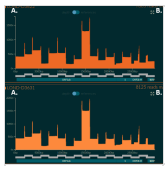

Version 3

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Long reads nanopore sequencing to recover SARS-CoV-2 whole genome V.3

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In Development dx.doi.org/10.17504/protocols.io.bfy7jpzn

Coronavirus Method Development Community

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ABSTRACT

This protocol describes step-by-step instructions for building long (~2kb) amplicon libraries to recover SARS-CoV-2 genomes using the nanopore sequencing.

It can be applied for sequencing on the MinION device or GridION.

This protocol was developed in collaboration with the Laboratory of Respiratory Viruses and Measles, Oswaldo Cruz Institute, FIOCRUZ, Brazil, and the two sequencing facilities at Pathogen Genomic Unit (PGU) and UCL Genomics, University College London (UCL), United Kingdom.

This document describes the manual steps to perform the protocol, but for further information about the automation of the protocol, please, contact Pathogen Genomics Unit (PGU) - UCL.
Manager: Rachel Williams
https://www.ucl.ac.uk/infection-immunity/pathogen-genomics-unit

This protocol is based on the amplicon tiling strategy described previously by Quick J et al 2017. However, we have applied this strategy to recover long reads (2kb), then some adjustments were performed in the protocol.

Quick J, Grubaugh ND, Pullan ST, Claro IM, Smith AD, Gangavarapu K, Oliveira G, Robles-Sikisaka R, Rogers TF, Beutler NA, Burton DR, Lewis-Ximenez LL, de Jesus JG, Giovanetti M, Hill SC, Black A, Bedford T, Carroll MW, Nunes M, Alcantara LC Jr, Sabino EC, Baylis SA, Faria NR, Loose M, Simpson JT, Pybus OG, Andersen KG, Loman NJ (2017). Multiplex PCR method for MinION and Illumina sequencing of Zika and other virus genomes directly from clinical samples.. Nature protocols.
https://doi.org/10.1038/nprot.2017.066

If you have doubts, we are happy to help you to test or implement the protocol.

MATERIALS

NAME	CATALOG #	VENDOR
NEBNext Ultra II End Repair/dA-Tailing Module - 96 rxns	E7546L	New England Biolabs
NEBNext Ultra II Ligation Module - 96 rxns	E7595L	New England Biolabs
Qubit® dsDNA HS Assay kit	Q32854	Thermo Fisher Scientific
NEB Blunt/TA Ligase Master Mix	M0367	
Agencourt AMPure XP beads	A63881	Beckman Coulter
Ethanol	100983	Merck Millipore
SuperScript™ IV First-Strand Synthesis System	18091200	Thermo Fisher

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

NAME	CATALOG #	VENDOR
ONT MinION Flow Cell R9.4.1	FLO-MIN106D	Oxford Nanopore Technologies
Ligation sequencing kit 1D	SQK-LSK109	Oxford Nanopore Technologies
Flowcell Wash Kit	EXP-WSH003	Oxford Nanopore Technologies
NEB Q5® Hot Start High-Fidelity 2X Master Mix	M0494L	New England Biolabs
Native Barcoding Expansion 1-12 (PCR-free)	EXP-NBD104	Oxford Nanopore Technologies
Native Barcoding Expansion 13-24 (PCR-free)	EXP-NBD114	Oxford Nanopore Technologies
Primer set SARS-CoV-2_v1		

MATERIALS TEXT

RT (Optional)

- SuperScript™ IV First-Strand Synthesis System. (200 reactions) Cat: 18091200 Invitrogen

PCR (Optional)

- Q5® Hot Start High-Fidelity 2X Master Mix Cat: M0494
- Primer set SARS-CoV-2_v1 Pool 1 and Pool 2 (2kb) Paola Resende

Clean-up and QC

- Qubit ds DNA HS kit. Cat: Q32854 Thermofisher
- Ampure XP. Cat: A63881 Beckman Coulter

End-Prep, Barcoding, Adapter Ligation and Sequencing

- NEBNext® Ultra™ II Ligation Module. Cat: E7595L NEB
- NEBNext® Ultra™ II End Repair/dA-Tailing Module. Cat: E7546L NEB
- NEB Blunt/TA Ligase Master Mix. Cat: M0287L NEB
- Ligation Sequencing Kit. Cat: SQK-LSK109 ONT
- Native Barcoding Expansion 1-12 (PCR-free). Cat: EXP-NBD104 ONT
- Native Barcoding Expansion 13-24 (PCR-free). Cat: EXP-NBD114 ONT
- Flow Cell (R9.4.1) ONT

Flow cell wash

- Flow Cell Wash Kit. Cat: EXP-WSH003 ONT

Real-time data analysis

- Computer operational system LINUX
- MinKnow
- RAMPART (https://github.com/artic-network/rampart)

Primers dilution

1 Instructions for Primer Scheme dilution:

The primer scheme dilution should be prepared in **Master Mix Hood** or **Clean Room**.
To avoid cross-contamination make sure that your original stock reagents have no contact with RNA or any amplified DNA material.

If lyophilised each primer should be resuspended to [M]**100 Micromolar (µM)** **(Stock dilution 1)**

The primers from **Primer Scheme 2kb_v1 Pool 1** and **Primer Scheme 2kb_v1 Pool 2** are:

Primer Scheme 2kb_v1 Pool 1: F1, R1, F3, R3, F5, R5, F7, R7, F9, R9, F11, R11, F13, R13, F15, R15, F17, R17
(9 primers pairs)

Primer Scheme 2kb_v1 Pool 2: F2, R2, F4, R4, F6, R6, F8, R8, F10, R10, F12, R12, F14, R14, F16, R16
(8 primers pairs)

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

	Primer Scheme 2kb_v1 Pool 1		Primer Scheme 2kb_v1 Pool 2
Oligo name	**Oligo sequence (5' to 3')**	**Oligo name**	**Oligo sequence (5' to 3')**
hCoV_F1_2kb	ACCAACCAACTTTCGATCTCTTGT	hCoV_F2_2kb	CTGCTCAAAATTCTGTGCGTGT
hCoV_R1_2kb	ACACCACCTGTAATGTAGGCCA	hCoV_R2_2kb	GGTCAGCACCAAAAATACCAGCT
hCoV_F3_2kb	AGCGGACACAATCTTGCTAAACA	hCoV_F4_2kb	TTGTGCACTTATCTTAGCCTACTGT
hCoV_R3_2kb	GGTTGTCTGCTGTTGTCCACAA	hCoV_R4_2kb	TGCCAAAAACCACTCTGCAACT
hCoV_F5_2kb	CACTATTGCAACCTACTGTACTGGT	hCoV_F6_2kb	GTACACTGACTTTGCAACATCAGC
hCoV_R5_2kb	CGTGTGTCAGGGCGTAAACTTT	hCoV_R6_2kb	AACGGCAATTCCAGTTTGAGCA
hCoV_F7_2kb	TGTACGCTGCTGTTATAAATGGAGA	hCoV_F8_2kb	TGGTACAACATTTACTTATGCATCAGC
hCoV_R7_2kb	TTTGACAGCAGAATTGGCCCTT	hCoV_R8_2kb	TGGGTGGTATGTCTGATCCCAA
hCoV_F9_2kb	CCTTGACCAGGGCTTTAACTGC	hCoV_F10_2kb	AGCAAAATGTTGGACTGAGACTGA
hCoV_R9_2kb	ATCATCTACAAAACAGCCGGCC	hCoV_R10_2kb	CCAAGCAGGGTTACGTGTAAGG
hCoV_F11_2kb	GCTGAAATTGTTGACACTGTGAGT	hCoV_F12_2kb	TGCATTCCACACACCAGCTTTT
hCoV_R11_2kb	AGCACCACCTAAATTGCAACGT	hCoV_R12_2kb	TAACAAAGGCTGTCCACCATGC
hCoV_F13_2kb	ACAAAAGAAAATGACTCTAAAGAGGGTTT	hCoV_F14_2kb	CAGGCTGCGTTATAGCTTGGAA
hCoV_R13_2kb	TGTGCTACCGGCCTGATAGATT	hCoV_R14_2kb	CATGACAAATGGCAGGAGCAGT
hCoV_F15_2kb	TCAGAGTGTGTACTTGGACAATCAA	hCoV_F16_2kb	ACGTGAGTCTTGTAAAACCTTCTTTTT
hCoV_R15_2kb	GTACCGTTGGAATCTGCCATGG	hCoV_R16_2kb	ACTGCCAGTTGAATCTGAGGGT
hCoV_F17_2kb	GGAATCATCACAACTGTAGCTGCA		
hCoV_R17_2kb	TAGGCAGCTCTCCCTAGCATTG		

Primer scheme to recover 2 kilobases amplicon of SARS-CoV-2 genome.

1.1 Prepare the **Primer Scheme 2kb_v1 Pool 1 and Pool 2** **[M]100 Micromolar (µM)** (stock dilution 2)

Add **20 µl** of each primer **[M]100 Micromolar (µM)** (stock dilution 1) to a 1.5mL tube labelled as **Primer Scheme 2kb_v1 Pool 1**. The final volume will be **360 µl**

Add **20 µl** of each primer **[M]100 Micromolar (µM)** (stock dilution 1) to a 1.5mL tube labelled as **Primer Scheme 2kb_v1 Pool 2.** The final volume will be **320 µl**

1.2 Prepare the **Primer Scheme 2kb_v1 Pool 1 and Pool 2** **[M]10 Micromolar (µM)** (concentration to be used)

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

Add **20 µl** of each primer **100 Micromolar (µM)** of **Primer Scheme 2kb_v1 Pool 1** (stock dilution 2) to a 1.5mL tube labelled as **Primer Scheme 2kb_v1 Pool 1** **10 Micromolar (µM)**

Add **180 µl** of water nuclease-free. The final volume will be **200 µl**

Add **20 µl** of each primer **100 Micromolar (µM)** of **Primer Scheme 2kb_v1 Pool 2** (stock dilution 2) to a 1.5mL tube labelled as **Primer Scheme 2kb_v1 Pool 2** **10 Micromolar (µM)**

Add **180 µl** of water nuclease-free**.** The final volume will be **200 µl**

Master Mix for cDNA and PCR steps 30m

2 Instructions to prepare the Maxter Mix for cDNA and PCR steps

You can save time if you prepare both Master Mix cDNA and PCR to be used on the same day.

The master mix for cDNA and PCR step should be prepared in **Master Mix Hood** or **Clean Room**. To avoid cross-contamination make sure that your original stock reagents have no contact with RNA or any amplified DNA material.

A **Negative Control** (H_2O nuclease free) should be included from cDNA step until the end.

Keep the enzymes on ice and thaw the other reagents at room temperature before placing on ice.

2.1 Master Mix RT_1:

This master Mix is prepared with components from:

SuperScript™ IV First-Strand Synthesis System (Catalog number: 18091050 [50 reactions] or 18091200 [200 reactions]).

However, you can use separated items using the enzyme: SuperScript™ IV Reverse Transcriptase (Catalog number: 18090200).

Prepare the following components in a 0.2mL 8-strip tube for the number of samples that will be tested (positive samples + a negative control)

VOLUME	COMPONENT

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

1 µl	50µM random hexamers
1 µl	10mM dNTPs mix (10mM each)

2.2 Master Mix RT_2:

Prepare the following components in a 1.5mL tube and keep the Master Mix2-RT on ice. (7uL per sample)

VOLUME	COMPONENT
4 µl	5x SSIV Buffer
1 µl	100mM DTT
1 µl	RNAseOUT RNase Inhibitor
1 µl	SSIV Reverse transcriptase

2.3 Master Mix PCR Pool 1 and Pool 2:

We have tested two catalog numbers of Q5®Hot Start High-Fidelity enzyme options:
Option 1 - Q5®Hot Start High-Fidelity 2X Master Mix (Catalog number: M0494)*
*Advantage: the master mix is ready to be used.

Option 2 - Q5®Hot Start High-Fidelity DNA Polymerase (Catalog number: M0493)

Bellow, we can find the master mix recipe of both. Please choose just one to continue the protocol.

Option 1 - Q5®Hot Start High-Fidelity 2X Master Mix (Catalog number: M0494)

Prepare the following components in two 1.5mL tubes and keep the Master Mix PCR Pool 1 and Pool 2 on ice

VOLUME Poo1	VOLUME Pool 2	COMPONENT
12.5 µl	**12.5 µl**	Q5 High-Fidelity 2X Master Mix
3.6 µl	**3.6 µl**	Primer Pool 1 or Primer Pool 2 ([M] **10 Micromolar (µM)**)
6.4 µl	**6.4 µl**	H_2O Nuclease free

Mix the master mix by inversion several times, briefly spin to collect the contents at the bottom of the tube.

Dispense **22.5 µl** per tube, **0.2mL 8-strip PCR tubes Pool 1** and **0.2mL 8-strip PCR tubes Pool 2.**

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

Option 2 - Q5®Hot Start High-Fidelity DNA Polymerase (Catalog number: M0493)

VOLUME Poo1	VOLUME Pool 2	COMPONENT
5 µl	**5 µl**	5X Q5 Reaction Buffer
0.5 µl	**0.5 µl**	10 mM dNTPs
0.25 µl	**0.25 µl**	Q5 Hot Start DNA Polymerase
3.6 µl	**3.6 µl**	Primer Pool 1 or Primer Pool 2 ([M] **10 Micromolar (µM)**)
13.15 µl	**13.15 µl**	H_2O Nuclease free

Mix the master mix by inversion several times, briefly spin to collect the contents at the bottom of the tube.

Dispense **22.5 µl** per tube, **0.2mL 8-strip PCR tubes Pool 1** and **0.2mL 8-strip PCR tubes Pool 2.**

Label the side of the tubes, not the lids as the marker may be removed by the heat in the PCR machine.

cDNA 1h 30m

3 Instructions for the cDNA step:

This step should be conducted in the pre PCR area.

Keep all the Master Mix (cDNA_2 and PCR) in the fridge.

3.1 Set up the thermocycler for the following condition:

65 °C

3.2 Spin down the **Master Mix RT_1**;

3.3 Add **11 µl** RNA to each 0.2 mL tube containing the **Master Mix RT_1**;

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

3.4 Mix by pipetting; and pulse centrifuge the tubes to collect the contents at the bottom of the tube.

3.5 Incubate the reaction for **65 °C** **00:05:00** 5m

3.6 Once this step is completed add **7 µl** of **Master Mix RT_2** in each tube

3.7 Mix by pipetting and pulse centrifuge the tubes to collect the contents at the bottom of the tube.

3.8 Incubate the reaction in the thermocycler for: 1h

42 °C **00:50:00**

70 °C **00:10:00**

4 °C hold

The cDNA can be stored in **-20 °C** . If needed, it is a safe stop point.

The remaining RNA should be stored in **-80 °C**

PCR

4 Instructions for the PCR step:

This step should be conducted in the pre PCR area.

4.1 Set up the thermocycler for the following conditions:

1 cycle

98 °C **00:02:00** Heat Activation

35 cycles

98 °C **00:00:10** Denaturation

65 °C **00:05:00** Annealing

1 cycle

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

4 °C Hold

4.2 Add **2.5 µl** cDNA of each sample to each 0.2mL 8-strip PCR tube containing the **Master Mix PCR Pool 1** and **Master Mix PCR Pool 2**

4.3 Mix by pipetting and pulse centrifuge the tubes to collect the contents at the bottom of the tube.

4.4 Incubate in the thermocycler following the conditions described in **substep 4.1**

Clean-up

5 Instructions for the DNA purification using magnetic beads AMPURE

This step should be conducted in the PCR room

Keep the Ampure beads and Qubit standards in room temperature **00:15:00** before start.

Prepare FRESH **[M]80 % (v/v)** ethanol. Do not use **[M]80 % (v/v)** Ethanol prepared in the previous day.

6 After the RT-PCR, Pool 1 and Pool 2 can be mixed (final volume **50 µl**)

7 Add an equal volume of AmpureXP PCR Clean-up beads (**50 µl**) to the tube (ratio of 1:1 of Ampure beads).

8 Mix gently by either flicking or pipetting 8-10 times.

If long reads (2 Kb) avoid the vortex

9 Incubate for **00:05:00** at room temperature.

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

10 Pulse centrifuge the tubes to remove any beads or solution from the lid or side of the tube.

11 Place on a magnetic rack and incubate for **00:02:00** or until the beads have pelleted against the magnet and the solution is completely clear.

12 Carefully remove and discard the solution, being careful not to displace the bead pellet.

13 Add **200 µl** of fresh room-temperature **80 % (v/v)** Ethanol to the pellet.

14 Incubate for **00:01:00** .

15 Carefully remove and discard Ethanol, being careful not to displace the bead pellet.

16 Repeat Ethanol wash steps 13-15 to wash the pellet again and continue from step 17.

17 Briefly pulse centrifuge the pellet and carefully remove as much ethanol as possible using a **10 µl** tip.

18 Allow the pellet to dry for **00:02:00** , being careful not to over-dry (if the pellet is cracking, then it is too dry). Pellet should appear opaque and slightly shiny.

19 Resuspend the pellet thoroughly in **32 µl** of water, and incubate for **00:02:00**

20 Pulse centrifuge to remove content in the lid.

21 Place on magnet and CAREFULLY remove water and transfer **32 µl** to a clean 1.5 mL Eppendorf tube.

MAKE SURE that no beads are transferred into this tube. In some cases, pulse centrifugation can be used to pellet residual beads.

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

Quality Control (QC)

22 **Instructions to measure the amount of DNA**

Quantify **2 µl** of the amplicon library using the Qubit fluorometer following the dsDNA protocol.

To avoid any cross-contamination, we recommend that you remove the total amount of working solution required for your samples and standards from the working solution bottle and then add the required volume to the appropriate tubes instead of pipetting directly from the bottle to each tube.

23 Set up the required number of 0.5mL tubes for standards and samples. The Qubit™ 1X dsDNA HS Assay requires 2 standards.

24 Label the tube lids. Do not label the side of the tube as this could interfere with the sample read. Label the lid of each standard tube correctly. Calibration of the Qubit™ Fluorometer requires the standards to be inserted into the instrument in the right order.

25 Add the Qubit™ 1X dsDNA 1X buffer to each tube standard tube (**190 µl**) and each sample tube (**198 µl**)

26 Add **10 µl** of each Qubit™ standards 1 and 2 to the appropriate tube.

27 Add **2 µl** of each sample to the appropriate tube

28 Vortex all tubes and incubate at room temperature for **00:02:00** , then proceed to “Read standards and samples”.

29 Carefully record all results to perform the DNA normalisation to prepare the library.

Normalisation 30m

30 **Instructions to normalise the DNA to prepare the library**

For the barcoding step is needed **12.5 µl** of DNA in a concentration of 60ng (long reads) per sample.

For an efficient barcoding step, we observe that DNA in the barcoding step should range of 25 to 50 fmol

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

(2kb). Then, we are using 60ng per sample.

This is a safe stop point.

End-prep and barcoding Master Mix

31 Instructions to prepare the End-prep and barcoding Master Mix

The **End-prep and barcoding Master Mix** should be prepared in **Master Mix Hood** or **Clean Room**. To avoid cross-contamination make sure that your original stock reagents have no contact with RNA or any amplified DNA material.

31.1 Master Mix End-prep:

Prepare the following components in a 1.5mL tube and keep the Master Mix PCR on ice.

VOLUME	COMPONENT
1.75 µl	Ultra II End Prep Reaction Buffer
0.75 µl	Ultra II End Prep Enzyme Mix

31.2 Master Mix barcoding:

Prepare the following components in a 1.5mL tube and keep the Master Mix PCR on ice.

VOLUME	COMPONENT
17.5 µl	Ultra II Ligation Master Mix
0.5 µl	Ligation Enhancer

End-prep

32 Instructions to End-prep reaction

This is a 'one-pot ligation' protocol for native barcoded ligation libraries.

This step should be conducted in the PCR room

33 Add **2.5 µl** of **Master Mix End-prep** to each tube containing **12.5 µl** of pre-normalised DNA (~ 60ng for 2 kb) and mix well by pipetting.

34 Incubate in a thermocycler at **20 °C** for **00:10:00** then **65 °C** for **00:05:00**

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

35 Place on ice for **00:00:30**

Barcoding 25m

36 **Instructions to Barcoding reaction**

Use a SINGLE native barcode (NBXX) per biological sample.

This step should be conducted in the PCR room

37 Add **2.5 µl** NBXX barcode to each tube containing **15 µl** DNA end-prepped.

38 Add **18 µl** of the barcoding mix to each tube and mix well by pipetting. The total volume will be **35.5 µl** .

25m

39 Incubate:

20 °C **00:15:00**

70 °C **00:10:00**

Then place on ice.

The **70 °C** incubation is to inactivate the DNA ligase to prevent barcode cross-ligation when reactions are pooled in the next step

Pooling and clean-up 25m

40 **Instructions to pool all samples and perform a clean-up**

Pool all barcoded samples together into a clean 1.5 ml Eppendorf tube. **35 µl** per sample

If the number of samples processed is more than 20 samples this pooling needs to be done in a 2.0mL Eppendorf tube.

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

41 Add a proportion 1:1 of AMPURE beads in the barcoded DNA pooled.
If necessary measure the volume of the above reaction and add the same volume of AMPURE beads, to get a 1:1 solution.

42 Incubate for **00:05:00** in the HulaMixer.

43 Spin down the liquid and place on a magnet rack for **00:03:00** or until clear.

44 Remove all the solutions without touch in the pellet

45 Add **500 µl** of [M]**80 % (v/v)** ethanol to the tube still on the magnetic rack to wash.

46 Remove the tube from the magnet rack and remove and turn 180º and place the tube again in the magnetic rack.

47 Remove and discard [M]**80 % (v/v)** ethanol without disturbing the pellet.

48 Repeat steps 45 and 47 and after continue from step 49.

49 Spin down and remove residual [M]**80 % (v/v)** ethanol and air dry for **00:02:00** . 2m

50 Resuspend in **32 µl** EB.

51 Incubate off the magnetic rack for **00:02:00** and after spin down. 2m

52 Replace the magnetic rack for **00:02:00**

53 Wait until clear, then carefully remove the solution and transfer to a clean 1.5 mL Eppendorf tube labeled such as the name of your processing file.

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

QC 25m

54 Remove **2 µl** and assess concentration by Qubit as described in the previous section. Keep the records in your processing file.

Normalisation 25m

55 Dilute 100-200 fmol (~ 150ng for 2kb) pooled sample to **30 µl** in Elution Buffer (EB).

Adapter ligation 25m

56 Set up the following adapter ligation reaction in a 1.5mL tube:

VOLUME	COMPONENT
30 µl	Cleaned-up barcoded amplicon pools (100-200 fmol)
10 µl	NEBNext Quick Ligation Reaction Buffer (5X)
5 µl	AMII Adapter mix II
5 µl	Quick T4 DNA Ligase

57 Incubate at RT for **00:30:00**

Remove the **SFB** from the freezer and let equilibrate to room temperature to be used in the next step

Clean-up 25m

58 **Clean-up**

After **00:30:00** incubation add **50 µl** Ampure beads (1x the volume of the total reaction volume)

59 **30 µl** Incubate for **00:05:00**

60 Spin down.

61 Place on a magnetic rack until clear.

62 Remove supernatant.

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

63 Add **200 µl** **SFB**. Remove and turn the tube in the magnetic rack.

CAUTION: **DO NOT USE** **[M]80 % (v/v)** ETHANOL

64 Place on a magnetic rack until clear.

65 Remove supernatant.

66 Repeat **SFB** wash

67 Spin down and remove residual SFB .

SFB is used for short fragments < 3kb.
LFB is used just for long fragments > 3Kb.

68 Add **14 µl** EB and resuspend by flicking.

69 Incubate at RT for **00:02:00**

70 Place on a magnetic rack.

71 Carefully transfer the solution to a clean 1.5 mL Eppendorf tube.

QC 25m

72 Remove **2 µl** and assess concentration by Qubit fluorometer- recovery aim 50-100 fmol, 61.80 - 123.6 ng for 2Kb amplicons

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

The library can be now stored at 4°C overnight if required, but for best results, proceed immediately to sequencing.

Testing the flow cell 25m

73

During this testing to save time you can thaw the following reagents at room temperature before placing on ice: Sequencing buffer (SQB); Loading beads (LB); Flush buffer (FB); and Flush tether (FLT). They will be used in the next step

Choose a flow cell, record the name of the flow cell on your working sheet

74 Insert the flow cell in the MinION device or GridION.

75 Test the flow cell using the MinKnow software to observe the number of active pores, keep this record on your working sheet.

76 Select the flowcell ticking the box

77 Click “check flowcell”

78 Click “start test”.

Loading the flow cell 25m

79 For this step please follow the protocol described by Josh Quick below:

Josh Quick. Priming and loading a MinION flowcell.
http://dx.doi.org/10.17504/protocols.io.7q5hmy6

MinKnow software 25m

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

80 Double–click the MinKNOW icon located on the desktop to open the MinKNOW software.

81 If your MinION was disconnected from the computer, plug it back in.

82 Choose the following flow cell type from the selector box: FLO-MIN106 : R9.4.1 flow cell

83 Then mark the flow cell as Selected.

84 Click the New Experiment button at the bottom left of the screen.

85 On the new experiment popup screen select the running parameters for your experiment from the individual tabs:

Experiment

On the New experiment popup screen, select the running parameters for your experiment from the individual tabs

Experiment: Name the run in the experiment field, leave the sample field blank.

Kit: Select LSK109. Do not select barcoding kits.

Run Options: Set the run length to 12 hours (you can stop the run once sufficient data has been collected as determined using RAMPART software).

Basecalling: Leave base calling turned but select 'high accuracy basecalling'.

Click 'Start run'.

Wash a flow cell 40m

86 **Washing the flow cell:**

This step should be performed to reuse the flow cell after a previous run.

The flow cell should be washed on the same day or on the following day of the run. Do not wait too much time to wash the Flow Cell.

For this step please follow the protocol described by Kirstyn Brunker bellow:

Kirstyn Brunker. Washing a MinION flowcell.
http://dx.doi.org/10.17504/protocols.io.bddzi276

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn

Citation: Paola Resende (05/05/2020). Long reads nanopore sequencing to recover SARS-CoV-2 whole genome . https://dx.doi.org/10.17504/protocols.io.bfy7jpzn