Jan 05, 2026

Improved Cas9-targeted nanopore sequencing facilitates ultra-deep analysis of genomic variation

DOI
https://dx.doi.org/10.17504/protocols.io.n92ldrq5ng5b/v1

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DOI: https://dx.doi.org/10.17504/protocols.io.n92ldrq5ng5b/v1

Protocol Citation: Nick Vereecke, Megan Behrmann, Anupama Khare, John Dekker 2026. Improved Cas9-targeted nanopore sequencing facilitates ultra-deep analysis of genomic variation. **protocols.io** https://dx.doi.org/10.17504/protocols.io.n92ldrq5ng5b/v1

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

Created: January 14, 2025

Last Modified: January 05, 2026

Protocol Integer ID: 118311

Keywords: R10.4.1, long-read sequencing, Cas9 targeted sequencing, Staphylococcus aureus, Bacteroides, library preparation, antimicrobial resistance, bead capture, heteroresistance, gene amplification, whole genome sequencing, nanopore adapter, nanopore, whole genome, read sequencing, terminal thymine nucleotides of the ont ligation adapter, sequencing method, enriched cas9, separation from background genomic dna, background genomic dna, sequencing efficiency, improved cas9, genomic variation, sequencing yield, dynamic gene amplification, cas9, ligated target, unamplified dna, microbiology, dna, previous cas9, bacteroide, gene, ont ligation adapter, thymine nucleotide

Funders Acknowledgements:
Division of Intramural Research of the National Institute of Allergy
Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research

Abstract

Here we present nanopore adapter-enriched Cas9-targeted sequencing (nAECATS), a method permitting inexpensive, ultra-deep, selective long-read sequencing of targeted regions in native, unamplified DNA. To develop this approach, we modified previous Cas9-targeted sequencing methods, adding a novel bead-based capture step that utilizes the 5′ stretch of eight terminal thymine nucleotides of the ONT ligation adapter for additional purification. This poly(T) region distinguishes the Cas9-enriched adapter-ligated target from non-ligated material, allowing highly specific enrichment and separation from background genomic DNA. Following extensive optimization, we tested the nAECATS protocol on a fixed 10 kb region of interest (ROI) within the *Bacteroides fragilis* genome and achieved 90% on-target sequencing yield with a mean target coverage of 51,000x from a single Flongle flow cell (353-fold increase in mean ROI coverage versus whole genome sequencing). We next applied nAECATS to a more challenging target of variable length (>20 kb) in a *Staphylococcus aureus* strain containing a dynamic gene amplification previously demonstrated to confer resistance to the fluoroquinolone antibiotic delafloxacin. Absolute ROI coverage of 46,000x and 74% on-target sequencing yield were achieved with a MinION flow cell, revealing individual *S. aureus* populations with 2-4 tandem amplifications at the single cell level, and demonstrating the potential of this approach to reveal novel underlying biology. While loss of sequencing efficiency and potential shearing were observed with progressively longer fragments (up to 41 kb), significant improvements in enrichment were still achieved. We anticipate that nAECATS ultra-deep sequencing will find broad application for a wide range of biological questions in pro- and eukaryotic (epi)genomics and microbiology.

Protocol materials

- streptavidin magnetic beads **New England Biolabs** **Catalog #**S1420S
- rCutSmart Buffer **New England Biolabs** **Catalog #**B6004S
- Alt-R® CRISPR-Cas9 tracrRNA **IDT Technologies** **Catalog #**1072532
- Nuclease Free Duplex Buffer **IDT Technologies** **Catalog #**11-01-03-01
- Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT** **Catalog #**1081060
- Quick CIP **New England Biolabs** **Catalog #**M0525S
- dATP Solution (100 mM) **New England Biolabs** **Catalog #**N0440S
- Taq DNA Polymerase with Standard Taq Buffer **New England Biolabs** **Catalog #**M0273S
- Qubit dsDNA BR Assay kit **Invitrogen - Thermo Fisher** **Catalog #**Q32850
- Genomic DNA ScreenTape Analysis **Agilent Technologies** **Catalog #**5067-5365
- Quick Ligation Kit - 150 reactions **New England Biolabs** **Catalog #**M2200L
- Maxwell® HT 96 gDNA Blood Isolation System **Promega** **Catalog #**A2670
- Nanobind CBB kit **PacBio** **Catalog #**102-301-900
- 1.5mL DNA LoBind tubes **Eppendorf** **Catalog #**0030108051
- AMPure XP Beads (AXP) **Beckman Coulter** **Catalog #**AXP
- Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific** **Catalog #**Q32851
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies** **Catalog #**SQK-LSK114

Preparation of High Molecular Weight (HMW) gDNA

2h

1 For the preparation of bacterial HMW gDNA we refer to either of following kits and associated extraction protocols.

2h

Note *- care should be taken to always follow good practices to prevent shearing of HMW gDNA (e.g., excessive mixing, vortexing, and the use of small pipetting volumes)*
Note *- always check that the composition of the elution buffer does not contain EDTA as a simple Tris-HCl elution buffer is preferred for ONT long-read downstream applications*
Note *- HMW gDNA can be stored at* 4 °C *for several weeks. Fresh material is recommended and any freezing/thawing should be avoided for long-read sequencing purposes.*
Note *- Always check the quality and quantity of the HMW gDNA using different methods, including NanoDrop QC, Qubit quantification, and TapeStation QC.*

1.1 Maxwell® HT 96 gDNA Blood Isolation System **Promega** **Catalog #**A2670

1.2 Nanobind CBB kit **PacBio** **Catalog #**102-301-900

2 Proceed to **Cas9 target enrichment - Preparing the Cas9 ribonucleoprotein complexes (RNPs)**

Cas9 target enrichment - Preparing the Cas9 ribonucleoprotein complexes (RNPs)

1h 7m 10s

3 Thaw an aliquot of rCutSmart Buffer **New England Biolabs** **Catalog #**B6004S, mix by vortexing, spin down in a microfuge to collect any liquid in the bottom of the tube, and place on ice.

5m

4 Thaw the crRNA probes and Alt-R® CRISPR-Cas9 tracrRNA **IDT Technologies** **Catalog #**1072532 on ice, mix by vortexing, spin down in a microfuge to collect any liquid in the bottom of the tube, and place on ice.

5 Pool the crRNA probes for each cleavage reaction in a 1.5mL DNA LoBind tubes **Eppendorf** **Catalog #**0030108051 tube by combining

5m

equal volumes of each crRNA probe ([M] 100 micromolar (µM) in TE (pH 7.5)).

Note *- for a double-flanked target, pool* 1 µL *of each crRNA into a 0.2 mL thin-walled PCR tube single crRNA or many crRNA probes (up to ~100) may be used in a single cleavage reaction.*
Note *- unused crRNA probe mix may be stored at* -80 °C *and minimal freeze thaw recommended (e.g., aliquots of* 1 µL *)*

6 Anneal the pooled crRNAs with tracrRNA in Nuclease Free Duplex Buffer **IDT Technologies Catalog #**11-01-03-01 by assembling the following in a 0.2 ml thin-walled PCR tube, as follows: 5m

	Reagent	Volume
	nuclease-free Duplex Buffer (IDT)	8 µL
	crRNA pool (100 µM, equimolar; see above)	1 µL
	tracrRNA (100 µM)	1 µL
	Total	10 µL

7 Mix well by pipetting and spin down in a microfuge to collect any liquid in the bottom of the tube. 1m

8 Start the thermal cycler protocol (95 °C for 00:05:10) and only place the tubes in the thermal cycler when the first 00:00:10 have passed. 10s

9 Incubate the crRNA:tracrRNA reaction mix at 95 °C for 00:05:00 , **but only place the tubes in the thermal cycler when** 95 °C **has been reached**. 10m

10 Remove the tube from the thermal cycler and allow it to **cool to room temperature** and spin down to collect any liquid in the bottom of the tube. 5m

Note *- Do not use a thermal cycler for this step.*
Note *- Storage and reuse of the annealed mix is not recommended.*

11 Spin down the Alt-R® S.p. HiFi Cas9 Nuclease V3 **IDT Catalog #**1081060 in a microfuge and place on a freezer block (-20 °C). 30s

12 To form Cas9 RNPs, assemble the following components in a 1.5 ml Eppendorf DNA LoBind tube in the following order: 5m

	Reagent	Volume
	Nuclease-free water	79.2 µL
	Annealed crRNA:tracrRNA mix (10 µM)	10 µL
	NEB rCutSmart Buffer (10X)	10 µL
	HiFi Cas9 (62 µM)	0.8 µL
	Total	100 µL

13 Mix well by flicking the tube and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

14 Form the RNPs by incubating the tube at 21 °C for 00:30:00 in a thermal cycler (lid heating off), then **return the RNPs on ice** until required. 30m

Note *- Proceed to the he next step (Dephosphorylating genomic DNA) during the* 00:30:00 *RNP incubation step*

Cas9 target enrichment - Dephosphorylating genomic DNA 46m 30s

15 Thaw the Quick CIP **New England Biolabs Catalog #**M0525S, mix the *Quick* CIP in the tube by pipetting up and down, and **keep at room temperature.** 5m

16 Prepare the HMW gDNA in nuclease-free water by transferring 5 µg HMW gDNA into a 0.2 ml thin-walled PCR tubes. 5m

Note *- Adjust to* 24 µL *with nuclease-free water if highly concentrated.*

17 Mix thoroughly by flicking the tube avoiding unwanted shearing and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

18 Assemble the following components in a clean 0.2 ml thin-walled PCR tube: 5m

	Reagent	Volume
	NEB rCutsmart buffer (10X)	3 µL
	HMW gDNA (at >210 ng/µL)	24 µL
	Total	27 µL

19 Ensure the components are thoroughly mixed by pipetting five times using a P200 pipet, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

20 Add 3 µL of *Quick* CIP to the tube, mix gently by flicking the tube, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

21 Using a thermal cycler, incubate at 37 °C for 00:20:00, followed by 80 °C for 00:04:00 and **hold at** 21 °C **(room temperature).** 30m

Cas9 target enrichment - Cleaving and dA-tailing target DNA 1h 8m 30s

22 Thaw the dATP Solution (100 mM) **New England Biolabs Catalog #**N0440S tube, vortex to mix thoroughly, and place on ice. 5m

23 Spin the Taq DNA Polymerase with Standard Taq Buffer **New England Biolabs Catalog #**M0273S down in a microfuge and place on a freezer block (-20 °C). 30s

24 Prepare a [M] 10 millimolar (mM) dATP solution in a fresh 1.5 ml DNA LoBind tube by adding 2 µL of the [M] 100 millimolar (mM) dATP solution to 18 µL of nuclease-free water, vortex to mix, spin down in a minifuge to collect any liquid in the bottom of the tube, and keep on ice. 2m

25 Add the following to the 0.2 mL thin-walled PCR tubes, containing 30 µl dephosphorylated DNA sample: 5m

	Reagent	Volume
	Dephosphorylated HMW gDNA	30 µL
	Cas9 RNPs	10 µL
	10 mM dATP	1 µL
	NEB Taq polymerase	1 µL
	Total	42 µL

26 Carefully mix the contents of the tube by gentle inversion, spin down in a microfuge to collect any liquid in the bottom of the tube, and place the tube in the thermal cycler. 1m

27 Using the thermal cycler, incubate at 37 °C for 00:20:00, followed by 72 °C for 00:10:00, and **hold at** 4 °C or return the tube on ice. 35m

Note *- The Cas9 enzyme is active at* 37 °C *and denatured at* 72 °C *.*

Note *- A 15 minute (range between 10-60 minutes) cut time is recommended by default. Longer 37ºC incubations may increase the amount of off-target reads without increasing the yield of on-target reads, while shorter incubations may result in incomplete target cleavage. However, some regions may benefit from a longer incubation at* 37 °C *.*

28 Perform quality and quantity control on 1 µL of the Cas9 enriched product using Qubit dsDNA BR Assay kit **Invitrogen - Thermo Fisher Catalog #**Q32850 and Genomic DNA ScreenTape Analysis **Agilent Technologies Catalog #**5067-5365 . 20m

29 Keep samples on ice and proceed to **Preparation of buffers for Poly(A) enrichment** and **Long-read Ligation Library preparation (SQK-LSK114)**.

Preparation of buffers for Poly(A) enrichment 20m

30 Prepare **Wash/Bind buffer (** [M] 20 millimolar (mM) **Tris-HCl,** [M] 0.5 Molarity (M) **NaCl,** [M] 1 millimolar (mM) **EDTA @** pH 7.5 **)** by adding: 5m

	Reagent	Volume
	nuclease-free water	4.6 mL
	NaCl (1M)	5 mL
	Tris-HCl (1M, pH 7.5)	0.2 mL
	EDTA (0.05M, pH 8.0)	0.2 mL
	Total	10 mL

31 Prepare **2X Wash/Bind buffer (** [M] 40 millimolar (mM) **Tris-HCl,** [M] 1 Molarity (M) **NaCl,** [M] 2 millimolar (mM) **EDTA @** pH 7.5 **)** by adding: 5m

	Reagent	Volume
	NaCl (1M)	9.2 mL
	Tris-HCl (1M, pH 7.5)	0.4 mL
	EDTA (0.05M, pH 8.0)	0.4 mL
	Total	10 mL

32 Prepare **Low-Salt buffer (** [M] 20 millimolar (mM) **Tris-HCl,** [M] 0.15 Molarity (M) **NaCl,** [M] 1 millimolar (mM) **EDTA @** pH 7.5 **)** by adding: 5m

	Reagent	Volume
	nuclease-free water	8.1 mL
	NaCl (1M)	1.5 mL
	Tris-HCl (1M, pH 7.5)	0.2 mL
	EDTA (0.05M, pH 8.0)	0.2 mL

	Reagent	Volume
	Total	10 mL

Long-read Ligation library preparation (SQK-LSK114) 34m

33 Thaw Ligation Buffer (LNB; part of SQK-LSK114) and *Quick* T4 Ligase Buffer from the Quick Ligation Kit - 150 reactions **New England Biolabs Catalog #**M2200L at room temperature, mix by pipetting, spin down in a microfuge to collect any liquid in the bottom of the tube, and keep on ice. 5m

34 Thaw Elution Buffer (EB; part of SQK-LSK114) at room temperature, mix by vortexing, spin down in a microfuge to collect any liquid in the bottom of the tube, and keep on ice. 5m

35 Thaw Long Fragment Buffer (LFB; part of SQK-LSK114) at room temperature, mix by vortexing, spin down in a microfuge to collect any liquid in the bottom of the tube, and keep on ice. 5m

36 Compose the following ligation reaction based on the number of Poly(A)35 you would like to pool: 3m

Note *- When 10 Poly(A) RXN are pooled, split the reaction into 2×5 Ligation RXN for convenience.*

	Reagent	1 Poly(A) RXN	3 Poly(A) RXN	5 Poly(A) RXN
	Poly(A) enriched Cas9 sample	42 µL	126 µL	210 µL
	Ligation Buffer (LNB)	20 µL	30 µL	50 µL
	Quick T4 Ligase Buffer	NA	30 µL	50 µL
	Quick T4 Ligase	10 µL	30 µL	50 µL
	Ligation Adapter (LA)	5 µL	5 µL	5 µL
	Nuclease-free Water	NA	10 µL	20 µL
	Total	77 µL	231 µL	385 µL

37 Mix by flicking the tube and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

38 Incubate the Ligation reaction for 00:15:00 at room temperature on a Hula mixer. 15m 30s

Ligation Adapter (LA) enrichment using Poly(A) paramagnetic beads 2h 21m 30s

39 Vortex the streptavidin magnetic beads **New England Biolabs Catalog #**S1420S well to homogenize them. 30s

40 Transfer 125 µL beads (= 500 µg beads) into a 1.5 mL LoBind Tube. 30s

41 Add 100 µL **Wash/Binding Buffer (** [M] 20 millimolar (mM) **Tris-HCl,** [M] 0.5 Molarity (M) **NaCl,** [M] 1 millimolar (mM) **EDTA @** pH 7.5 **),** vortex to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

42 Place the tube on a magnetic rack and leave for 00:00:30 to pellet beads 1m

43 Remove supernatant and discard. 1m

44 Add 100 µL **Wash/Binding Buffer,** vortex to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

45 Place the tube on a magnetic rack and leave for 00:00:30 to pellet beads. 1m

46 Remove supernatant and discard. 30s

47 Repeat step 44-46 **TWICE** with fresh **Wash/Binding Buffer**. 5m

48 Thaw the **TEG-biotinylated poly(A)35 (at 8 pmol/µL in** [M] 20 millimolar (mM) **Tris-HCl,** [M] 0.5 Molarity (M) **NaCl,** [M] 1 millimolar (mM) **EDTA @** pH 7.5 **)** on ice, mix by vortexing, spin down in a microfuge to collect any liquid in the bottom of the tube, and place on ice.

49 Add 25 µL of **TEG-biotinylated poly(A)35 (at 8 pmol/µL in** [M] 20 millimolar (mM) **Tris-HCl,** [M] 0.5 Molarity (M) **NaCl,** [M] 1 millimolar (mM) **EDTA @** pH 7.5 **)** and vortex to resuspend the beads and oligos. 30s

50 Incubate at Room Temperature for 00:30:00 on a Hula Mixer. 30m

51 Place the tube on a magnetic rack and leave for 00:00:30 to pellet beads. 1m

52 Remove supernatant and keep in a fresh 1.5 mL Eppendorf DNA LoBind tube. 30s

Note *- this will allow to assess how much biotinylated bait is left using the Qubit ssDNA kit.*

53 Wash beads by adding 100 µL **Wash/Binding Buffer,** vortex to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

54 Place the tube on a magnetic rack and leave for 00:00:30 to pellet beads. 1m

55 Remove supernatant and discard. 30s

56 Repeat Step 53-55 **TWICE** with fresh **Wash/Binding Buffer**. 4m

57 Add 77 µL / 231 µL / 385 µL **2X Wash/Binding buffer (** [M] 40 millimolar (mM) **Tris-HCl,** [M] 1 Molarity (M) **NaCl,** [M] 2 millimolar (mM) **EDTA @** pH 7.5 **)** to the magnetic beads mixture, flick the tube to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

Note *- Volume depends on number of pooled reactions in the Long-read Ligation library preparation (SQK-LSK114) procedure*

58 Add 77 µL / 231 µL / 385 µL of the Cas9 enriched and adapter ligated DNA sample (*i.e.*, ~ 5 µg in 50 µL EB (part of SQK-LSK114)) to the magnetic beads mixture, flick the tube to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

Note *- Volume depends on number of pooled reactions in the Long-read Ligation library preparation (SQK-LSK114) procedure*

59 Incubate at Room Temperature for 00:30:00 on a Hula Mixer. 30m

60 Place the tube on a magnetic rack and leave for 00:02:00 to pellet beads. 2m 30s

61 Remove supernatant and keep in a fresh 1.5 mL Eppendorf DNA LoBind tube. 30s

Note *- this will only serve as control for QC using Qubit dsDNA BR and TapeStation gDNA ScreenTape.*

62 Wash beads by adding 100 µL **Wash/Binding Buffer,** flick the tube to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

63 Place the tube on a magnetic rack and leave for 00:02:00 to pellet bead. 2m 30s

64 Remove supernatant and discard. 30s

65 Repeat Step 62-64 for a second wash with fresh **Wash/Binding Buffer**. 2m

66 Add 100 µL **cold Low Salt Buffer (** 20 millimolar (mM) **Tris-HCl,** 0.15 Molarity (M) **NaCl,** 1 millimolar (mM) **EDTA @** 7.5 **; kept on ice)** to the magnetic beads mixture, flick the tube to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

67 **Immediately** place the tube on a magnetic rack and leave for 00:00:30 to pellet beads. 1m

68 **Immediately** remove supernatant and discard. 30s

69 **Immediately** add 20 µL **pre-warmed (** 37 °C **) Elution Buffer (** [M] 10 millimolar (mM) **Tris-HCl @** pH 7.5 **)** to the magnetic beads mixture, flick the tube to resuspend the beads, and spin down in a microfuge to collect any liquid in the bottom of the tube. 30s

Note *- Volume can be changed depending on downstream application.*

70 Incubate for 00:30:00 at 37 °C on a Hula Mixer. 30m

71 Place the tube on a magnetic rack and leave for 00:02:00 to pellet beads. 2m 30s

72 Transfer supernatant to a fresh 1.5 mL Eppendorf DNA LoBind Tube and keep on ice. 30s

Note *– This is the fraction of our interest.*

73 **Optional** - Repeat Step 69-72 for a second eluate. Though this generally does not result in extra recovery. 3m

74 Perform quality and quantity control on 1 µL of the resulting supernatants and retained fractions (See above) using Qubit dsDNA BR/HS and TapeStation gDNA ScreenTape. 15m

Note *- For optimal recovery and sequencing efficiency it is recommended to omit quality and quantity control on the eluate to optimize input for sequencing.*

75 Proceed immediately to **Adapter removal using AMPure XP Beads.**

Adapter removal using AMPure XP Beads 1h 5m 30s

76 Allow the AMPure XP Beads (AXP) **Beckman Coulter Catalog #AXP** to get to room temperature. 15m

77 Vortex the AXP Beads to homogenize them. 30s

78 Add 8 µL AXP Beads to the Poly(A) enriched Cas9 sample, mix by flicking the tube, and spin down in a microfuge to collect any liquid in the bottom of the tube. 1m

Note *- This is a 0.4X clean-up if a 20 µL elution was done in the previous step. Whenever volumes in the previous procedure are changed, change them here as well to guarantee optimal adapter removal.*

79 Incubate on a Hula Mixer for 00:05:00 at room temperature. 5m 30s

80 Spin the sample down in a microfuge to collect any liquid in the bottom of the tube and pellet on a magnet. 30s

81 Keep the tube on the magnet for 00:02:00 (or until clear and colourless) and remove supernatant. 2m 30s

82 Add 250 µL LFB to the tube and gently rotate the 1.5 mL Eppendorf LoBind tube 180º and allow the beads to migrate through the LFB. Once migrated, turn the tube another 180º and allow the beads to migrate through the LFB for a second time. 2m

Note *- For some sample types it might be required to resuspend the beads by flicking rather than having them migrate through the LFB. In this case resuspend the beads and allow to pellet against the magnet for 2 minutes.*

83 Keep the tube on the magnet for 00:02:00 (or until clear and colourless) and remove supernatant. 2m 30s

Note *- Some samples require flicking of the tube*

84 Repeat Step 82-83 with fresh LFB. 5m

85 Spin the tube down in a microfuge to collect any liquid in the bottom of the tube, place back on the magnet, and remove residual LFB buffer using a P20 tip. 30s

86 Allow the tube to dry (lid open) for 00:00:30 . 1m

Note *- Do not dry the pellet to the point it shows cracks.*

87 Remove the tube from the magnetic rack, add 7 µL Elution Buffer (EB) onto the pellet, and resuspend by flicking the tube. 1m

Note *- For MinION/GridION flow cell library preparation a volume of* 12 µL *is desirable here as* 1 µL *is required for the final library loading.*

88 Spin down in a microfuge to collect any liquid in the bottom of the tube and incubate for 00:10:00 at 37 °C on a Hula mixer. 10m 30s

89 Place the tube on the magnetic rack and pellet the beads for 00:02:00 (or until clear and colourless). 2m 30s

90 Retain the eluted adapter-depleted Poly(A) enriched Cas9 products and transfer into a fresh 1.5 mL Eppendorf DNA LoBind tube. 30s

91 Perform quality and quantity control on 1 µL of the adapter-depleted Poly(A) enriched Cas9 product using
Qubit dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #**Q32851
and TapeStation gDNA ScreenTape. 15m

Note *- For optimal recovery and sequencing efficiency it is recommended to omit quality and quantity control on the eluate to optimize input for sequencing.*

92 Immediately proceed to **Long-read Sequencing on Flongle.**

Long-read Sequencing on Flongle 10m

93 For the sequencing on Flongle please refer to the respective protocols for Flongle and MinION/GridION flow cell library prepration and loading as part of the 10m

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

- **Link**