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Poliovirus direct detection and nanopore sequencing (DDNS) FAQs

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Poliovirus Sequencing Consortium

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This short FAQ document summarises the poliovirus direct detection and nanopore sequencing (DDNS) protocol, the equipment and reagents required, the cost and staff-time involved (Feb 2022).

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Poliovirus Direct Detection and Nanopore Sequencing (DDNS) FAQs

Overview

Direct Detection by Nanopore Sequencing (DDNS) allows rapid molecular detection and sequencing of poliovirus from stool samples in a simple integrated protocol [1]. It replaces cell culture, intratypic differentiation (ITD) and Sanger sequencing, allowing case confirmation by generating a VP1 sequence within 3 days of sample processing [2]. It can be performed in any laboratory with experience conducting PCR, bringing sequencing capacity to many more Global Polio Laboratory Network (GPLN) labs. This short FAQ document summarises the method, the equipment and reagents required, the cost and staff-time involved.

What is DDNS?

Poliovirus DDNS involves a nested PCR reaction [3] using custom barcoded primers, followed by nanopore sequencing of the VP1 product. Unlike traditional Sanger sequencing, nanopore allows samples containing multiple polioviruses or other enteroviruses to be correctly identified and sequenced. This is because individual, full length DNA strands are read as they pass through pores on a nanopore flow cell. Each flow cell contains 512 nanopores that rapidly read DNA strands, typically generating 1 million VP1 reads per hour. Flow cells can be re-used several times if washed with a nuclease solution after use. The protocol is summarised in the Appendix below and is available on protocols.io from the poliovirus sequencing consortium (https://dx.doi.org/10.17504/protocols.io.bf8zjrx6).

What do I need?

Laboratory Space: The DDNS protocol should be performed in a Biosafety level 2 (BSL-2, or equivalent) laboratory and ideally in three separate working areas:

- Area 1 RNA extraction is performed in a dedicated Class I or II biosafety cabinet
- Area 2 Nested PCR is performed in a dedicated PCR hood with a separate “clean” hood for the preparation of master mixes
- Area 3 Library preparation and sequencing area can be performed on the benchtop

Areas 2 and 3 could be combined if necessary.

Equipment: The equipment and ‘one-off’ purchases required to implement the DDNS protocol are:

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A	B	C
Item	**Number**	**Total cost (US$)**
Plate centrifuge	1	900
Vortex	1	270
Mini centrifuge	1	280
Benchtop centrifuge	1	1,800
Micropipette p10	3	780
Micropipette p20	3	780
Micropipette p200	3	780
Micropipette p1000	3	780
Multichannel P10	1	1,690
Multichannel P200	1	1,690
Thermocycler	1	5,200
Gel electrophoresis tank	1	370
Laptop and MinION sequencer+	1	5,000
Kingfisher Duo (optional)*	*1*	*22,100*
Total (with Kingfisher Duo)		**$42,630**
Total (without Kingfisher Duo)		**$20,530**

+Can be replaced with a Nanopore MinION Mk1C and a low-specification laptop at a similar price. *Helpful for high-throughput labs, allowing processing of 12 samples simultaneously for RNA extraction (or 96 samples for a Kingfisher Flex at USD$ 57,000). Otherwise, manual RNA extractions can be performed.

Consumables: In addition to the setup costs, consumables and reagents are:

A	B	C
Items	**Storage/Shipping**	**Supplier**
SuperScript III One-Step RT-PCR System (Platinum Taq)	Frozen	Thermo Fisher, Carramore
DreamTaq	Frozen	Thermo Fisher, Carramore
NEBNext Companion Module	Frozen	New England Biolabs, Carramore
Agencourt AMPure XP beads	Refrigerated	Beckman Coulter, Carramore
Ligation Sequencing Kit 1D	Refrigerated	Oxford Nanopore, Carramore
MinION Flow cells	Refrigerated	Oxford Nanopore, Carramore
Flow cell wash kits	Refrigerated	Oxford Nanopore, Carramore
PanEV primers and barcoded VP1 primers	Room temp	Eurofins, IDT, etc.
Plasticware (tips etc.)	Room temp	Local

How much does it cost?

Estimated costs without any bulk discounts are given below, based on automated RNA extraction via the Kingfisher. Sequencing batches containing greater numbers of samples (up to 96, including controls) give better value per sample, as the cost of performing a sequencing run is fixed, regardless of

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the number of samples included.

A	B
Samples processed per week	**Cost per sample ($US)**
25	21
50	15
90+	12

*Low through-put laboratories can perform manual RNA extractions (e.g. via the Roche High Pure Viral RNA kit) for a similar price.

How sensitive and specific is it?

Preliminary data from pilot GPLN laboratories indicate that DDNS has equivalent sensitivity and specificity to cell culture when testing fresh or recently frozen samples (<2 months old).

What QA/QC procedures are in place?

The protocol has been streamlined to mitigate human error and the techniques involved will be familiar to staff who have performed ITD by qPCR. Once RNA extraction is completed, samples and barcoded primers can be handled with multichannel pipettes, facilitating accurate and high-throughput sample processing, minimising risks of cross contamination. So far, a positive control for RNA extraction and PCR has not been required although we are developing controls that can be used at the individual sample or batch level if desired (we favour a batch control to minimise sample handling and costs). A detailed quality assurance program is in development.

Can it be used for environmental surveillance samples?

The method can be used with RNA extracted from environmental surveillance samples, although sensitivity compared with culture is slightly lower given the smaller volumes process (0.3 vs 3 ml of sample). Further work is underway to test larger sample volumes or multiple aliquots.

References

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Appendix: protocol summary

Citation: Alex Shaw, Catherine Troman, Joyce Akello, Erika Bujaki, Manasi Majumdar, Javier Martin, Nick Grassly Poliovirus direct detection and nanopore sequencing (DDNS) FAQs https://dx.doi.org/10.17504/protocols.io.b5ggq3tw

The DDNS method can be performed directly on stool suspension and involves the following steps (summarised in Figure 1):

1) RNA extraction– Extraction can be manual or automated (e.g. using a Kingfisher machine). We recommend the Roche High Pure Viral RNA kit, the QIAamp Viral RNA kit, the Thermofisher MagMAX Viral RNA Isolation kit, or MagMAX Pathogen RNA/DNA kit.

2) Nested RT-PCR- Amplification of the VP1 region is performed using a nested PCR; the sensitivity of this approach has been validated for a wide range of polioviruses. First, the enterovirus capsid region is amplified by RT-PCR, then the product is added directly to a VP1 PCR using barcoded primers. A set of 96 barcoded primers in plate format allow multiplexing of up to 96 samples.

3) Sample pooling- 2µL of all samples tested are pooled together. This reduces both the cost and time required for sample cleaning and quantification, and the associated potential for error or cross-contamination during these steps. Including samples negative for poliovirus does not use significant sequencing chemistry (other than to identify any non-polio enteroviruses amplified by the nested PCR).

4) Sequencing library preparation- The sample pool is cleaned with AMPure beads to remove enzymes and primers, and the sequencing adaptor ligated onto the amplified DNA.

5) Nanopore sequencing- Sequencing of the VP1 amplicons can be performed on any Oxford Nanopore Technologies device. For data to be processed in real-time, a MinION Mk1B controlled by a high-specification GPU laptop, or a MinION Mk1C is recommended.

6) Data analysis- Sequencing reads are split between samples and mapped to a poliovirus reference database via the freely available RAMPART software. A custom module, realtime-polio, then generates consensus sequences and identifies mutations in comparison to reference strains (https://github.com/polio-nanopore/realtime-polio). The software is currently run in the Linux or Mac operating system, but a version for Windows is being developed.

The complete protocol is maintained and freely available at:
https://dx.doi.org/10.17504/protocols.io.bf8zjrx6

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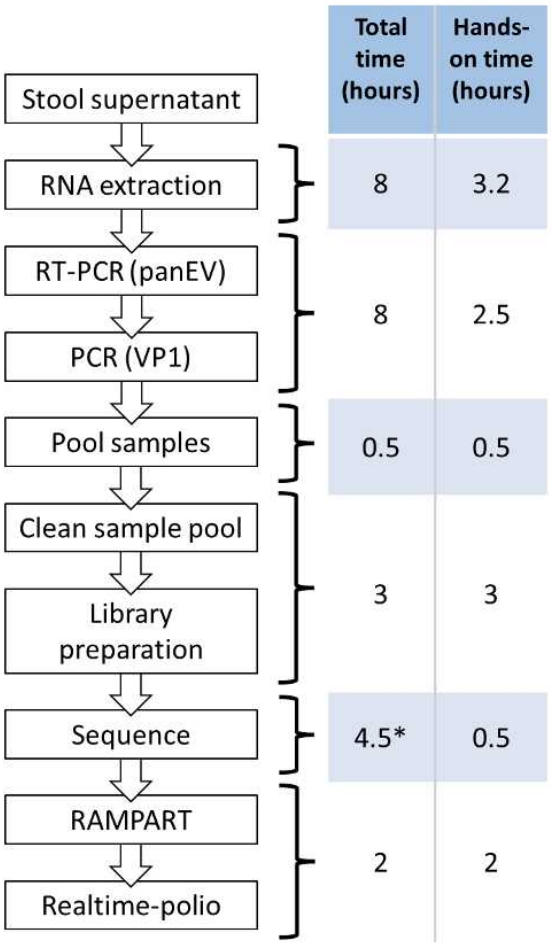

Figure 1 Steps of the DDNS protocol. Timings shown are for 96 samples with automated extraction (batches of 12, Kingfisher Duo). Manual extractions will require 1.25 hours per batch of 12 (kit dependent). High throughput extraction (e.g. Kingfisher FLEX) can process 96 samples in one hour. * Run duration can be varied.

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