JAN 11, 2023

OPEN ACCESS

DOI:
dx.doi.org/10.17504/protocols.io.3byl4jzb8lo5/v1

Protocol Citation: pramadaprasad, Harsha P.K., Chitra Pattabiraman 2023. MinION Sequencing protocol for Rabies virus of Arctic lineage. **protocols.io** https://dx.doi.org/10.17504/protocols.io.3byl4jzb8lo5/v1

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

Created: Dec 23, 2022

Last Modified: Jan 11, 2023

PROTOCOL integer ID: 74417

Keywords: MinION, Sequencing, Rabies

MinION Sequencing protocol for Rabies virus of Arctic lineage

Forked from MinION Sequencing protocol for Rabies virus of Indian subcontinental lineage

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ABSTRACT

An ONT MinION-based sequencing protocol to retrieve whole genomes of Rabies virus of the Artic lineage. Primers were selected using PrimalScheme (https://primalscheme.com/) and theprotocol was modified from the Zika and SARS-CoV-2 sequencing protocols using the same tool.

RNA Extraction

1 Extract and purify RNA from Brain tissue homogenate using QIAamp Viral RNA Mini Kit **Qiagen Catalog #52904** .

Note

Removal of genomic DNA by on column DNase treatment (optional)
Genomic DNA can be removed by on column
DNase I (RNase-free) - 5,000 units **New England Biolabs Catalog #M0303L** treatment for 30 min at room temperature before the wash steps.

cDNA Preparation

2 20m

Note

Extracted RNA samples are mixed thoroughly by brief vortex and spin down to collect all liquid at the bottom.

2.1 Prepare the master mix for cDNA Prep as follows in a PCR plate/ tubes for 10µL reaction.

A	B
Component	Volume
Lunascrpit RT Supermix (5X)	2µl
Template	8µl
Total	10 µl

LunaScript RT SuperMix Kit - 100 rxns **New England Biolabs Catalog #E3010L**

Note

Prepare all reagents in a biosafety cabinet designated for clean work.

2.2 Incubate the reaction mix following the conditions mentioned below in a thermo cycler:

25 °C for 00:02:00

55 °C for 00:10:00

95 °C for 00:01:00

Hold at 4 °C

Amplicon PCR

3 Primers are designed using Primal Scheme. Resuspend the lyophilized oligos fully in Nuclease free water / 1X TE to a concentration of 100µM, vortex thoroughly,Nuclease-free, and spin down. 5h

RABV_Arctic_primers.csv

4 Separate odd and even primer sets. Add 5µl of each odd primer to a 1.5mL LoBind tube labelled Pool 1. Repeat the same for all even primers for Pool 2. These are the 100µM stocks of each primer pool.

5 Dilute 100µM stock pools (1:10) in Nuclease free water, to generate 10µM primer stock. A final concentration of 15 nM per primer is used. Here we use a total of 39 primers in Pool 1 and 39 primers in Pool 2. Here 0.75µl of per primer pool (10µM) is required for a 25µl reaction.

6 Set up two PCR reactions per sample (for Pool 1 and 2) as follows in tubes or plates. Mix by gentle pipetting and pulse spin to collect liquid at the bottom.

A	B	C
Component	Reaction Mix 1	Reaction Mix 2
5X Q5 Reaction Buffer	5µL	5µL
10 mM dNTPs	0.5µL	0.5µL
Q5 Hot Start DNA Polymerase	0.25µL	0.25µL
Pool 1 (10µM)	0.75µL	0µL
Pool 2 (10µM)	0µL	0.75µL
Nuclease-free water	18.5µL	18.5µL

Q5 High-Fidelity DNA Polymerase - 500 units **New England Biolabs Catalog #M0491L**

7 Add 2.5µl cDNA to each of the PCR reactions, mix by gentle pipetting and spin the tube to collect all liquid at the bottom of the tube.

8 Set up the following PCR conditions in the thermal cycler.

Stage	0 °C	00:00:00	Cycles
Heat Activation	98 °C	00:00:30	1
Denaturation	98 °C	00:00:15	25-35
Annealing	65 °C	00:05:00	25-35
Hold	4 °C	Indefinite	1

CITATION

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.

LINK

https://doi.org/10.1038/nprot.2017.066

PCR clean-up

9 Label the tubes/plates and pool the PCR reactions for each samples into a single LoBind tube .

A	B
PCR pool 1	25µL
PCR pool 2	25µL
Nuclease Free water	0µL
Total	50µL

Note

When higher viral loads are anticipated based on Ct values < 20 then reduce the amount of amplicons to

15-20µL each and make up the volume to 50µl with Nuclease free water.

10 Clean-up the amplicons using AMPure XP beads **Beckman Coulter** .

Thaw the AMPure beads to room temperature and vortex thoroughly to ensure that the beads are well suspended/ until the solution is uniformly brown in colour.

10.1 Add 0.4x volume (0.4:1) of AMPure beads to the sample tube and mix gently by either flicking or pipetting. For example add 20,µl AMPure beads to a 50 µl reaction.

10.2 Spin the tubes to collect entire liquid at the bottom and incubate the tubes at room temperature for 00.05.00.

10.3 Place the tubes on magnetic stand and incubate for 00:02:00 or until the beads have settled and the supernatant is completely colourless.

10.4 Carefully remove and discard the supernatant, without disturbing the bead pellet.

10.5 Add 200µl of 70% ethanol or enough to cover the pellet and wait for 30s.Carefully remove and discard ethanol delicately without disrupting the bead pellet.

10.6 and repeat Spin to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible.

10.7 Incubate the tube with open lid for 00:00:30 or until the pellet is dried (if the pellet dries completely it will crack and become difficult to resuspend)

Resuspend pellet in 30µl Elution Buffer (EB), mix gently by either flicking or pipetting and incubate for

10.8 00:02:00.Make sure that the pellet is completely resuspended.

10.9 Place on magnetic stand and transfer sample to a clean 1.5mL LoBind tube ensuring no beads are transferred into this tube.

10.10 Quantify 1µl product using the Qubit Fluorometer using the
Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854** .

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

End-Prep Reaction

11 In a new PCR plate/ tubes set up the following reaction for each sample. Prepare Master mix and aliquote it into tubes . If the Qubit reading is > 15ng/ul ,

A	B
Component	Volume
PCR dilution from previous step	3.3µL
Ultra II End Prep Reaction Buffer	1.2µL
Ultra II End Prep Enzyme Mix	0.5µL
Nuclease-free water	5µL
Total	10µL

If the Qubit reading is 5ng/ul -15ng/ul

A	B
Component	Volume
PCR dilution from previous step	5µL
Ultra II End Prep Reaction Buffer	1.2µL
Ultra II End Prep Enzyme Mix	0.5µL

A	B
Nuclease-free water	3.3µL
Total	10µL

If the Qubit reading is <5ng/ul,

A	B
Component	Volume
PCR dilution from previous step	8.3µL
Ultra II End Prep Reaction Buffer	1.2µL
Ultra II End Prep Enzyme Mix	0.5µL
Nuclease-free water	0µL
Total	10µL

NEBNext Ultra II End Prep Reaction Buffer **New England Biolabs Catalog #E7647**

NEBNext Ultra II End Prep Enzyme Mix **New England Biolabs Catalog #E7646**

11.1 Reaction conditions for End Prep:

Incubate

25 °C for 00:15:00

65 °C for 00:15:00

On ice for 00:01:00

Native Barcoding

12 In a new labelled tube/plate prepare the following components.

A	B
Component	Volume
End-preparation reaction mixture	0.75µL
NBXX barcode	1.25µL
Blunt/TA Ligase Master Mix	5µL
Nuclease-free water	3µL
Total	10µL

Native Barcoding Expansion 1-12 (PCR-free) **Contributed by users Catalog #EXP-NBD104**

Native Barcoding Expansion 13-24 (PCR-free) **Oxford Nanopore Technologies Catalog #EXP-NBD114**

NEB Blunt/TA Ligase Master Mix **Contributed by users Catalog #M0367**

12.1 Incubate at

25 °C for 00:20:00

65 °C for 00:10:00

On ice for 00:01:00

12.2 In a new 1.5mL LoBind tube pool all barcoded reactions together.

12.3 Add 0.4x volume of AMPure beads to the sample tube and mix by either flicking or gentle pipetting. For example add 96µl AMPure beads to 240µl pooled barcoding reactions.

12.4 Briefly vortex and mix the reactions and incubate at room temperature for 00.05.00.

12.5 Place on magnetic stand and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

12.6 Remove the tube from the magnetic stand and add 200µl SFB and resuspend beads completely using pipette. Spin down to collect liquid at the bottom of the tube and place on the magnetic stand. Remove supernatant and discard.

12.7 Add 200µl of 70% ethanol to wash the pellet. Carefully remove and discard ethanol, being careful not to touch the bead pellet.

12.8 Spin to collect all liquid at the bottom of the tube and carefully remove as much residual ethanol as possible.

12.9 With the tube lid open incubate for 00:00:30 or until the pellet is dried (if the pellet dries completely it will crack and become difficult to resuspend).

12.10 Resuspend pellet in 30µl Elution Buffer(EB), mix gently by either flicking or pipetting and incubate for 00:02:00.

12.11 Place on magnet stand and transfer sample to a clean 1.5mL Eppendorf tube ensuring no beads are transferred into this tube.

12.12 Quantify 1µl of the barcoded amplicons using the Qubit Flurometer.

Equipment

Qubit	NAME
Flurometer	TYPE
Invitrogen	BRAND
Q33228	SKU
https://www.thermofisher.com/order/catalog/product/Q33228	LINK

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Adapter Ligation

13 Prepare the following reaction for AMII Adapter ligation in a PCR tube.

A	B
Component	Volume
Barcoded amplicon pool	30µL
NEBNext Quick Ligation Reaction Buffer (2X)	40µL

A	B
Adapter Mix (AMII)	5µL
Quick T4 DNA Ligase	5µL
Total	80µL

13.1 Incubate at 25 °C for 00:20:00, possibly in a thermo cycler.

Post adapter ligation Clean-up

14 Add 80µl (1:1) of AMPure beads to the sample tube and mix gently by either flicking or pipetting. Spin to collect all liquid at the bottom of the tube.

14.1 Incubate for 00:05:00 at room temperature.

14.2 Place on magnetic rack and incubate for 00:02:00 or until the beads have pelleted and the supernatant is completely clear. Carefully remove and discard the supernatant, being careful not to touch the bead pellet.

14.3 Add 200µl SFB and resuspend beads completely by pipette mixing. Brief spin to collect all liquid at the bottom of the tube. Remove supernatant and discard.

14.4 Repeat step 14.3 to perform a second SFB wash.

14.5 Brief spin and remove any residual SFB. Add 15µl EB (ONT) and resuspend beads by gentle flicking or pipette mixing.

14.6 Incubate at room temperature for 00:02:00 and place on magnetic stand until clear. Transfer final library to a new 1.5mL LoBind tube

14.7 Quantify 1µl of the barcoded amplicons using the Qubit Fluorometer.

Equipment

Qubit	NAME
Flurometer	TYPE
Invitrogen	BRAND
Q33228	SKU
https://www.thermofisher.com/order/catalog/product/Q33228	LINK

Qubit® dsDNA HS Assay Kit **Thermo Fisher Scientific Catalog #Q32854**

Flow cell Priming and Sequencing

15 Thaw the following reagents.

Sequencing buffer (SQB)
Loading beads (LB)
Flush buffer (FB)
Flush tether (FLT)

15.1 Add 30µl FLT to the FB tube and mix well by vortex and spin down.

15.2 Prepare the following library dilution for sequencing in a LoBind tube:

A	B
SQB	37.5µL
LB	25.5µL
Library	12-14µL

A	B
Total	75-80µL

Ligation sequencing kit 1D **Oxford Nanopore Technologies Catalog #SQK-LSK109**

Flow Cell Priming Kit **Oxford Nanopore Technologies Catalog #EXP-FLP002**

ONT MinION Flow Cell R9.4.1 **Oxford Nanopore Technologies Catalog #FLO-MIN106D**

15.3 Flowcell Priming, loading of the library and sequencing are performed based on the following protocol: dx.doi.org/10.17504/protocols.io.bbmuik6w