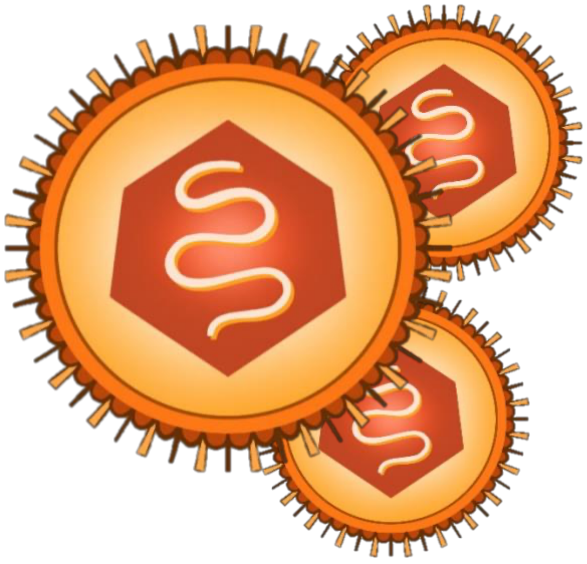
eawag
aquatic research

Wastewater-based poliovirus surveillance using digital PCR

Final Version: January 2025

Authors: Charles Gan, Melissa Pitton, Jolinda de Korne, Rainer Gosert, Karoline Leuzinger, Tamar Kohn, Christoph Ort, Timothy R. Julian.
Olga Schubert (Internal Review).

DOI: http://doi.org/10.55408/eawag:34819



Summary

Poliovirus is the causative agent of poliomyelitis, a potentially debilitating disease that is a target for global eradication. Vaccination against poliomyelitis is an important tool in the eradication effort, but one of the available vaccines, the oral poliovirus vaccine, contains live, attenuated strains that can revert to virulent forms. Community transmission of so-called vaccine-derived polioviruses (VDPVs) can contribute to outbreaks, including in areas with no recently reported poliomyelitis cases. Wastewater-based surveillance (WBS) is a widely applied method to monitor communities for outbreaks through the detection and characterization of VDPVs in wastewater including sewage. Results of WBS can help to target interventions, such as increased vaccination efforts. Switzerland is a Polio-Free country, with no evidence of community circulation of poliomyelitis. However, Polio-Free countries remain at risk of importation of wild poliovirus and VDPVs. This document has two parts: Part I describes the development of a digital PCR pan-poliovirus assay to screen wastewater for the presence of poliovirus. Our pan-poliovirus assay has been sufficiently developed to allow application when required. In Part II, the need to further characterize detected poliovirus through strain-specific differentiation assays is discussed. A literature review of available differentiation assays complements this part. Together, the report documents wet-lab methodological approaches to implement poliovirus screening in wastewater within the scope of the ongoing WBS methods targeting respiratory viruses (Influenza A, Influenza B, SARS-CoV-2, and Respiratory Syncytial Virus) and offers a resource to help aid response and surveillance decisions.

PART I: Development of a digital PCR pan-poliovirus assay for screening wastewater for putative poliovirus

Background

Poliovirus (PV) is a highly contagious non-enveloped virus with a single-stranded positive-sense RNA genome[1]. This virus can cause poliomyelitis (or simply polio), a debilitating disease that may lead to paralysis or even death[2]. However, the majority of people infected with PV show no symptoms, and a small proportion (~25%) only experience flu-like symptoms[3]. PV is a member of the *Enterovirus* genus in the *Picornaviridae* family, and three serotypes have been described: PV1, PV2, and PV3. Prevention against polio can be achieved through vaccination, and two forms of vaccine are available: the inactivated PV vaccine, which is the dominant type applied in Switzerland; and the oral PV vaccine, which contains live, attenuated strains that might revert to virulent forms, leading to VDPVs[4]. A world without polio is the goal of the Global Polio Eradication Initiative (GPEI), launched in 1988[5]. Achieving this objective relies on key strategies, including high vaccination coverage and continuous surveillance[6]. Monitoring can be achieved through different approaches, such as acute flaccid paralysis (AFP) surveillance, which consists of identifying children with muscle weakness or paralysis and analyzing their stool, enterovirus surveillance, which consists of screening stools of those with PV-like symptoms for enteroviruses and typing to confirm if it is PV, and wastewater surveillance. In settings with high vaccination coverage, wastewater surveillance can 1) detect wild poliovirus (WPV) circulating long before detection of an AFP case or a confirmed PV typing, reducing the time to detection, and 2) inform about the extent of potential ongoing transmission in response to clinical-based detection of an AFP or a polio case. Within the scope of Switzerland's ongoing WBS, which targets respiratory pathogens[7], Eawag has developed a molecular assay using digital PCR (dPCR) to detect PV RNA in wastewater. Development of the assay will enable rapid scaling up of environmental monitoring in the event of a suspected or confirmed PV outbreak in Switzerland, such as reports of positive clinical cases from the National Reference Laboratory.

Material and Methods

Experimental design

In collaboration with the National Reference Laboratory (NRL), Eawag has adapted a published pan-poliovirus (pan-PV) assay, developed by Kilpatrick and colleagues[8]. It is designed to detect multiple types of PVs, including WPVs and VDPVs. In this study, the pan-PV assay was developed and optimized using two distinct, but comparable, positive controls, a plasmid and a synthetic DNA construct. Subsequently, assay linearity was tested using serially diluted positive materials, and the limit of detection (LoD) and limit of quantification (LoQ) were determined. The assay's specificity was investigated by testing a panel of non-PV enteroviruses. Finally, inhibitory effects of positive material by wastewater extracts were determined using spike-in experiments.

Pan-PV positive controls, primers, and probe

Assay validation was performed using two types of positive controls: i) a pan-PV plasmid provided from the NRL (i.e., pUC57Pan_Polio), and ii) a synthetic DNA construct (i.e., gBlock®) manufactured by Integrated DNA Technologies (IDT, USA) (Supplemental Table S1).

The sequences of primers and probe were sourced from Kilpatrick, et al.[8]. Primers were manufactured by Microsynth AG (Switzerland), and the probe was purchased from IDT (Table 1).

Table 1. Primers and probe used in the pan-PV dPCR assay in this study. *Bases follow IUPAC conventions, with instances of "I" bases representing deoxyinosines which pair with A, C, G and T bases. "Y" bases representing pyramidines which pair with G and A bases. "M" bases representing aminos which pair with T and G. "R" bases representing purines which bind to C and T. †Contains a SUN® fluorophore (SUN), IDT ZEN® internal quencher (/ZEN/) and an Iowa Black Hole Quencher® (IABk-FQ) at the terminal end.

Name	Sequence
Kilpatrick Pan-PV Forward*	5'-CITAITCIMGITTYGAYATG-3'
Kilpatrick Pan-PV Reverse*	5'-AYRTACATIATYTGRTAIAC-3'
Kilpatrick Pan-PV Probe*†	5'-SUN-TGRTTNARI/ZEN/GCRTGICCRTTRTT-IABkFQ-3'

Digital PCR assay

The single-plex pan-PV dPCR assay was developed on the Naica® system 3-color dPCR platform (Stilla Technologies, France). PCR mixtures were performed in a total pre-reaction volume of 27 µL, which consisted of 21.6 µL of mastermix and 5.4 µL of template (Supplemental Table S2). The reaction volume (25 µL) was loaded onto a chamber of a Sapphire chip (Stilla Technologies), which was then transferred to a Geode (Stilla Technologies). Thermal cycling was performed using the following conditions: partitioning (40°C for 12 min), reverse transcription (50°C for 10 min), enzyme activation (95°C for 1 min), and 45 cycles of denaturation (95°C for 7 sec), annealing (42°C for 1 min), and extension (60°C for 1 min). Thresholding for discriminating positive and negative partitions was done manually by placing a line midway between the two high-density clusters, or groups of partitions with similar fluorescence amplitude, present in the green channel.

Limit of Detection (LoD) and Limit of Quantification (LoQ)

LoD and LoQ were determined using a two-step approach, as was done in the 2024 report titled "Wastewater-based surveillance for measles, mumps and rubella in Switzerland". First, at least 24 replicates of five dilutions of pan-PV gBlock® ranging from a target concentration of 3.36 genome copies (gc) per reaction to 9'030 gc/reaction were measured using dPCR. Reaction chambers only passed quality control if the chamber contained at least 15'000 partitions. Target concentrations were estimated based on the manufacturer's specifications; for analysis, the average concentration of all replicates measured by dPCR was used. Second, LoD and LoQ were then interpolated based on model fitting the empirical data.

The LoD was defined as the lowest concentration of target template per reaction that can be detected in 95% of the reactions. The LoD was computed using a model-based approach as described by Klymus et al.[9], with some modifications. Specifically, parameters were optimized to "best," corresponding to the model with the lowest residual standard error. For adaptation to dPCR, concentrations were used directly, instead of letting the model calculate the concentrations based on C_q values, as done for qPCR. The LoD is dependent on the number of replicates processed per sample, as the likelihood of detection becomes greater with increasing number of replicates. As such, the LoD estimates for one replicate are extrapolated to estimates for multiple replicate levels (i.e., one, two, three, four, five, and eight replicates). The detection probability for each replicate amount was represented as a one-replicate detection probability by using the nth-root of the probability of non-detection (e.g., for 95% detection: $1 - (0.05)^{1/n}$ for n replicates).

The LoQ is the lowest quantifiable estimate for the concentration of PV in wastewater for which we have an acceptable level of precision. Here, we define the LoQ as the lowest concentration at which the coefficient of variation (CoV) is at or below 30%. Since no universally accepted guidelines exist for defining the LoQ threshold, a 30% threshold was selected for this study, aligning with recommendations commonly found in the literature[10,11].

To estimate the LoQ with a CoV of 30%, we derived the CoV based on interpolation of empirical data, using a power law model (Equation 1):

$$y = a * x^{-k} \quad (1)$$

where y is CoV, a is a scaling constant, x is the concentration measured, and k is the exponent defining the power relationship. The power law model was selected because of its suitability in characterizing the relationship between uncertainty and concentration levels in a Poisson-distributed process. Power law model depicts a system in which CoV is inversely related to concentration. That is, as concentration increases, the CoV decreases in a non-linear manner. Both LoD and LoQ values were first expressed in gc/reaction and then multiplied by 1'200 reactions/L, which is a conversion factor to units of gc/L wastewater. The conversion factor is specific to our nucleic acid extraction, dilution factor, and dPCR detection protocols (Equation 2).

$$\left[\frac{gc}{L}\right] = \left[\frac{gc}{reaction}\right] * 3\ (dilution) * \frac{1}{5\frac{\mu L\ extract}{reaction}} * 80\ \mu L\ extracted * \frac{1}{40mL\ wastewater} * \frac{1000mL}{1L} \quad (2)$$

Assay specificity

To test the specificity of our assay, we tested the assay against a panel of non-PV enteroviruses isolated from wastewater. Enteroviruses echovirus 6 (EV6), coxsackievirus B5 (CVB5), coxsackievirus B3 (CVB3), coxsackievirus B1 (CVB1), and coxsackievirus A9 (CVA9) were kindly provided by Dr. Aina Astorch Cardona and Prof. Tamar Kohn (EPFL, Switzerland) in the form of extracted RNA. The non-PV enteroviruses were supplied at estimated concentrations of 10^5 gc/µL, confirmed by dPCR and most probable number cell infectivity assay, and were tested with our PV assay at a concentration of 10^3 gc/µL template, equivalent to 5×10^3 gc/reaction.

Matrix effects of wastewater on positive material

Wastewater contains a multitude of components including organic and inorganic substances that can inhibit PCR amplification and/or fluorescence-based detection. To quantify the impact of inhibition on sample estimates, we tested samples for the ability to accurately estimate concentrations using spike-in controls. Pan-PV gBlock® and pan-PV plasmid were both spiked into wastewater extracts to quantify the inhibitory effects of the wastewater matrix on PCR amplification. To account for diversity in inhibition level, five times diluted wastewater extracts from Lugano and Basel and three times diluted wastewater from Lucerne, Altenrhein, and Laupen were tested. The dilutions chosen were based on previous tests to minimize inhibition for respiratory virus quantification[12]. All tested samples were collected on the 5th of November 2023. Wastewater extracts were produced by extracting nucleic acids from 24-hr composite influent samples using the Wizard® Enviro Total Nucleic Acid Kit (Promega Corporation, USA) as described previously[12]. Inhibition value is defined as one minus the ratio between the concentration measured in a spiked in extract divided by the concentration spiked in plus the endogenous PV concentration within the extract (Equation 3). For the purposes of this report, endogenous PV was not directly measured and so is assumed to be below the limit of detection. Estimated inhibition, expressed in percentage, can range from 0 to 100%, with 100% implying a complete inhibition and 0% implying no inhibition. Inhibition below 40% is considered acceptable.

$$Inhibition\ (\%) = \left(1 - \frac{Spiked\ wastewater\ extract\left[\frac{gc}{reaction}\right]}{Amount\ spiked\ in\left[\frac{gc}{reaction}\right] + Endogenous\ polio\ (assumed\ 0)}\right) \times 100 \quad (3)$$

Performance of the dPCR assay was also assessed for its ability to distinguish positive fluorescent droplets from negative non-fluorescent droplets using the separation score. The separation score was determined using positive controls (pan-PV gBlock® and pan-PV plasmid) in wastewater matrices (same samples used for inhibition testing) and positive controls in RNase free water. The two were compared because wastewater matrices can affect droplet fluorescence. Separation score was determined directly from the Naica® Crystal Miner software (Stilla Technologies). Higher separation scores suggest superior performance with a value of 2.5 considered the minimum needed for clear thresholding[13].

Results

Validation of a pan-PV single-plex dPCR assay and linearity testing

We validated our pan-PV assay by assessing amplification at different concentrations. Following initial assay testing to ensure sufficient separation (i.e., discrimination) between positive and negative clusters, we tested assay linearity using serially diluted pan-PV gBlock® (10-fold dilutions) as a positive control. The dPCR assay showed excellent separation (separation score 6.85 ± 0.26) (Figure 1A) and linearity from this dilution series (slope = 0.98, R^2 = 0.98) (Figure 1B).

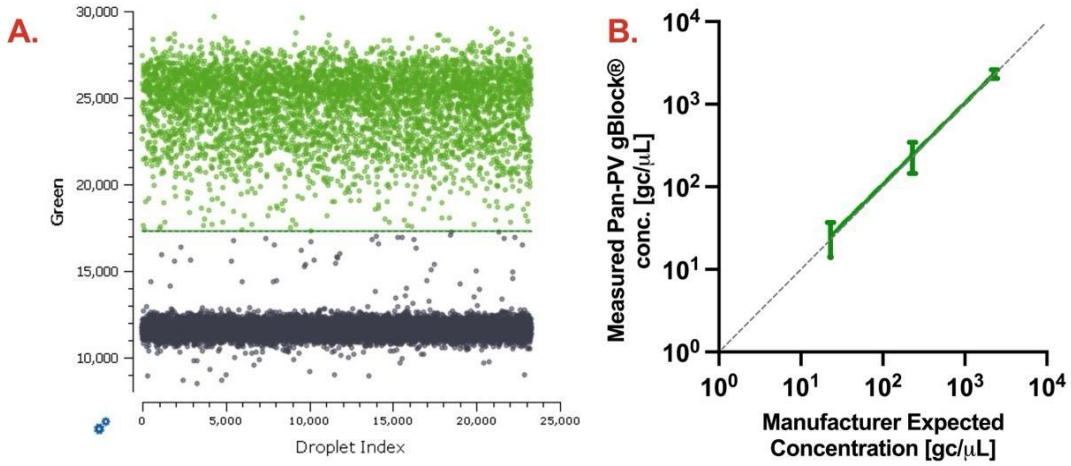


Figure 1. Validation of a pan-PV single-plex dPCR assay. (A) One-dimensional visualization of partition classification taken from Naica® Crystal Miner software (Stilla Technologies). Vertical axis ("Green") shows fluorescence intensity with the title indicating the fluorescent channel. Horizontal axis ("Droplet Index") represents the number of partitions in the selected chamber. Each dot corresponds to a partition. The separation of positive partitions is performed by drawing a line (green horizontal line) midway in between the positive (amplified fluorescence) partitions and the negative (background fluorescence) partitions. (B) A linear regression line was fitted to concentrations determined by dPCR (vertical axis) vs. given by the manufacturer (horizontal axis, expressed in gene copies [gc] per µL). The grey dashed line indicates the 1:1 identity. Error bars indicate the standard deviation around the mean of replicated measurements (n = 3).

Determination of Limit of Detection

The Limit of Detection (LoD) was determined by measuring the pan-PV gBlock® at five different concentrations (9'030, 1'180, 803, 63.3, and 3.36 gc/reaction) using a minimum of 24 replicates for each concentration (Supplemental Table S3). Subsequently, empirical data were fitted to a Weibull type 2, 2-parameter function, identified as the model with the lowest residual standard error and good fit (Bates and Watts classical lack-of-fit test[14], p = 0.98). Based on this model fit, the LoD was interpolated to be 469 gc/reaction for one replicate. By increasing the number of replicates, the effective LoD decreases. Specifically: 116 gc/reaction for two replicates, 51.2 gc/reaction for three replicates, 28.7 gc/reaction for four replicates, 18.3 gc/reaction for five replicates, and 7.08 gc/reaction for eight replicates (Figure 2).

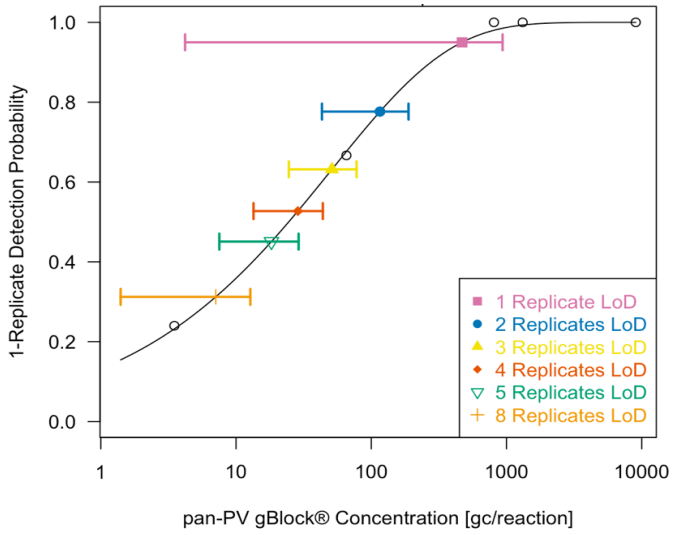


Figure 2. Detection probability as a function of pan-PV gBlock® concentration (gc/µL). The modelled curve was fit to 1-replicate detection probabilities of five measured concentrations derived from averaging a minimum of 24 replicates (open circles). Colored symbols represent the modeled LoD for different replicate numbers: 1 replicate (magenta square), 2 replicates (blue circle), 3 replicates (yellow triangle), 4 replicates (red diamond), 5 replicates (green triangle), and 8 replicates (orange cross). Error bars are capped above 0 and represent the asymptotic Wald-type confidence interval around the mean concentration for each set of replicate interpolations.

In the context of wastewater monitoring and based on our nucleic acid extraction, dilution, and dPCR detection methodology, the LoD is equivalent to 563'000 gc/L wastewater for one replicate. With increasing numbers of replicates, the LoD decreases to 139'000 for two replicates, 61'400 for three replicates, 34'400 for four replicates, 21'900 for five replicates, and 8'500 gene copies per liter wastewater for eight replicates.

Determination of Limit of Quantification

The LoQ with a corresponding CoV of 30% was estimated based on the results of empirical estimates for the concentration of 9'030, 1'180, 803, 63.3, and 3.36 gc/reaction (Table 2). Fitting the data to the power law model yielded an estimated power law coefficient (k) of –0.38 and a width of the scaling relationship (a) of 587. Based on this power law model, the LoQ was found to be 2'510 gc/reaction, equivalent to 3×10^6 gc/L wastewater using our nucleic acid extraction, dilution, and dPCR detection protocol (

Figure 3).

Table 2. Coefficient of variation in relation to pan-PV gBlock® concentration. gc: genome copies.

Concentration Expected from Manufacturer [gc/reaction]	dPCR Concentration from Averaged Replicates [gc/reaction]	# of Replicates	Coefficient of Variation (%)
15.0	3.36	25	270
45.0	63.3	24	180
1'000	803	25	50
1'250	1'180	30	42
10'000	9'030	30	14

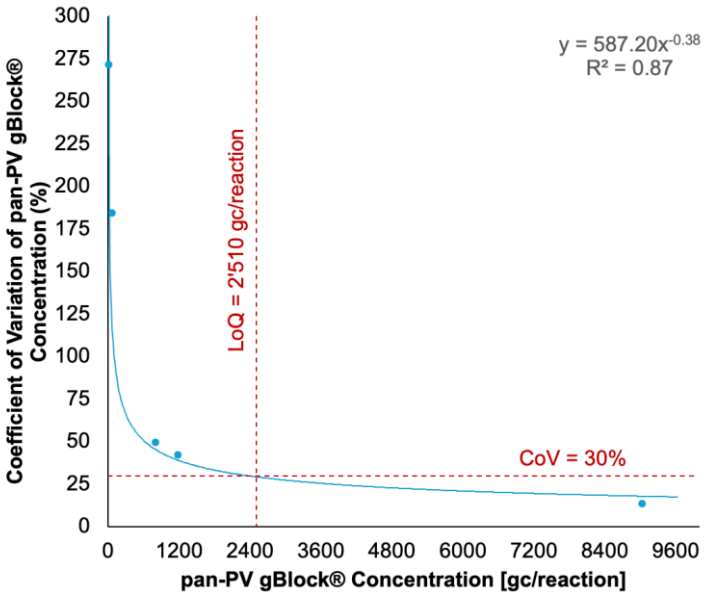


Figure 3. The relationship between pan-PV gBlock® concentration and coefficient of variation (CoV). Data points show the CoV (%) at varying concentrations of pan-PV gBlock® [gc/reaction], which were fitted to a power model. The curve follows the equation $y = 587 * x^{-0.38}$, with an R^2 value of 0.87, indicating an inverse relationship between pan-PV gBlock® concentration and CoV. The dashed red horizontal line marks a CoV of 30%, and the dashed red vertical line marks a concentration of 2'510 gc/reaction.

Testing wastewater matrix effects on positive material

To evaluate the impact of the wastewater matrix on detection, we compared the separation scores of pan-PV synthetic DNA and plasmid spiked into nuclease-free water and wastewater using the same mastermix for all cases. When pan-PV synthetic DNA was spiked into nuclease-free water

at an average concentration of 87 gc/reaction, the separation score was on average (± standard deviation) 6.57 ± 0.06 (Figure 4A), whereas spike-in wastewater samples had an average separation score of 6.46 ± 0.83, equivalent to a 1.7% decrease (Figure 4B). Further, we saw a more notable difference between the use of pan-PV synthetic DNA and pan-PV plasmid. When the pan-PV plasmid was spiked in at a concentration of 382 gc/reaction, the separation score, when spiked in nuclease-free water (5.45 ± 0.01), was lower compared to pan-PV synthetic DNA and even more so when spiked in wastewater (4.46 ± 0.35) (Figure 4C and Figure 4D).

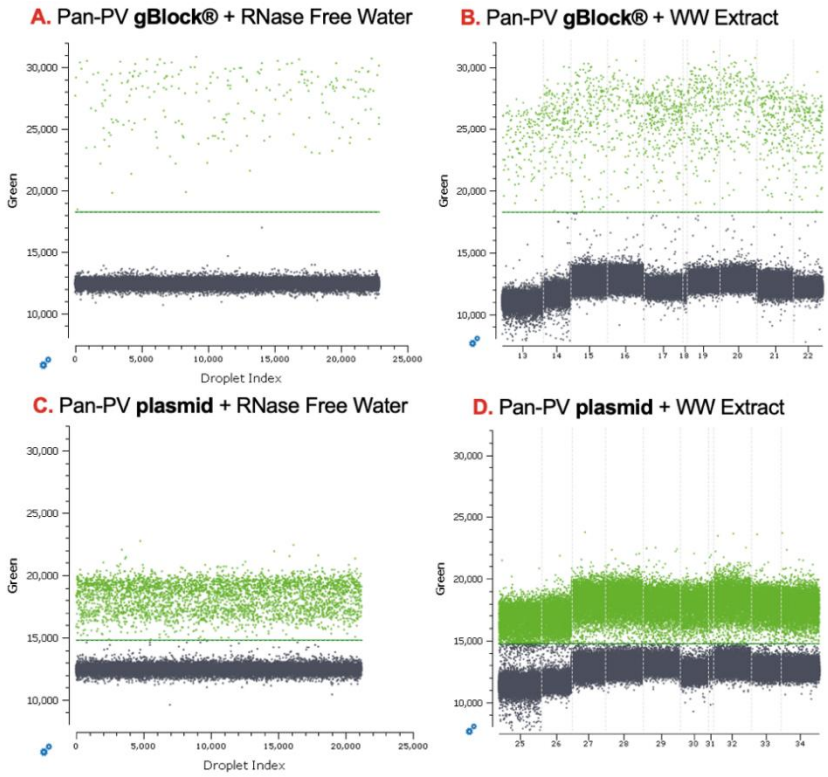


Figure 4. Spike-in of PV positive controls in wastewater. One-dimensional visualizations of partition classification taken from Naica® Crystal Miner software (Stilla Technologies). Vertical axes show fluorescence intensity in the green channel. Horizontal axes display the number of partitions in a single chamber (A and C), or in different samples (B and D). Each dot corresponds to a partition. Clustering of positive partitions is performed by setting a threshold (green line) midway in between the two clusters. (A) Separation of positive and negative partitions when pan-PV gBlock® is spiked in RNase free water, (B) when pan-PV plasmid is spiked in RNase free water, (C) when pan-PV gBlock® is spiked in wastewater extracts (chamber 13 and 14 from Lugano, 15 and 16 from Basel, 17 and 18 from Lucerne, 19 and 20 from Altenrhein, 21 and 22 from Laupen), and (D) when pan-PV plasmid is spiked in wastewater extracts (chamber 25 and 26 from Lugano, 27 and 28 from Basel, 29 and 30 from Lucerne, 31 and 32 from Altenrhein, 33 and 34 from Laupen).

Despite the differences in cluster separation, the positive fluorescing droplets could still be distinguished from the negative droplets for all cases. Furthermore, no inhibition above the acceptable threshold was observed for any of the spiked wastewater samples when using both types of positive controls (Table 3, Table 4). Specifically, for the pan-PV gBlock®, the sample from Basel showed the most inhibition with a value of 23%, and the sample of Lucerne had the least inhibition (i.e., -3%). For pan-PV plasmid, the sample from Lugano showed the most inhibition with

a value of 18%, and the sample of Altenrhein had the least inhibition (i.e., -3%). Notably, none of these inhibition values is above our acceptable threshold of 40%.

Table 3. Effect of wastewater matrix on pan-PV gBlock® material. WWTP: wastewater treatment plant, AVG: mean, STD: standard deviation, C: concentration. Concentration is expressed in genome copies (gc) per reaction (rxn). We define an inhibition value below 40% as acceptable. Above 40% the sample is inhibited to an extent that reduces confidence in the estimated concentration.

WWTP	Date	Dilution	AVG of C_{spike} [gc/rxn]	STD of C_{spike} [gc/rxn]	AVG of C_{sample} [gc/rxn]	STD of C_{sample} [gc/rxn]	Inhibition (%)	STD of Inhibition (%)
Lugano	05.11.23	5x	87.0	8.00	68.5	17.5	21	28
Basel	05.11.23	5x	87.0	8.00	67.3	5.25	23	13
Lucerne	05.11.23	3x	87.0	8.00	89.3	20.8	-3	34
Altenrhein	05.11.23	3x	87.0	8.00	84.5	12.5	3	23
Laupen	05.11.23	3x	87.0	8.00	87.3	0.25	0	10

Table 4. Effect of wastewater matrix on pan-PV plasmid material. WWTP: wastewater treatment plant, AVG: mean, STD: standard deviation, C: concentration. Concentration is expressed in genome copies (gc) per reaction (rxn). We define an inhibition value below 40% as acceptable. Above 40% the sample is inhibited to an extent that reduces confidence in the estimated concentration.

WWTP	Date	Dilution	AVG of C_{spike} [gc/rxn]	STD of C_{spike} [gc/rxn]	AVG of C_{sample} [gc/rxn]	STD of C_{sample} [gc/rxn]	Inhibition (%)	STD of Inhibition (%)
Lugano	05.11.23	5x	382	20.4	314	26.4	18	11
Basel	05.11.23	5x	382	20.4	358	2.15	6	6
Lucerne	05.11.23	3x	382	20.4	336	17.4	12	9
Altenrhein	05.11.23	3x	382	20.4	394	44.5	-3	17
Laupen	05.11.23	3x	382	20.4	364	2.35	5	6

Specificity of the pan-PV single-plex dPCR assay

We assessed the specificity of our pan-PV single-plex dPCR assay by testing it against a panel of non-PV enteroviruses, covering five distinct serotypes, alongside positive and no-template controls. Selected strains included: echovirus 6 (EV6), coxsackievirus B5 (CVB5), coxsackievirus

B3 (CVB3), coxsackievirus B1 (CVB1), and coxsackievirus A9 (CVA9). All strains were tested at a concentration of 10^3 gc/µL or 5×10^3 gc/reaction. We observed no amplification of targets that were not PV, as none of the serotypes showed a positive signal in our assay, whereas the positive control showed amplification. This suggests that our assay is specific enough to avoid detecting some of the most commonly circulating strains within the *Enterovirus* genus (Figure 5).

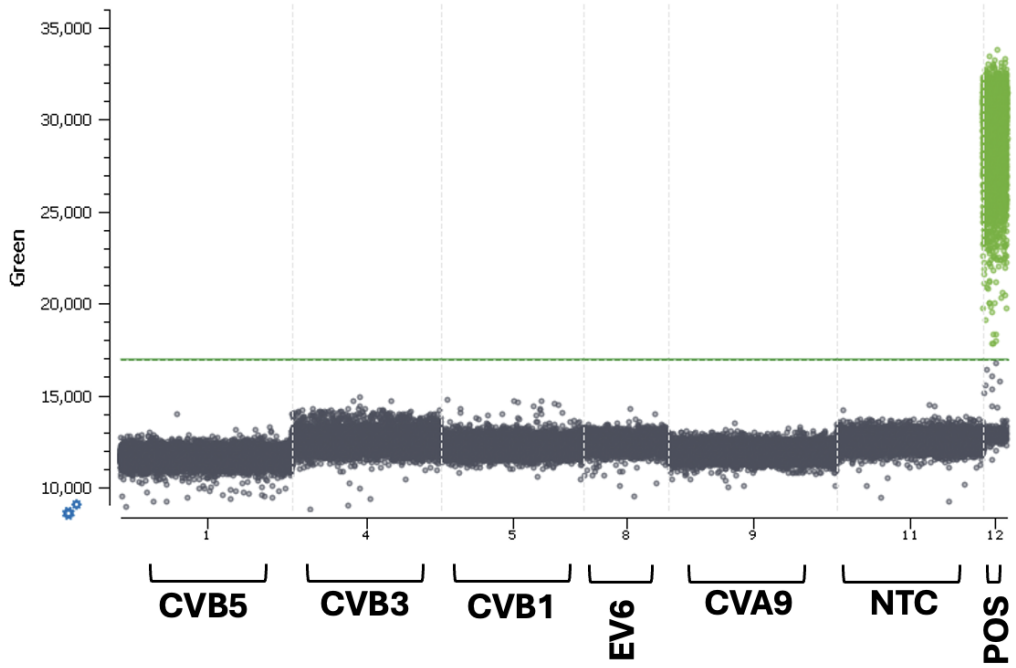


Figure 5. Specificity of the pan-PV dPCR assay. The vertical axis ("Green") indicates the fluorescent intensity detected in the green channel, and the horizontal shows the samples tested with the pan-PV assay, as well as positive control (POS) and no-template control (NTC). CVB5, CVB3, CVB1, EV6, and CVA9 belong to the *Enterovirus* genus, but are non-PV enteroviruses. For the positive control (POS), the pan-PV gBlock® was used, whereas for the no-template control (NTC) RNase-free water was used. Each dot indicates a partition. Green partitions are positive, whereas black partitions are negative. The green line indicates the threshold for PV positivity.

Discussion

Assay accuracy, precision, and sensitivity

Our assay displays LoD and LoQ values that are higher than observed for other dPCR assays, which we hypothesize is caused by the inclusion of multiple degenerate bases in both primers and probe. We note that the high LoD value refers to the confidence with which we can detect PV RNA in wastewater; lower concentrations can be detected in wastewater but not as reliably. Degenerate bases have the benefit of allowing the detection of multiple serotypes, but it may be limiting our assay sensitivity. Assays targeting serotypes with greater specificity may also more reliably detect PV RNA at lower concentrations.

During testing, we observed discrepancies between manufacturer-reported concentrations for the pan-PV gBlock® positive control and our dPCR measurements. Indeed, the dPCR assay underestimated the concentration stated by the manufacturer. These differences were more pronounced at lower concentrations, likely due to the high coefficient of variation (CoV) of our assay, and may be the result of inefficient PCR amplification, inaccuracy in manufacturer provided concentration estimates, or pipetting error. Notably, concentrations reported by manufacturers could not be confirmed (for example, using a fluorimeter) due to the low molecular weight of the target sequence which is, for example, below the limit of detection for Qubit.

When testing spiked-in wastewater extracts, we observed a shifted positive cluster, closer to the negative cluster, but clusters could still be differentiated. The reduced separation between clusters when measuring the pan-PV plasmid control in wastewater may be due to suboptimal amplification efficiency, leading to decreased fluorescence. A contributing factor to the suboptimal amplification efficiency may have been the circular nature of pan-PV plasmid which could be linearized through digestion. If the plasmid is in undigested form, some 3D structures may persist, which can interfere with primer and probe binding and subsequently impact the detectability when using dPCR.

In our study, assay specificity was tested to maximize the certainty that if a positive signal is detected in the wastewater, we can confidently confirm that it is a serotype of PV. For specificity testing, we selected distinct enteroviruses because of their close phylogenetic relationship to PVs within the *Enterovirus* genus. Indeed, these non-PV enteroviruses are abundantly present within wastewater and are genetically and structurally similar to PVs, which allows us to evaluate the assay's ability to specifically detect PV without cross-reacting with other enterovirus serotypes. We found no positive signal when testing five common non-PV enteroviruses with our assay which demonstrates a degree of specificity that can at least exclude widely circulating strains within the same *Enterovirus* genus.

Deriving cases from wastewater loads

PV is known to be a persistent pathogen, surviving on surfaces and in soil for up to a year, and in wastewater and sediments for several days[15]. While this feature makes PV difficult to eradicate, it could lead to advantages when implementing wastewater-based surveillance (WBS). In our study, we determined the LoD, which we translate to concentrations of PV reliably detected in wastewater. However, we cannot accurately relate this to the minimum number of cases needed to detect PV RNA in wastewater. Importantly, implementation of WBS for PV elsewhere provides

important insights. In New York, in 2022, one case of paralytic PV with some underlying circulation of symptomless PV infections, resulted in detection in 34% of 126 samples taken over a 6-month period using polyethylene glycol (PEG) precipitation and ultracentrifugation extraction, and RT-qPCR with a similar set of primers and probe to that which we use[16]. We expect our proposed method would achieve similar or superior performance, as our method of nucleic acid extraction using direct capture is generally more sensitive than PEG precipitation and ultracentrifugation[17], and dPCR is also more sensitive than qPCR[18]. Finally, we could also increase our likelihood to detect cases by increasing the number of replicates, as shown in our calculations of LoD and LoQ.

PART II: Exploring possibilities for differentiating PV types without cultivation

Background

While PV can be detected through generic molecular assays such as the developed pan-PV dPCR assay, these assays can often not differentiate PV types. However, intratypic differentiation (ITD) plays a crucial role in the surveillance, which aims at distinguishing between WPVs, vaccine strains (also known as Sabin strains), and VDPVs[19]. This differentiation is essential for guiding the public health response as only detection of WPV or VDPV would suggest transmission of PVs. In the context of wastewater surveillance in Switzerland, ITD through culturing presents logistical challenges, as the enrichment of PV is strongly discouraged globally and the practice of doing so would require a higher biosafety level than what is currently available at Eawag. Therefore, culture-free methods to differentiate vaccine strains from WPVs and VDPVs are valuable.

Intratypic differentiation using molecular assays

Various studies have investigated the possibility of differentiating PV types by developing molecular assays, including traditional PCR and quantitative PCR (qPCR). The Global Polio Eradication Initiative (GPEI) has released guidelines and details about available qPCR assays for PV ITD[20]. Specifically, six molecular assays have been described and validated by Gerloff and colleagues[21]. Notably, these ITD assays have been developed for differentiating subtypes after culturing, therefore, it is challenging to predict how these assays would perform when using them directly on wastewater samples, particularly in terms of detection sensitivity.

Intratypic differentiation using sequencing approaches

The current gold standard method for PV detection includes a combination of targeted molecular assays and Sanger sequencing. Furthermore, genome sequencing can monitor genetic evolution and potentially track samples back to specific patients, and it can identify VDPVs which are currently the highest risk genotypes for countries with waning vaccination rates[22]. Direct sequencing for PV in wastewater is a possibility, as shown with methods for the direct detection in mixed stool samples by Shaw et al. (2020)[23]. However, this method remains a research approach that has not yet been widely implemented. What limits the potential utility of direct PV sequencing from wastewater is the low concentrations of PV in wastewater samples, which may fall below the detection or sequencing limit. Should PV surveillance in wastewater become an essential component of surveillance in Switzerland, additional resources devoted to further developing direct sequencing from wastewater are needed, in line with the current program to monitor SARS-CoV-2 variants directly from wastewater[24]. Such a program would necessarily require partnerships between the Wastewater Monitoring Laboratory at Eawag and the National Reference Laboratory (NRL). Direct sequencing could be useful for identifying the lineage, transmission characteristics, and potentially the source.

Overall conclusions and recommendations

We present and describe the development and validation of a dPCR pan-PV assay. We validated the assay through linearity, determination of LoD and LoQ, and specificity, and we provide robust data on the assay's performance using two types of positive controls. The dPCR is readily implementable in response to perceived need. Importantly:

i. Despite our efforts to validate the pan-PV assay, its true performance for wastewater surveillance can only be assessed when applying it to actual scenarios (i.e., wastewater samples).

ii. Due to the low assay sensitivity (i.e., high LoD and LoQ thresholds), a negative result does not guarantee the absence of PV in the community.

iii. To maximize detection probability in view of high LoD and LoQ, we suggest running the pan-PV assay on multiple replicates of wastewater samples, and we recommend 3-5 to optimize detectability and laboratory processing efforts.

The pan-PV assay may be useful for informing responses to potential outbreaks in Switzerland. For example, this assay may be able to highlight geographic regions or time periods where increased clinical surveillance, expanded WBS, or targeted vaccination campaigns are needed. Ultimately, this assay could guide public health decisions which could reduce or ameliorate the impact of poliomyelitis outbreaks. In the event of PV detection in wastewater with the pan-PV assay, we recommend the following:

i. Given the high coefficient of variation observed at low concentrations, additional caution is advised whenever low levels of PV are detected with the assay to ensure accurate interpretation.

ii. Sabin-like PVs were frequently detected in Zurich wastewater from 2001-2005[25], and intratypic differentiation (ITD) highlighted that these were introduced independently and did not support ongoing transmission or concern. Therefore, we recommend:

    a. Using the pan-PV assay in wastewater in the context of reactive surveillance (i.e., after a clinical AFP case/outbreak), and

    b. Using an ITD methodology in the context of routine wastewater surveillance to correctly interpret a positive wastewater signal.

Contributors

This report was prepared by Charles Gan, Melissa Pitton, Jolinda de Korne, Rainer Gosert, Karoline Leuzinger, Tamar Kohn, Christoph Ort, Timothy R. Julian. The first draft was reviewed by Olga Schubert (Internal Review). For further information or clarification about the contents of the report, please contact covid.abwasser@eawag.ch.

Funding

The project is funded by the Swiss Federal Office of Public Health through the grant “Abwassermonitoring für übertragbare Krankheiten - Qualitätssicherung und Pilotprojekt” to Christoph Ort and Timothy R. Julian.

Acknowledgments

We thank the National Reference Laboratory for generously providing us with the pan-PV plasmid used in this study and for their insights regarding the optimization of thermal cycling conditions.

Data Availability

All data are available at [https://data.eawag.ch/dataset/ww-based-polio-surv-dpcr]

References

1 Racaniello, V. R. One hundred years of poliovirus pathogenesis. *Virology* **344**, 9-16 (2006). https://doi.org/10.1016/j.virol.2005.09.015

2 Melnick, J. L. Current status of poliovirus infections. *Clinical Microbiology Reviews* **9**, 293-300 (1996). https://doi.org/doi:10.1128/cmr.9.3.293

3 *Clinical Overview of Poliomyelitis*, <https://www.cdc.gov/polio/hcp/clinical-overview/index.html> (Accessed on: 20/11/2024).

4 *Vaccine-Derived Poliovirus*, <https://www.cdc.gov/vaccines/vpd/polio/hcp/vaccine-derived-poliovirus-faq.html> (Accessed on: 14/10/2024).

5 *Global Polio Eradication Initiative (GPEI)*, <https://polioeradication.org/> (Accessed on: 14/10/2024).

6 *Poliomyelitis Eradication*, <https://www.bag.admin.ch/bag/en/home/gesund-leben/umwelt-und-gesundheit/biosafety/polio-ausrottung.html> (Accessed on: 14/10/2024).

7 *WISE Dashboard - Wastewater-based Infectious Disease Surveillance and Epidemiology*, <https://wise.ethz.ch/> (Accessed on: 01/11/2024).

8 Kilpatrick, D. R. *et al.* Rapid Group-, Serotype-, and Vaccine Strain-Specific Identification of Poliovirus Isolates by Real-Time Reverse Transcription-PCR Using Degenerate Primers and Probes Containing Deoxyinosine Residues. *Journal of Clinical Microbiology* **47**, 1939-1941 (2009). https://doi.org/doi:10.1128/jcm.00702-09

9 Klymus, K. E. *et al.* Reporting the limits of detection and quantification for environmental DNA assays. *Environmental DNA* **2**, 271-282 (2020). https://doi.org/10.1002/edn3.29

10 Forootan, A. *et al.* Methods to determine limit of detection and limit of quantification in quantitative real-time PCR (qPCR). *Biomolecular Detection and Quantification* **12**, 1-6 (2017). https://doi.org/10.1016/j.bdq.2017.04.001

11 Kralik, P. & Ricchi, M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. *Front Microbiol* **8**, 108 (2017). https://doi.org/10.3389/fmicb.2017.00108

12 Nadeau, S. *et al.* Influenza transmission dynamics quantified from RNA in wastewater in Switzerland. *Swiss Medical Weekly* **154**, 3503 (2024). https://doi.org/10.57187/s.3503

13 Lievens, A., Jacchia, S., Kagkli, D., Savini, C. & Querci, M. Measuring Digital PCR Quality: Performance Parameters and Their Optimization. *PLOS ONE* **11**, e0153317 (2016). https://doi.org/10.1371/journal.pone.0153317

14 Bates, D. M. & Watts, D. G. *Nonlinear Regression Analysis and Its Applications*. (John Wiley & Sons, Inc., 1988).

15 Kline, A. *et al.* Persistence of poliovirus types 2 and 3 in waste-impacted water and sediment. *PLoS One* **17**, e0262761 (2022). https://doi.org/10.1371/journal.pone.0262761

16 Ryerson, A. B. *et al.* Wastewater Testing and Detection of Poliovirus Type 2 Genetically Linked to Virus Isolated from a Paralytic Polio Case - New York, March 9-October 11, 2022. *MMWR Morb Mortal Wkly Rep* **71**, 1418-1424 (2022). https://doi.org/10.15585/mmwr.mm7144e2

17 Dimitrakopoulos, L. *et al.* Evaluation of viral concentration and extraction methods for SARS-CoV-2 recovery from wastewater using droplet digital and quantitative RT-PCR. *Case Studies in Chemical and Environmental Engineering* **6**, 100224 (2022). https://doi.org/https://doi.org/10.1016/j.cscee.2022.100224

18 Ahmed, W. *et al.* Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Science of The Total Environment* **739**, 139960 (2020). https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.139960

19 Sun, H. *et al.* Validation of a redesigned pan-poliovirus assay and real-time PCR platforms for the global poliovirus laboratory network. *PLoS One* **16**, e0255795 (2021). https://doi.org/10.1371/journal.pone.0255795

20 *Poliovirus rRT-PCR ITD 4.1 Addendum Kit*, <https://polioeradication.org/wp-content/uploads/2020/01/GPLN-GP2-appendix-English.pdf> (Accessed on: 24/09/2024).

21 Gerloff, N. *et al.* Diagnostic Assay Development for Poliovirus Eradication. *J Clin Microbiol* **56** (2018). https://doi.org/10.1128/jcm.01624-17

22 Lai, Y. A., Chen, X., Kunasekaran, M., Rahman, B. & MacIntyre, C. R. Global epidemiology of vaccine-derived poliovirus 2016-2021: A descriptive analysis and retrospective case-control study. *EClinicalMedicine* **50**, 101508 (2022). https://doi.org/10.1016/j.eclinm.2022.101508

23 Shaw, A. G. *et al.* Rapid and Sensitive Direct Detection and Identification of Poliovirus from Stool and Environmental Surveillance Samples by Use of Nanopore Sequencing. *Journal of Clinical Microbiology* **58**, 10.1128/jcm.00920-00920 (2020). https://doi.org/doi:10.1128/jcm.00920-20

24 Jahn, K. *et al.* Early detection and surveillance of SARS-CoV-2 genomic variants in wastewater using COJAC. *Nature Microbiology* **7**, 1151-1160 (2022). https://doi.org/10.1038/s41564-022-01185-x

25 Zurbriggen, S. *et al.* Isolation of Sabin-Like Polioviruses from Wastewater in a Country Using Inactivated Polio Vaccine. *Applied and Environmental Microbiology* **74**, 5608-5614 (2008). https://doi.org/doi:10.1128/AEM.02764-07

Supplemental Material

Supplemental Table S1. Characteristics of poliovirus positive material used in this study.

Name	Type/Description	Sequence	Source
pUC57Pan_Polio	Poliovirus sequence was inserted in the multiple cloning site (MCS) of the pUC57 plasmid	ATTACGCCAAGCTTGCATGCAGGCC TCTGCAGTCGACGGGCCCGGGATC CGATccaatGAATTGTAATACGACTCA CTATAGGGCCATGGagtttttcacgtattcc agatttgacatggaattcacctttgtaatcactgcca attttacagaaaccaataatgggcacgctttaaatc aggtataccaaatcatgtacgtaccaccaggcC TCGAGTCTATAGTGTCACCTAAATAG CTTGGCattggATCTAGATGCATTCGC GAGGTACCGAGCTCGAATTC[#]	National Reference Laboratory (NRL)
pan-PV gBlock®	gBlock®	ACAAATCCACTAGTCCCTTCTGATAC AGTGCAAACCAGACATGTTGTACAAC ATAGGTCAAGGTCAGAGTCTAGCATA GAGTCTTTCTTCGCGCGGGGTGCAT GCGTGACCATTATGACCGTGGATAA CCCAGCTTCCACCACGAATAAGGAT AAGCTATTTGCAGTGTGGAAGATCAC TTATAAAGATACTGTCCAGTTACGGA GGAAATTGGAGTTCTTCACCTATTCT AGATTTGATATGGAACTTACCTTTGTG GTTACTGCAAATTTCACTGAGACTAA CAATGGGCATGCCTTAAATCAAGTGT ACCAAATTATGTACGTACCACCAGGC GCTCCAGTGCCCGAGAAATGGGAC GACTACACATGGCAAACCTCATCAAA TCCATCAATCTTTTACACCTACGGAA CAGCTCCAGCCCGG	Integrated DNA Technologies (IDT, US)

[#] Sequence inserted between M13 primers

Supplemental Table S2. Reaction composition for pan-PV dPCR assay. *Volume for one reaction. #Manufactured by Quantabio (USA).

Reagent	Volume (µL)*
qScript® XLT 1-step RT-PCR Tough Mix#	13.5
Forward Primer (50µM)	0.54
Reverse Primer (50µM)	0.54
Probe (20µM)	0.54
RNase Free Water	19.98
Template	5.4

Supplemental Table S3. Positivity rate in relation to polio gBlock® concentration. gc: genome copies.

Concentration [gc/reaction]	# of Replicates	# of Positive Chambers	Positivity Rate (%)
9'030	31	31	100
1'180	30	30	100
803	25	25	100
63.3	24	24	100
3.36	25	7	28