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Apr 04, 2022

prime-seq V.2

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Cost-efficient library generation by early barcoding has been central in propelling single-cell RNA sequencing. Here, we optimize and validate prime-seq, an early barcoding bulk RNA-seq method. We show that it performs equivalently to TruSeq, a standard bulk RNA-seq method, but is fourfold more cost-efficient due to almost 50-fold cheaper library costs. We also validate a direct RNA isolation step, show that intronic reads are derived from RNA, and compare cost-efficiencies of available protocols. We conclude that prime-seq is currently one of the best options to set up an early barcoding bulk RNA-seq protocol from which many labs would profit.

DOI

dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

https://genomebiology.biomedcentral.com/articles/10.1186/s13059-022-02660-8

Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth 2022. prime-seq. **protocols.io** https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2
Aleksandar Janjic

protocol

Janjic, A., Wange, L.E., Bagnoli, J.W. et al. Prime-seq, efficient and powerful bulk RNA sequencing. Genome Biol 23, 88 (2022). https://doi.org/10.1186/s13059-022-02660-8

- Updated volumes for double size selection - Changes in language to make sections clearer - Updated publication link

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

protocol ,

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- 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:

A	B	C	D	E	F
Oligo	**Vendor**	**Purification**	**Working Conc.**	**Sequence**	**Notes**
Barcoded Oligo-dT (E3V7NEXT)	Sigma	Cartridge	10 µM	ACACTCTTTCCCTACACGACGCTCTTCCGATCT[12 bp BC]NNNNNNNNNNNNNNNVTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTVN	
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 (Nextera)	IDT	Trugrade	5 µM	CAAGCAGAAGACGGCATACGAGAT[i7]GTCTCGTGGGCTCGG	
i5 Index Primer (TruSeq)	IDT	Trugrade	5µM	AATGATACGGCGACCACCGAGATCTACAC[i5]ACACTCTTTCCCTACACGACGCTCTTCCGATCT	
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

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 **Sigma Aldrich Catalog #M3148**

QuantiFluor(R) RNA System **Promega Catalog #E3310**

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

☒ Proteinase K solution, 20 mg ml –
1 **Ambion Catalog #AM2546**
☒ 5 M Sodium chloride (NaCl) **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 **Sigma**
Aldrich Catalog #E7889
☒ Ethanol absolute **Carl**
Roth Catalog #9065.4
☒ Igepal **Sigma**
Aldrich Catalog #I8896
☒ KAPA HiFi 2x RM **Kapa**
Biosystems Catalog #KR0370
☒ Poly(ethylene glycol) **Sigma**
Aldrich Catalog #89510
☒ UltraPure DNase/RNase Free Distilled Water **Contributed by**
users Catalog #10977-049
☒ Trizma hydrochloride solution **Sigma**
Aldrich Catalog #T2694
☒ Aluminium seals for cold storage **Contributed by**
users Catalog #391-1275
☒ Filter tips 96 low retention 10 uL **Contributed by**
users 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 **Sigma**
Aldrich Catalog #S2002-100G

Please follow all Manufacturer safety warnings and recommendations.

:

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

4 **When running the protocol for the first time prepare Cleanup Beads (see end of the protocol)!** 45m

Lysate vs Extracted RNA

5 ⚠

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 5 includes a Step case.
Lysate (similar input), Direct Lysis
Lysate (variable input)
Extracted RNA

First Time Setup

step case

Lysate (similar input), Direct Lysis

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

6 When running the direct lysis protocol for the first time, prepare Bead Binding Buffer (see end of the protocol)!

Sample Collection

7 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

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

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9 ⚠

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.

10 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

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

12 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

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

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

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

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

After adding EtOH, incubate for 30 s so that all beads are bound to magnet.

17 Repeat wash step once more 2m

18 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

19 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.

20 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**

21 Add **15 µL** of **DNase I Mix** and mix by pipetting 2m

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

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

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

26 Repeat wash step once more 2m

27 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**.

Reverse Transcription 5m

28 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**

29 Add **4 µL** **H2O** to the beads 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.

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

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

cDNA Pooling & Purification 5m

33 Place the plate on a magnet 3m

34 Pool the supernatant of all wells into a 2 mL tube 10m

35 Add **10 µL** of **Cleanup Beads (22% PEG)** ***per 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.

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

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

38 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.

39 Repeat wash step once more 1m

40 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.

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

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

Exonuclease I Treatment 35m

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

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

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

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

47 Wash with **50 µL** of **80% EtOH** while the tube is on the magnet, discard the supernatant 1m

48 Repeat wash step once more 1m

49 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.

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

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

Full length cDNA Amplification 1m

52 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**

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

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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	∞	

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

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

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

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

58 Wash with **100 µL** of **80% EtOH** while the tube is on the magnet, discard the supernatant 1m

59 Repeat wash step once more 1m

60 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.

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

62 5m

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

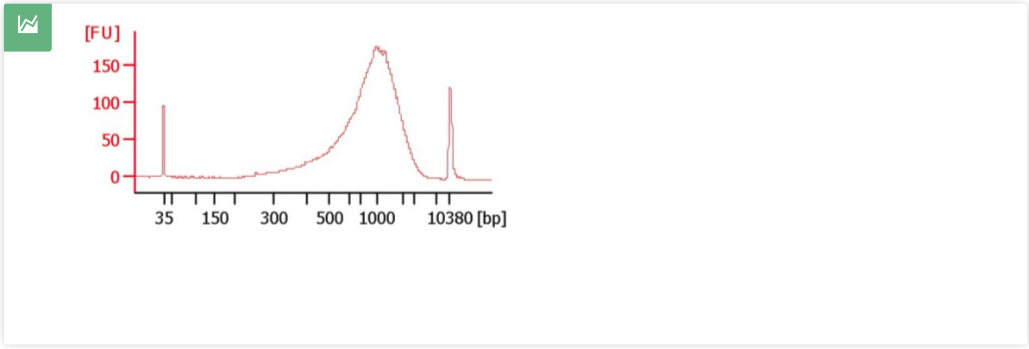
63 10m

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.

64 45m

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

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

65

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

66 1m

Prepare **Fragmentation Mix**

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**

Ensure that the Ultra II FS Reaction Buffer is completely thawed. If a precipitate is seen in the buffer, pipette up

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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.

67 Vortex the **Fragmentation Mix** for **00:00:05** and immediately proceed to step 67 10s

68 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 20m

69 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**

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

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

Turn off heated lid

72 Add **37.3 µL** Buffer EB to Samples 1m

73 Mix Sample with **26 µL** **SPRI select beads** 1m

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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

The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

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

75 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!

76 Mix supernatant with **10 µL** **SPRI select beads** 1m

The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

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

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

79 Wash with **150 µL** of **80% EtOH** while the plate is on the magnet, discard the supernatant 1m

80 Repeat wash step once more 1m

81 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.

82 Elute samples in **11 µL** **0.1X TE** (dilute 1X TE Buffer 1:10 in water) for **00:05:00** 5m

Library PCR 45m

83 Transfer **10.5 µL** of samples to clean wells

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

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

85 Add **1 µL** of **Index Primer (TruSeq i5, 5 uM)** to each well

Alternatively the universal primer P5NEXTPT5 can be used in case the second index will not be sequenced.

86 Prepare **Library PCR Mix** by adding **12.5 µL**

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

87 Incubate the **Library PCR** reaction as follows:

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 cDNA input.

As a general guide we recommend:

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

Double Size Selection 25m

88 Add **25 µL** Buffer EB to Index PCR

89 Mix Index PCR with **26 µL** **SPRI select beads**

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

selection properties.

The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

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

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

Be careful not to discard! This is your library.

92 Mix supernatant with **10 µL** **SPRI select beads**

The volume of SPRI select beads used during library size selection can be adjusted based on desired library size. Optimization for your samples may be required.

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

94 Place the plate on the magnet stand until clear and discard supernatant.

95 Wash with **150 µL** of **80% EtOH** while the plate is on the magnet, discard the supernatant

96 Repeat wash step once more

97 Air dry beads for **00:05:00**

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

98 Elute in **15 µL** **UltraPure Water**.

99 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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

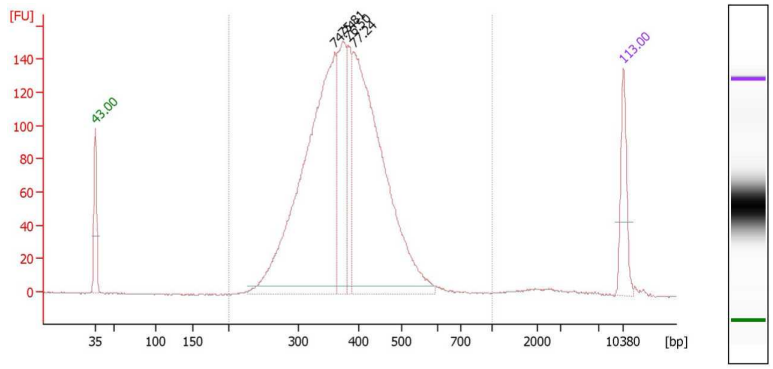
Stopping point: The libraries can be safely stored at -8 -20 °C until they will be QCed and sequenced.

QC and quantification 45m

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

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.

Libraries will typically exceed 1-5 ng/µl concentration



Sequencing 1m

101 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 Read (i7) and 28 cycles for the Read 1 (BC+UMI). Dual index sequencing can be done when using patterned flowcells. 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:

A	B	C	D	E	F
Sequencer	**Read 1**	**Read 2**	**Index Read (i7)**	**Index Read (i5)**	**Kit**
NovaSeq	28	94	8	8	NovaSeq SP v1.5 100 cycle
NextSeq 500/550	28	56	8	0	NextSeq 500/550 HiOut v3 75 cycle
NextSeq 1000/2000	28	94	8	8	NextSeq 1000/2000 P2 100 cycle
NextSeq 2000	28	52	8	0	NextSeq 2000 P3 50 cycles
HiSeq	28	114	8	0	HiSeq 3000/4000 150 cycles

Prepare Cleanup Beads (22% PEG) 10m

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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**

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 10m

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

105 Place on magnet stand and remove storage buffer 1m

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

107 Place on magnet stand and remove supernatant 30s

108 Repeat wash step one more time 1m

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

110 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

Prepare Bead Binding Buffer 10m

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

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2

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.

Citation: Aleksandar Janjic, Lucas Esteban Wange, Johannes JWB Bagnoli, Johanna Geuder, Phong Nguyen, Daniel Richter, Christoph Ziegenhain, Wolfgang Enard, Beate Vieth prime-seq https://dx.doi.org/10.17504/protocols.io.81wgb1pw3vpk/v2