WastewaterSCAN Measles Assay Development and Validation

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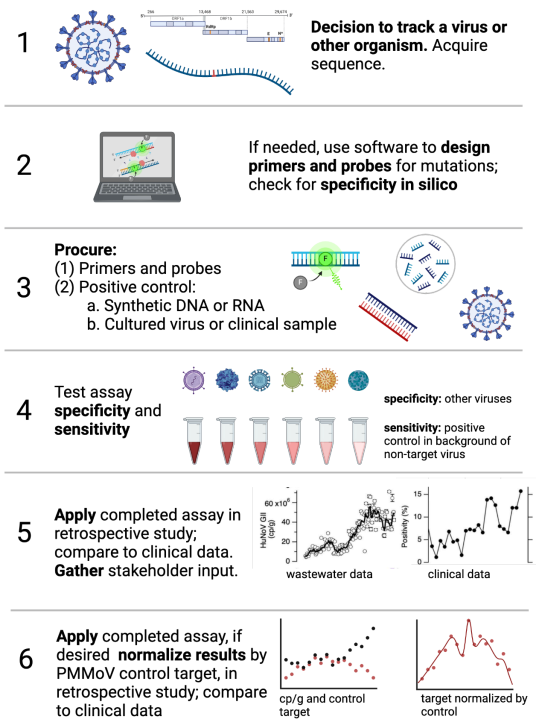
Background & Summary

The purpose of this document is to describe the methodology used by WastewaterSCAN to design and validate a hydrolysis probe-based assay that is specific and sensitive to wild type (WT) measles virus RNA in wastewater, that does not cross-react with the vaccine strain of the measles virus. We also evaluate an existing assay that has been used to identify WT measles in wastewater but shows some cross reactivity with measles vaccine RNA (Wu et al.)[1] The newly presented WT-specific assay is a modified version of a previously published assay (Roy et al.)[2] and is referred to as the "modified Roy et al." assay. The approach we use generally follows the outline presented in the schematic to the right.



Measles wild type-specific assay design (modified Roy et al. assay)

Roy et al.[2] report a measles RT-QPCR assay targeting the 3′ region of the measles N gene. We downloaded 97 (43 genotype B3 and 54 genotype D8) measles sequences from NCBI in March 2025 and obtained the Edmonston-Enders and Edmonston-Zagreb vaccine sequences. We then aligned the sequences of the Roy et al. primers and probe with the sequences. The in silico results suggested that the Roy et al. assay could be made specific to circulating WT measles genotypes B3 and D8, and not specific to the vaccine sequences, by changing the sequence of the probe and the reverse primer. The newly designed assay (hereafter, modified Roy et al. assay) is shown in Table 1. Note that although the original Roy et al. assay used a locked nucleic-acid probe, the modified assay in Table 1 does not.

Probe	CATGATGATCCAAGTAGTAGTGA
Forward Primer	AGGATGAGGCGGACCARTACTT
Reverse Primer	CRATATCTGAGATTTCCTTGTTCTC

Table 1. Modified Roy et al. assay.

In silico testing for specificity and sensitivity

We used NCBI BLAST to confirm specificity of the modified Roy et al. assay to WT genotypes B3 and D8. Specificity was checked through an exclusionary BLAST that excludes the intended

assay target, allowing for the determination of any potential off target amplification. Based on this in silico testing, the modified Roy et al. assay (Table 1) is specific to all known and circulating WT variants of measles, and is not expected to amplify nucleic-acids from the vaccine strains.

In vitro testing for specificity and sensitivity

Specificity against common wastewater pathogen targets

The modified Roy et al. assay was tested in vitro against nucleic acids from a panel of respiratory pathogens (Figure 1). Nucleic-acids were extracted and purified as described below for the wastewater solids samples and then used neat as template in droplet digital 1-step RT-PCR assays. Assays were run in a single well using the cycling conditions and post processing using a droplet reader as for the wastewater samples in singleplex. No cross-reactivity was observed from either assay against the panel of non-target organisms.

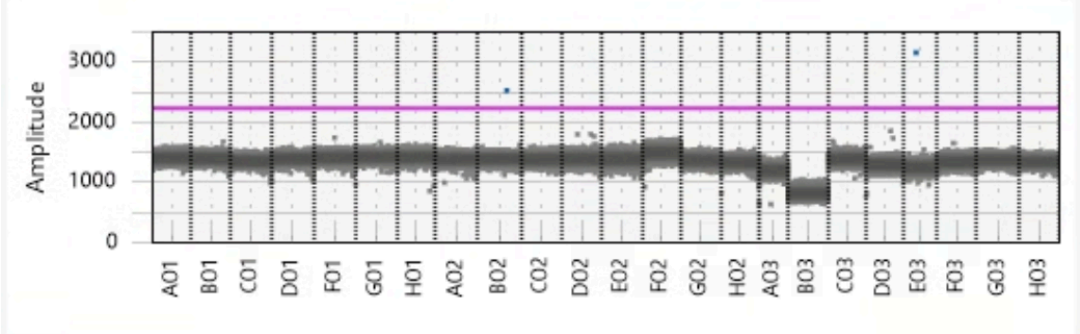


Figure 1. Amplitude plot for in vitro specificity testing. The amplitude of fluorescence for each sample is shown in the y-axis. The pink line separates positive (above line) droplets in blue and negative (below line) droplets in grey. From left to right, A01 is Parainfluenza 1, B01 is parainfluenza 2, C01 is parainfluenza 3, D01 is parainfluenza 4, E01 is influenza A H1N1pdm, F01 is influenza A H1, G01 is influenza A H3, H01 is influenza B, A02 is adenovirus 1, B02 is adenovirus 3, C02 is adenovirus 31, D02 is Rhinovirus Type 1A, E02 is RSV A, F02 is SARS-CoV-2, G02 is *M. pneumoniae,* H02 is C. pneumoniae, A03 is Metapneumovirus 8, B03 is coronavirus HKU-1, C03 is coronavirus 229E, D03 is coronavirus NL63, E03 is coronavirus OC43, F03 is *B. parapertussis*, G03 is *B. pertussis*, H03 is a no template control. One positive droplet is observed in locations B02 and E03, containing adenovirus 3 and coronavirus OC43, but three droplets must be positive to score a sample as positive, so these are negatives. All targets are part of a panel purchased from Zeptometrix (Buffalo, NY) and are inactivated pathogens.

Specificity against measles targets

The modified Roy et al. assay and the Wu et al. assay were tested in vitro against measles positive controls: synthetic gBlocks for D8 and B3 (Table 2), and genomic RNA from Edmonston vaccine strain (obtained from ATCC). Assays were run in a single well using the cycling

conditions and post processing using a droplet reader as for the wastewater samples in singleplex.

D8 gene block	ATCTGGCCTCACCTTCGCATCAAGAGGTACCAACATGGAGGATGAGGC GGACCAATACTTTTCACATGATGATCCAAGTAGTAGTGATCAATCCAGG TTCGGATGGTTCGAGAACAAGGAAATCTCAGATATCGAAGTGCAAGAC CCTGAGGGATTTAACATGATTCTGGGTACCATTCTAGCCCAAATTTGGG TCTTGCTCGCAAAGGCGGTTACGGCCCCAGACACGGCAGCTGATTCG GAGCTAAGAAGGTGGATAAAGTACACCCAACAAAGAAGGGTAGTTGGT GAATTTAGATTGGAGAGAAAATG
B3 gene block	GCCTTACCTTCGCATCGAGAGGTACTAATATGGAGGATGAGGCGGACC AGTACTTTTCACATGATGATCCAAGTAGTAGTGATCAATCCAGGTTCGG GTGGTTTGAGAACAAGGAAATCTCAGATATTGAAGTGCAAGACCCTGA GGGCTTCAACATGATTCTGGGTACCATCTTAGCTCAAATTTGGGTCTTG CTCGCAAAGGCGGTTACGGCTCCAGACACAGCAGCTGATTCAGAGCT AAGAAGGTGGATCAAATACACCCAACAAAGAAGAGTAGTTGGTGAATT

Table 2. Gene block sequences for wild type measles genotypes D8 and B3.

The modified Roy et al. assay detected both B3 and D8 but did not produce any positive droplets for the vaccine nucleic-acids. The Wu et al. assay performed similarly; the vaccine nucleic acid template amplified but produced fluorescence at a lower level than the B3 and D8 nucleic-acids. Cross-reactivity was also reported by Wu et al. in their original description of the assay.

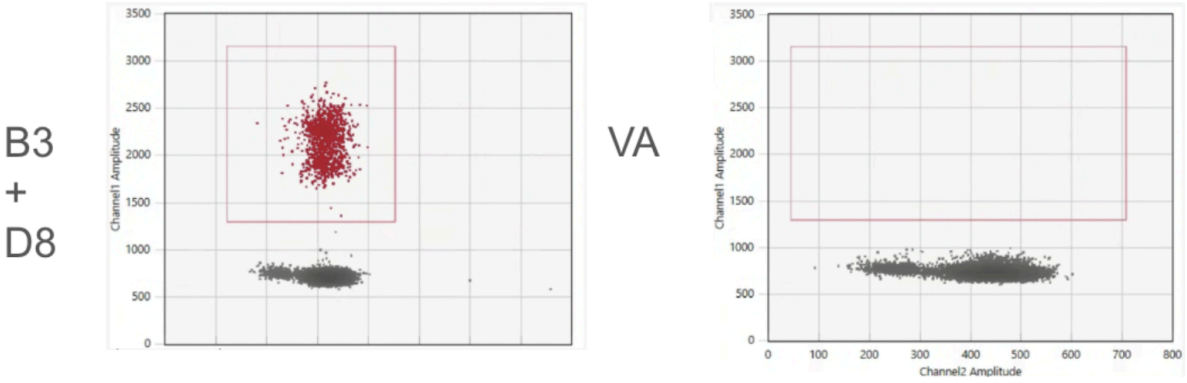


Figure 2. Amplitude plot showing droplets from digital PCR testing for sensitivity for the **modified Roy et al. assay**. Positive droplets are shown in red and negative are shown as grey. The left panel shows results for equal molar concentrations of gblocks for B3 and D8 (approximately 1000 copies of each per reaction) as template. The right panel shows results for vaccines nucleic acids (approximately 1000 copies per reaction). The red boxes indicate the region of the plots where positive droplets are expected based on thresholding.

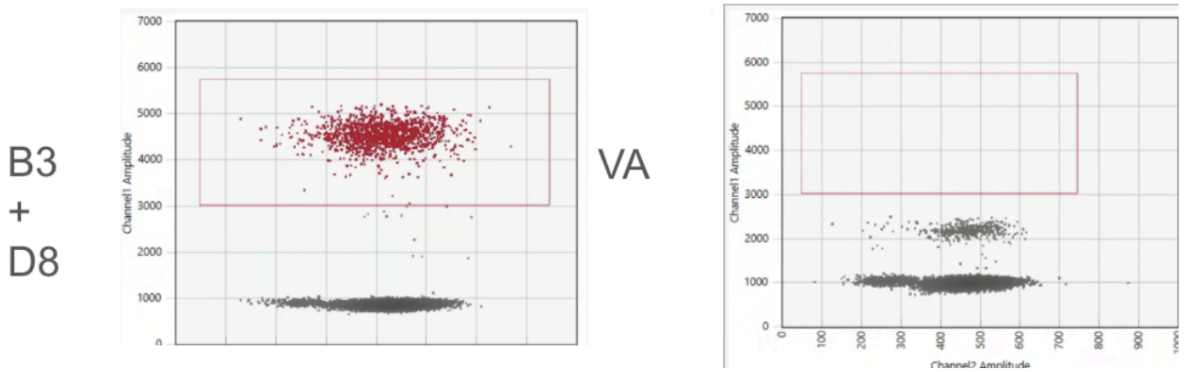


Figure 3. Amplitude plot showing droplets from digital PCR testing for sensitivity for the **Wu et al. assay**. Positive droplets are shown in red and negative are shown as grey. The left panel shows results for equal molar concentrations of gblocks for B3 and D8 (approximately 1000 copies of each per reaction) as template. The right panel shows results for vaccines nucleic acids (approximately 1000 copies per reaction). The red boxes indicate the region of the plots where positive droplets are expected based on thresholding.

Sensitivity

Nucleic-acids representing genotypes B3 and D8, and from the measles vaccine were serially diluted in sterile, nucleic-acid free water to achieve template concentrations between 0 and 200

copies per reaction and run in triplicate using droplet digital RT-PCR using the modified Roy et al. assay (Table 1).

All negative controls were negative. The modified Roy et al. assay detected the intended targets (B3 and D8) across all dilutions. The lowest concentration at which all 3 replicates were positive was 6.25 gene copies per reaction for the D8, and 25 gene copies per reaction for the B3 gblock. The modified Roy et al. assay produced zero positive droplets for all concentrations of the vaccine strain control (VA). These results support the specificity of the assay to genotypes B3 and D8.

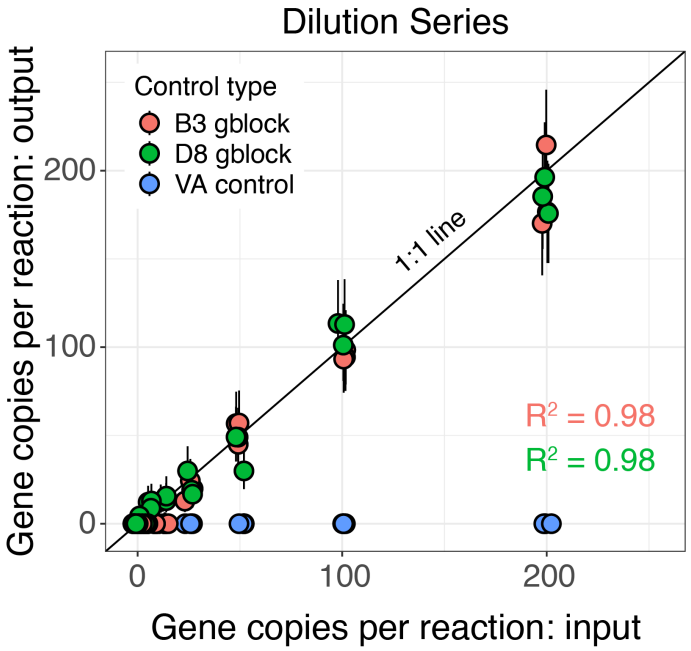


Figure 4. Sensitivity testing using the **modified Roy et al. assay**. Expected concentrations based on preparation of gblock control material and measured concentrations with and without the RT step for digital PCR. Error bars represent the standard deviation. If error bars cannot be seen, they are smaller than the symbols.

Prospective limited pilot testing in wastewater

Pathogen nucleic acid targets are strongly associated with the solid phase of wastewater (Wolfe et al 2023)[3], so we make measurements in the solid phase of wastewater. The following methods were used for prospective testing with a group of pilot plants and will be implemented by WastewaterSCAN in further measles testing of wastewater.

Methods

Sample collection

Samples were obtained from 6 different WWTPs across the country between 23 and 27 April 2025. A total of 9 samples were collected representing either 24-hr composited raw influent or settled solids from the primary clarifier. Samples were shipped on blue ice to the lab, stored at 4°C, and then processed as described below, within 48 hours of receipt. Samples from 2 different days were collected at three of the plants (Plants A, B, and C) and samples from one day were collected at the remaining three plants (Plants D, E, and F).

Pre-analytical processing

Briefly, solids are isolated from wastewater samples and re-suspended in a buffer at a low enough concentration so as to minimize inhibition, and homogenized. Samples were then subjected to nucleic-acid extraction and purification (Chemagic Viral DNA/RNA 300 Kit H96, PerkinElmer, Shelton, CT), and inhibitor removal (Zymo OneStep PCR Inhibitor Removal Kit, Irvine, CA). Dry weight was determined using another aliquot of solids and drying in an oven. Nucleic-acids from 10 aliquots of each sample are obtained and each used neat as template in 10 replicate droplet digital 1-step RT-PCR (dd-RT-PCR) reaction wells to measure measles RNA using the modified Roy et al. assay (Table 1). The measles assay was run in multiplex with assays for Dengue virus RNA types 1, 2, 3 and 4- the results of which are not reported herein. The modified Roy et al. assay was run using a probe labeled with the fluorescent molecule FAM. The process includes negative and positive extraction and PCR controls, as well as a measure of recovery as determined from spiking exogenous bovine coronavirus (BCoV). Further details are described in a protocol on protocols.io[4,5] and in Boehm et al 2024[6].

Droplet digital PCR

Concentrations of measles RNA in controls and samples were measured in multiplex droplet digital 1-step RT-PCR (dd-RT-PCR) reactions using an AutoDG Automated Droplet Generator (Bio-Rad, Hercules, CA), Mastercycler Pro (Eppendorf, Enfield, CT) thermocycler, and a QX200 Droplet Reader (Bio-Rad).

Digital droplet RT-PCR was performed on 20 µl samples from a 22 µl reaction volume, prepared using 5.5 µl template, mixed with 5.5 µl of One-Step RT-ddPCR Advanced kit for Probes (catalog no. 1863021; Bio-Rad), 2.2 µl reverse transcriptase, 1.1 µl dithiothreitol (DTT), and primers and probes at a final concentration of 900 nM and 250 nM, respectively. Droplets were generated using the AutoDG Automated Droplet Generator (Bio-Rad).

PCR was performed using Mastercycler Pro with the following protocol: reverse transcription at 50 °C for 60 min, enzyme activation at 95 °C for 5 min, 40 cycles with 1 cycle consisting of denaturation at 95 °C for 30 s and annealing and extension at 59 °C for 30 s, enzyme deactivation at 98 °C for 10 min, and then an indefinite hold at 4 °C. The ramp rate for temperature changes was set at 2 °C/s, and the final hold at 4 °C was performed for a minimum of 30 min to allow the droplets to stabilize. Droplets were analyzed using the QX200 Droplet

Reader (Bio-Rad). All liquid transfers were performed using the Agilent Bravo (Agilent Technologies).

A well had to have over 10,000 droplets for inclusion in the analysis. Extraction and PCR positive and negative controls were run on each 96-well plate. Results from replicate wells were merged for analysis. Concentrations of the targets in wastewater samples are presented as copies per gram dry weight. For a sample to be scored as a positive, there had to be at least 3 positive droplets. The lowest measurable concentration is approximately 1000 copies/g dry weight (corresponds to three positive droplets). Errors are reported as standard deviations of the measurements as obtained from vendor's software.

Results

The measles positive control was positive and the negative controls had zero positive droplets. Of the 9 wastewater samples tested for measles using the modified Roy et al. assay, eight of them were negative and one was positive. The positive sample contained 13,710 (+4350/-3750) copies per gram solids dry weight measles RNA (numbers in parenthesis are 68% confidence intervals), representing a value approximately ten times the lowest detectable concentration of measles RNA using this method. Travel-associated measles cases have been reported in the county where the positive sample was collected, but it is unknown at the time of writing this document if a positive case is currently present in the sewershed.

References

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