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Poliovirus Sequencing Co…

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We use this protocol and it's working

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Abstract

This protocol is an update from the protocol described in the paper "Rapid and sensitive direct detection and identification of poliovirus from stool and environmental surveillance samples using nanopore sequencing" by Shaw et al in the Journal of Clinical Microbiology (2020), DOI: 10.1128/JCM.00920-20 and is commonly known as Direct Detection of Poliovirus by Nanopore Sequencing (DDNS).

The protocol aims to amplify the VP1 region of poliovirus through a nested PCR using panEV primers followed by amplification of the VP1 sequence using the Q8/Y7 primer set. We use barcoded primers as this greatly simplifies the subsequent library preparation process. Primer sequences for the panEV primers, Q8/Y7 primers and barcoded Q8/Y7 primers are found in Dataset S1 of the publication.

This protocol is for use with Oxford Nanopore kit14 chemistry sequencing reagents and MinION Mk1B, MinION Mk1C, or GridION sequencer.

Guidelines

Steps 12 onwards are based on protocols from Oxford Nanopore Technologies.

Materials

- NEBNext Ultra II End Repair/dA-Tailing Module - 24 rxns **New England Biolabs Catalog #**E7546S
- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S
- Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114
- Ethanol, Absolute, Molecular Biology Grade **Thermo Fisher Scientific Catalog #**BP2818500

0.2ml PCR tubes

- 1.5 mL LoBind tubes **Eppendorf Catalog #**022431021
- UltraPure™ DNase/RNase-Free Distilled Water **Thermo Fisher Scientific Catalog #**10977023
- Ultrapure BSA **Ambion Catalog #**AM2616
- Agencourt AMPure XP **Beckman Coulter Catalog #**A63880
- Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114

Before start

This protocol describes the amplification of the VP1 sequence, sample barcoding and library preparation. We anticipate users will have performed an RNA extraction prior to this protocol to extract Poliovirus RNA. We recommend either the Roche High Pure Viral RNA Kit (with added proteinase K), QIAamp Viral RNA Mini Kit or the MagMAX Viral RNA Isolation Kit for this process.

Barcoded VP1 Primers:

For high throughout sequencing in 96 well plates, we recommend the purchase and assembly of a 96-well primer plate with 10 µM of barcoded Y7 primer and 10 µM of barcoded Q8 primer in each well.
Each well contains Q8 and Y7 primers with the same unique barcode e.g A1 = Y7 with barcode 1 and Q8 with barcode 1, A2 = Y7 with barcode 2 and Q8 with barcode 2, etc.

The full set of 96 barcoded primer sequences are shown in Dataset_S1 of Shaw et al, 2020.

Nested PCR First Round (PanEV)

1 Prepare a master mix using the reaction volumes detailed in the table below for the number of samples you have plus negative controls. 20m

Forward primer: 5'NTR [TGGCGGAACCGACTACTTTGGGTG] (Arita et al. 2015)

Reverse Primers: Cre [TCAATACGGTGTTTGCTCTTGAACTG] (Arita et al. 2015)
nOPV-MM-R [TCGATACGGTGCTTGGATTTAAATTG]

Note: The reverse primers include both a Pan-enterovirus primer, and a primer which allows amplification of nOPV2. **These are mixed in equally to make a 10µM working solution.**

	A	B
	Reagent	1 reaction (µL)
	2x Master mix	12.5
	SSIII Platinum Taq mix	1
	Reverse primers (10µM)	1
	Nuclease free water	4.5

Table1: Mastermix contents for a single PanEV rt-PCR reaction. This can be multiplied up to fit the number of reactions you will be carrying out.

2 Vortex the mastermix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the tube. Aliquot 19µL to each PCR tube and add 5µL of sample RNA or nuclease free water for negative controls.

3 Incubate at 50°C for 30 minutes. 30m

4 Add 1µL of the forward primer to each reaction.

5 Amplify using the following cycling conditions: 4h 30m

	A	B	C	D
	Cycle	Step	Temperature (°C)	Time
	1	Initial denaturation	94	2 minutes
	42	Denaturation	94	15 seconds
		Annealing	55	30 seconds
		Extension	68	4 minutes 30 seconds
	1	Final extension	68	5 minutes
	-	Hold	10	-

Table 2: Cycling conditions for the first round PanEV PCR

Nested PCR Round 2 (VP1) 2h 30m

6 VP1 amplification is performed using barcoded primers as described in Dataset_S1 in Shaw *et al* 2020. These are in a 96-well plate layout and the forward and reverse primers can be premixed to make a 10uM working stock. 30m

Prepare a mastermix as described below for the number of reactions you require:

	A	B
	Reagent	1 Reaction (µL)
	DreamTaq 2x mastermix	12.5
	Nuclease free water	8.5

Table 3: Mastermix contents for second round PCR using barcoded primers.

7 Vortex the mastermix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the tube.

8 Aliquot 21µL for each well of a 96-well PCR plate and add 2µL of 10uM barcoded primers (ensuring a different barcode is used for each sample) and 2µL of first round PCR product or nuclease free water for negative controls.

9 Amplify using the following cycling conditions: 2h

	A	B	C	D
	Cycle	Step	Temp (C)	Time
	1	Initial Denaturation	95	2 minutes
	35	Denaturation	95	30 seconds
		Annealing	55	30 seconds
		Extension	72	1 minute
	1	Final Extension	72	10 minutes
	-	Hold	10	

Table 4: Cycling conditions for VP1 PCR

10 You can check a representative set of samples on an agarose gel or Tapestation (Agilent) to confirm success of the PCR. A band is expected around 1.2kb

Library Preparation for ONT MinION: Pooling, End-prep, and Adapter ligation

11 Pool 2µL of each VP1 PCR product into a 1.5mL tube and concentrate with Ampure beads

Note: We have found the pooling by volume rather than quantifying and equimolar pooling works well for routine surveillance where many samples may be negative. It also cuts down the library preparation time.

11.1 Add a volume of Ampure beads equal to the volume of the pooled VP1 products and incubate at room temperature for 5 minutes

e.g. 50 samples, 2ul each pooled = 100ul pool, so add 100ul Ampure beads

11.2 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear.

11.3 Pipette off the solution, avoiding disturbing the bead pellet.

11.4 Add 200µL of 80% Ethanol to the tube, leave for 30seconds, then remove and discard.

Repeat.

11.5 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining Ethanol.

Allow the pellet to air dry for 1minute or until dry but not cracking

11.6 Take the tube off the magnet and add 51µL of nuclease free water. Flick the tube to resuspend the beads and incubate at room temperature for 2 minutes.

11.7 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely.

11.8 Remove 50µL of the eluted DNA and add to a clean 0.2mL PCR tube.

12 End-preparation:
Add the following reagents to the 0.2mL tube containing the cleaned DNA pool.

	A	B
	Component	Volume (µL)
	UltraII End-prep reaction buffer	7
	UltraII End-prep enzyme mix	3

Table 5: Reaction for end-prep of your pooled library

13 Mix gently by flicking the tube and spin down for 3 seconds.

14 Incubate for 5 minutes at 20C followed by 5 minutes at 65C 10m

15 Transfer to a 1.5mL tube and perform an AMPure bead clean. 15m

15.1 Vortex the Ampure beads until all the beads are well mixed.

15.2 Add 60µl of resuspended beads to the tube and flick the tube to mix.

Incubate at room temperature for 5 minutes

15.3 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear.

15.4 Pipette off the solution, avoiding disturbing the bead pellet.

15.5 Add 200µL of 80% Ethanol to the tube, leave for 30seconds, then remove and discard.

Repeat.

15.6 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining Ethanol.

Allow the pellet to air dry for 1minute or until dry but not cracking

15.7 Take the tube off the magnet and add 61µL of nuclease free water. Flick the tube to resuspend the beads and incubate at room temperature for 2 minutes.

15.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely.

15.9 Remove 60µL of the eluted DNA and add to a clean 1.5mL tube.

16 Spin down and thaw Ligation Adapter (LA) on ice.
Spin down the NEB Quick T4 Ligase and place on ice
Thaw Ligation Buffer (LNB) at room temperature, spin down, mix by pipetting, then place on ice.
Thaw Elution Buffer (EB), and Short Fragment Buffer (SFB) at room temperature, mix by vortexing then place on ice.

Remove your flow cell from the fridge to allow it to get to room temperature.

17 Prepare the following reaction mix adding reacgents to the 1.5mL tube with end-prepped DNA:

	A	B
	Component	Volume (µL)
	End-prepped DNA	60
	Ligation buffer (LNB)	25
	Quick T4 Ligase	10

	A	B
	Ligation Adapter (LA)	5

Table6: Reaction mix for sequencing adapter ligation

18 Mix gently by flicking the tube then spin down.

19 Incubate at room temperature for 10 minutes.

19.1 During this time, you can run your flow cell check

Plug in your sequencing device, open the lid and insert your flowcell. In the MinKNOW software, navigate to the start panel then select flowcell check, then start. This will tell you how many pores are available for sequencing.

20 Carry out an AMPure bead purification using 40µL of resuspended AMPure XP beads.

Note: This clean-up is different to previous as it uses the ONT Short Fragment Buffer (SFB) and Elution buffer (EB) instead of 80% ethanol and water.

20.1 Vortex the Ampure beads until all the beads are well mixed.

20.2 Add 40µL of resuspended beads to the 1.5mL tube and mix by flicking the tube.

Incubate at room temperature for 5 minutes

20.3 Spin down the tube for 3 seconds then place on a magnetic rack until all the beads have formed a pellet and the solution is clear.

20.4 Pipette off the solution, avoiding disturbing the bead pellet.

20.5 Add 250µL of Short Fragment Buffer (SFB). Remove the tube from the magnet and resuspend the beads in the SFB by flicking the tube.
Spin down for 3 seconds then return the tube to the magnet.
Allow the beads to pellet, then remove and discard.

Repeat.

20.6 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining SFB.

Allow the pellet to air dry for 1minute or until dry but not cracked

20.7 Take the tube off the magnet and add 15µL of Elution buffer (EB). Pipette to resuspend the beads and incubate at room temperature for 10 minutes.

20.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely.

20.9 Remove the eluted DNA and transfer to a clean 1.5ml tube.

21 Quantify your adapted library using a Tapestation or Qubit Fluorometer

21.1 Transfer 20fmol (16ng) of your library into a 0.2mL PCR tube and make up to 12uL using Elution Buffer, then place on ice until ready for loading.

If you need to dilute the library for easier pipetting, you can dilute it in Elution Buffer.

Priming and Loading of the MinION Flowcell

22 Thaw the Sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flush (FCF) at room temperature then place on ice.

Mix the SQB, FCF, and FCT by vortexing, spin down, and return to ice.

23 To create the priming mix, add 30µL of FCT and 5uL of BSA (50mg/ml) to the tube of FCF then mix by pipetting to create the priming mix

24 Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so that the priming port is visible. After opening the priming port, check for any bubbles under the cover. Draw back a small volume to remove any bubbles (a few µLs). Visually check that there is continuous buffer from the priming port across the sensor array.

25 Using a P1000 pipette, slowly load 800µL of the priming mix into the flow cell via the priming port.

Leave a small amount of liquid in the end of the pipette tip to ensure you do not introduce air into the flowcell.

Leave for 5 minutes.

26 Mix the contents of the LIB tube by pipetting just before adding to the following library mix in a 1.5ml tube:

	A	B
	Reagent	Volume (µL)
	DNA library	12
	Sequencing b uffer (SB)	37.5
	Library beads (LIB)	25.5

27 Complete the flowcell priming by opening the SpotOn port cover and carefully loading 200µL of the priming mix into the **priming port.** As before, leave a small amount of liquid in the bottom of the tip to avoid the introduction of air bubbles.

When adding the priming mix, you may see a small amount of liquid come up through the SpotOn port. If you do, pause and allow the liquid to flow back into the flowcell before continuing putting through the priming mix.

28 Mix the prepared library mix gently by pipetting.

Add the library mix to the flowcell via the SpotOn port in a dropwise fashion, allowing each drop to flow into the flowcell before adding the next.

29 Replace the SpotOn port cover and close the priming port, then replace the lid of your sequencing device.

30 Open the ONT MinKNOW software and follow the steps to set up and start your sequencing run.

In the Start section, select start run and follow the prompts to select the kit used, set the run time, and set basecalling and demultiplexing.