Jul 25, 2024

CTLR-Seq Protocol

DOI
dx.doi.org/10.17504/protocols.io.q26g71d7kgwz/v1

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DOI: dx.doi.org/10.17504/protocols.io.q26g71d7kgwz/v1

Protocol Citation: Bo Zhou, GiWon Shin, Yiling Huang, Raegan N. Wood, Hanlee P. Ji, Alexander E. Urban 2024. CTLR-Seq Protocol. **protocols.io** https://dx.doi.org/10.17504/protocols.io.q26g71d7kgwz/v1

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

Created: April 15, 2024

Last Modified: July 25, 2024

Protocol Integer ID: 104107

Keywords: High Molecular Weight (HMW) DNA, Nanopore Sequencing, High Molecular Weight Library Systems (HLS)

Funders Acknowledgement:
Dr. Alexander E Urban
Grant ID: NIH grants: P50HG00773506, R01MH100900, and HG010359. Additional funding from Bruce Blackie, the Jaswa Innovator Award, the Stanford Psychiatry Genetics of Schizophrenia fund, NIH DP2 New Innovator Award (DP2 MH100010-01), Uytengsu-Hamilton 22q11 Neuropsychiatry Research Award
Dr. Hanlee P Ji
Grant ID: NIH grants: R01HG006137-04, P01HG00205ESH, and U01HG010963. Additional support from the Research Scholar Grant, RSG-13-297-01-TBG (American Cancer Society), National Science Foundation Award 1807371, and the Clayville Foundation.
Dr. Bo Zhou
Grant ID: NIH grants: K01MH129758 and T32HL110952. Additional funding from the Stanford Maternal and Child Health Research Institute Instructor K Support Award, Uytengsu-Hamilton 22q11 Neuropsychiatry Research Award

Abstract

We developed a generally applicable method CRISPR/Cas9-targeted long read sequencing (CTLR-Seq) to resolve, haplotype-specifically, and at base-pair resolution, large, complex, and highly repetitive genomic regions that had been previously impenetrable to next-generation sequencing analysis, i.e. large segmental duplication (SegDup) regions and their associated genome rearrangements that often stretch hundreds of kilobases. CTLR-Seq combines in vitro Cas9-mediated cutting of the genome and pulse-field gel electrophoresis to haplotype-specifically isolate intact large (100-2000 kb) regions that encompass previously unresolvable genomic sequences. These targets are then sequenced (amplification-free) with up to 250x on-target coverage using nanopore sequencing, allowing for their complete sequence assembly.

Guidelines

- High Molecular Weight (HMW) DNA is long and fragile. Pipetting DNA elutions should always be very slow and very gentle, using wide orifice (WO) pipette tips.
- Over-drying beads during cleanup steps will result in a decreased yield.
- Use a fresh box of pipette tips for steps involving RNA.

Additional Notes:

1. Custom gRNAs are assay-specific. Design gRNAs to be specific to targets.
2. Sage Science recommends NEB EnGen Spy Cas9 HF1 for the Cas9 nuclease, but NEB wild-type Cas9 nuclease was used in this protocol.
3. Sage Science recommends the final total concentration of Cas9 complexes to be at 1uM in 1X HLS Enzyme Buffer.
4. Sage Science's PCR protocol does not call for dilution, but instead uses bCD reagent at 0.1%.

Data Analysis

required software:

minimap2 (version 2.26)

https://github.com/lh3/minimap2

seqtk

https://github.com/lh3/seqtk

samtools (version 1.19)

https://github.com/samtools/

pod5 (version 3.10)

https://github.com/nanoporetech/pod5-file-format

flye (vesion 2.6)

https://github.com/fenderglass/Flye

dorado (version 0.5.3)

https://github.com/nanoporetech/dorado

bedtools

https://github.com/arq5x/bedtools2

```
# align reads to reference genome

ref=GCA_000001405.15_GRCh38_no_alt_plus_hs38d1_analysis_set.fna
minimap2 -t 4 -ax map-ont combined_nanopore_reads.fastq.gz | samtools
sort -@ 30 - | samtools view -@ 30 -bh - >
combined_nanopore_reads.hg38.bam


# select target region of interest

samtools view -bh combined_nanopore_reads.hg38.bam chr:start-end > target.hg38.bam

# select read names in target region

samtools view target.hg38.bam | cut -f1 | sort | uniq >
target.hg38.list

# obtain pod5 files for these regions

pod5 filter ${pod5_directory} --ids target.hg38.list -M -o
target.hg38.pod5

# basecall selected reads into super accuracy

dorado basecaller sup target.hg38.pod5 > target.hg38.sup.bam

# convert bam to fastq

bedtools bamtofastq -i target.hg38.sup.bam -fq target.hg38.sup.fastq

# (optional) select reads from specific regions within target region

bedtools intersect -a target.hg38.bam -b ${specific_region}.bed -wa -f
0.99 | samtools view - | cut -f1 | sort | uniq > target.specific_region.list

seqtk subseq target.hg38.sup.fastq > target.specific_region.fastq

# assemble reads

flye --threads 12 --nano-corr target.specific_region.fastq -g
${estimated_target_size} -o ${output_directory} --min-overlap 5000
```

Materials

A	B	C
Material	**Vendor**	**Catalog Number**
Qubit 1X DS DNA High Sensitivity Assay Kit	Thermo Fischer Scientific (Waltham, MA, USA)	Q32851
Alt-R® CRISPR-Cas9 tracrRNA, 20 nmol	Integrated DNA Technologies (Coralville, IA, USA)	1072533
Custom Alt-R® gRNA, 10 nmol	Integrated DNA Technologies (Coralville, IA, USA)	
HLS-CATCH Cassette Kit.	Sage Science (Beverly, MA, USA)	HIT0004 or HIT0012
Hi-Bead HMW DNA Concentration Kit	Sage Science (Beverly, MA, USA)	HBK0012
AMPure XP Bead-Based Reagent	Beckman Coulter Life Sciences (San Jose, CA, USA)	A63880
KAPA HyperPlus Kit	Roche Holding AG (Basel, Switzerland)	KK8514
Duplex Buffer	Integrated DNA Technologies (Coralville, IA, USA).	11-04-02-01
TaqMan™ Universal PCR Master Mix	Applied Biosystems by Thermo Fischer Scientific (Waltham, MA, USA)	4364340
StepOne Real-Time PCR System	Thermo Fischer Scientific (Waltham, MA, USA)	4376357
1.5 ml PCRclean tube DNA LoBind	Eppendorf (Hamburg, Germany)	0030108051
UltraPure DNase/RNase-Free Distilled Water	Invitrogen – Thermo Fischer Scientific (Waltham, MA, USA)	10977-049
0.75% agarose cassette	Sage Science (Beverly, MA, USA)	HIT0004
NEB EnGen Spy Cas9 HF1, alternatively use wild-type Cas9 nuclease	New England Biolabs (Ipswich, MA, USA)	M0667M
TaqMan™ Copy Number Reference Assay, human, RNase P (VIC probe)	Thermo Fischer Scientific (Waltham, MA, USA)	4403328
Custom TaqMan™ Copy Number Assay (FAM probe)	Thermo Fischer Scientific (Waltham, MA, USA)	4400296

Qubit™ dsDNA HS Assay Kit **Invitrogen - Thermo Fisher Catalog #**Q32851

- Alt-R CRISPR-Cas9 system **IDT Catalog #**1072533
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- Nuclease Free Water **IDT Technologies Catalog #**11-04-02-01
- TaqMan™ Universal PCR Master Mix **Thermo Fisher Catalog #**4364340
- StepOne™ Real-Time PCR System **Thermo Fisher Catalog #**4376357
- DNA LoBind Tubes, 1.5 mL **Eppendorf Catalog #**0030108051
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Catalog #**10977049
- EnGen® Spy Cas9 HF1 | - 2500 pmol **New England Biolabs Catalog #**M0667M
- TaqMan™ Copy Number Reference Assay, human, RNase P **Thermo Fisher Catalog #**4403328
- Custom TaqMan™ Copy Number Assay **Thermo Fisher Catalog #**4400296
- HLS-CATCH Cassette Kit **sage science Catalog #**HIT0004

Equipment:

This protocol requires a Sage HLS HMW Library system (Sage Science), Oxford Nanopore Technologies Sequencer, and a StepOne Real-Time PCR System or equivalent.

crRNA pools can also be used for multiple target enrichment:

	A	B	C	D
	Tube 1	**Tube 2**		
	crRNA 1	4 µL	crRNA 2	4 µL
	tracrRNA	2.6 µL	tracrRNA	2.6 µL
	Duplex buffer	19.4 µL	Duplex Buffer	19.4 µL
	Total Volume	**22 µL**	**Total Volume**	**22 µL**

Adjust volume accordingly to not exceed what is recommended below.

Add these to the respective PCR tubes:

	A	B	C	D
	Tube 1		**Tube 2**	

	A	B	C	D
	crRNA 1 with tracr RNA (annealed)	22 µL	crRNA 2 with tracr RNA (annealed)	22 µL
	4X enzyme buffer	10 µL	4X enzyme buffer	10 µL
	Cas9 (20 µM)	8 µL	Cas9 (20 µM)	8 µL
	Total Volume	**40 µL**	**Total Volume**	**40 µL**

qPCR Assay

	A	B	C
	Component	**Volume per reaction**	**Volume total (for 25 reactions + 10%)**
	Master mix	5µL	137.5µL
	bCD	2µL	55µL
	probe	0.5µL	13.75µL
	RNase P	0.5µL	13.75µL
	Total	8µL	220µL

Prepare the Priming Mix according to the table:

	A	B
	Flow Cell Flush (FCF)	1175µL
	Flow Cell Tether (FCT)	30µL
	Total Volume	1200µL

Prepare the Library according to the table:

A	B
DNA Library	32µL
Sequencing buffer (SB)	100µL
Library Beads (LIB)	68µL
Total volume	200

Add the master mixes to a 96 amp FAST plate according to the following layout:

P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
									Ctrl	Ctrl	Ctrl

Probe 1 master mix (8µL)
Probe 2 master mix (8µL)
Probe 3 master mix (8µL)

Prepare one master mix for each probe used.

Before start

This protocol takes at least 5 days to complete, due to overnight incubation steps.

Sample Handling:

10m

1 Count number of cells in sample.

2 Wash cells 3X with phosphate-buffer

15m

saline (PBS). Centrifugation should be done at 100 g - 200 g for 00:05:00 - 00:10:00, depending on cell type.

3 Resuspend pellet in 60 µL of Sage Science M2 Buffer per million cells counted prior to washing.

4 Store On ice.

Qubit Quantification:

3m 35s

5 Gently mix the cell suspension, slowly with a WO pipette tip, to encourage solution homogeny.

6 Obtain two 1.5 mL Eppendorf tubes and transfer 10 µL of cell suspension to each of the two tubes.

7 Add 190 µL of Qubit Lysis Buffer to each tube, and vortex at maximum speed for 00:00:45.

45s

8 Briefly spin down (< 00:00:01).

1s

9 Add 600 µL of TE buffer to each tube and vortex at maximum speed for 00:00:45.

45s

10 Briefly spin down (< 00:00:01).

1s

11 Add 5 µL of lysate from each tube to a corresponding Qubit Assay tube, along with 195 µL of Qubit 1X dsDNA High Sensitivity Working Solution.

12 Briefly vortex (~ 00:00:03) to mix and spin down. 3s

13 Incubate at Room temperature for 00:02:00 before quantifying. 2m

14 Quantify with Qubit machine for 1x dsDNA High Sensitivity and record the concentration per mL.

15 Calculate the average concentration between the two replicates.

16 Multiply the average concentration per mL by 3,200 to calculate the total concentration of DNA in the sample.

17 Dilute the cell suspension in M2 buffer so that 70 µL contains up to 5 µg of genomic DNA.

Sage Science HLS CATCH

18 Prepare cassettes:

- Prepare cassettes in accordance with the Sage Science HLS-CATCH Protocol.
- Select the positions being used and run the “check current” protocol.

Extraction 1h

19 Remove the contents of the elution wells and replace with 80 µL running buffer.

20 Remove the contents of the sample well and replace with 70 µL of cell suspension.

21 Remove the contents of the reagent well and replace with 180 µL of 3% SDS HLS Lysis Reagent.

22 Tape the cassettes and close the lid.

23 Run the appropriate workflow depending on size of target of interest (e.g., workflow 'CATCH 300-1000kb inj80V 2m sep3h.shflow.')

24 After approximately one hour of run time, begin preparing the guide RNAs (can also be prepared up to 72 hours prior to start of experiment and store

at 4 °C).

Guide RNA Preparation

20m

25 Ensure that the tracrRNA and gRNA is diluted in duplex buffer to 100 undetermined (recommended to measure RNA concentration using Qubit RNA kits to ensure correct concertation.

26 In clean RNase-free PCR tubes, prepare the following On ice :

> **Note**
>
> If using sgRNA, use the volume suggested for tracrRNA.

27 crRNA pools can also be used for multiple target enrichment, but adjust volume accordingly to not exceed what is recommended below.

A	B	C	D
Tube 1		**Tube 2**	
crRNA 1	4 µL	crRNA 2	4 µL
tracrRNA	2.6 µL	tracrRNA	2.6 µL
Duplex buffer	19.4 µL	Duplex Buffer	19.4 µL
Total Volume	**22 µL**	**Total Volume**	**22 µL**

28 Mix well and spin down.

29 Incubate the guide RNAs at 95 °C for 00:05:00 . 5m

30 Allow the guide RNAs to cool at Room temperature for 00:05:00 . 5m

31 Add the following to the respective PCR tubes:

	A	B	C	D
	Tube 1		**Tube 2**	
	crRNA 1 with trac r RNA (annealed)	22 µL	crRNA 2 with tracr RNA (annealed)	22 µL
	4X enzyme buffer	10 µL	4X enzyme buffer	10 µL
	Cas9 (20 µM)	8 µL	Cas9 (20 µM)	8 µL
	Total Volume	**40 µL**	**Total Volume**	**40 µL**

32 Mix well and spin down.

33 Incubate the tubes at 37 °C for 00:10:00 . 10m

34 Combine **Tube 1** and **Tube 2** as **Cas9 complex mixture** and leave On ice (or 4 °C for longer storage until needed).

35 When ready for treatment step below (i.e., 5-10 minutes prior to start of Treatment step below), dilute **Cas9 complex mixture** 4-fold with 1x Enzyme Buffer (e.g., add 240 µL of 1x Enzyme Buffer to 80 µL of **Cas9 complex mixture**).

Treatment

4m

36 After the extraction phase is complete, remove 5 mL of buffer from the (+) electrode port of each cassette lane, where the SDS is concentrated, and replace with 5mL of fresh running buffer.

37 Carefully remove the tape from each cassette (slowly peel starting from the upper right corner to avoid contaminating elution wells) and remove the contents of the sample and reagent wells.

38 Add 80 µL of the **diluted Cas9 complex mixture** to the sample wells of each cassette.

39 Add 220 µL of Sage 1X enzyme buffer to the reagent wells of each cassette.

40 Make sure that there is no meniscus or protrusion by adding or removing running buffer from cassettes if needed.

41 Close the lid and press 'OK.'

> **Note**
>
> Do not tape the cassettes.

42 Injection of Cas9 complex will start and last for approximately 00:02:00 - 00:04:00 (depending on selected workflow. When complete, open the lid and remove the contents of the sample wells.

4m

43 Add 80 µL of enzyme buffer to the sample wells. Do not tape the cassettes.

44 Close the lid and press 'OK.'

45 After 30 minutes, the machine will pause. Open the lid and empty the reagent wells.

46 Add 180 µL of SAGE SCIENCE 1% SDS Lysis Reagent to the reagent wells.

47 Seal the cassettes with tape.

48 Top off the running buffer such that there is no meniscus or protrusion.

49 Close the lid and press 'OK.'

Collection

8h

50 The run will complete after several hours. For optimal elution, allow the cassettes to sit undisturbed Overnight . 8h

51 Open the lid and remove the tape from the cassettes.

52 Use a WO pipette tip to remove the contents of the elution wells. Pipette very slowly by hand.

53 Place the contents into labeled PCR strip tubes.

qPCR Assay

11m 15s

54 Perform a 1:30 dilution by adding 1 µL of elution to 29 µL of water.

55 Briefly vortex the dilutions to mix. Spin down.

56 Prepare Master Mixes according to the following volumes:

57 Beta-cyclodextrin (bCD) is provided in HLS-CATCH Cassette Kit (Sage Science).

58

A	B	C
Component	**Volume per reactio n**	**Volume total (for 25 reactions + 10%)**
Master mix	5µL	137.5µL
bCD	2µL	55µL
probe	0.5µL	13.75µL
RNase P	0.5µL	13.75µL
Total	8µL	220µL

59 Prepare one master mix for each probe used (using 3 probes here as example).

60 Add the master mixes to a 96 amp FAST plate according to the following layout:

P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
P1E1	P1E2	P1E3	P1E4	P1E5	P1E6	P3E1	P3E2	P3E3	P3E4	P3E5	P3E6
P2E1	P2E2	P2E3	P2E4	P2E5	P2E6	P4E1	P4E2	P4E3	P4E4	P4E5	P4E6
									Ctrl	Ctrl	Ctrl

Probe 1 master mix (8µL)
Probe 2 master mix (8µL)
Probe 3 master mix (8µL)

61 Add 2 µL of dilution and 2.5 µL control DNA according to the schematic above.

62 Mix with a pipette set to 8µL.

63 Seal plate and spin down.

64 Run qPCR protocol on StepOne according to the following protocol:

64.1 Denature 95 °C for 00:10:00 . 10m

64.2 50 cycles of 00:00:15 at 95 °C and 00:01:00 at 60 °C . 1m 15s

Qubit Measurement

65 Based on the results of the qPCR, combine the elutions that have target enrichment in an Eppendorf LoBind 1.5 mL tube.

66 Quantify target elution using Qubit 1X dsDNA High Sensitivity kit. Combine elution modules with target enrichment if appropriate.

Hi Bead Cleanup 9h 8m 1s

67 Bring Binding Buffer and Hi-Bead suspension to Room temperature .

68 Add an equivalent volume of Binding Buffer to the combined elution. Allow the Binding Buffer to gently drip into the elution.

69 Gently incorporate the buffer into the elution by rocking back and forth. Avoid flicking or tapping.

70 Place the tube in a rotator at a 45° - 90° angle to ensure gentle mixing. Mix on the rotator for 00:05:00 . 5m

71 Vortex the Hi-Bead suspension.

72 Add the Hi-Bead Suspension in a 0.6:1 ratio of bead volume to elution volume. Add the beads to the side of the tube and allow it to gently drip into the elution.

73 Gently rock the tube back and forth 5 times to incorporate the beads.

74 Place the tube in the rotator at a 45° - 90° angle for 00:20:00 . 20m

75 Briefly spin (< 00:00:01), and place on a magnet. 1s

76 When the solution is clear, discard the supernatant.

77 Add 80% ethanol to the tube in a 3:1 ratio of ethanol volume to elution volume. Let it sit for 00:03:00 . 3m

78 Remove the supernatant.

79 Repeat the wash step for a total of 2 washes.

80 Spin the tube and re-magnetize to remove residual ethanol. Do not allow the beads to dry out.

81 Add 50 µL 10mM Tris buffer and gently rock back and forth to resuspend the beads.

82 Incubate at 55 °C for 00:10:00 . 10m

83 Decrease the heat to 37 °C and shake at 300 rpm for 00:15:00 . 15m

84 Gently resuspend the beads using a magnet and gentle rocking.

85 Shake at 300 rpm, 37°C, 00:15:00 . 15m

86 Place the tube at 4 °C Overnight . 8h

87 Magnetize the beads to disturb the bead pellet and gently rock the tube back and forth to resuspend the beads.

88 Magnetize and transfer the supernatant (50 µL) to a pcr tube by pipetting very slowly with a WO pipette tip.

89 Quantify elution using Qubit 1X dsDNA High Sensitivity kit.

ER&AT 1h

90 Add 7 µL of KAPA ERAT buffer to the 50µL of eluted DNA from the beads.

91 Add 3 µL ERAT enzyme to the DNA, such that the total volume now is 60µL.

92 Mix well by pipetting very slowly with a WO pipette tip.

93 Incubate at 20 °C for 00:30:00 and then at 65 °C for 00:30:00 . 1h

Ligation 10h

94 Mix the Ligation buffer (LNB) because it is viscous.

95 Add 25 µL of LNB to the 60 µL ERAT product.

96 Add 10 µL of KAPA Ligase.

97 Add 5 µL of Ligation Adapter (LA).

98 Mix well by pipetting very slowly with a WO pipette tip.

99 Incubate at 20 °C for 05:00:00 and then at 4 °C Overnight . 13h

AMPure XP Bead Cleanup 8h 56m

100 Transfer the ligation product from the PCR strip tube to a 2.0 mL round bottom LoBind tube. The total volume is 100µL.

101 Vortex AMPure XP Beads (AXP) and add 50 µL AXP beads to the ligation product.

102 Gently rock the tube back and forth to incorporate.

103 Mix on the rotator at a 45° – 90° degree angle for 00:20:00 . 20m

104 Spin down and magnetize.

105 Once the solution is clear, remove the supernatant.

106 Add 250 µL of Long Fragment Buffer (LFB) and gently rock the tube back and forth to resuspend the beads.

107 Spin down and return the tube to the magnet.

108 Once the solution is clear, discard the supernatant.

109 Add another 250 µL of LFB and repeat the previous wash step for a total of 2 washes.

110 Discard the supernatant and resuspend the pellet in 33 µL of Elution Buffer (EB).

111 Place the tube on the rotator horizontally for 00:05:00 . 5m

112 Spin down and incubate at 37 °C for 00:15:00 with 300 rpm shaking. 15m

113 Gently resuspend the beads using a magnet and gentle rocking.

114 Shake at 300 rpm, 37°C, 00:15:00 . 15m

115 Place the tube at 4 °C Overnight . 8h

116 Magnetize the beads for at least 00:01:00 . 1m

117 Transfer 33 µL of supernatant to a new 1.5 mL LoBind tube.

Qubit Measurement

118 Quantify target elution using Qubit 1X dsDNA High Sensitivity kit (optional). *

Priming and Loading the Flow Cell 1h 10m

119 Allow an Oxford Nanopore R10 Flow Cell to reach Room temperature .

120 Insert the flow cell into the ONT promethION Sequencing Machine.

121 Select the flow cell position and start the flow cell check.

122 Thaw, vortex, and spin down the Sequencing Buffer (SB), Library Beads (LIB) and Flow Cell Tether (FCT).

123 Prepare the Priming Mix according to the following table:

	A	B
	Flow Cell Flush (FCF)	1175µL
	Flow Cell Tether (FCT)	30µL
	Total Volume	1200µL

124 Record the number of available pores from the flow cell check.

125 Open the flow cell port and turn a P1000 to 200µL.

126 Slowly rotate up the volume from 200 µL to 230 µL so that a small amount of liquid enters the tip. Discard the extracted liquid.

127 Add 550 µL of priming mix, and incubate for 00:05:00 .

5m

128 Mix the Library Beads (LIB) well by pipette.

129 Prepare the Library according to the following table:

	A	B
	DNA Library	32µL
	Sequencing buffer (SB)	100µL
	Library Beads (LIB)	68µL
	Total volume	200

130 Rotate up another small volume from the flow cell port and discard the liquid.

131 Add another 550 µL of priming mix and incubate for 00:05:00 . 5m

132 Enter the run parameters such that the following are selected:

- DNA
- PCR-free
- LSK114 kit
- Minimum read length 1000
- High accuracy base calling
- 99 hour run time

133 Rotate up another small volume from the flow cell port and discard the liquid.

134 Load the 200µL Library into the flow cell port.

135 Allow the Library to incubate in the flow cell for 01:00:00 . 1h

136 Begin sequencing.

Protocol references

Citations:

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