weight and be the same type of tissue.

Large differences between tissue samples will impact distribution of sequencing reads and can potentially affect normalization.

If you are unsure if the samples will contain the same amount of RNA, it is best to switch to the "*Lysate (variable)*" case in Step 13.

18 Transfer 50 µL of **lysate** to a new plate, return one plate immediately to -80 C freezer to save as a backup 1m

Note

Conversely, one can prepare two plates during sorting with 50 µL of lysis buffer.

Proteinase K Digest 30m

19 Add 1 µL **Proteinase K** (20 mg/mL) and 1 µL **EDTA** (25 mM) to each well 1m

20 Incubate for 00:15:00 at 50 °C and then heat inactivate the Proteinase K for 00:10:00 at 75 °C 25m

Bead Clean Up 20m

21 Mix each bulk sample (50 µL per well) with 100 µL of **Cleanup Beads (22% PEG)** 1m

22 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

Note

While binding, prepare **DNase I Mix** (Step 28)

23 Place on magnet stand until clear (~3 min) and then discard supernatant 3m

24 Wash with 100 µL of **80% EtOH** while the plate is on the magnet. Discard the supernatant 2m

25 Repeat wash step once more 2m

26 Air dry beads for 00:03:00 3m

Note

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

DNAse I Digest 1m

27 Add 5 µL H2O and **resuspend beads by vortexing vigorously** 2m

Note

If you encounter bead clumping at this step, try to resuspend the beads by vigorously pipetting the samples. We have generated high quality prime-seq libraries despite heavy clumping.

28 Prepare **DNase I Mix** 3m

	Reagent	Well	Plate
	DNase I	1 µL	110 µL
	DNase I Buffer (10x)	2 µL	220 µL
	Bead Binding Buffer (2x)	10 µL	1.1 mL
	H2O	2 µL	220 µL
	Total	**15 µL**	**1.65 mL**

29 Resuspend beads in 15 µL of **DNase I Mix** 2m

30 Incubate DNase I Mix and beads for 00:10:00 at 20 °C (Room Temp) 10m

31 Heat inactivate the DNase I by adding 1 µL of **EDTA (100 mM)** and incubating for 00:05:00 at 65 °C 6m

32 Place plate on magnet stand until clear (~3 min) and discard the supernatant. 3m

33 Wash with 100 µL of **80% EtOH** while the plate is on the magnet. Discard the supernatant 2m

34 Repeat wash step once more 2m

35 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.

Note

While drying, prepare **Reverse Transcription Mix** (Step 36).

Reverse Transcription

5m

36 Prepare **Reverse Transcription Mix** 5m

	Reagent	Well	Plate
	Maxima H Minus RT	0.15 µL	16.5 µL
	Maxima RT Buffer (5x)	2 µL	220 µL
	dNTPs (25 mM)	0.4 µL	44 µL
	TSO (E5V7NEXT) (100 uM)	0.1 µL	11 µL
	UltraPure Water	2.35 µL	258.5 µL
	Total	**5 µL**	**550 µL**

37 Add 4 µL **H2O** 1m

Note

The 4 µL of water can be combined with the Reverse Transcription Mix by increasing the water in Row 6 from 2.35 µL to 6.35 µL.

If working with many samples, or if using a stepper pipette or robot, we find that it is better to add some water separately to prevent the beads from drying too much.

38 Add 5 µL **Reverse Transcription Mix** 1m

39 Add 1 µL of **Barcoded oligodT (E3V7NEXT) (10 µM)** per well 2m

40 Incubate for 01:30:00 at 42 °C 1h 30m

cDNA Pooling & Purification

5m

41 Place the plate on a magnet 3m

42 Pool all wells of one plate into a 2 mL tube 10m

43 Add 10 µL of **Cleanup Beads (22% PEG)** ***for each sample*** for a 1:1 ratio (e.g. 240 µL for 24 samples) 5m

Note

The EDTA in the **Cleanup Beads (22% PEG)** will inactivate the RT and make pooling easier due to the color.

44 Incubate for 00:05:00 at Room temperature to allow binding of the cDNA onto beads 5m

45 Place the tube on the magnet stand until clear (~3 min) and discard supernatant 3m

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

Note

Volume of EtOH should be adjusted depending on the number of samples. More samples will require more EtOH to cover the beads completely.

47 Repeat wash step once more 1m

48 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.

49 Elute the beads in 17 µL of **UltraPure Water** 1m

50 Incubate for 00:05:00 at RT and transfer to a new PCR tube or plate 5m

Exonuclease I Treatment 35m

51 Add 2 µL of **ExoI Buffer (10x)** and 1 µL of **Exonuclease I**. Incubate as follows: 35m

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

52 Mix each sample (20 µL per well) with 16 µL of **Cleanup Beads (22% PEG)** for a 1:0.8 ration 1m

53 Incubate for 00:05:00 at Room temperature to allow binding of the cDNA onto beads 5m

54 Place the tube on the magnet stand until clear (~3 min) and discard supernatant 3m

55 Wash with 50 µL of **80% EtOH** while the tube is on the magnet. Discard the supernatant 1m

56 Repeat wash step once more 1m

57 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.

58 Elute the beads in 20 µL of **UltraPure Water** 1m

59 Incubate for 00:05:00 at RT and transfer to a new PCR tube or plate 5m

Full length cDNA Amplification 1m

60 Prepare **Pre Amplification Mix** 1m

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

61 Add 30 µL **Pre Amplification Mix** to sample 1m

62 Incubate the Pre Amplification PCR as follows: 1h 30m

	Step	Temperature	Time	Cycles

	Initial Denaturation	98 C	3 min	1 cycle
	Denaturation	98 C	15 sec	10 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 (sample number, cell number, or concentration).

As a rule of thumb we assume big cells like embryonic stem cells to contain 10 pg of total RNA and small cells like T-cells ~ 1-2 pg

As a general guide we recommend:

	Total RNA Input	**Cycles**
	10 ng	16
	50 ng	14
	100 ng	12
	500 ng	10
	1000 ng	9

cDNA Bead Purification

1m

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

64 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

65 Place the tube on the magnet stand until clear (~3 min) and discard supernatant 3m

66 Wash with 100 µL of **80% EtOH** while the tube is on the magnet. Discard the supernatant 1m

67 Repeat wash step once more 1m

68 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.

69 Elute cDNA in 10 µL **UltraPure Water** 1m

70 Incubate for 00:05:00 at RT and transfer to a new PCR tube or plate 5m

Note

Stopping Point. Samples can be safely stored at -20 °C and protocol can be continued at a later date.

cDNA Quantification and Quality Check 45m

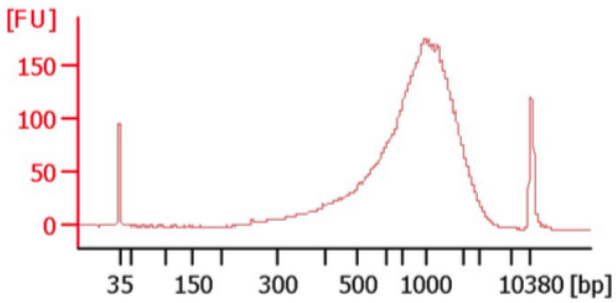
71 Quantify the cDNA using the **Quant-iT PicoGreen dsDNA assay kit** or equivalent Qubit following the manufacturer's protocol. Use 1 µl of clean cDNA for quantification. 10m

72 Quality check the cDNA using the Agilent 2100 Bioanalyzer with **High Sensitivity DNA Analysis Kits**. 45m

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



Library Preparation

73

Note

Before starting, read the library preparation section carefully as there are a few steps that are very time sensitive.

74 Prepare **Fragmentation Mix** 1m

	Reagent	1x
	Ultra II FS Reaction Buffer	1.4 µL
	Ultra II FS Enzyme Mix	0.4 µL
	cDNA (4-8 ng/µL)	2.5 µL

	TE	1.7 µL
	Total	**6 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.

Note

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

75 Vortex the **Fragmentation Mix** for 00:00:05 and immediately proceed to step 70 10s

76 Incubate the Fragmentation reaction as follows: 40m

	Step	Temperature	Time
	Pre-Cool	4 C	∞
	Fragmentation	37 C	5 min
	A Tailing and Phosphorylation	65 C	30 min
	Storage	4 C	∞

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.

Adapter Ligation

1m

77 Prepare **Adapter Ligation Mix** 1m

	Reagent	1x
	NEBNext Ultra II Ligation Master Mix	6 µL
	NEBNext Ligation Enhancer	0.2 µL
	prime-seq Adapter (1.5 µM)	0.5 µL
	Total	**6.7 µL**

78 Add 6.7 µL **Adapter Ligation Mix** to each replicate 1m

79 Incubate for 00:15:00 at 20 °C 15m

Note

Turn off heated lid

80 Add 37.3 µL Buffer EB to Samples 1m

81 Mix Index PCR with 26 µL **SPRI select beads** (ratio of 1:0.52) 1m

Note

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

82 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

83 Place the plate on the magnet stand until clear and **transfer 76 µl supernatant to clean well**. 3m

> Note
>
> Be careful not to discard! This is your sample!

84 Mix supernatant with 10 µL **SPRI select beads** (ratio of 1:0.72) 1m

85 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

86 Place the plate on the magnet stand until clear and discard supernatant. 3m

87 Wash with 150 µL of **80% EtOH** while the plate is on the magnet. Discard the supernatant 1m

88 Repeat wash step once more 1m

89 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.

90 Elute samples in 10.5 µL **0.1X TE** (dilute 1X TE Buffer 1:10 in water) for 00:05:00 5m

Library PCR 1m

91 Transfer samples to clean wells 1m

92 Add 1 µL of **Index Primer (i7, 5 uM)** to each well 1m

Note

This is the unique index that will be used for demultiplexing libraries.

93 Prepare **Library PCR Mix** 1m

	Reagent	**1x**	**5x**
	NEBNext Ultra II Q5 Master Mix	12.5 µL	62.5 µL
	P5NEXTPT5 primer (5uM stock)	1 µL	5 µL
	Total	**13.5 µL**	**67.5 µL**

Note

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

Note

Instead of the universal P5NEXTPT5 primer, one can use unique i5 Indices for each sample. This is recommended if using a sequencer with patterned flow cells to account for index hopping.

94 Add 13.5 µL of **Library PCR Mix** to each well 1m

95 Incubate the **Library PCR** reaction as follows: 20m

	Step	Temperature	Time	Cycles
	Initial Denaturation	98 C	30 sec	1 cycle
	Denaturation	98 C	10 sec	10 cycles *
	Annealing/Elongation	65 C	1 min 15 sec	
	Final Elongation	65 C	5 min	1 cycle
	Storage	4 C	∞	

Note

Adjust the number of cycles based on total cDNA input.

As a general guide we recommend:

	cDNA Input	Cycles
	20 ng	10
	10 ng	11
	5 ng	12

Library Double Size Selection

30s

96 Add 25 µL Buffer EB to Index PCR 1m

97 Mix Index PCR with 26 µL **SPRI select beads** (ratio of 1:0.52) 1m

Note

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

98 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

99 Place the plate on the magnet stand until clear and **transfer** 76 µL **supernatant to clean well**. 30s

Note

Be careful not to discard! This is your library.

100 Mix supernatant with 10 µL **SPRI select beads** (ratio of 1:0.72) 1m

101 Incubate for 00:05:00 at 20 °C (Room Temp) 5m

102 Place the plate on the magnet stand until clear and discard supernatant. 3m

103 Wash with 150 µL of **80% EtOH** while the plate is on the magnet. Discard the supernatant 1m

104 Repeat wash step once more 1m

105 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.

106 Elute in 15 µL **UltraPure Water**. 1m

107 Incubate for 00:05:00 and then place on magnet until clear. Transfer eluted library to new well. 5m

Note

Stopping point. The libraries can be safely stored at -20 °C until they wil be QCed and sequenced.

Library QC 45m

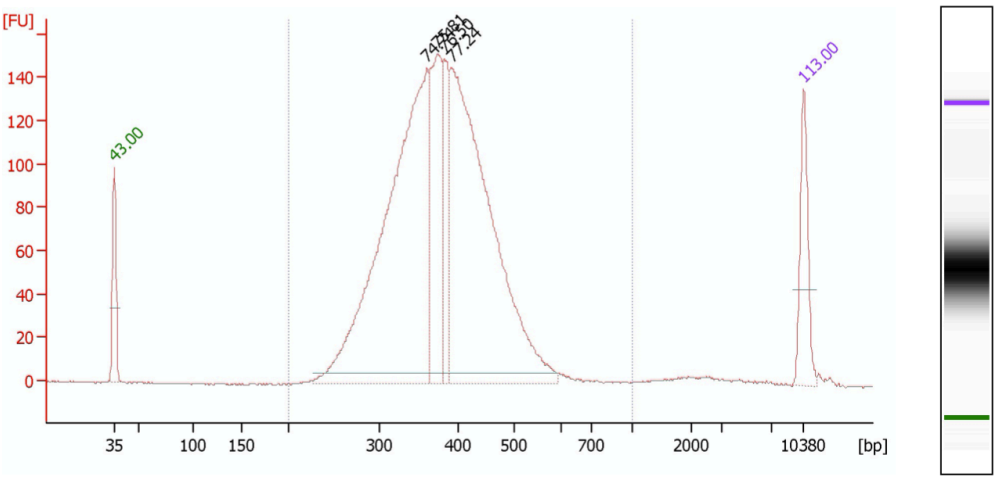
108 Quantify and quality control the library using the Agilent 2100 Bioanalyzer with **High Sensitivity DNA Analysis Kits**. 45m

Note

Bulk libraries often yield high concentrations, which should be diluted to get accurate molarity measurements on the Bioanalyzer. Ideally, do not load more than 2 ng onto the chip.

Expected result

Successful libraries will typically be 2-5 ng/µl concentration



Sequencing

109 Samples should be submitted according to your Sequencing Facility specifications. At least 8 cycles are required for the Index Read (i7) 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 is sufficient.

Some potential sequencing options:

	Read	Read 1	Read 2	Index Read (i7)	Index Read (i5)	Kit
	HiSeq	28	114	8	0	HiSeq 3000/4000 150 cycles
	NextSeq	28	56	8	0	NextSeq 500/

						550 HiOut v3 75 cycle
	NovaSeq	28	94	8	8	NovaSeq SP v1.5 100 cycle