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2 **Supplementary Materials**

3 **Assessment and application of GeneXpert rapid testing for respiratory viruses**

4 **in school wastewater**

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S1. School enrollment information

Table S1. Schools sampled (n = 169) with school code, type, and enrollment information.

School	Type	Enrollment
A	Elementary school	405
B	Middle school	509
C	Middle school	1,034
D	High school	2,434

S2. RT-ddPCR assays setup and thermal cycling conditions

Table S2. Primers and probes used for quantification of SARS-CoV-2 with RT-ddPCR

Target	Assay Name	Sequence (5'-3')	Reference
N1 gene	Forward Primer	GACCCCAAAATCAGCGAAAT	(Lu et al., 2022)
	Reverse Primer	TCTGGTTACTGCCAGTTGAATCTG	
	Probe	Cy5/ACCCCGCAT/TAO/TACGTTTGGTGGACC/3IAbRQSp	
	Amplicon length	72	
	Gblock sequence	ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGTCTTGGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAATAGCAGTCCAGATGACCAAA	
N2 gene	Forward Primer	TTACAAACATTGGCCGCAAA	(Lu et al., 2022)
	Reverse Primer	GCGCGACATTCCGAAGAA	
	Probe	Cy55/ACAATTTGCCCCCAGCGCTTCAG/3IAbRQSp	
	Amplicon length	67	
	Gblock sequence	CATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCCAAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCCCCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACACAGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTC	

Table S3. Primers and probes used for quantification of influenza A and influenza B with RT-ddPCR

Target	Assay Name	Sequence (5’-3’)	Reference
M1 protein (Influenza A)	Forward Primer	CTTCTAACCGAGGTCGAAACGTA	(Whiley and Sloots, 2005)
	Reverse Primer	GGTGACAGGATTGGTCTTGTCTTTA	
	Probe	SUN/TCAGGCCCC/ZEN/CTCAAAGCCG AG/3IABkFQ	
	Amplicon length	155	
	Gblock sequence	AGGGTCTCGCGACATGAGTCTTCTAA CCGAGGTCGAAACGTACGTTCTCTCT ATCGTCCCGTCAGGCCCCCTCAAAGC CGAGATCGCGCAGAGACTTGAAGATG TGTTTGCAGGGAAGAACACCGATCTT GAGGCACTCATGGAATGGCTAAAGAC AAGACCAATCCTGTCACCTCTGACTA AGGGGATTTTAGGATTTGTGTTCACGC TCACCGTGCCCAGTGAGCGAGGACTG CAGCGTAGACGCTTTGTCCAAAATGC CCTTAATGGGAATGGGGATCCAAACA ACATGGACAGAGCGGTCAAACTGTAC ATGGCAGAGACCTA	
HA glycoprotein (Influenza B)	Forward Primer	AAATACGGTGGATTAAACAAAAGCAA	(van Elden et al., 2001)
	Reverse Primer	CCAGCAATAGCTCCGAAGAAA	
	Probe	ROXN/CACCCATATTGGGCAATTTCCT ATGGC/3IAbRQSp	
	Amplicon length	170	
	Gblock sequence	AGGGTCTCGCGACGTAATAAAAGGGT CCTTGCCTTTAATTGGTGAAGCAGATT GCCTCCATGAAAAATACGGTGGATTA AACAAAAGCAAGCCTTACTACACAGG AGAACATGCAAAAGCCATAGGAAATT GCCCAATATGGGTGAAAACACCCTTG AAGCTGGCCAATGGAACCAAATATAG ACCGCCTGCAAAACTATTAAAGGAAA GGGGTTTCTTTGGAGCTATTGCTGGTT TCTTGGAAGGAGGATGGGAAGGAATG ATTGCAGGTTGGCACGGATACACATC TCATGGAGCACATGGAGTGGCAGTGG CTGGCAGAGACCTA	

26 **Table S4.** Primers and probes used for quantification of respiratory syncytial virus (RSV) with
27 RT-ddPCR

Target	Assay Name	Sequence (5’-3’)	Reference
N gene	Forward Primer	CTCCAGAATAYAGGCATGAYTCTCC	(Hughes et al., 2022)
	Reverse Primer	GCYCTYCTAATYACWGCTGTAAGAC	
	Probe	FAM/TAACCAAAT/ZEN/TAGCAGCAGG AGATAGATCAG/3IABkFQ	
	Amplicon length	121	
	Gblock sequence	CTAGAAAATCCTACAAAAAAATGCTA AAAGAAATGGGAGAGGTAGCTCCAG AATACAGGCATGACTCTCCTGATTGT GGGATGATAATATTATGTATAGCGGC ATTAGTAATAACCAAATTAGCAGCAG GAGATAGATCAGGTCTTACAGCTGTG ATTAGGAGGGCTAATAATGTCCTAAA AAATGAAATGAAACGTTATAAAGGCT TACTACCCAAGGATATAGCCAACAGC TTCTATGAAGTGTTTGAAAAATATCCT CACTTTATAGATGTTTTTGTTCATTTT GGTATAGCACAATCTTCTA	

28
29 **Table S5.** Final concentrations of primer-probe mix for each target

Assay Name	Final concentration (µM)	40x concentration (µM)
Forward Primer	0.9	36
Reverse Primer	0.9	36
Probe	0.25	10

30
31 **Table S6.** Reaction composition for RT-ddPCR assay

Reagent	Volume (µL)
One-step RT-ddPCR supermix	5.5
Reverse Transcript (10x)	2.2
300 mM DTT	1.1
Primer-Probe mix of each target	0.55*
RNase/DNase-free water	0.45
RNA template	10

32 *Final concentrations in reaction: 0.25µM (probe) and 0.9µM (forward and reverse primers)

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Table S7. Thermal cycling conditions for respiratory virus ddPCR assay

Cycling Step	Temperature °C	Time	Number of Cycles
Reverse transcription	50	60 min	
Enzyme activation	95	10 min	
Denaturation	94	30 sec	40
Annealing/Extension	60	60 sec	
Enzyme Deactivation	98	10 min	
Hold (optional)	4	Infinite	

S3. Quality Control Measures and Limit of Detection (LOD) Calculation

The quality control measures and the calculation of LOD are described by Lou et al. (2022) and are summarized briefly as follows. Duplicates of negative control samples were included in the concentration, extraction, and quantification steps to assess potential contamination (Borchardt et al., 2021). Specifically, two 50 mL aliquots of deionized (DI) water were processed in the same way as wastewater samples and used as negative controls for concentration. Two bead tubes containing glass beads and lysis buffer were included as extraction negative controls. The negative controls for concentration and extraction were included in all ddPCR quantification plates containing the wastewater samples that were processed together with the controls. In addition, each ddPCR quantification plate included at least four no-template controls (NTCs) with RNAse-free water and two positive controls using gBlock Gene Fragments (IDT, USA; sequence provided in Tables S1-S3).

All valid quantification wells required the number of total generated droplets to be greater than 10,000 droplets. For each target, the LOD was deteremined for each quantification plate. The LOD was determined as three positive droplets plus the maximum number of positive droplets among the negative controls. The LOD was then converted to copies per µL of DNA template and copies per liter of wastewater based on the estimated droplet volume (0.86 nL), the averaged number of total droplets throughout the plate, the volume fraction of DNA template

within a droplet (10/22), and the concentration factor during sample processing (Eq S1-3). The LOD is calculated as 1,320 copies/L-wastewater assuming no positive droplets in the negative controls and 19,000 total droplets as the average number of total droplets in each well.

Eq S1:

$$LOD_{droplet} = 3 + maximum\ number\ of\ positive\ droplets\ across\ all\ process\ blanks^{*}$$

[*] Process blanks include: two concentration blanks, two extraction blanks, and no less than four no template controls per plate included in ddPCR quantification.

Eq S2:

$$LOD_{\mu L-RNA\ template} = \frac{LOD_{droplet}}{0.86\frac{nl}{droplet} \times n\ total\ droplets^{*} \times \frac{10}{22} fraction\ of\ template\ within\ droplet} = \frac{LO\ldots}{\ldots}\ copies\ per\ \mu L\ of\ DNA\ template$$

[*] Use the average total droplets across all samples in the ddPCR plate

Eq S3:

$$LOD_{L-wastewater} = LOD_{\mu L-DNA\ template} \times 50\ \mu L\ total\ extraction \times \frac{1000\ \mu L\ of\ lysis\ buffer\ with\ wa\ldots}{300\ \mu L\ of\ lysis\ buffe\ldots} \times \frac{1}{50\ mL\ wastewater\ concentrated} \times \frac{1000\ mL}{1\ L} = LOD_{\mu L-DNA\ template} \times \frac{1000000}{300} copies\ per\ L\ of\ wastewater$$

67 **S4. Recovery rates of respiratory virus in wastewater using filtration-ddPCR workflow**

68 Eq S4:

$$Recovery\ rate\ (\%) = \frac{C_{pcr}}{\frac{C_{stock} \times V_{stock}}{50\ mL} \times \frac{1000\ mL}{1\ L}} \times 100\%$$

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70 Where:

71 C_{pcr}: viral concentration from ddPCR, copies/L

72 C_{stock}: viral concentration of virus stock, copies/µL

73 V_{stock}: volume of virus stock spiked into 50 mL of wastewater sample, µL

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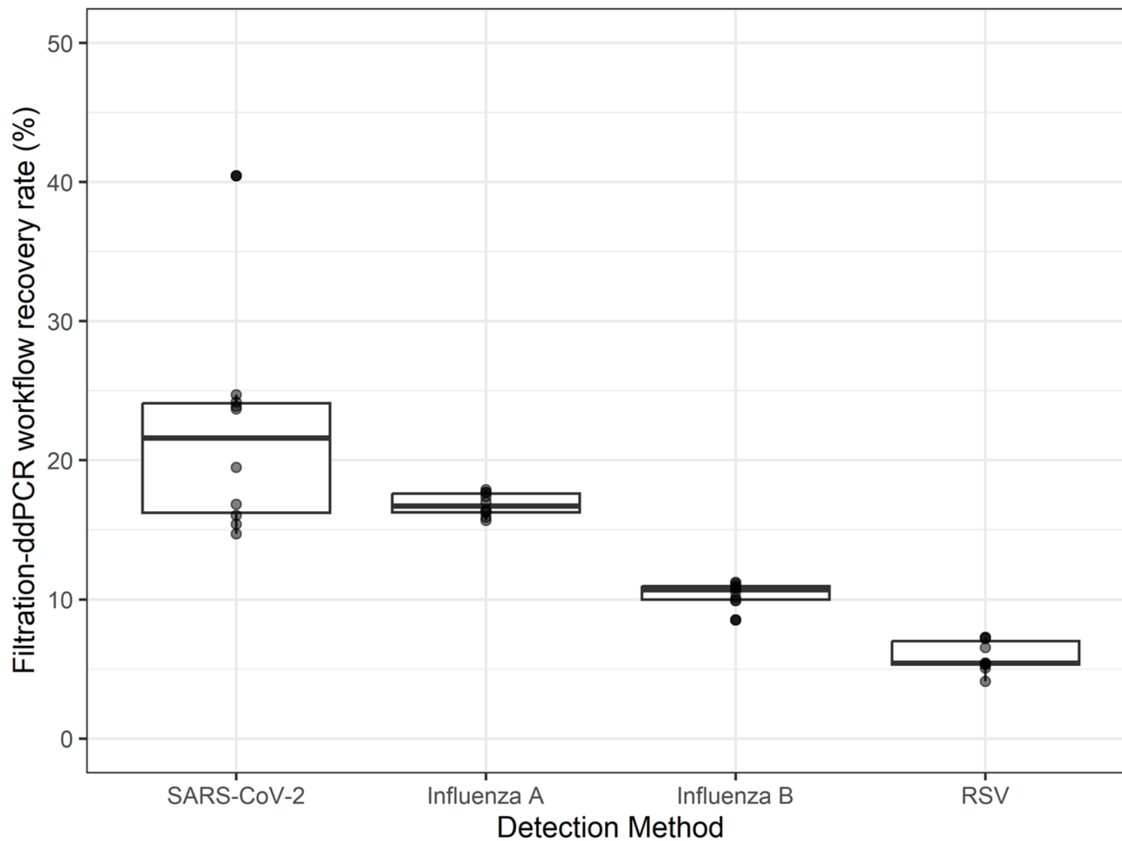

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76 **Figure S1.** Filtration-ddPCR workflow recovery rate of SARS-CoV-2, influenza A, influenza B,
77 and RSV. The boxplots show the measured recovery rate of 10 replicates of wastewater samples
78 spiked with target viruses. Dots indicate the calculated recovery rate of each measurement.

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81 **S5. The amount of nucleic acid extracts used in filtration-ddPCR workflow for target viral**
82 **concentration quantification.**

83 Eq S5

$$50\ mL\ wastewater\ concentrated \times \frac{300\ \mu L\ of\ lysis\ buffer\ used\ for\ extraction}{1000\ \mu L\ of\ lysis\ buffer\ with\ wastewater\ fraction\ resuspended} \times \frac{10\ \mu L\ RNA\ template\ for\ quantification}{50\ \mu L\ total\ extraction} = 3\ mL\ wastewater$$

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