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Nested VP1 PCR and Nanopore Sequencing from Stool and ES Samples V.1

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Poliovirus Sequencing C...

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Working and published, but also under development. Updates will be added as newer versions.

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Abstract

This protocol is published 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.

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.

Guidelines

The section “Library Preparation for the ONT MinION” is based on the Oxford Nanopore Technologies 1D PCR barcoding amplicon/cDNA (SQK-LSK109).

Materials

MATERIALS

- NEBNext Quick Ligation Module - 20 rxns **New England Biolabs** **Catalog #**E6056S
- NEBNext FFPE DNA Repair Mix - 24 rxns **New England Biolabs** **Catalog #**M6630S
- Agencourt AmPure XP beads **Catalog #**A63880
- NEBNext End repair / dA-tailing Module (E7546)
- SuperScript™ III One-Step RT-PCR System with Platinum™ Taq DNA Polymerase **Thermo Fisher** **Catalog #**12574018
- Flow Cell Priming Kit (EXP-FLP002) **Oxford Nanopore Technologies** **Catalog #**EXP-FLP002
- Ligation sequencing kit 1D **Oxford Nanopore Technologies** **Catalog #**SQK-LSK109

Blunt/TA Ligase Master Mix (NEB, M0367) *Only required for standard primers*
PCR Barcoding Kit (Oxford Nanopore, EXP-PBC001) or PCR Barcoding Expansion Pack 1-96 (Oxford Nanopore, EXPPBC096) *Not required for barcoded primers*
LongAmp Taq 2X Master Mix (NEB, M0287) *Not required for barcoded primers*

Troubleshooting

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 recommened either the Roche High Pure Viral RNA Kit, QIAamp Viral RNA Mini Kit or the MagMAX Viral RNA Isolation Kit for this process as fragmentation of the RNA will prevent succesful amplification during the panEV PCR.

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: TGGCGGAACCGACTACTTTGGGTG (Arita et al. 2015)
Reverse Primer: TCAATACGGTGTTTGCTCTTGAACTG (Arita et al. 2015)

		1 Reaction (µL)
	2x Master Mix	12.5
	SS III Platinum Taq mix	0.5
	Reverse Primer (10 µM)	1
	Nuclease Free Water	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.

5 Amplify using the following cycling conditions:

	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 the barcode adaptor attached (BCA primers) or with barcodes attached (sequences in Dataset_S1 of Shaw *et al* 2020)

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

		1 Reaction (µL)
	DreamTaq 2x master mix	12.5

	Water	9.5
	Forward primer (10 µM)	1
	Reverse primer (10 µM)	1
	PCR product	1*
	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 1 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 1 µL of barcoded forward primer and 1 µL of barcoded reverse primer if necessary.

9 Add 1 µL of PCR product.

10 Amplify using the following cycling conditions:

	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	-

PCR Steps

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

12 **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.

Library Preperation for the ONT MinION: Tailing and barcoding

13 For Standard Primers- Complete all steps
For BCA primers- Complete steps 24 to 28
For Barcoded primers- Skip to Library Preperation for the ONT MinION: Pooling and adapting

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.

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:

	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 Preperation for the ONT MinION: Pooling and adapting

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

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
1 µL DNA CS

32 Mix gently by flicking the tube, and spin down

33 Incubate for 10 minutes at 20 °C and 10 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) 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)

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.
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.