Electronic Supplementary Material (ESI) for Environmental Science: Water Research & Technology.

Supporting Material for

Solid-liquid partitioning of Dengue, West Nile, Zika, Hepatitis A, Influenza A, and SARS-CoV-2 viruses in wastewater samples from across the United States

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Number of pages: 11

Number of Tables: 4

Number of Figures: 3

ITEM TO CHECK	PROVIDED	COMMENT
	Y/N	
1. SPECIMEN		
Detailed description of specimen type and numbers	Y	included in methods section
Sampling procedure (including time to storage)	Y	included in methods section
Sample aliquotation, storage conditions and duration	Y	included in methods section
2. NUCLEIC ACID EXTRACTION		
Description of extraction method including amount of sample processed	Y	included in methods section
Volume of solvent used to elute/resuspend extract	Y	included in methods section
Number of extraction replicates	Y	included in methods section
Extraction blanks included?	Y	included in methods section
3. NUCLEIC ACID ASSESSMENT AND STORAGE		
Method to evaluate quality of nucleic acids	N	NA
Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass)	N	NA
Storage conditions: temperature, concentration, duration, buffer, aliquots	Y	included in methods section
Clear description of dilution steps used to prepare working DNA solution	Y	included in methods section
4. NUCLEIC ACID MODIFICATION	Y	
Template modification (digestion, sonication, pre-amplification, bisulphite etc.)	N	NA
Details of repurification following modification if performed	Y	zymo kit; included in methods section
5. REVERSE TRANSCRIPTION		
cDNA priming method and concentration	N	NA
One or two step protocol (include reaction details for two step)	Y	included in methods section
Amount of RNA added per reaction	Y	included in supplementary information
Detailed reaction components and conditions	Y	included in supplementary information
Estimated copies measured with and without addition of RT*	N	NA
Manufacturer of reagents used with catalogue and lot numbers	Y	included in the methods section
Storage of cDNA: temperature, concentration, duration, buffer and aliquots	N	NA
6. dPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION		
Sequence accession number or official gene symbol	N	NA
Method (software) used for design and *in silico* verification	Y	we used previously published assays; in-silico verification of assays described in methods section
Location of amplicon	N	NA
Amplicon length	Y	included in supplementary information
Primer and probe sequences (or amplicon context sequence)**	Y	included in supplementary information
Location and identity of any modifications	N	NA
Manufacturer of oligonucleotides	Y	included in methods section
7. dPCR PROTOCOL		
Manufacturer of dPCR instrument and instrument model	Y	included in methods section
Buffer/kit manufacturer with catalogue and lot number	Y	included in methods section
Primer and probe concentration	Y	included in supplementary information
Pre-reaction volume and composition (incl. amount of template and if restriction enzyme	Y	included in supplementary information
Template treatment (initial heating or chemical denaturation)	N	NA
Polymerase identity and concentration, Mg++ and dNTP concentrations***	N	NA
Complete thermocycling parameters	Y	included in supplementary information
8. ASSAY VALIDATION		
Details of optimisation performed	N	we used previously published/validated assays
Analytical specificity (vs. related sequences) and limit of blank (LOB)	N	NA
Analytical sensitivity/LoD and how this was evaluated	Y	we determined a theorically lowest concentration limit; included in methods section
Testing for inhibitors (from biological matrix/extraction)	Y	we used BCoV as an inhibitor control; included in methods section
9. DATA ANALYSIS		
Description of dPCR experimental design	Y	included in methods section
Comprehensive details negative and positive of controls (whether applied for QC or for estimation of error)	Y	included in methods section
Partition classification method (thresholding)	Y	included in methods section
Examples of positive and negative experimental results (including fluorescence plots in	Y	included in supplementary information
Description of technical replication	Y	included in supplementary information
Repeatability (intra-experiment variation)	N	NA
Reproducibility (inter-experiment/user/lab etc. variation)	N	NA
Number of partitions measured (average and standard deviation)	Y	included in supplementary information
Partition volume	Y	included in supplementary information
Copies per partition (λ or equivalent) (average and standard deviation)	Y	included in supplementary information
dPCR analysis program (source, version)	Y	included in methods section
Description of normalisation method	N	NA
Statistical methods used for analysis	Y	included in methods section
Data transparency	raw data available on request:	raw data available on request

Figure S1: Checklist for Minimum Information for Publication of Quantitative Digital PCR Experiments (dMIQE2020)

Additional information on RT ddPCR assays

For each duplex assay, each PCR well was a 20 µL reaction with One-Step RT-ddPCR Advanced Kit for Probes Supermix, 15 mM 140 DTT, 1X primer/probe assay mix, and 5 µL RNA template. Final concentrations of the primers and probes were 900 nM and 250 nM, respectively.

A subset of aliquots (70) was selected at random to determine the average and standard deviations of number of partitions and copies per partition.

For the SARS-CoV-2 and Influenza A duplex assay, the average (standard deviation) number of partitions (droplets) across two replicate wells was 38,835 (1,780). The volume of the partitions, as reported by the machine vendor is 0.00085 µL. The average (standard deviation) of copies per partition for the SARS-CoV-2 RNA was 5.8×10^{-3} (7.2×10^{-3}), and the average (standard deviation) of copies per partition for Influenza A RNA was 2.6×10^{-2} (3.2×10^{-2}). An example fluorescent plot from the QX200 reader in shown in Figure S2. Results from the other assays are similar.

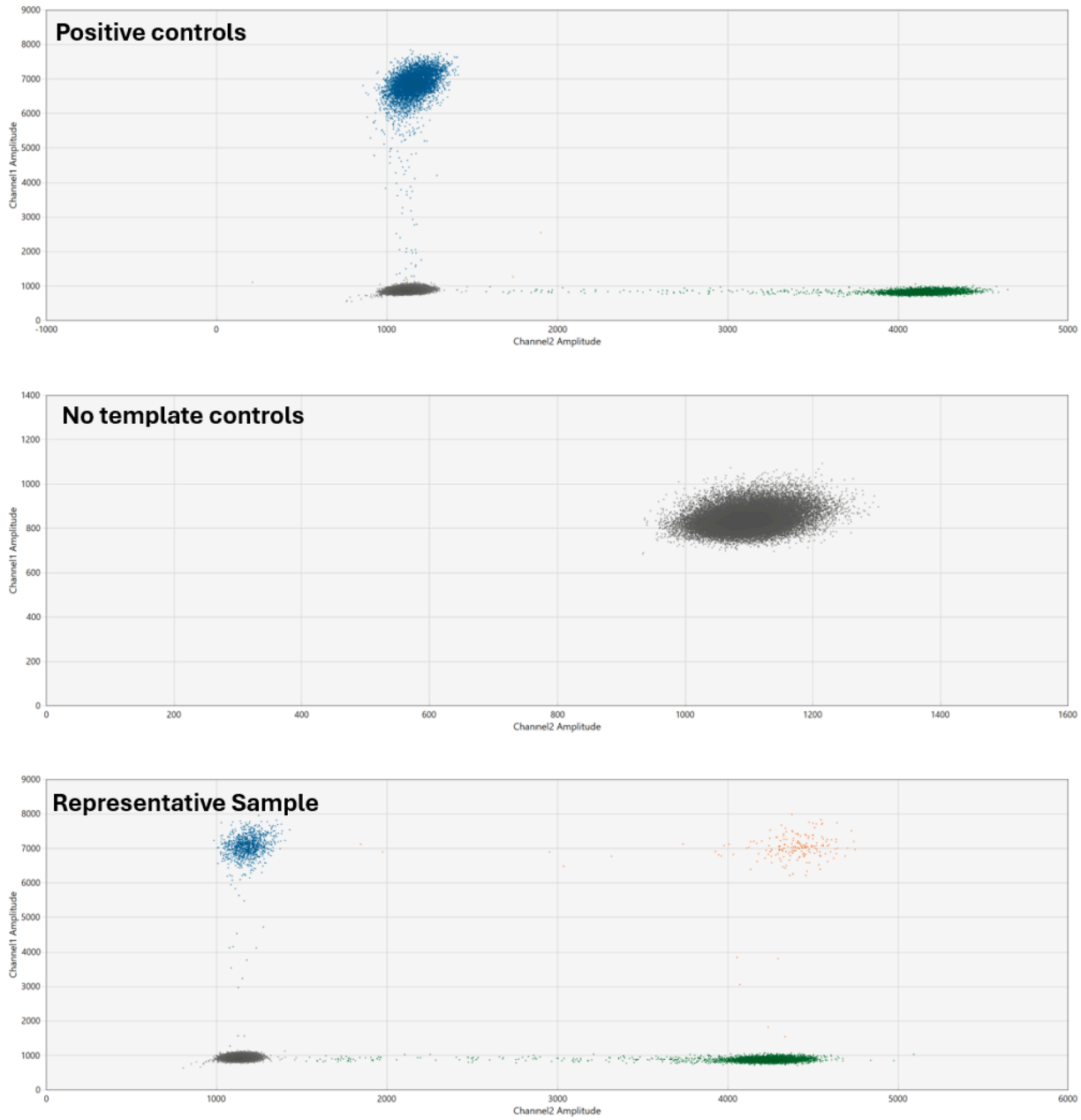

Figure S2: Examples of ddPCR experimental results for the SARS-CoV-2 and Influenza A duplex assay. Top plot represents positive controls for SARS-CoV-2 (green) and Influenza A (blue), middle plot depicts no template controls (grey), and bottom plot shows a representative sample.

Table S1: Name of the plant, population served, plant capacity (MGD), sample collection date, and description of chemicals added upstream of the sample collection point for each wastewater treatment plant.

Plant	Name	State	Approximate number of people served	Plant capacity (MGD)	Sample collection date	Chemicals added at the plant (upstream of sample collection site)
A	San José-Santa Clara Regional Wastewater Facility	CA	250,000	167	10/3	$FeCl_3$ and NaOCl
B	Jackson Wastewater Treatment Plant	MI	90,000	24	10/26,11/30	$FeCl_2$
C	City of Youngstown Wastewater Treatment Plant	OH	174,000	102	10/27,12/1	No
D	Passaic Valley Sewerage Commission	NJ	1,500,000	330	10/23	No
E	Akron Water Reclamation Facility	OH	365,000	280	10/27, 12/1	No
F	South Monmouth Regional Sewerage Authority	NJ	52,672	9	10/27, 12/1	No
G	Lawrence Kansas River Wastewater Treatment Facility	KS	80,000	65	10/27, 12/1	Microsand, polymer, and ferric chloride during high flow events
H	Bayshore Regional Sewerage Authority	NJ	100,000	16	10/27, 12/1	No
I	City of Wilson-Hominy Creek Water Reclamation Facility	NC	50,000	14	10/27, 12/1	No
J	City of Stamford, Water Pollution Control Authority	CT	140,000	24	10/27	No
K	City of Coeur d'Alene Water Resource Recovery Facility	ID	50,540	6	10/27, 12/1	Aluminum sulfate

Table S2: Concentrations of Dengue, Zika, WNV, Hepatitis A, Influenza, and SARS-CoV-2 in virus cocktails for Batch 1 and 2

Batch	Cocktail	Viral RNA Concentrations (cp/ml)					
		Dengue	Zika	WNV	Hepatitis A	Influenza	SARS-CoV-2
1	VC0	2.46×10^{8}	9.19×10^{7}	2.85×10^{8}	4.10×10^{6}	8.60×10^{5}	1.56×10^{6}
	VC1	5.47×10^{7}	2.05×10^{7}	5.96×10^{7}	3.00×10^{6}	7.04×10^{5}	1.21×10^{6}
	VC2	5.63×10^{6}	3.77×10^{6}	5.85×10^{6}	1.86×10^{6}	4.05×10^{5}	1.40×10^{6}
	VC3	1.05×10^{6}	4.70×10^{5}	5.94×10^{5}	1.19×10^{6}	1.96×10^{5}	1.50×10^{6}
	VC4	2.67×10^{5}	9.37×10^{4}	1.44×10^{5}	5.94×10^{5}	1.15×10^{5}	9.70×10^{5}
	VC5	5.85×10^{4}	1.98×10^{4}	2.80×10^{4}	3.15×10^{5}	5.70×10^{4}	3.71×10^{5}
	VC6	1.04×10^{4}	4.10×10^{3}	5.60×10^{3}	1.54×10^{5}	2.37×10^{4}	1.27×10^{5}
2	VC7	2.22×10^{7}	4.56×10^{6}	2.48×10^{7}	4.94×10^{6}	1.11×10^{6}	1.87×10^{6}
	VC8	2.17×10^{6}	1.60×10^{6}	2.14×10^{6}	1.61×10^{6}	4.34×10^{5}	1.49×10^{6}
	VC9	1.79×10^{5}	3.04×10^{5}	1.66×10^{6}	7.65×10^{5}	1.94×10^{5}	1.17×10^{6}

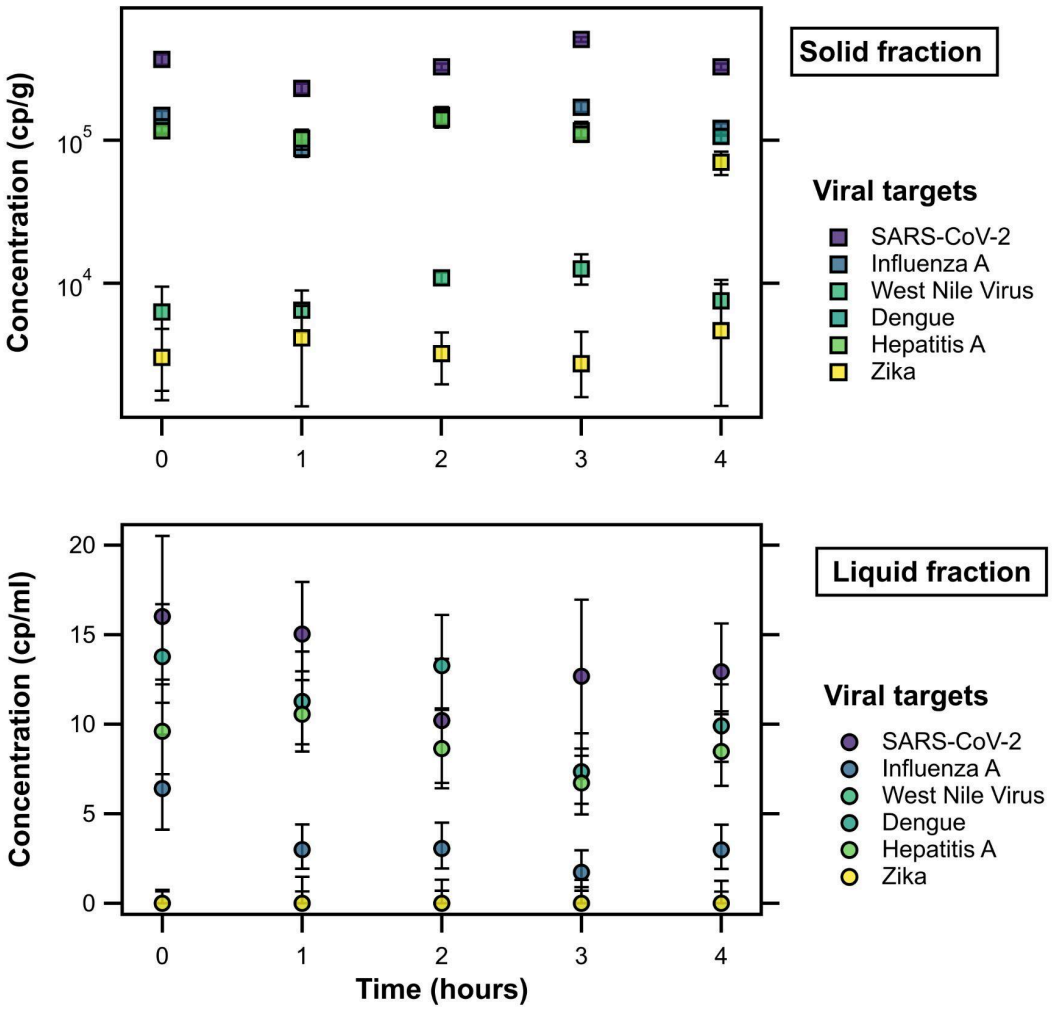

Figure S3:Kinetic experiment to determine the time needed for the spiked viruses to reach apparent equilibrium. Viral RNA concentrations measured in the liquid and solid fractions of wastewater spiked from PLANT A, measured every hour for up to 4 hours.

Table S3: Primers and probes for RT-ddPCR assays

Duplex Assay	Target	Sequence	Reference
1	Dengue Type 1	Forward primer: 5′-CAAAAGGAAGTCGYGCAATA-3' Reverse primer: 5′-CTGAGTGAATTCTCTCTGCTRAAC-3' Probe: CATGTGGYTGGGAGCRCGC (5' FAM/ZEN/3' IBFQ)	CDC[1]
	West Nile virus	Forward primer: 5′-TCAGCGATCTCTCCACCAAAG-3' Reverse primer: 5′-GGGTCAGCACGTTTGTCATTG-3' Probe: TGCCCGACCATGGGAGAAGCT (5' HEX/ZEN/3' IBFQ)	Lanciotti et al. (2000)[2]
2	Zika	Foward primer: 5′-GGCRTTRGCCATCAGTCG-3' Reverse: 5′-ATGGAGCATCCGKGAGACT-3' Probe: TGGCAGCTYCTTTATTTCCACARAAG (5' FAM/ZEN/3' IBFQ)	Hui et al. (2018)[3]
	Hepatitis A	Foward primer: 5′-GGTAGGCTACGGGTGAAAC-3' Reverse primer: 5′-AACAACTCACCAATATCCGC-3' Probe: CTTAGGCTAATACTTCTATGAAGAGATGC (5' HEX/ZEN/3' IBFQ)	Jothikumar et al. (2005)[4]
3	Influenza A	Foward primer: 5′-CAAGACCAATCYTGTCACCTCTGAC-3' Reverse primer: 5′-GCATTYTGGACAAAVCGTCTACG-3' Probe: TGCAGTCCTCGCTCACTGGGCACG (5' FAM/ZEN/3' IBFQ)	CDC[5]
	SARS-CoV-2	Forward primer: 5′-CATTACGTTTGGTGGACCCT-3' Reverse primer: 5′-CCTTGCCATGTTGAGTGAGA-3' Probe: CGCGATCAAAACAACGTCGG (5' HEX/ZEN/3' IBFQ)	Wolfe et al. (2021)[6]
4	BCoV	Forward primer: 5'-CTGGAAGTTGGTGGAGTT-3' Reverse primer: 5'-ATTATCGGCCTAACATACATC-3' Probe: CCTTCATATCTATACACATCAAGTTGTT (5' FAM /ZEN/3' IBFQ)	Decaro et al. (2008)[7]

All probes contained fluorescent molecules and quenchers (5′ FAM and or HEX/ZEN/3′ IBFQ); FAM, 6-fluorescein amidite; HEX, hexachloro-fluorescein; ZEN, a proprietary internal quencher from Integrated DNA Technologies (Coralville, IA, USA); and IBFQ, Iowa Black FQ.

For batch 1, the following duplex assays were prepared: 1) Dengue (FAM) + West Nile Virus (HEX), 2) Zika (FAM) + Hepatitis A (HEX), and 3) Influenza A (FAM) + SARS-CoV-2 (HEX).

For batch 2, we prepared the same duplex assays for the first experiment. For the second experiment, we prepared a duplex assay for Zika (FAM) and West Nile Virus (HEX)

Table S4: Thermal cycling conditions for SARS-CoV-2, RSV, RV, MS2, and BCoV

Cycling Step	Temperature °C	Time	Number of Cycles
Reverse transcription	50	60 min	1
Enzyme activation	95	10 min	1
Denaturation	95	30 sec	40
Annealing/Extension	59	1 min*	40
Enzyme deactivation	98	10 min	1
Hold	4	Infinite	1

***BCoV**

Dimensional analysis[8] for liquid and solid fractions of spiked wastewater samples

$$Concentration\ in\ solids\ (cp/g) = \frac{x\ copies}{\mu l\ rxn} \times \frac{B\ \mu l\ rxn}{A\ \mu l\ template} \times \frac{C\ \mu l\ total\ eluent\ from\ extraction\ kit}{Z\ g\ wet\ mass\ solids\ added\ to\ extraction\ kit} \times \frac{1}{\%\ of\ solids}$$

$$Concentration\ in\ liquid\ (cp/ml) = \frac{x\ copies}{\mu l\ rxn} \times \frac{B\ \mu l\ rxn}{A\ \mu l\ template} \times \frac{C\ \mu l\ total\ eluent\ from\ extraction\ kit}{D\ ml\ in\ liquid}$$

Table S5: Partition coefficients (K_F) of Dengue, Hepatitis A, Influenza A, SARS-CoV-2, West Nile Virus, and Zika spiked into wastewater samples.

Wastewater Treatment Plant	Dengue	Hepatitis A	Influenza A	SARS-CoV-2	West Nile Virus	Zika
A*	1,900	1,060	7,560	4,820	3,470	750
B*	11,600	12,700	5,800	52,900	22,900	14,400
C	7,800	102,900	-	-	1,911,700	2,300
D	15,700	102,200	15,800	155,400	-	-
E	1,800	12,700	15,600	58,300	18,800	400
F	7,000	2,900	14,700	1,800	120,400	9,700
G	2,200	12,700	-	-	1,374,700	6,200
H	4,600	4,000	9,900	7,000	9,300	18,700
I	14,600	57,000	4,600	35,600	3,912,000	15,100
J	1,800	10,000	40,400	7,600	-	-
K*	5,900	27,600	5,300	25,300	23,600	2,100

Notes:

* Plants add chemicals upstream of sample collection point

- Partition coefficient could not be estimated

Table S6: Intensity of adsorption (n) of Dengue, Hepatitis A, Influenza A, SARS-CoV-2, West Nile Virus, and Zika spiked into wastewater samples.

Wastewater Treatment Plant	Dengue	Hepatitis A	Influenza A	SARS-CoV-2	West Nile Virus	Zika
A*	1.0	0.9	0.9	0.9	1.1	1.1
B*	0.6	1.1	1.6	1.0	2.0	1.9
C	1.8	0.9	-	-	1.3	2.8
D	0.8	0.7	1.0	0.6	-	-
E	1.6	1.4	1.9	1.0	1.5	2.6
F	0.7	0.9	0.7	1.0	1.1	1.2
G	3.8	3.9	-	-	1.0	1.6
H	1.1	1.0	1.8	1.2	1.3	0.8
I	0.9	0.7	1.9	0.6	1.0	1.7
J	1.5	1.1	0.8	1.4	-	-
K*	1.1	0.8	1.5	0.9	1.4	2.0

Notes:

* Plants add chemicals upstream of sample collection point

- Intensity of adsorption could not be estimated

References:

(1) Santiago, G. A.; Vergne, E.; Quiles, Y.; Cosme, J.; Vazquez, J.; Medina, J. F.; Medina, F.; Colón, C.; Margolis, H.; Muñoz-Jordán, J. L. Analytical and Clinical Performance of the CDC Real Time RT-PCR Assay for Detection and Typing of Dengue Virus. *PLoS Negl Trop Dis* **2013**, *7* (7), e2311. https://doi.org/10.1371/journal.pntd.0002311.

(2) Lanciotti, R. S.; Kerst, A. J.; Nasci, R. S.; Godsey, M. S.; Mitchell, C. J.; Savage, H. M.; Komar, N.; Panella, N. A.; Allen, B. C.; Volpe, K. E.; Davis, B. S.; Roehrig, J. T. Rapid Detection of West Nile Virus from Human Clinical Specimens, Field-Collected Mosquitoes, and Avian Samples by a TaqMan Reverse Transcriptase-PCR Assay. *J Clin Microbiol* **2000**, *38* (11), 4066–4071. https://doi.org/10.1128/JCM.38.11.4066-4071.2000.

(3) Hui, Y.; Wu, Z.; Qin, Z.; Zhu, L.; Liang, J.; Li, X.; Fu, H.; Feng, S.; Yu, J.; He, X.; Lu, W.; Xiao, W.; Wu, Q.; Zhang, B.; Zhao, W. Micro-Droplet Digital Polymerase Chain Reaction and Real-Time Quantitative Polymerase Chain Reaction Technologies Provide Highly Sensitive and Accurate Detection of Zika Virus. *Virol. Sin.* **2018**, *33* (3), 270–277. https://doi.org/10.1007/s12250-018-0037-y.

(4) Jothikumar, N.; Cromeans, T. L.; Sobsey, M. D.; Robertson, B. H. Development and Evaluation of a Broadly Reactive TaqMan Assay for Rapid Detection of Hepatitis A Virus. *Appl Environ Microbiol* **2005**, *71* (6), 3359–3363. https://doi.org/10.1128/AEM.71.6.3359-3363.2005.

(5) CDC. *Research Use Only CDC Flu SC2 Multiplex Assay Primers and Probes. Centers for Disease Control and Prevention.* Centers for Disease Control and Prevention. https://www.cdc.gov/coronavirus/2019-ncov/lab/multiplex.html (accessed 2024-02-20).

(6) Wolfe, M. K.; Topol, A.; Knudson, A.; Simpson, A.; White, B.; Vugia, D. J.; Yu, A. T.; Li, L.; Balliet, M.; Stoddard, P.; Han, G. S.; Wigginton, K. R.; Boehm, A. B. High-Frequency, High-Throughput Quantification of SARS-CoV-2 RNA in Wastewater Settled Solids at Eight Publicly Owned Treatment Works in Northern California Shows Strong Association with COVID-19 Incidence. *mSystems* **2021**, *6* (5), e00829-21. https://doi.org/10.1128/mSystems.00829-21.

(7) Decaro, N.; Elia, G.; Campolo, M.; Desario, C.; Mari, V.; Radogna, A.; Colaianni, M. L.; Cirone, F.; Tempesta, M.; Buonavoglia, C. Detection of Bovine Coronavirus Using a TaqMan-Based Real-Time RT-PCR Assay. *Journal of Virological Methods* **2008**, *151* (2), 167–171. https://doi.org/10.1016/j.jviromet.2008.05.016.

(8) Graham, K. E.; Loeb, S. K.; Wolfe, M. K.; Catoe, D.; Sinnott-Armstrong, N.; Kim, S.; Yamahara, K. M.; Sassoubre, L. M.; Mendoza Grijalva, L. M.; Roldan-Hernandez, L.; Langenfeld, K.; Wigginton, K. R.; Boehm, A. B. SARS-CoV-2 RNA in Wastewater Settled Solids Is Associated with COVID-19 Cases in a Large Urban Sewershed. *Environ. Sci. Technol.* **2021**, *55* (1), 488–498. https://doi.org/10.1021/acs.est.0c06191.