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Guidelines for verification of the DDNS method for poliovirus direct detection

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Poliovirus Sequencing Co…

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We use this protocol and it's working

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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 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 (Coxsackievirus 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 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 recorded in the verification report document, 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.

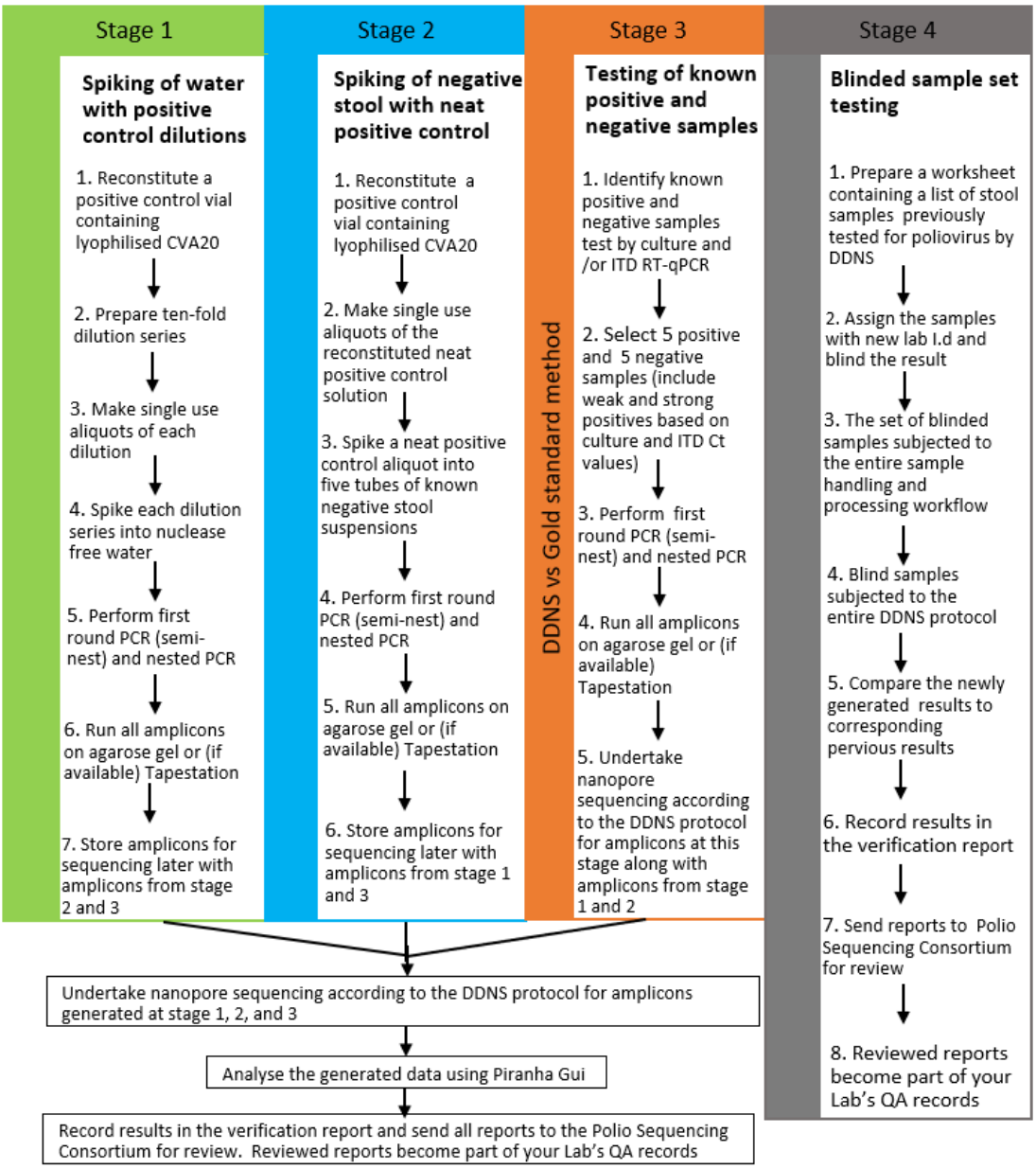


Figure 1. overview of the In-house verification of DDNS method

Attachments

DOCX

Verification_Report_...

84KB

Guidelines

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

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

1 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.

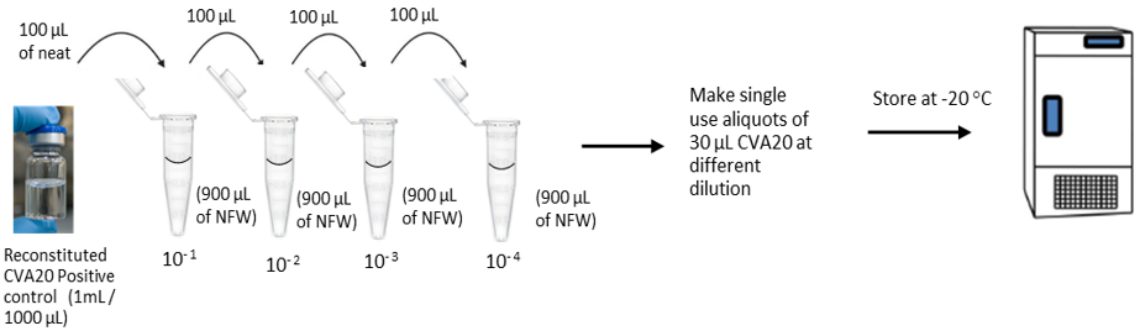
1.1 Working in the MSCII, reconstitute the vial containing the lyophilised CVA20 (freeze dried material) by adding 1mL of NFW

Lyophilised CVA20 positive control

1.2 Vortex briefly to ensure that the material completely dissolves in water giving a colourless liquid.

Reconstituted CVA20 positive control in 1mL of NFW

1.3 Prepare ten-fold dilution series (neat to 10^{-4}) of the CVA20 positive control as shown below and make single use aliquots (30 µL) in sterile microcentrifuge tube and store at -20 °C until ready for use.



1.4 Thaw two aliquots of each dilution including the neat solution.

1.5 Prepare two parallel sample series by spiking each ten-fold dilution into two tubes with 270 µL aliquot of NFW, including the neat solution.

1.6 Extract the Viral RNA using the MagMAX Viral RNA Isolation kit.

Note

Ensure to include negative extraction control (NFW)

1.7 Perform the first round PCR (semi-nest) on each RNA extract (same day of extraction).

Note

Include PCR negative control by using NFW

1.8 Perform nested VP1 on all the first round PCR (semi-nest) products.

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

1.10 Store the amplicons in fridge for sequencing to be performed along aside amplicons generated in Stage 2 and 3

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

2 To show if the assay worked efficiently with the stool matrix.

2.1 Thaw five aliquots of the neat positive control solution prepared in step 1.3 above

2.2 Spike a neat positive control aliquot (30 µL) into five tubes with 270 µl aliquots of five different negative stool suspensions (prepared according to the WHO protocol).

2.3 Extract the Viral RNA using the 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.

2.4 Perform the first round PCR (semi-nest) on each RNA extract (same day of extraction).

> **Note**
>
> Include PCR negative control by using NFW instead of RNA

2.5 Perform nested VP1 on all the first round PCR (semi-nest) products.

> **Note**
>
> Use different barcodes than those used for samples in stage 1

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

2.7 Store the amplicons in the fridge or (if available) freeze for sequencing to be performed along aside amplicons generated in Stage 1 and 3

> **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.

Stage 3. Testing of known positive and negative samples

3 DDNS vs gold standard method result comparison.

3.1 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.

3.2 Perform the first round PCR (semi-nest) on each RNA extract (same day of extraction)

> **Note**
>
> Include PCR negative control by using NFW instead of RNA.

3.3 Perform the nested VP1 on all the first round PCR (semi-nest) products

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

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

3.5 Undertake nanopore sequencing according to the DDNS protocol for amplicons generated at this stage 1, along with amplicons from stage 1 and 2 on one flow cell 2.

3.6 Analyse the generated sequence data using the Piranha GUI software

3.7 Record the results for each stage in the verification report. Here is the verification report template: Verification_Report_V2.docx 84KB

Stage 4. Blinded sample set testing

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

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

4.1 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

4.2 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.

4.3 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.

4.4 Subject the samples to the entire DDNS protocol.

4.5 Analyse the data using the Piranha GUI software.

4.6 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.

4.7 Record the results in the verification report. Verification_Report_V2.docx 84KB

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