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Guidelines for in-house verification of the DDNS method for poliovirus direct detection V.3

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Poliovirus Sequencing C...



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We use this protocol and it's working

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Abstract

This document describes a pragmatic approach to conducting in-house verification of the DDNS method for detection of polioviruses from stool samples, evaluating and verifying technical performance of each process. Laboratories should conduct in-house verification of the complete DDNS method to confirm that the required performance characteristics can be met within the settings. Verification of the DDNS method will be assessed based on the following parameters: accuracy and sensitivity. To support DDNS method performance, the laboratory should ensure that lab personnel are adequately trained, competent, and qualified to perform the protocol.

Note: We recommend working closely with members of the Polio Sequencing Consortium (PSC) before and during in-house verification of the DDNS method to ensure that the laboratory has all the required components and competency to perform the full protocol.

Purpose

To outline the key stages in conducting in-house verification of the DDNS method for poliovirus detection from stool suspensions proving technical abilities and performance. These will include proving sufficient recovery of viral nucleic acid during RNA extraction and achieving efficient PCR amplification during STAGE 1 and STAGE 2. STAGES 3 and 4 further target sample handling, accuracy and adherence to protocol QC recommendations. Library preparation, sequencing, data analysis and interpretation are also assessed during verification. Performance characteristics are evaluated based on results from spiking experiments using preparations of the assay positive control (Coxsackievirus A20 material), testing known positive and negatives samples, and testing a set of blinded samples. The completed in-house verification will demonstrate that assay results are in line with how the DDNS method has been designed to perform. This will allow obtaining reliable data and establishing a QA process to ensure data integrity. Results from the consecutive stages of the in-house verification should be recorded in the verification report document, discussed with the Polio Sequencing Consortium and the reports become part of the laboratory's QA records. Overview of the procedure is shown in figure below.

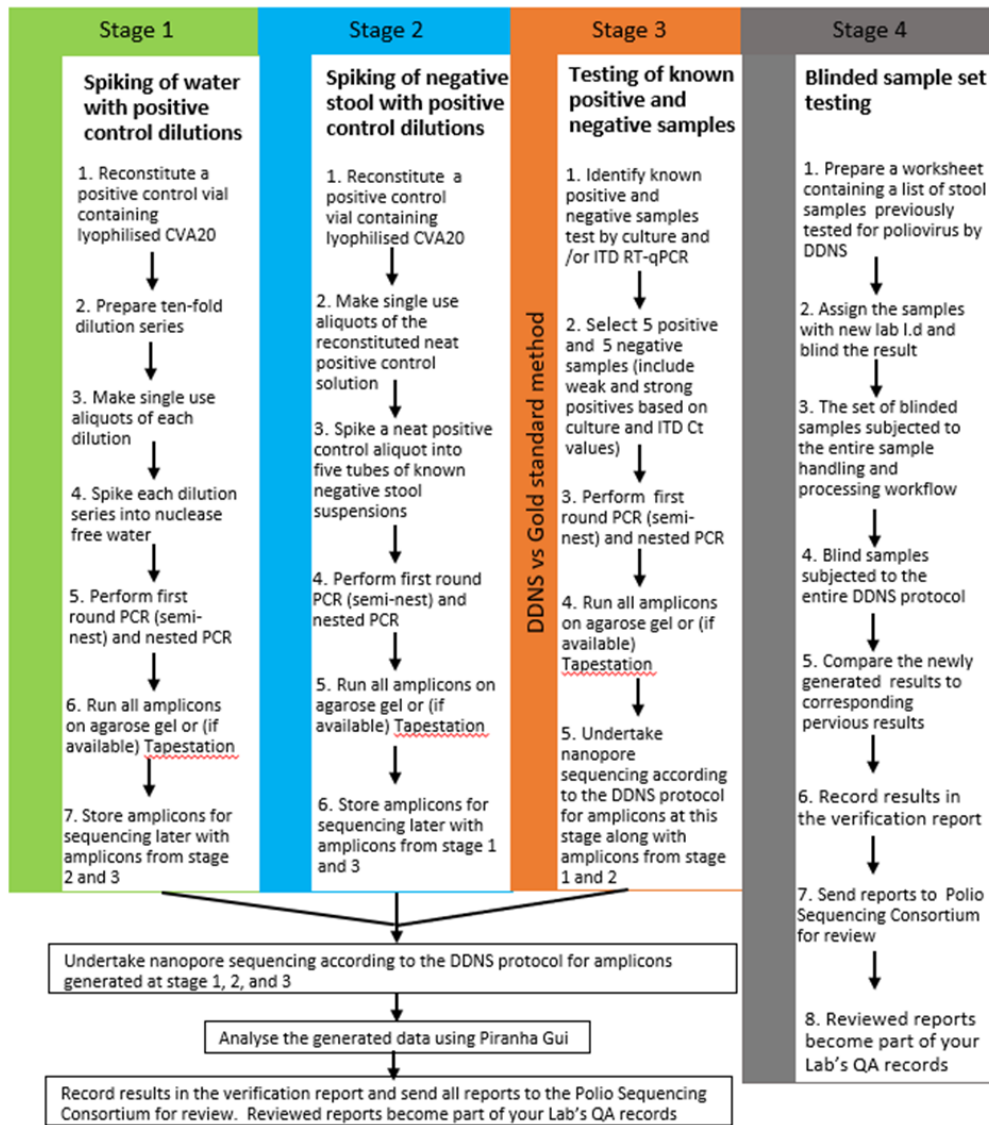


Figure 1. Overview of the DDNS in-house verification process

Detailed protocols

Viral RNA extraction: dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2

DDNS: dx.doi.org/10.17504/protocols.io.rm7vzbbyyxvx1/v6

Attachments



DDNS_Verification_Re...

89KB

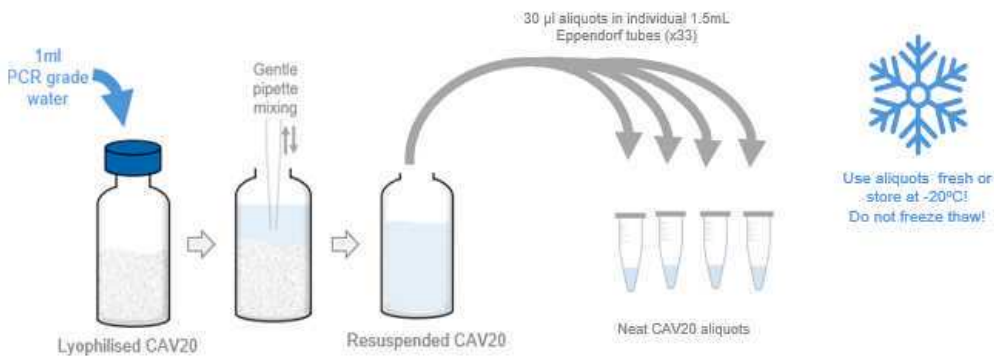
Guidelines

This protocol needs prior approval by the users' institutional review board (IRB) or equivalent ethics committee(s).

Reconstitution of CVA20 lyophilised positive control material

- 1 Working in the MSCII, reconstitute the vial containing the lyophilised CVA20 (freeze dried material) by adding 1mL of Nuclease Free Water (NFW).

Resuspension and aliquoting of lyophilised positive control



Control material resuspension

Note

ACDP Hazard group 2 infectious material - Handle at Biosafety Level 2 and perform procedures that may generate aerosols in a Class II Microbiological Safety Cabinet (MSCII).

No attempt should be made to weigh out or transfer the freeze-dried material.

- 1.1 Mix gently by pipetting up and down to ensure that the material completely dissolves in water giving a colourless liquid. Check and ensure that any powder on the seal insert or on the wall is also rinsed into the vial.
- 1.2 Prepare single use aliquots (30 μ L) in labelled sterile microcentrifuge tubes and keep on ice to use fresh or store at -20 °C until further use. Ensure tube labels have accurate name from vial and preparation date recorded. One vial of lyophilised material should yield ~33 vials of 30 μ L reconstituted viral preparations.

STAGE 1. Spiking of nuclease free water with positive control dilutions

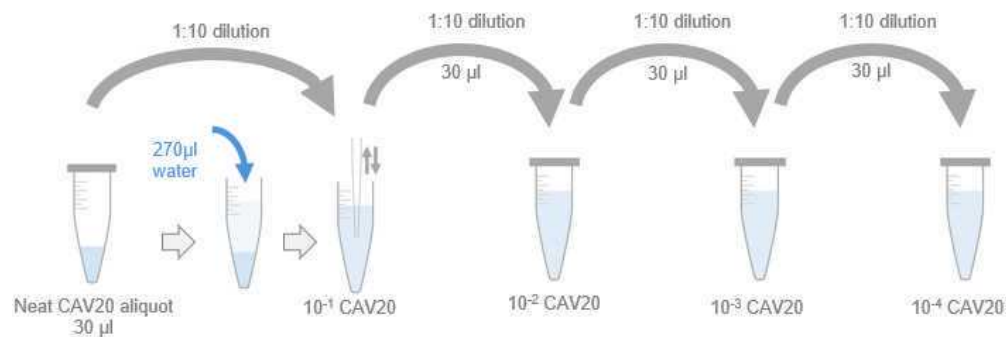
- 2 STAGE 1 is completed using nuclease free water spiked with the positive control material dilutions. Results of this experiment will show if RNA extraction and PCR amplification

work efficiently, establishing detection sensitivity for the viral material in the particular laboratory settings using the equipment available.

Note

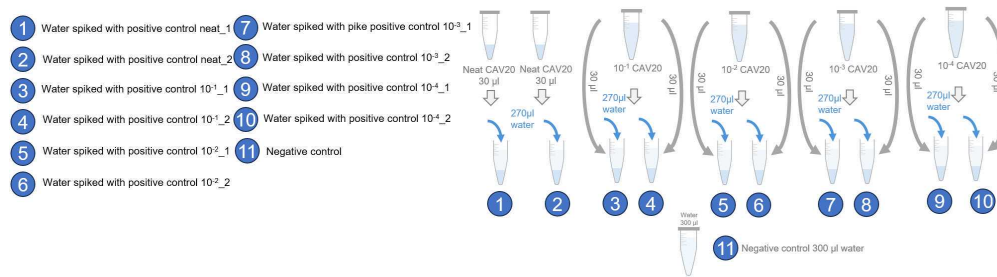
Using fresh, unfrozen aliquots of the positive control from Step 1 is recommended for STAGE 1 experiment.

Prepare ten-fold dilution series (neat to 10^{-4}) of the CVA20 positive control as shown below.



- 2.1 Label 3 individual microcentrifuge tubes 10^{-2} to 10^{-4} and add 270 µL NFW to each one.
- 2.2 Use 1 tube of freshly prepared aliquot (30 µL) of the reconstituted positive control from Step 1 and add 270 µL NFW. Label this tube as 10^{-1} .
- 2.3 Mix gently, and add 30 µL of the prepared 10^{-1} dilution to 270 µL water in tube labelled 10^{-2} .
- 2.4 Mix gently, and add 30 µL of the prepared 10^{-2} dilution to 270 µL water in tube labelled 10^{-3} .
- 2.5 Mix gently, and add 30 µL of the prepared 10^{-3} dilution to 270 µL water in tube labelled 10^{-4} .

3 Prepare sample series as depicted below.



Note

STAGE 1 experiment is performed with duplicate samples yielding two results for each dilution point.

Add 270 µL of NFW to two neat 30 µL aliquots from Step 1 and label tubes 1 and 2.

- 3.1 Spike each ten-fold dilutions in tubes 10⁻¹ to 10⁻⁴ from Step 2 into labelled tubes containing 270 µL of NFW.

This should result in 10 prepared samples for viral RNA extraction.

- 3.2 Prepare negative extraction control (300 µL pure NFW) as sample 11.

- 4 Extract the Viral RNA using the MagMAX Viral RNA Isolation kit. Follow steps described here for small volume RNA extraction:

dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2

Keep RNA on ice and use fresh to set up RT-PCR reactions. Store left over RNA frozen.

- 5 Perform the first round RT-PCR on each RNA extract (same day as RNA extraction) as per DDNS protocol: dx.doi.org/10.17504/protocols.io.rm7vzbbyyxvx1/v6

Note

Include PCR negative control by using NFW instead of RNA.

- 5.1 Perform barcoded VP1 PCR on all the first round PCR products as templates and adding an extra PCR negative control, resulting in 13 reactions.

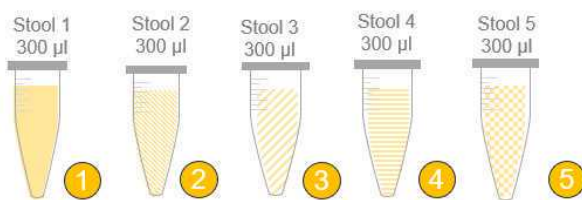
Note

Record what barcode is used for each sample.

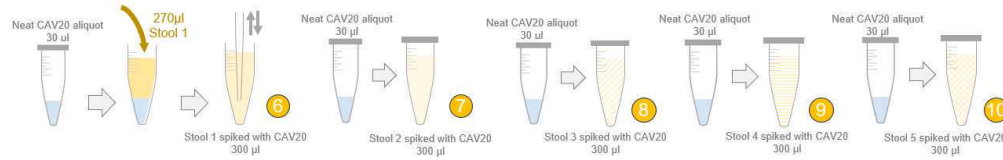
- 6 Run all amplicons on agarose gel or (if available) Tapestation to confirm successful amplification.
- 6.1 Share results with PSC team for review before progressing to next stage.
- 6.2 Store the PCR products in fridge for sequencing to be performed alongside amplicons generated in subsequent STAGE 2 and 3.

STAGE 2. Spiking of negative stool suspensions with neat positive control

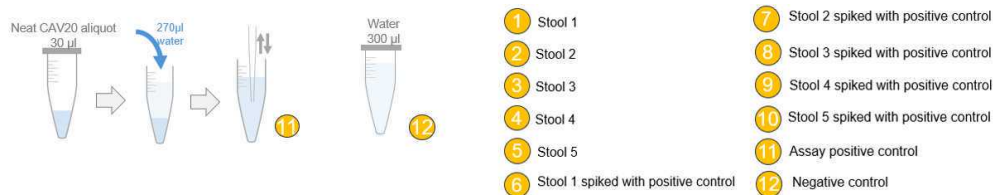
- 7 STAGE 2 is designed to show if the assay can be performed efficiently with stool matrix. Individual positive control aliquots are spiked into 5 different, previously tested poliovirus and non-polio enterovirus negative stool suspensions (prepared according to the WHO laboratory protocol). Un-spiked stool samples and regular positive and negative controls complete the sample set for this experiment.
- 7.1 Thaw 6 aliquots of the neat positive control solution prepared in Step 1 of this protocol.
- 7.2 Transfer 300 µl of each stool suspension into separate labelled tubes. (Un-spiked stools, Samples 1-5)



- 7.3 Spike neat positive control aliquots (30 μ L) into five tubes with 270 μ L of five different negative stool suspensions as shown below and mix gently by pipetting.



- 7.4 Prepare assay controls to complete the sample list.
Add 270 μ L of water to one aliquot of 30 μ L positive control - Assay positive control (Sample 11)
300 μ L PCR grade water - Negative control (Sample 12)



This should result in a total of 12 samples prepared for viral RNA extraction.

- 8 Extract the Viral RNA using the MagMAX Viral RNA Isolation kit. Follow steps described here for small volume RNA extraction:
dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2

Note

Ensure to include the regular positive assay control, negative extraction control and aliquots of the un-spiked stool samples.

Keep RNA on ice and use fresh to set up RT-PCR reactions. Store left over RNA frozen.

- 9 Perform the first round RT-PCR on each RNA extract (same day of extraction) as per DDNS protocol: dx.doi.org/10.17504/protocols.io.rm7vzbxyxvx1/v6

**Note**

Include PCR negative control by using NFW instead of RNA.

- 10 Perform barcoded VP1 PCR on all the first round PCR products as templates and adding an extra PCR negative control resulting in 14 reactions.

Note

Use different barcodes than those used for samples in stage 1. Record accurately what barcode is used for each sample.

- 11 Run all amplicons on agarose gel or (if available) Tapestation to confirm successful amplification.

- 11.1 Share results with PSC team for review before progressing to next stage.

- 11.2 Store the amplicons in the fridge or (if available) freeze for sequencing to be performed along side amplicons generated in STAGE 1 and 3.

Note

Individual stool samples might contain PCR inhibiting compounds, resulting in negative result for the spiked sample. In such case, an additional experiment can be set up using 1:10 dilution of the original stool suspension and successful amplification can confirm inhibition in the original spiked sample.

Note

A perceived negative stool might come up positive for enterovirus due to sampling effect or viral load around the limit of detection of virus isolation. In such case, an additional experiment will need to be set up with a different negative stool sample.

STAGE 3. Testing of known positive and negative samples



- 12 STAGE 3 experiments provide DDNS vs virus isolation and intratypic differentiation method result comparison for a previously characterised set of stool samples.
- 12.1 Identify 5 known positive and 5 negative stool suspensions that have been tested by the gold standard method (virus Isolation and ITD-qPCR) and test them using the DDNS method. We recommend including a mixture of weak and strong positive samples.

Note

A negative control consisting of NFW, and the assay positive control (CVA20) must be included in all runs to control for cross-contamination.

- 12.2 Extract the Viral RNA using the MagMAX Viral RNA Isolation kit. Follow steps described here : dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2
- 12.3 Perform the first round PCR on each RNA extract (same day of extraction) dx.doi.org/10.17504/protocols.io.rm7vzbyyxvx1/v6

Note

Include PCR negative control by using NFW instead of RNA.

- 12.4 Perform the nested VP1 on all the first round PCR products

Note

Use different barcodes than those used for samples in stage 1 and 2.

- 12.5 Run all amplicons on agarose gel or (if available) Tapestation to confirm successful amplification.
- 13 Share results with PSC team for review before progressing to pooling and library preparation.

- 13.1 Undertake library preparation and nanopore sequencing according to the DDNS protocol for PCR amplicons generated at this stage, along with amplicons from STAGE 1 and STAGE 2 on by pooling all three sets of barcoded VP1 products.
- 13.2 Analyse the generated sequence data using the Piranha GUI software and complete QC checks.
- 13.3  DDNS_Verification_Report_V3.docx 89.8KB Record the results for each stage in the verification report (template attached). Share all documentation along with the report with the PSC team for review.

STAGE 4. Blinded sample set testing

- 14 Testing of blinded samples will allow the laboratory to test its entire quality system and provide a real time assessment of the laboratory's proficiency in performing DDNS.

Note

Stage 4 should be performed after at least 3 – 6 months of ongoing DDNS prospective testing.

- 14.1 Prepare a worksheet containing a list of stool samples that have previously been tested for poliovirus by DDNS method. At least 5 to 10 samples containing different combinations of PV, NPEV alone and /or in mixtures including weak and strong positive samples.
- 14.2 Assign the sample with a new lab identification number and blind the previous results.

Note

Senior scientist overseeing the DDNS testing is responsible for blinding the results and ensuring that lab staff performing the DDNS method receive blinded samples.

- 14.3 The blinded samples should go through the entire sample handling and processing workflow as though the samples have just been received in the lab for testing.
- 14.4 Subject the samples to the entire DDNS protocol.
dx.doi.org/10.17504/protocols.io.rm7vzbyyvx1/v6

14.5 Analyse the data using the Piranha GUI software.

14.6 Compare the newly generated results to the previous results.

Note

The newly generated results should match the previously obtained results. If there is a discrepancy in any of the results, it would be regarded as a failed internal quality assurance and the process must be repeated. Occasionally, a sample with viral load close to the limit of detection might swing between positive and negative result on retesting, so results need careful evaluation.

14.7  DDNS_Verification_Report_V3.docx 89.8KB Record the results in the verification report (template attached).

14.8 Share the verification report as well as the gel images, run reports and Piranha reports with PSC for review. Once the report is discussed and reviewed, this will become part of your laboratory's QA records.

Protocol references

Joyce Akello, Alex Shaw, Catherine Troman, Erika Bujaki, Manasi Majumdar, Javier Martin, Nick Grassly 2024. Guidelines for verification of the DDNS method for poliovirus direct detection. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.eq2lyw5jrvx9/v1>

Alex Shaw, Catherine Troman, Joyce Akello, Erika Bujaki, Manasi Majumdar, Shannon Fitz, Ben Bellekom, Aine OToole, c.ansley, rachel.colquhoun, YASIR ARSHAD, khurshida, alammu, Andrew Rambaut, Javier Martin, Nick Grassly 2025. Direct Detection of poliovirus and Nanopore Sequencing (DDNS) - Stool. **protocols.io** <https://dx.doi.org/10.17504/protocols.io.rm7vzbyyxvx1/v6> Version created by **Catherine Troman**

Erika Bujaki, Joyce Akello, Alex Shaw, Catherine Troman, khurshida, YASIR ARSHAD, alammu, Nick Grassly, Javier Martin 2025. Small volume viral RNA extraction using MagMAX Viral RNA Isolation Kit . **protocols.io** <https://dx.doi.org/10.17504/protocols.io.81wgbzpyogpk/v2> Version created by **Erika Bujaki**