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

Nanopore Sequencing of poliovirus isolates

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ABSTRACT

This protocol is adapted from "Direct Detection of poliovirus and Nanopore Sequencing (DDNS) - Stool" to make it suitable for use with cell culture isolates in either a liquid form or that have been transported to the lab via FTA card.

The protocol aims to amplify the VP1 region of poliovirus through a single PCR using the Q8/Y7 primer set. We use barcoded primers as this greatly simplifies the subsequent library preparation process. Primer sequences for the barcoded Q8/Y7 primers are found in Dataset S1 of Shaw et al (2020).

This protocol is for use with Oxford Nanopore v14 chemistry sequencing reagents and can be used with the MinION Mk1B or GridION sequencer.

Within the protocol steps, quality control checks are included and follow the workflow set out in the document "Quality Control and Data Recording for DDNS".

MATERIALS

Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114

Nuclease-free Water **Contributed by users**

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PROTOCOL MATERIALS

ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #**EXP-WSH004

Step 28

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880 Step 9

NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S

Step 14

Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114

Step 14

SuperScript III One-Step RT-PCR System with Platinum Taq **Invitrogen - Thermo Fisher Catalog #**12457-026

Step 3

NEBNext Ultra End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #**E7442L

Step 10

Ultrapure BSA **Ambion Catalog #**AM2616 Step 14

Nanopore Flow Cell R10.4.1 **Oxford Nanopore Technologies Catalog #**FLO-MIN114

Materials

Nuclease-free Water Materials

BEFORE START INSTRUCTIONS

This protocol describes the amplification of the VP1 region and library preparation for sequencing. We anticipate users will have performed an RNA extraction prior to this protocol to extract poliovirus RNA. We recommend the MagMAX Viral RNA Isolation Kit for this process.

Barcoded VP1 Primers:

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.

These primers can be ordered premixed or as two separate plates then mixed in the lab.

Sample Organisation

30m

1 Pairs of samples (with the same EPID) can have consecutive barcodes but try not to group samples from the same geographic area together. This helps detect any potential cross-contamination because identical sequences are then unlikely to be detected in samples with consecutive barcodes that are adjacent to one another on the 96-well plate.

1.1 Record sample data, and the order for the samples in your csv file. At this point you can also a 30m any other metadata that you have for the samples.

Here is an example of a barcode csv file: barcodes.csv 0B
It is advised that you edit the name of the file so it is unique for each run you analyse.

You should also include any positive and negative controls in your list of samples. A positive control (resuspended Coxsackievirus A20 provided by NIBSC) and negative control (nuclease free water) should each be included on the first and last RNA extraction batches of the day at least.

If any samples are repeats from a previous run, note this down in the appropriate column.

If there has been a delay in the processing of the sample e.g. due to a lack of extraction kits or software updates preventing the run, note "Yes" in the column "DelaysInProcessingForDDNS" and enter the type of delay in the column "DetailsOfDelays."

Note

Do not include any personal information from the patients in your csv file, and avoid using special characters in any of the metadata columns, including the sample names (stick to - or _).

2 For batching samples into a run, we recommend performing a sequencing run at least once every two weeks with up to 48 samples (including any positive and negative controls).

This frequency avoids causing too long a delay between sequencing runs when waiting for sample numbers to build up for batching together.

rt-PCR (barcoded VP1)

6h 30m

3 Prepare a master mix using the reaction volumes detailed in the table below for the number of samples yo 30m have plus negative controls. The reaction mix and SSIII enzyme are provided in

SuperScript III One-Step RT-PCR System with Platinum Taq **Invitrogen - Thermo Fisher Catalog #**12457-026

	A	B
	Reagent	1 reaction (µL)
	2x Reaction mix	12.5
	Nuclease Free Water	4.5
	SSIII Platinum Taq mix	1

4 Vortex the mastermix for 3 seconds and spin down for 5 seconds to gather contents at the bottom of the 20m tube. Aliquot 18µL to each 0.2mL PCR tube.

Add 2µL of barcoded primer to each well, ensuring you add a different barcode for each reaction.

Add 5µL sample RNA to each reaction, and 5µL of nuclease free water for negative controls, and pipette 5 times to mix

5 Spin down for 5 seconds to make sure all reagents are in the bottom of the tube. 10s

6 Amplify using the following cycling conditions: 3h

	A	B	C	D
	Cycle	Step	Temperature (°C)	Time
	1	Reverse transcription	50	30 minutes
	1	Initial denaturing	94	2 minutes
	42	Denaturing	94	15 seconds
		Annealing	55	30 seconds
		Extension	68	2 minutes
	1	Final extension	68	5 minutes
	-	Hold	10	-

7 Once the PCR is finished, check to see if any reactions have evaporated, if so, note this down in the sample csv. 5m

8 Check all positive and negative controls on a 1% agarose gel. The expected band for the positive control is around 1.2kb. 1h

All samples can be marked as “Pass” for the PositiveControlCheck if all positive controls extracted on the same day show a VP1 band on the gel.

All samples can be marked as “Pass” for the NegativeControlCheck if all negative controls extracted on the same day show no VP1 band on the gel.

If any positive controls fail, or any negative controls have a band, all samples must be marked as fail.

8.1 If there is no band for the positive control, repeat the VP1 reaction for the controls only. If a band is visible after repeating, discard the previous VP1 amplicons and repeat the VP1 reactions for all samples. 1h

If there is no band visible after repeating the PCR, repeat the RNA extractions after checking the RNA extraction kit is being used correctly and has not expired.

8.2 If the negative control check is failed, repeat the rtPCR. 30m

If the negative control still shows a band on a gel or tapestation:

1. Thoroughly clean the PCR and RNA extraction workstations.
2. Replace each of the PCR reagents in turn whilst performing blank reactions to determine a contaminated reagent.
3. Perform an additional Negative RNA extraction to confirm that that RNA extraction kit is not contaminated.

Library Preparation for ONT MinION: Pooling, End-Prep and Adapter Ligati… 1h 45m

9 Pool 2µL of each VP1 product into a clean 1.5mL tube and concentrate with AMPure beads 5m

Agencourt AMPure XP **Beckman Coulter Catalog #**A63880

9.1 Add a volume of AMPure beads equal to the volume of the pooled VP1 products and incubate a room temperature for 5 minutes. Flick gently after 2 minutes to aid binding. 6m

e.g. 40 samples, 2µL each pooled = 80µL pool, so add 80µL AMPure beads

9.2 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. 3m

9.3 Pipette off the solution, avoiding disturbing the bead pellet. 1m

9.4 Add 200µL of 80% Ethanol to the tube, leave for 30 seconds, then remove and discard. 2m

Repeat this step.

9.5 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining Ethanol. 1m

Allow the pellet to air dry for 30 seconds or until dry but not cracking

9.6 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. 3m

9.7 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely. 30s

9.8 Remove 50µL of the eluted DNA and add to a clean 0.2mL PCR tube. 30s

10 End-preparation: 2m
Add the following reagents from
NEBNext Ultra End Repair/dA-Tailing Module - 96 rxns **New England Biolabs Catalog #**E7442L to the 0.2mL tube containing the cleaned DNA pool.

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

11 Mix gently by flicking the tube and spin down for 3 seconds. 1m

12 In the thermocycler, incubate for 5 minutes at 20°C followed by 5 minutes at 65°C 10m

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

13.1 Vortex the AMPure beads until all the beads are well mixed. 30s

13.2 Add 60µl of resuspended beads to the tube and flick the tube to mix. 5m

Incubate at room temperature for 5 minutes. Flick gently after 2 minutes to aid binding.

13.3 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. 1m

13.4 Pipette off the solution, avoiding disturbing the bead pellet. 1m

13.5 Add 200µL of 80% Ethanol to the tube, leave for 30seconds, then remove and discard. 2m

Repeat.

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

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

13.7 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. 3m

13.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet completely. 1m

13.9 Remove 60µL of the eluted DNA and add to a clean 1.5mL tube. 30s

> **Note**
>
> At this point you can store the end-prepared library at 4°C. This can be stored for up to one week before continuing to step 14, however it is advised to continue the library preparation protocol as soon as possible.
>
> Ensure you label the tube clearly with the run name and what stage of the library preparation you were at e.g. 20240229 DDNSrun10 end-prep

14 3m

From NEBNext Quick Ligation Module - 20 rxns **New England Biolabs Catalog #**E6056S
Spin down the NEB Quick T4 Ligase and place on ice.

From Ligation Sequencing Kit V14 **Oxford Nanopore Technologies Catalog #**SQK-LSK114
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.

If you plan to start the run on the same day, remove the Flush buffer, flush tether (FLT) and Ultrapure BSA **Ambion Catalog #**AM2616 from the freezer and thaw at room temperature. Once thawed, place on ice.

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

15 Prepare the following reaction mix adding reagents to the 1.5mL tube with 60µL end-prepped DNA: 2m

	A	B
	Reagent	Volume (µL)
	Ligation buffer (LNB)	25

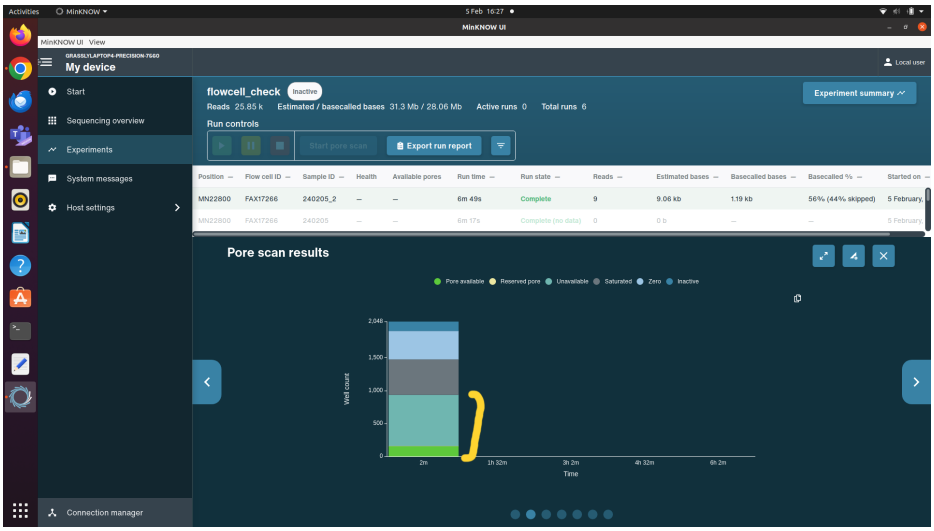
	A	B
	Ligation adapter (LA)	5
	NEB Quick T4 Ligase	10

16 Mix gently by flicking the tube then spin down for 3 seconds 1m

17 Incubate at room temperature for 10 minutes 10m

17.1 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.
If your flow cell has been used before, instead of running a flow cell check, start a dummy sequencing run by selecting Start Sequencing, name the run "flowcell_check", select any kit, then set the time to 10 minutes, skip to final review then start 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 (calculated by adding together the available and unavailable pores in the pore status graph (see example image below)).



If a flow cell has less than 700 pores, do not use it for a 48 sample DDNS isolate run. Take out a different flow cell and perform a flow cell check. The number of pores available in the flow cell you use should be noted down in the sample csv in the column “PoresAvilableAtFlowCellCheck” and the flow cell ID should be recorded in the “FlowCellID” column. Also record the number of times the flow cell has been used in the column “FlowCellUses”.

18 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.

18.1 Vortex the AMPure beads until all the beads are well mixed. 30s

18.2 Add 40µL of resuspended beads to the 1.5mL tube and mix by flicking the tube. 6m

Incubate at room temperature for 5 minutes. Flick gently after 2 minutes to aid binding.

18.3 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. 1m

18.4 Pipette off the solution, avoiding disturbing the bead pellet. 30s

18.5 Add 250µL of Short Fragment Buffer (SFB). Remove the tube from the magnet and resuspend th 4m
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.

18.6 Spin down the tube for 2 seconds, place back on the magnet, then remove any remaining SFB. 1m

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

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

18.8 Spin down the tube for 3 seconds then place back on the magnet, allowing the beads to pellet 1m
completely.

18.9 Remove 12µL of the eluted DNA and transfer to a clean 1.5mL tube. 30s

> **Note**
>
> At this point you can keep your prepared library at 4°C overnight with minimal loss of throughput. This can be kept for up to two weeks before sequencing, however we recommend that you continue to flow cell loading as soon as possible.
>
> Ensure you label the tube clearly with the run name and what stage of the library preparation you were at e.g. 20240229 DDNSrun10 final library.

Priming and Loading the flowcell

30m

19 Thaw the Sequencing buffer (SB), Library beads (LIB), Flow Cell Tether (FCT) and one tube of Flow Cell Flu 10m
(FCF) at room temperature then place on ice.

Mix the SQB, FCF, and FCT by vortexing, spin down, and return to ice. Spin down the LIB then place back on ice.

20 To create the priming mix, combine the following reagents in a clean 1.5mL tube: 2m

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

Mix by pipetting and spin down. Place on ice until ready to use.

21 Open the lid of the nanopore sequencing device and slide the flow cell's priming port cover clockwise so th 3m
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.

22 Using a P1000 pipette, slowly load 800µL of the priming mix into the flow cell via the priming port. 2m

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.

23 Mix the contents of the LIB tube by pipetting just before adding to the following library mix in the 1.5ml tub 2m
with your DNA library:

	A	B
	Reagent	Volume (µL)
	Sequencing buffer (SB)	37.5
	Library beads	25.5

	A	B
	(LIB)	

24 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. 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.

25 Mix the prepared library mix gently by pipetting. 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.

26 Replace the SpotOn port cover and close the priming port, then replace the lid of your sequencing device. 1m

Starting the sequencing run

5m 30s

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

27.1 Click start, then start sequencing. 1m
Create a name for you sequencing run, it is good practise to make this unique and identifiable for if you ever need to revisit the data. 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.

27.2 Select the kit used - this is SQK-LSK114. Once you click this the barcoding options will appear. 30s
Select EXP-PBC096, then click continue

27.3 In the run length options, set the run time to 7 hours for a run of 40 samples (approximately 10 minutes per sample rounded up to the nearest hour). Click continue. 30s

27.4 In the basecalling options, select high accuracy basecalling. In the barcoding options, make sur barcoding is enabled and toggle to use barcode at both ends. Click continue until you reach the run overview, where you can double check the selected options, then click start run. 1m

27.5 In your sample csv, record the run number in the column "RunNumber", the date in "DateSeqRunLoaded", and the run duration in "RunHoursDuration". 2m

Washing the flow cell

1h 25m

28 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. The wash uses the reagents supplied in the ONT wash kit:

ONT Flow Cell Wash Kit **Oxford Nanopore Technologies Catalog #**EXP-WSH004

28.1 Thaw the Wash Diluent at room temperature and mix briefly by vortexing. 10m

Spin down the tube of wash mix (WMX) and place on ice.

28.2 Prepare the following wash solution in a clean 1.5mL tube 5m

	A	B
	Reagent	Volume (µL)
	Wash diluent	398
	Wash mix (WMX)	2

29 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

30 Carefully add the 400µL wash solution through the priming port, leaving a small amount of liquid in the t 1h 1m
towards the end to avoid introducing an air bubble.

Close the priming port and incubate at room temperature for one hour.

30.1 At this point you can remove all waste from the waste channel, ensuring that the Priming Port is 2m
closed before doing so.

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, and for how long e.g. 13/03/2024 DDNS-run30 7 hours

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.

31 After incubation you can either follow the flow cell priming and loading steps starting from Step 19 to load a new run, or the following steps for storing the flow cell for future use.

31.1 Briefly vortex the thawed Storage Buffer to mix, then add 500µL slowly through the Priming Port 5m

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.