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Direct Detection of poliovirus and Nanopore Sequencing (DDNS) - Environmental Samples V.1

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Poliovirus Sequencing C...



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We are still developing and optimizing this protocol

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Abstract

This protocol covers amplification of poliovirus nucleic acid from concentrated sewage samples and the subsequent sequencing of barcoded VP1 amplicons.

The protocol was successfully tested on concentrates prepared with two-phase separation method, spin-column concentration and bag-mediated filtration system. Poliovirus molecular detection sensitivity will inherently depend on what viral content had been successfully captured in the starting wastewater concentrate sample.

Viral RNA should be purified in duplicates from each concentrate following the method for MagMAX Viral RNA Isolation Large volume extraction protocol using 1.2 mL of concentrate for each extraction. It is recommended to perform the extraction fresh so the RNA can be used immediately after purification. Each RNA sample is then subjected to two step amplification in triplicates using a semi-nested PCR approach. Once controls are confirmed by gel electrophoresis, the PCR products are carried forward for nanopore sequencing library preparation. The PCR amplicons are pooled and purified before end-repair and dA-tailing. Following sequencing adapter ligation, the sequencing mix is prepared and loaded onto the flow cell.

This protocol is for use with Oxford Nanopore kit14 chemistry ligation sequencing reagents and the prepared sequencing mix can be run on MinION Mk1B or GridION sequencer.



Materials

Equipment required:

Pipettes P10, P20, P200, P1000

Pipette tips P10, P20, P200, P1000

1.5 ml Eppendorf DNA LoBind microcentrifuge tubes

Thermal cycler

0.2 ml thin-walled PCR tubes/strips/plate

Microfuge

Vortex mixer

Microplate centrifuge

Magnetic racks of two types: accommodating PCR plate/strips and 1.5 mL tube(s)

Gel electrophoresis equipment or Tapestation

Qubit fluorometer, with assay tubes

Nanopore Sequencing device (Mk1B/Mk1C or GridION) with FLO-MIN114 / R10.4.1 flow cell

Gel electrophoresis capacity to visualise PCR products

Reagents required:

Primers

SuperScriptTM III One-Step RT-PCR System with PlatinumTM Taq DNA Polymerase (ThermoFisher, 12574026)

DreamTaq Hot Start PCR Mastermix (ThermoFisher, K9011 or K9012)

Oxford Nanopore Technologies Ligation Sequencing kit SQK-LSK114

Agencourt AMPure XP beads

Ultrapure BSA 50mg/ml (Invitrogen, AM2616)

NEBNext Quick Ligation Module (E6056)

NEBNext Ultra II End repair/dA-tailing Module (E7546)

Freshly prepared 80% ethanol in nuclease-free water

Nuclease-free water

Qubit Broad Range dsDNA kit or Tapestation DNA kit

Gel electrophoresis agarose gel kit and buffers, DNA dye and 1 kb DNA ladder or Tapestation DNA kit



Before start

For direct detection of poliovirus in waste water we recommend to perform the large volume viral RNA extraction protocol on each sewage concentrate in duplicates. Each RNA sample should be analysed in triplicate PCRs, resulting in a total of 6 sequencing results for each sewage concentrate.

This protocol describes steps following viral enrichment by sewage concentration and RNA purification.

Notes for using this protocol:

Avoid pipette mixing of samples whenever possible, especially for RNA and long amplicons to minimise unwanted shearing and mix instead by gently flicking tubes or manually rotating plates.

Do not vortex enzyme tubes and always add ligase enzymes to the reaction mixtures last to reduce homopolymer formation. When precipitate is visible in buffers, warm to room temperature and dissolve by pipetting or vortexing.

Overdrying the pelleted beads can result in loss of long fragments due to inefficient elution as the nucleic acid will be bound to the beads too strongly. Always try to remove excess alcohol or Short Fragment Buffer at the end of wash steps using a small pipette tip and dry pellets for a short period of 30s to 1 min only to avoid cracking and overdrying.

Apart from individual amplicon quantitation, at least one quantitation of the pool is recommended during library preparation to ensure there is no significant loss of DNA and to avoid overloading the flow cell.

This can be done after pooling the barcoded samples and taking forward a maximum of 1000ng of DNA for adapter ligation. Any left-over pool can be stored at -20°C to be used later if needed.

The adapter ligated pool can be quantitated and checked for size distribution in troubleshooting to investigate if loss/fragmentation of amplicons occur during library preparation.

Viral Nucleic Acid Amplification - First Round, RT-PCR

- 1 Calculate the number of reactions to prepare.

2m

Each sewage concentrate RNA should be tested for amplification in triplicates to increase detection sensitivity. Positive and negative RNA extraction controls should also be included, along with additional PCR positive and negative controls. For PCR positive control use RNA that was previously successfully amplified and stored appropriately, this can ideally be your positive control RNA from your previous run.

- 1.1 Prepare enough RT-PCR Master mix for the number of reactions with allowing extra for pipetting loss by combining the reagents shown in the table below in a labelled microcentrifuge tube:

20m

A	B
RT-PCR	Per reaction (µL)
2x Reaction Mix	12.5
SSIII Platinum Taq Enzyme mix	1
Reverse Primers working stock (10 µM)	1
Nuclease free Water	1.5
Total volume	16

Reverse transcription reaction mix

RT-PCR primers (to be used as 10 µM working stocks):

Forward: Y7 [TCAATACGGTGTTTGCTCTTGAAGT]

Reverse: AriR/Cre [TCAATACGGTGTTTGCTCTTGAAGT] (Arita et al. 2015)

& nOPV-MM-R [TCGATACGGTGCTTGGATTAAATTG]

Note

The reverse primers include both a Pan-enterovirus primer and a primer which allows amplification of nOPV2. These are mixed to make a working solution with each oligo at 10 μ M .

For example, 10 μ L of each 100 μ M reverse primer stock is added to 80 μ L of nuclease free water to create 100 μ L of a working solution with each primer at 10 μ M. Any leftover primer working stock can be frozen for future use.

1.2 Briefly vortex and centrifuge the Master mix and aliquot 16 μ L into each PCR tube.

5m

1.3 Add **8 μ L** of sample RNA/control RNA or nuclease free water for PCR negative control.

5m

1.4 Flick tubes to gently mix the content, then centrifuge them briefly and incubate at 50 $^{\circ}$ C for 30 minutes, with cooling down to 10 $^{\circ}$ C at the end of the program.

33m

Note

Only the reverse primers are used for the Reverse Transcription step. The forward primer is added before starting the PCR cycles.

1.5 Briefly centrifuge tubes after the RT step and add 1 μ L of the forward primer 10 μ M working stock to each tube using new pipette tip for each addition.

5m

1.6 Amplify using the following cycling conditions:

3h 20m

	A	B	C	D
	CYCLE	STEP	TEMP ($^{\circ}$ C)	TIME
	1	Initial Denaturation	94	2 minutes

II

	A	B	C	D
	42	Denaturation	94	15 seconds
		Annealing	55	30 seconds
		Extension	68	2minutes 30 seconds
	1	Final Extension	68	5 minutes
	-	Hold	10	-

Thermal profile for first round of PCR - half capsid

Amplicon size of the first PCR is ~2kb.

- 1.7 Use your first PCR reactions to set up the second PCR amplification immediately or store them in the fridge for short term or frozen for long term.

PCR Round Two - semi-nested VP1 amplification

2



DDNS_Primer_Sequences.xlsx 20.3KB

Second PCR reaction mix

VP1 amplification is performed using barcoded primers as described in Dataset_S1 in Shaw et al 2020. These should be ordered in a 96-well plate layout and the forward and reverse primers premixed to make an overall 10µM working stock.

Semi-nested VP1 primers:

Forward: barcoded Y7 [GGGTTTGTGTCAGCCTGTAATGA]

Reverse: barcoded Q8 [AAGAGGTCTCTRTTCCACAT]

To allow a simplified protocol, we use a 96-well primer plate with 5µM barcoded Y7 primer and 5µM 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 and in the attached spreadsheet. To prepare 200 µL primer working stock at 5 µM

for each oligo, add 10 μL of the 100 μM master primer stocks to 180 μL nuclease free water. Any leftover primer working stock can be frozen for future use.

- 2.1 Prepare enough PCR mastermix as shown below for the number of reactions performed, allowing extra for pipetting loss :

20m

A	B
Second PCR	Per reaction (μL)
DreamTaq 2x Hot Start Master mix	12.5
Water	8.5
Total volume	21

Second PCR reaction mix

- 2.2 Vortex the mastermix briefly and centrifuge to collect content.

10s

- 2.3 Aliquot 21 μL of mastermix for each reaction well and add 2 μL of the combined forward and reverse barcoded primers ensuring a different barcode is used for each sample.

5m

- 2.4 Add 2 μL of first round PCR product from the centrifuged RT-PCR wells. Add nuclease free water for the PCR negative control.

3m

- 2.5 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



	A	B	C	D
		Extension	72	1 minute
1		Final Extension	72	10 minutes
	-	Hold	10	

Thermal profile for second round of PCR - VP1

- 2.6 Run and check all your PCR controls from both reactions on gel electrophoresis to ensure successful amplification of the assay positive control and no contamination of the assay and PCR negative controls.

1h 20m



Library Preparation for Nanopore Sequencing: Pooling, End-prep, Adapter ligation

- 3 **Library Pooling:**
Pooling and purifying barcoded amplicons.

Note

Even pooling volumes are used to avoid time consuming individual purification and quantification steps. An alternative method to the one presented below is to perform AMPure bead clean for each sample with a 1:1 ratio of beads, quantify, and create an equimolar pool with a total of 1000 µg of DNA.

- 3.1 Pool 2 µL of each barcoded amplicon into a 1.5 mL tube.

5m

Note

Change tips between the individual PCR reactions to avoid cross-contaminating them.

- 3.2 Add a volume of room temperature AMPure beads equal to the volume of the pooled VP1 amplicons.
e.g. 50 samples, 2µL each pooled = 100µL pool, so add 100uL AMPure beads

10s



3.3 Mix the pool with the beads by gently flicking the tube and incubate at room temperature for 5 minutes. Flick again to keep the mixture a homogenous colour if beads appear to settle.

5m

3.4 Spin down the tube briefly then place on a magnetic rack until all the beads have formed a pellet and the solution is clear. Pipette off the supernatant without disturbing the bead pellet.

3m

Note

If beads are accidentally collected during removal of supernatant, just empty the pipette tip back into the tube. Centrifuge the tube again if necessary, place back on the magnet and wait for full separation of the pellet before trying removal again.

3.5 Keeping the tube on the magnet, rinse the pellet by adding 200µL of 80% Ethanol to the tube, leave for 30 seconds, then remove the supernatant and discard.

1m

3.6 Repeat Step 3.5 to complete two rinses with 80% Ethanol.

1m

3.7 Spin down the tube for 3 seconds, place back on the magnet, then remove any remaining Ethanol with a small volume, fine pipette tip.

30s

3.8 Allow the pellet to air dry for 30 seconds or until all alcohol evaporated , but avoid over drying to the point of cracking or crumbling.

1m

3.9 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.

3m

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

30s

II

4 End-preparation:

Preparing the pooled and purified amplicons for sequencing adapter ligation.

4.1 Add the following reagents to the PCR tube containing the 50 µL purified pool from Step 3.12 :

1m



Component	Volume (μL)
Ultrall End-prep reaction buffer	7
Ultrall End-prep enzyme mix	3

Reaction mix for end-prep of pooled library.

4.2 Mix gently by flicking the tube and spin down briefly to collect content.

10s

4.3 In the thermocycler, incubate for 5 minutes at 20°C followed by 5 minutes at 65°C

11m

Note

Create an End-prep incubation program in the thermocycler: 5 min at 20°C followed by 5 minutes at 65°C, followed by cooling and holding at 10°C.

4.4 Resuspend room temperature AMPure beads by vortexing and add 60 μL to a labelled clean 1.5 mL tube.

30s

4.5 Transfer the 60 μL End-prep reaction to the same tube and mix gently with the beads to allow the nucleic acid to bind. Incubate at room temperature for 5 minutes with flicking the tube gently after 2 minutes again to keep the mixture homogenous.

6m

4.6 Spin the tube briefly to collect content then place on the magnet, allowing the beads to pellet completely.

1m

4.7 Pipette off the supernatant without disturbing the pelleted beads.

30s

4.8 Keeping the tube on the magnet, rinse the pellet by adding 200μL of 80% Ethanol to the tube, leave for 30 seconds, then remove the supernatant and discard.

1m



- | | | |
|------|--|-----|
| 4.9 | Repeat Step 4.8 to complete two rinses with 80% Ethanol. | 1m |
|
 | | |
| 4.10 | Spin down the tube for 3 seconds, place back on the magnet, then remove any remaining Ethanol with a small volume, fine pipette tip. | 30s |
|
 | | |
| 4.11 | Allow the pellet to air dry for 30 seconds or until all alcohol evaporated , but avoid over drying to the point of cracking or crumbling. | 1m |
|
 | | |
| 4.12 | Take the tube off the magnet and add 61 µL of nuclease free water. Flick the tube to resuspend the beads and incubate at room temperature for 2 minutes. | 3m |
|
 | | |
| 4.13 | Spin down the tube briefly then place back on the magnet, allowing the beads to pellet completely until eluate is clear and colourless. | 1m |
|
 | | |
| 4.14 | Remove 60µL of the eluted DNA and add to a clean, labelled 1.5 mL Eppendorf DNA LoBind tube. | 30s |

Note

End-prepped DNA library can be stored at 4°C for up to one week, however it is advised to continue the library preparation protocol as soon as possible.

5 **Sequencing adapter ligation:** Motor protein ligation to end-prepped library.

- | | | |
|-----|--|----|
| 5.1 | Prepare reagents and materials for the next steps: Spin down the NEB Quick T4 Ligase and place on ice. Spin down and thaw Ligation Adapter (LA) 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. | 3m |
|-----|--|----|



In preparation for the sequencing run, remove the Flush buffer, flush tether (FLT) and BSA from the freezer and thaw at room temperature. Once thawed, place on ice.

Take the flow cell out of the fridge, remove it from the black pouch and open the plastic case to allow it reaching room temperature and letting any moisture evaporate.

- 5.2 Prepare the following reaction mix for adapter ligation, by adding reagents to the 1.5mL tube with end-prepped DNA:

2m

A	B
Component	Volume (μL)
End-prepped DNA	60
Ligation buffer (LNB)	25
Quick T4 Ligase	10
Ligation Adapter (LA)	5

Reaction mix for sequencing adapter ligation

- 5.3 Mix gently by flicking the tube and collect the content.

1m

- 5.4 Incubate the reaction at room temperature for 10 minutes.

10m

6 Library purification

Final bead cleaning of the library using Short Fragment Buffer (SFB) instead of ethanol and Elution Buffer (EB) instead of water.

Note

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



- 6.1 Vortex the AMPure beads to mix and add 40µL of the resuspended beads to the adapter ligation reaction tube from step 5 and mix the content by flicking the tube. 1m
- 6.2 Incubate mixture at room temperature for 5 minutes. Flick gently after 2 minutes ensuring homogenic mixture to aid binding. 5m
- 6.3 During this time, you can run your flow cell check
- Start MinKNOW 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.

Note

If your flow cell has been used before and the flow cell check returns an unexpectedly low pore number, instead of running a flow cell check you can start a dummy sequencing run. Do this by selecting Start Sequencing, name the run "flowcell_check", select any kit, then set the time to 0.2 hours, then start the run. At the beginning of the run it will do a short flow cell check and give a more accurate number for the available pores. The result is shown on the pore scan results panel (the pore count is the sum of available pores and unavailable pores shown on the graph) and also in system messages.

- 6.4 Spin down the tube briefly then place on a magnetic rack allowing the beads to pellet completely. The solution should become clear. 1m
- 6.5 Pipette off the supernatant without disturbing the pelleted beads. 30s
- 6.6 Remove the tube from the magnet and add 250µL of Short Fragment Buffer (SFB). Resuspend the beads in the SFB by flicking the tube. 2m
- 6.7 Spin down for 3 seconds then return the tube to the magnet. Allow the beads to pellet, then remove the supernatant and discard. 30s
- 6.8 Repeat steps 6.6 and 6.7 to complete two washes. 2m 30s
- 6.9 Spin down the tube for 3 seconds, place back on the magnet, then remove any remaining SFB with a small volume, fine pipette tip. 30s



- 6.10 Allow the pellet to air dry for 1 minute. 1m
- 6.11 Take the tube off the magnet and add 15µL of Elution buffer (EB). Flick gently to resuspend the beads and incubate at room temperature for 10 minutes. Flick again a few times during the incubation to ensure sufficient mixing. 10m 30s
- 6.12 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely. 1m
- 6.13 Remove 12µl of the eluted DNA and transfer to a clean 1.5ml tube. 30s

Note

It is recommended to load the library for sequencing as soon as possible, but it can be stored overnight in the fridge or frozen for up to two weeks if storage is unavoidable.

Priming and Loading of the MinION Flowcell

- 7 Thaw the Sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT), Bovine Serum Albumin (BSA) and the Flow Cell Flush (FCF) buffer at room temperature then place on ice. 10m
- Mix the SB, FCF, and FCT by vortexing, spin down, and return to ice. Spin down the LIB then place back on ice.
- 8 Prepare the priming mix by adding the following reagents in a clean 1.5mL tube or a 200µL PCR tube: 2m

A	B
Reagent	Volume (µL)
Flow cell flush (FCF)	1,170
Flow cell tether (FCT)	30
BSA (50mg/ml)	5

Flowcell priming mix



Mix by pipetting, spin down briefly and store on ice until ready to use.

- 9 Take the flow cell out from the sequencing device used for flow cell check and place it back in the plastic case. 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. Using an unlocked P1000 pipette and tip draw back a small volume to remove any bubbles (a few μL s). Visually check that there is continuous buffer from the priming port across the sensor array. 3m
- 10 Using a P1000 pipette, slowly load 800 μL of the priming mix into the flow cell via the priming port. 6m

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

Leave to incubate for 5 minutes.

- 11 Prepare the library mix shown below by adding the Sequencing buffer (SB) and Library beads (LIB) to the tube containing your adapter ligated and cleaned library from step 6.13. 2m

Reagent	Volume (μL)
DNA library	12
Sequencing buffer (SB)	37.5
Library beads (LIB)	25.5

Library mix, prepared for loading

- 12 Complete the flowcell priming by opening the SpotOn port cover and carefully loading 200 μL 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. 2m

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.

- 13 Mix the prepared library mix gently by pipetting and load immediately. 2m



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.

- 14 Replace the SpotOn port cover and close the priming port, then place the flowcell in your sequencing device. 1m
- 15 In the MinKNOW software follow the steps below to set up and start your sequencing run. 30s
- 16 Click start, then start sequencing.
Create a name for your sequencing run, it is good practise to make this unique and identifiable. The date and an experiment name are recommended. In sample name you can put a number or repeat the experiment name - this is not as important as the run name. Then click continue. 1m
- 17 Select the kit used - this is SQK-LSK114. Once you click this the barcoding options will appear. Select EXP-PBC096, then click continue 30s
- 18 In the run length options, set the run time to 6-12 hours depending on the number of samples and number of pores on the flowcell. 30s
- 19 In the basecalling options, select high accuracy basecalling. In the barcoding options, make sure barcoding is enabled and toggle to use barcode at both ends. Double check your selected options, then click start run. 1m

Washing your flow cell

1h 23m 30s

- 20 After the sequencing run is finished you can wash your flow cell to remove the remaining library and either prepare for another sequencing run or for storage at 4 °C.

Thaw the Wash Diluent at room temperature and mix briefly by vortexing. Spin down the tube of wash mix (WMX) and place on ice. 10m
- 21 Prepare the following wash solution in a clean 1.5ml tube: 2m

	Wash diluent	398µL
	Wash mix	2µL



Flow cell wash solution

- 22 Open the Priming port and using a P1000 pipette, carefully remove a small amount of liquid to remove any air bubbles under the port. 2m
- 23 Carefully add 200µL of the wash solution through the priming port, leaving a small amount of liquid in the tip at the end to avoid introducing an air bubble. Close the priming port and incubate for 5 minutes at room temperature. 6m
- 24 Add the remaining 200µL of wash solution through the priming port. Close the priming port and incubate at room temperature for one hour. 1h
- 25 At this point you can remove all waste from the waste channel, ensuring that the Priming Port is closed before doing so. 30s
- 26 You can also put a label on the packaging of the flow cell to detail the date it was run, what was run on it, for how long and which barcodes were used if not all 96. The results of the last pore count shown in the MinKNOW run report of the run can also be written on the label. 2m
- 27 If you will be storing the flow cell for future reuse, take out the bottle of Storage Buffer from the Wash Kit to thaw at room temperature.
- 28 After incubation you can either run a new library following the flow cell priming and loading steps starting from Step 7

Proceed with the putting the Storage buffer on for storing the flow cell for future use.
- 29 Briefly vortex the thawed Storage Buffer to mix, then add 500µL slowly through the Priming Port. 1m
- 30 Close the priming port before removing all waste from the waste channel. Place the flow cell back in its plastic box and envelope, then store at 4°C



Protocol references

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