VERSION 1

OCT 20, 2020

OPEN ACCESS

DOI:

dx.doi.org/10.17504/protocol.s.io.s9veh66

External link:

<https://doi.org/10.1038/s41598-021-82883-0>

Protocol Citation: Aleksandar Janjic, Lucas Esteban 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>

MANUSCRIPT CITATION:

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

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prime-seq V.1

Scientific Reports

In 1 collection

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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 mcSCR-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.

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

Created: Sep 05, 2018

Last Modified: Oct 20, 2020

PROTOCOL integer ID:
15381

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

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	ACACTCTTTCCCTACACGA CGCTCTTCCGATCT[12 bp BC]NNNNNNNNNNNNNNNN VTTTTTTTTTTTTTTTTTTT TTTTTTTTTTTVN	
Template Switching Oligo (TSO) (E5V7NEXT)	Sigma	RNase-Free HPLC	100 µM	Biotin- ACACTCTTTCCCTACACGA CGCrGrGrG	
Preamp Primer (SINGV6)	Sigma	Standard Desalting	10 µM	Biotin- ACACTCTTTCCCTACACGA CGC	
3' enrichment primer (P5NEXTPT5)	Sigma	Standard Desalting	5 µM	AATGATACGGCGACCACC GAGATCTACACTCTTTCCC TACACGACGCTCTTCCGAT CT	
i7 Index Primer (N7XX)	IDT	Trugrade	5 µM	CAAGCAGAAGACGGCATA CGAGAT[i7]GTCTCGTGGG CTCGG	
prime-seq Adapter AntiSense	IDT	Standard Desalting	1.5 µM	/5Phos/CTGTCTCTTATAC ACATCT	Duplexed DNA
prime-seq Adapter Sense	IDT	Standard Desalting	1.5 µM	GTCTCGTGGGCTCGGAGAT GTGTATAAGAGACAGT	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
β -mercaptoethanol 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) 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

⊗ NEBNext Ultra II FS DNA Library Prep Kit for Illumina New England Biolabs Catalog #E7805S

SAFETY WARNINGS

⚠ Please follow all Manufacturer safety warnings and recommendations.

BEFORE START INSTRUCTIONS

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

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 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

10m

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)**

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
H ₂ O	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 μ L 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 $-80\text{ }^{\circ}\text{C}$ 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
- 20 Incubate for 00:15:00 at $50\text{ }^{\circ}\text{C}$ and then heat inactivate the Proteinase K for 00:10:00 at $75\text{ }^{\circ}\text{C}$

1m

25m

Bead Clean Up

20m

- 21 Mix each bulk sample (50 μL per well) with 100 μL of **Cleanup Beads (22% PEG)**
- 22 Incubate for 00:05:00 at $20\text{ }^{\circ}\text{C}$ (Room Temp)

1m

5m

Note

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

- 23 Place on magnet stand until clear ($\sim 3\text{ min}$) 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 μ L	1.1 mL
H2O	2 μ L	220 μ L
Total	15 μ L	1.65 mL

- 29 Resuspend beads in 15 μ L 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 μ L 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 μ L 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 µM)	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 $^{\circ}\text{C}$ 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 μ L 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 μ L of **Exol Buffer (10x)** and 1 μ L 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 μM)	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 μ L 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 μ L **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 μ L 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 μ L
Ultra II FS Enzyme Mix	0.4 μ L
cDNA (4-8 ng/ μ L)	2.5 μ L

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 μ L **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 will 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/ μ l 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
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