

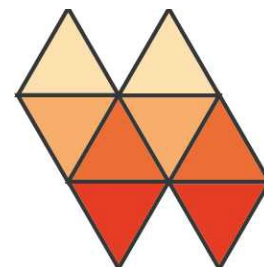
Oct 16, 2023

Version 1

Verification guidelines for the DDNS method for poliovirus direct detection V.1

DOI

<https://dx.doi.org/10.17504/protocols.io.rm7vzx54rgx1/v1>



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Poliovirus Sequencing C...



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Document Citation: Joyce Akello, Alex Shaw, Catherine Troman, Erika Bujaki, Manasi Majumdar, Javier Martin, Nick Grassly 2023. Verification guidelines for the DDNS method for poliovirus direct detection. **protocols.io**

<https://dx.doi.org/10.17504/protocols.io.rm7vzx54rgx1/v1>

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Created: October 13, 2023

Last Modified: April 19, 2024

Document Integer ID: 89250

Keywords: polio sequencing consortium, members of the polio sequencing consortium, detection of poliovirus, poliovirus direct detection this document, verification of the ddns method, house verification of the ddns method, verification of the ddn, guidelines for the ddns method, poliovirus, ddns training, house verification on the entire ddns method, ddns performance requirements for the sample matrix, attended ddns training, ddns method, ddns method performance, responsibility of each laboratory, ddns performance requirement, entire ddns method, lab personnel, ddn, laboratory, biosafety risk assessment, house verification, verification, detection, guideline

Funders Acknowledgements:

Bill and Melinda Gates Foundation

Abstract

This document describes a pragmatic approach to conduct in-house verification of the DDNS method for detection of polioviruses. A laboratory should conduct an in-house verification on the entire DDNS method to confirm that the required performance characteristics can be met within the settings. This can prove that the laboratory can adequately perform the method as intended achieving the DDNS performance requirements for the sample matrix (stool) to which the DDNS method is applied for. Verification of the DDNS method will be assessed based on the following parameters: accuracy and sensitivity

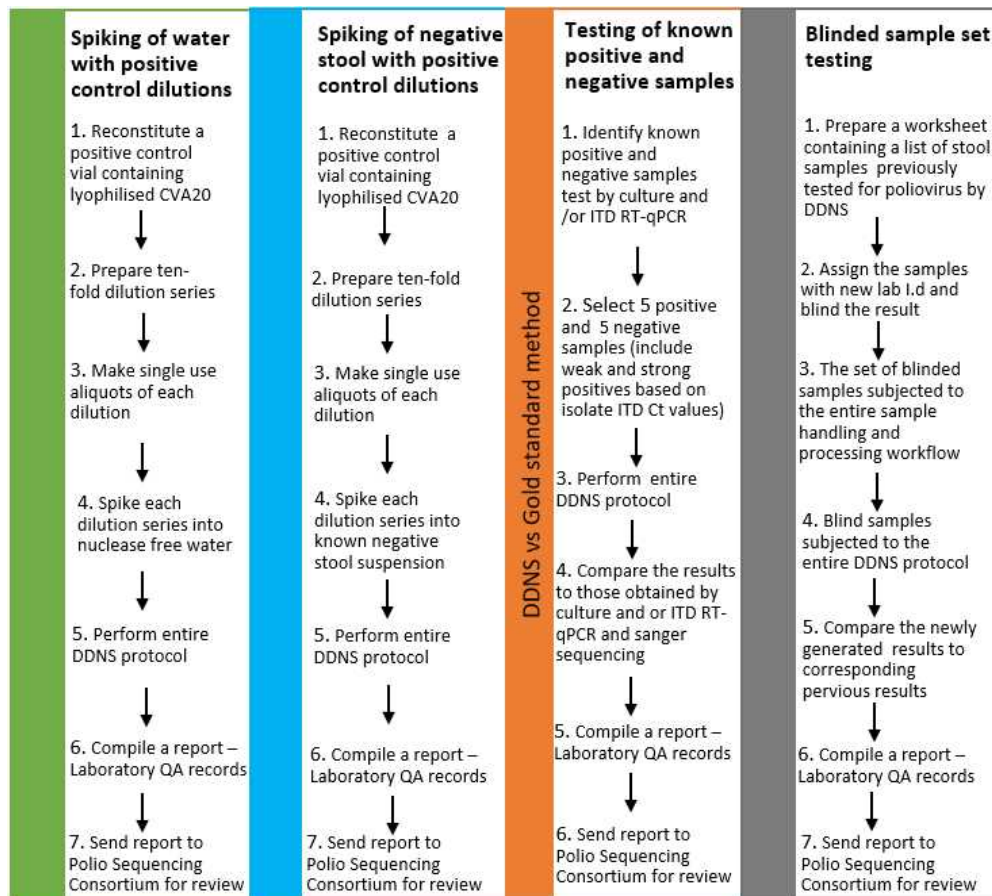
To verify the DDNS method performance, the laboratory should ensure that the lab personnel are adequately trained, competent, and qualified to perform the DDNS method. It is the responsibility of each laboratory to ensure that staff have attended DDNS training, biosafety risk assessment is performed, and adequate protective equipment is available to staff.

Note: We recommend working closely with members of the Polio Sequencing Consortium (PSC) before and during the 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 steps of how to conduct in-house verification of the DDNS method for poliovirus detection from stool suspensions. This will include establishing the laboratory's detection sensitivity by performing spiking experiments of water and negative stool using dilution series of the assay positive control (Coxsackie virus A20), testing known positive and negatives samples, and testing a set of blinded samples.

The completed in-house verification will demonstrate that your lab results are in line with how the DDNS method has been designed to perform. This will allow you to obtain more reliable data and a QA process that protects the integrity of your data overall. Results from the consecutive stages of the in-house verification should be compiled into reports, discussed with the Polio Sequencing Consortium and the reports become part of your laboratory's QA records. Overview of the procedure is shown in Figure 1 below.



Reagents

- Sample types – stool
- Positive control (Coxsackie virus A20) – Supplied by NIBSC, contact Dr. Erika Bujaki at erika.bujaki@mhra.gov.uk



- and copy Dr. Javier Martin at javier.martin@mhra.gov.uk) and Dr. Thomas Wilton at thomas.wilton@mhra.gov.uk
- Nuclease free water (NFW)
- RNA extraction reagents (Recommend using the MagMAX Viral RNA Isolation kit performed manual or automated [ThermoFisher], the QIAamp Viral RNA Kit [Qiagen], or Roche High Pure Viral RNA Kit with proteinase K [Roche])

Equipment

For details on the equipments, refer to the Equipment for DDNS_v2 excel worksheet.

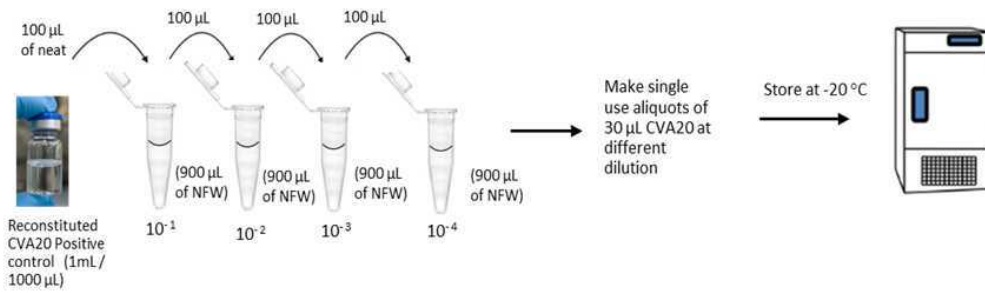
Procedure.

1. Spiking of nuclease free water with positive control dilutions

As the stool matrix to which the DDNS method is applied for is known to occasionally contain inhibitory substances interfering with PCR amplification, nuclease free water should be spiked with the positive control dilutions. This will show if the RNA extraction and the PCR amplification worked efficiently without the stool matrix effect. This experiment establishes detection sensitivity threshold in the particular laboratory settings, using the equipment available.

Note: The following experiment should be performed twice. Producing two independent tests, each containing duplicate results for each dilution point

- Working in the MSCII, reconstitute the vial containing the lyophilised CVA20 (freeze dried material) by adding 1mL of nuclease free water (NFW)**
- Vortex briefly to ensure that the material completely dissolves in water giving a colourless liquid.**
- Prepare ten-fold dilution series (neat to 10^{-4}) of the CVA20 positive control as shown below and make single use aliquots (30 μ L) in sterile microcentrifuge tube and store at -20 °C until ready for use.**



- d. Thaw two aliquots of each dilution including the neat solution
- e. Prepare two parallel sample series by spiking each ten-fold dilution into two tubes with 270 µL aliquot of NFW, including the neat solution.
- f. Extract the Viral RNA – MagMAX Viral RNA Isolation kit.

Note: A negative control consisting of NFW must be included in all runs to control for cross-contamination

- g. Perform PanEV RT-PCR on each RNA extract (same day of extraction). Note: include PCR negative control by using NFW
- h. Perform nested VP1 on all the PanEV PCR products.
- i. Run all amplicons on agarose gel/Tapestation to confirm successful amplification
- j. Undertake nanopore sequencing according to the DDNS protocol.
- k. Analyse the generated data using PIRANHA GUI software.
- l. Compile verification stage 1 report and share with PSC for review. This will become part of your laboratory's QA records

2. Spiking of negative stool suspensions with positive control dilutions

- a. Thaw one aliquot of each positive control dilution including the neat solution prepared in step 1c above.
- b. Spiked each ten-fold dilution into individual 270 µL aliquot of negative stool suspension (prepared according to the WHO protocol) ensuring to take an aliquot from the same stool.

- c. Extract the Viral RNA – MagMAX Viral RNA Isolation kit.

Note: Ensure to include the regular positive assay control, negative extraction control and an aliquot of the un-spiked stool sample

- d. Perform PanEV RT-PCR on each RNA extract (same day of extraction). Note: include PCR negative control by using (NFW)
- e. Perform nested VP1 on all the PanEV PCR products.
- f. Run all amplicons on agarose gel/Tapestation to confirm successful amplification
- g. Undertake nanopore sequencing according to the DDNS protocol.
- h. Analyse the generated data using PIRANHA GUI software.
- i. Compile verification stage 1 report and share with PSC for review. This will become part of your laboratory's QA records

Note: Individual stool samples might contain very low viral load just around the assay's limit of detection, so occasionally a perceived negative stool might come up positive for enterovirus. In such case, the experiment will need to be repeated using a different negative stool sample

3. Testing of known positive and negative samples

- a. Identify 5 known positive and 5 negative stool suspensions that have been tested by the gold standard method (virus isolation and / or 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

- b. Perform the entire DDNS protocol.
For details on the DDNS protocol, refer to the DDNS protocol V2 and for the RNA extraction refer to the MagMAX Viral RNA Isolation protocol
- c. Analyse the data using the PIRANHA GUI software.

- d. Compare the results generated by DDNS method to the gold standard method (virus Isolation and / or ITD-qPCR)
- e. Compile verification stage 3 report and share with PSC for review. This will become part of your laboratory's QA records.

Note: Samples with low viral load, very close to assay's limit of detection can occasionally swing between positive and negative on retesting, resulting in new or lost detection, so results need careful evaluation

4. Blinded sample set testing

Testing of blinded samples will allow the laboratory to test its entire quality system and provide a real time assessment of the laboratory proficiency.

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

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

- c. 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.
- d. Subject the samples to the entire DDNS protocol.
- e. Analyse the data using PIRANHA GUI software.
- f. 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.



- g. Compile verification stage 4 report and share with PSC for review. This will become part of your laboratory's QA records.