Version 3

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Nested VP1 PCR and Nanopore Sequencing from Stool and ES Samples v1.2 V.3

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Poliovirus Sequencing Consortium

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ABSTRACT

This protocol is updated 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 advise the use of barcoded primers where possible as this greatly simplifies the subsequent library preparation process. Additional steps have however been included in the case that the second PCR step is performed either with standard Q8/Y7 primers or using Q8/Y7 with the barcode adaptors (BCA) attached. Primer sequences for the panEV primers, Q8/Y7 primers and modified Q8/Y7 primers are found in Dataset S1 of the publication.

Sequencing of the panEV product is also possible through the removal of the VP1 nested PCR steps and the preparation of the panEV product for sequencing.

Version updates:
v1.1 – Updated to LSK-109
v1.2 – Minor formatting edits
v1.3 – Streamlining and minor alterations
v1.4 – Written inclusion of negative control in PCR
v1.5 – Updated to dreamtaq and Y7R primers
v1.6 – Updated MinKNOW instructions & minor edits
v1.7 – Changed back to Y7 primers
v1.10 – Updated to LSK-110, removed cleaning and quantification prior to pooling, updated taq volume in rtPCR, altered nested VP1 to use neat panEV product.
v1.11 – Updated RNA extraction to include Proteinase K, removed DNA CS addition, added pooling of Y7/Q8 primers.
v1.2 - Updated authorship, addition of nOPV2 primers

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PROTOCOL CITATION

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MANUSCRIPT CITATION please remember to cite the following publication along with this protocol

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GUIDELINES

Steps 29 onwards are based on protocols from Oxford Nanopore Technologies.

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MATERIALS TEXT

MATERIALS

For protocols with both barcoded and standard primers:

Agencourt AmPure XP beads **Contributed by users Catalog #A63880**

SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase **Thermo Fisher Catalog #12574018**

NEBNext® Companion Module for Oxford Nanopore Technologies® Ligation Sequencing (E7180S)
Ligation Sequencing Kit 1D (SQK-LSK110)
DreamTaq (K1071 or K1072)

PanEV primers: 5'NTR [TGGCGGAACCGACTACTTTGGGTG], Cre [TCAATACGGTGTTTGCTCTTGAACTG]
Optional- nOPV2-R [TCGATACGGTGCTTGGATTTAAATTG] (For nOPV2 detection)

Freshly prepared 70% ethanol in nuclease-free water
Nuclease-free water

Reagents required only for protocol with barcoded primers:

Barcoded primers, with the structure
Forward primer: Y7_VP1_BC#
GGTGCTG##########################TTAACCTGGGTTTGTGTCAGCCTGTAATGA

Reverse Primer: Q8_VP1_BC#
GGTGCTG##########################TTAACCTAAGAGGTCTCTRTTCCACAT

where # strings are Oxford Nanopore barcode sequences (see Shaw *et al*, 2020)

Reagents required only for protocol with standard primers:

Forward primer: Y7_VP1 [TTAACCTGGGTTTGTGTCAGCCTGTAATGA]
Reverse Primer: Q8_VP1 [TTAACCTAAGAGGTCTCTRTTCCACAT]
NEBNext®Ultra™ II End Repair/dA-Tailing Module (NEB, E7546)
Blunt/TA Ligase Master Mix (NEB, M0367)
PCR Barcoding Kit (Oxford Nanopore, EXP-PBC001) or PCR Barcoding Expansion Pack 1-96 (Oxford Nanopore, EXPPBC096)
LongAmp Taq 2X Master Mix (NEB, M0287)

BEFORE STARTING

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 as fragmentation of the RNA will prevent successful amplification during the panEV PCR.

PanEV primers

For the initial RT-PCR, uses the PanEV primers 5'NTR [TGGCGGAACCGACTACTTTGGGTG] and Cre [TCAATACGGTGTTTGCTCTTGAACTG] (Arita *et al,* 2015)

For nOPV2 detection a second Reverse primer, nOPV2-R, should be mixed with Cre in equal quantities to make

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up a 10 µM working solution.
nOPV2-R [TCGATACGGTGCTTGGATTTAAATTG]

Barcoded VP1 Primers

For high throughout sequencing in 96 well plates we recommend the purchase and assembly of 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.
Primer structure:
Forward primer:
Y7_VP1_BC#GGTGCTG##########################TTAACCTGGGTTTGTGTCAGCCTGTAATGA

Reverse Primer: Q8_VP1_BC#
GGTGCTG##########################TTAACCTAAGAGGTCTCTRTTCCACAT

Barcode sequences as Nanopore ligation sequencing barcodes and as shown in Shaw *et al,* 2020.

Nested PCR First Round (PanEV)

1 Nested PCR First Round (panEV primers):
Prepare a Master mix using reaction volumes as detailed below, **excluding forward primer and the RNA**:

Forward Primer (5'NTR): [TGGCGGAACCGACTACTTTGGGTG] (Arita et al. 2015)
Reverse Primer (Cre): [TCAATACGGTGTTTGCTCTTGAACTG] (Arita et al. 2015)

NB. For the detection of nOPV2, a second Reverse primer, nOPV2-R, should be mixed with Cre in equal quantities to make up a 10 µM working solution.

nOPV2-R: [TCGATACGGTGCTTGGATTTAAATTG]

A	B
	1 Reaction (µL)
2x Master Mix	12.5
SS III Platinum Taq mix	1
Reverse Primer (10 µM)	1
Nuclease Free Water	4.5
Forward Primer (10 µM)	1
RNA	5
Total volume	25

2 Briefly vortex and centrifuge down. Add 19 µL of master mix to each PCR tube and 5 µL of eluted RNA.

3 Incubate at 50 °C for 30 minutes.

4 Add 1 µL of the forward primer to the tubes.

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5 Amplify using the following cycling conditions:

A	B	C	D
CYCLE	STEP	TEMP (°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	-

* Extension time for panEV amplification

Nested PCR Round 2 (VP1)

6 VP1 amplification is performed using the Q8/Y7 primers. These can be modified to remove library preperation steps as shown in Shaw *et al* 2020, DOI: 10.1128/JCM.00920-20. The basic PCR uses the primers

Forward Primer (Y7): GGGTTTGTGTCAGCCTGTAATGA (Kilpatrick et al. 2011)
Reverse Primer (Q8): AAGAGGTCTCTRTTCCACAT (Yang et al. 1992)

These can be replaced with either Q8/Y7 primers with barcodes attached (sequences in Dataset_S1 of Shaw *et al* 2020). We recommend premixing these primers in a 96 well plate, with 10 µM forward primer and 10 µM of reverse primer in each well.

Prepare a Master mix using reaction volumes as detailed below, excluding the diluted PCR product (and primers if these are barcoded):

A	B
	1 Reaction (µL)
DreamTaq 2x master mix	12.5
Water	8.5
Forward primer (10 µM)	1
Reverse primer (10 µM)	1
PCR product	2*
Total volume	25

* Previous protocol editions have used a 5 µL of a 1 in 20 dilution of the PCR product (5 µL in 100 µL nuclease free water); we find that 2 µL of the neat product works as well, if not better, and reduces the potential for contamination.

7 Briefly vortex and centrifuge down the master mix and aliquot 20 µL into each PCR tube (or 18 µL if using barcoded primers).

8 Add 2 µL of premixed forward and reverse primers if necessary (i.e. if using barcoded VP1 primers).

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9 Add 2 µL of PCR product.

10 Amplify using the following cycling conditions:

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 minutes
1	Final Extension	72	10 minutes
-	Hold	10	-

11 PCR confirmation: Check a representative set of samples to confirm that the PCR has been successful.

12 For Standard Primers- Complete all steps
For Barcoded primers- Skip to Library Preparation for the ONT MinION: Pooling and adapting (step 29)

Library Preparation for the ONT MinION: Tailing and barcoding

13 AMPure bead purification using 15 µL (1 : 0.6 ratio) of resuspended AMPure XP beads and elute in 25 µL nuclease-free water, retaining 23 µL of eluate.

14 Standardise DNA:
Quantify 1 µL of purified PCR product using a Qubit fluorometer
Transfer 1 µg of DNA into a clean PCR tube.
Adjust the volume to 23 µL with nuclease-free water.

15 End-prep & dA-tailing:
Prepare the following reaction mix:

<1 µg DNA	23 µL
Ultra II End-prep reaction buffer	3.5 µL
Ultra II End-prep enzyme mix	1.5 µL
Nuclease-free water	2.5 µL

16 Mix gently by flicking, and spin down.

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17 Incubate for 5 minutes at 20 °C and 5 minutes at 65 °C

18 AMPure bead purification using 30 µL (1 : 1 ratio) of resuspended AMPure XP beads and elute in 20 µL nuclease-free water, retaining 16 µL of eluate.

19 Quantify 1 µL of end-prepped DNA using a Qubit fluorometer - recovery aim > 700 ng.

20 Ligation of Barcode Adapter
Add the reagents in the order given below:
15 µL End prep DNA
10 µL Barcode Adapter
25 µL Blunt/TA Ligase Master Mix

21 Mix gently by flicking the tube, and spin down.

22 Incubate the reaction for 10 minutes at room temperature.

23 AMPure bead purification using 20 µL (1 : 0.4 ratio) of resuspended AMPure XP beads and elute in 25 µL nuclease-free water, retaining 15 µL of eluate.

24 Standardise the DNA:
Quantify 1 µL of the adapted DNA using a Qubit fluorometer
Transfer 100 fmol of DNA into a clean PCR tube
Adjust the volume to 24 µL with nuclease-free water.

25 Barcoding PCR :
Set up a barcoding PCR reaction as follows for each sample:
1 µL PCR Barcode
24 µL 100 fmol PCR Product
25 µL LongAmp Taq 2x master mix

26 Mix gently by flicking the tube, and spin down.

27 Amplify using the following cycling conditions:

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A	B	C	D
CYCLE	STEP	TEMP (°C)	TIME
1	Initial Denaturation	95	3 minutes
12	Denaturation	95	15 seconds
	Annealing	62	15 seconds
	Extension	65	2 minutes*
1	Final Extension	65	2 minutes
-	Hold	4	-

* Extension time for VP1 sequencing

28 AMPure bead purification using 40 µL (1 : 0.8 ratio) of resuspended AMPure XP beads and elute in 25 µL nuclease-free water, retaining 23 µL of eluate.

Library Preparation for the ONT MinION: Pooling and adapting

29 Sample Pooling for standard VP1 primers:
Quantify 1 µL of the barcoded DNA using a Qubit fluorometer
Prepare 1 µg of pooled barcoded DNA in 47 µl Nuclease-free water.

Sample Pooling for barcoded VP1 primers:
There are two options for pooling the barcoded amplicons:
1) Equimolar pooling
Perform an AMPure bead purification using 15 µL (1 : 0.6 ratio)
Quantify 1 µL of the barcoded DNA using a Qubit fluorometer
Calculate the amount of each sample required to evenly pool 1 µg of DNA
Pool the samples accoridng to this caluclation and add nuclease-free water to a total volume of 47 µl
Note step 30, which is useful for low cocnentration samples where it may be difficult to pool 1 µg in a 47 µl volume.

OR

2) Equi-volume Pooling
This method avoids cleaning and quantification of samples and We typically perform this for routine testing of AFP stool samples where many will be negative.
Simply pool 2 µl of every sample and perform step 30.

30 Optional- If pool volume is >47 µL, concentrate an AMPure bead purification using a 1:1 ratio of resuspended AMPure XP beads and elute in 50 µL nuclease-free water, retaining 47 µL of eluate.

31 End-prep and dA-tailing:
Add the following reagents in a 0.2 mL PCR tube.
47 µL 1 µg DNA
3.5 µL NEBNext FFPE DNA Repair Buffer
2 µL NEBNext FFPE DNA Repair Mix
3.5 µL Ultra II End-prep reaction buffer
3 µL Ultra II End-prep enzyme mix

32 Mix gently by flicking the tube, and spin down

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33 Incubate for 5 minutes at 20 °C and 5 minutes at 65 °C.

34 Place on ice for 30 seconds.

35 Transfer sample to a 1.5 mL Eppendorf DNA LoBind tube.

36 AMPure bead purification using 60 µL (1 : 1 ratio) of resuspended AMPure XP beads and elute in 61 µL nuclease-free water, retaining 60 µL of eluate in a clean 1.5 mL Eppendorf DNA LoBind tube.

37 Adaptor ligation:
Thaw and prepare the kit reagents as follows:
Spin down and thaw Adapter Mix (AMX-F) on ice
Spin down T4 Ligase from NEBNext Quick Ligation Module (E6056) on ice
Thaw Ligation Buffer (LNB) at room temperature, spin down, mix by pipetting. Place on ice.
Thaw Elution Buffer (EB) at room temperature, mix by vortexing, spin down. Place on ice.
Thaw one tube of S Fragment Buffer (SFB)* for VP1 at room temperature, mix by vortexing, spin down and place on ice.

*For panEV sequencing, use L Fragment Buffer (LFB)

38 Prepare the following reaction mix in a 1.5 mL Eppendorf DNA LoBind tube:
60 µL DNA
25 µL Ligation Buffer (LNB)
10 µL NEBNext Quick T4 DNA Ligase
5 µL Adapter Mix (AMX-F)

39 Mix gently by flicking the tube, and spin down.

40 Incubate the reaction for 10 minutes at room temperature.

41 AMPure XP cleanup:
Prepare the AMPure XP beads for use; resuspend by vortexing.
Add 40 µL of resuspended AMPure XP beads to the adapter ligation reaction from the previous step and mix by pipetting.
Incubate on a rotator for 5 minutes at room temperature.
Place on magnetic rack, allow beads to pellet and pipette off supernatant.
Add 250 µL of the LFB/SFB to the beads.
Close the tube lid and resuspend the beads by flicking the tube.
Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.
Add 250 µL of the LFB/SFB to the beads.
Close the tube lid and resuspend the beads by flicking the tube.
Return the tube to the magnetic rack, allow beads to pellet and pipette off the supernatant.

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Spin down the tube and place back on the magnet.
Pipette off residual supernatant and briefly air dry.
Remove the tube from the magnetic rack and resuspend pellet in 15 µl Elution Buffer.
Incubate for 10 minutes at room temperature.
Pellet beads on magnet until the eluate is clear and colourless.
Remove and retain the eluate which contains the DNA library in a clean 1.5 mL Eppendorf DNA LoBind tube

42 Store the library on ice until ready to load into a nanopore flow cell.

Priming and loading the SpotON Flow Cell

43 A video explaining the setup and monitoring of a DDNS MinION sequencing run can be found at:
https://youtu.be/rYTKbLzNSog

Thaw the Sequencing Buffer (SQB), Loading Beads (LB), Flush Tether (FLT) and one tube of Flush Buffer (FLB) at *room temperature* before placing the tubes on ice as soon as thawing is complete.

Mix the Sequencing Buffer (SQB) and Flush Buffer (FLB) tubes by vortexing, *spin down* and return to ice.
Spin down the Flush Tether (FLT) tube, mix by pipetting, and return to ice.

44 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 small bubble under the cover. Draw back a small volume to remove any bubble (a few µLs). Visually check that there is *continuous buffer* from the priming port across the sensor array.

45 Prepare the flow cell priming mix: add 30 µL of thawed and mixed Flush Tether (FLT) directly to the tube of thawed and mixed Flush Buffer (FLB) and mix by pipetting up and down.

46 Load 800 µL of the priming mix into the flow cell via the priming port, avoiding the introduction of air bubbles. Wait for 5 minutes.

47 Thoroughly mix the contents of the LB tube by pipetting.

48 In a new tube, prepare the library for loading as follows:

A	B
Reagent	**Volume (µL)**
Sequencing Buffer (SQB)	37.5
Loading Beads (LB), mixed immediately before use	25.5
DNA library	12
Total	75

49 Complete the flow cell priming:
Gently lift the SpotON sample port cover to make the SpotON sample port accessible.
Load 200 µL of the priming mix into the flow cell via the priming port (**not** the SpotON sample port), avoiding the

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introduction of air bubbles.

50 Mix the prepared library gently by pipetting up and down just prior to loading.
Add 75 µl of sample to the flow cell via the SpotON sample port in a dropwise fashion. Ensure each drop flows into the port before adding the next.

51 Gently replace the SpotON sample port cover, making sure the bung enters the SpotON port, close the priming port and replace the MinION lid.

52 Use the Oxford Nanopore Ltd MinKNOW software to begin a sequencing run. Note that the piranha software package (step 53) will accept data that has been demultiplexed in MinKNOW.

Sequencing run and data analysis

53 For the analysis of DDNS data, we advise the use of the piranha (Poliovirus Investigation Resource Automating Nanopore Haplotype Analysis) software package which can be found at:
https://github.com/aineniamh/piranha

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