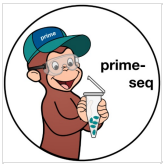

Oct 20, 2020

prime-seq

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2 *Works for me* dx.doi.org/10.17504/protocols.io.s9veh66

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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.

DOI

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PROTOCOL CITATION

Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard 2020. prime-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.s9veh66

KEYWORDS

bulk RNA sequencing, RNA-seq, transcriptomics, SCRB-seq

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PROTOCOL INTEGER ID

15381

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	ACACTCTTTCCCTACACGACGCTCTTCCG ATCT[12 bp BC]NNNNNNNNNNNNNNVTTTTTTTTTT TTTTTTTTTTTTTTTTTTTTVN	
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	AATGATACGGCGACCACCGAGATCTACAC TCTTTCCCTACACGACGCTCTTCCGATCT	
i7 Index Primer (N7XX)	IDT	Trugrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTC TCGTGGGCTCGG	
prime-seq Adapter AntiSense	IDT	Standard Desalting	1.5 µM	/5Phos/CTGTCTCTTATACACATCT	Duplexed DNA
prime-seq Adapter Sense	IDT	Standard Desalting	1.5 µM	GTCTCGTGGGCTCGGAGATGTGTATAAG AGACAGT	Duplexed DNA

Specific barcoded oligodT (E3V7NEXT) sequences:

E3V7_Set1.txt

E3V7_Set2.txt

MATERIALS

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

NAME	CATALOG #	VENDOR
Sera-Mag Speed Beads	65152105050250	Ge Healthcare
Sodium Azide	S2002-100G	Sigma Aldrich
NEBNext Ultra II FS DNA Library Prep Kit for Illumina	E7805S	New England Biolabs

SAFETY WARNINGS

Please follow all Manufacturer safety warnings and recommendations.

DISCLAIMER:

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.

BEFORE STARTING

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

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UltraPure Water	up to 49 mL
Total	**49 mL**

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

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

Lysate vs Extracted RNA

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

on your starting material, please follow one of the following cases:
Step 13 includes a Step case.
Lysate (similar input)
Lysate (variable input)
Extracted RNA

Prepare Bead Binding Buffer 10m

step case

Lysate (similar input)

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

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**

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**

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

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17 ⚠

Add cells or tissue to wells

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.

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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

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

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**

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

UltraPure Water	2.35 µL	258.5 µL
Total	**5 µL**	**550 µL**

37 Add **4 µl** **H2O** 1m

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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	∞	

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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 5m

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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

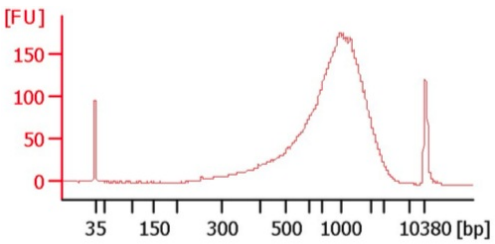
cDNA Quantification and Quality Check 45m

71 10m

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.

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

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.



Library Preparation

73

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

Ultra II FS Enzyme Mix	0.4 µL
cDNA (4-8 ng/µL)	2.5 µL
TE	1.7 µL
Total	**6 µL**

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.

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	∞

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

79 Incubate for **00:15:00** at **20 °C** 15m

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

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

89 Air dry beads for **00:05:00** 5m

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

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**

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

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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	∞	

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

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

Be careful not to discard! This is your library.

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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

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 5m

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 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

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.

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66

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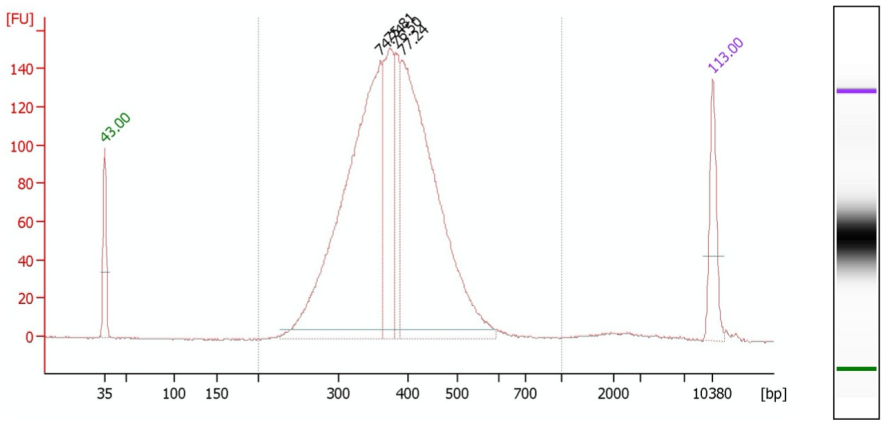

Sequencing

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

Citation: Aleksandar Janjic, Lucas Esteban LEW Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard (10/20/2020). prime-seq. https://dx.doi.org/10.17504/protocols.io.s9veh66