Supplemental Text

Wastewater surveillance using ddPCR reveals highly accurate tracking of omicron variant due to altered N1 probe binding efficiency

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Filtration, extraction, and quantification protocols are available on protocols.io: https://www.protocols.io/workspaces/mclellan-lab

Supplemental Text 1 Droplet digital PCR standards

The Exact Diagnostics SARS-CoV-2 standard (BioRad, SKU: COV019) were used as a positive control for the N1 and N2 assay (Figure S4). The standard was diluted in RNase-free water 1:8 to be quantified at approximately 8 cp/µL (~100 droplets) using Bio-Rad Digital Droplet PCR (ddPCR). The clinical residual standards for both Omicron and Delta were provided as extracted RNA from the Wisconsin State Lab of Hygiene. Both standards were diluted in RNase-free water 1:50 to be quantified at approximately 9 cp/µL (~ 150 droplets) using Bio-Rad ddPCR. Aliquots of diluted standards were limited to two freeze-thaws before being discarded.

Supplemental Text 2 Droplet digital PCR quality control

Positive controls (standards) and no template control (NTC) reactions (S4) were used to guide the threshold position(s) following the guidelines published in (1). Reactions were considered to have passed quality control if the droplet count was >10,000, and NTC on each reaction was below the LOD (3 droplets, ~0.2209 cp/µL), and the standards on reach reaction had approximately 100 droplets. For additional information on how the LOD or limit of quantification (LOQ) was determined, please refer to Feng et. al. (2). No samples in

this analysis failed these quality control measures. Additionally, all samples passed inhibition control consisting of spiking a known amount of Bovine Respiratory Syncytial Virus (approximately 4000 copies) into each sample, and then quantifying by RT-ddPCR according to Feng et al. (2). The .csv files were exported for further analysis in MS Excel 2019 and R (version 1.0.1)(3).

Supplemental Text 3 Variant assay validation

To validate the results of our variant concentration, we compared the trends of the N1 cloud splits with the TaqMan SARS-CoV-2 Mutation Panels S.P681R.CCT.CGT (Delta) and S.P681H.CCT.CAT (Omicron) (ThermoFisher Scientific, Waltham, MA) in a total of 41 samples from Jones Island and De Perre WWTPs between November 21, 2021, and January 30, 2022. Mutation assays for the Delta and Omicron variant (Applied Biosystems, Waltham, Massachusetts, USA) were made in 22 µL reactions as described in methodology. The concentration detected in each ddPCR reaction was compared to the concentration derived from the reclassification of the 2D amplitude cluster delineation by first confirming normality of the data sets using the Shapiro-Wilk normality test, then using Spearman's rank correlation rho using the function *cor.test* (stats version 3.6.2). Both data sets were graphed against each other to confirm paralleling trends of rising and falling concentrations of each variant using R (version 1.0.1)(3).

Supplemental Text 4 Data Transparency

All figures were created in R (version 1.0.1)(3), or taken directly from Bio-Rad QuantaSoft software (version 1.7). All data was obtained directly from Bio-Rad QuantaSoft software, or the GISAID database[4], and was analyzed using R (version 1.0.1).

Table S1 List of the ddPCR assays used in analysis

Target	Primer/Probe Sequence (5'-3')	Supplier	Ref
SARS-CoV-2 N1	F: GACCCCAAAATCAGCGAAAT	Eurofins	[1]
	R: TCTGGTTACTGCCAGTTGAATCTG	Eurofins	
	P: FAM/ACCCCGCAT/ZEN/TACGTTTGGTGGACC/lABkFQ	IDT	
SARS-CoV-2 N2	F: TTACAAACATTGGCCGCAAA	Eurofins	[1]
	R: GCGCGACATTCCGAAGAA	Eurofins	
	P: HEX/ACAATTTGCCCCCAGCGCTTCAG/BHQI and HEX/ACAATTTGC/ZEN/CCCCAGCGCTTCAG/lABkFQ	IDT	
Delta (P681R)	Reporter 1 dye: VIC, Reporter 1 Quencher: NFQ, Reporter 2 dye: FAM, Reporter 2 quencher: NFQ CTCAGACTAATTCTC[C/A]TCGGCGGGCACGTAG	Applied Biosystems	[3]
Omicron (P681H)	Reporter 1 dye: VIC, Reporter 1 Quencher: NFQ, Reporter 2 dye: FAM, Reporter 2 quencher: NFQ, CTCAGACTAATTCTC[C/G]TCGGCGGGCACGTAG	Applied Biosystems	[3]

*ddPCR primers and probes at a final concentration of 900 nM and 250 nM, respectively.
*Reporter 2 dye targets Mutant of TaqMan SARS-CoV-2 Mutation Panels.

Table S2 RT-ddCPR reaction assay volumes using one-step RT-ddPCR Advanced Kit for Probes (Catalog no. 1864022). All primers and probes are diluted to final concentration in RNase-free water.

Assay	Reagents	Volume per well (µL)	Final concentration
N1 N2 BCoV BRSV PMMoV*	Supermix	5.5	1X
	RT	2.2	20 U/µL
	DTT (300 nM)	1.1	15 mM
	FAM Primers (18 uM each)	1.1	900 nM each
	FAM Probe (5 uM)	1.1	250 nM
	HEX Primers (18 uM each)	1.1	900 nM each
	HEX Probe (5 uM)	1.1	250 nM
	H2O	3.3	-
	Sample	5.5	-
	Total	22	
Omicron Delta	Supermix	5.5	1X
	RT	2.2	20 U/µL
	DTT (300 nM)	1.1	15 mM
	Thermo Fisher Assay	0.55	-

H2O	3.3	-
Sample	5.5	-
Total	22	

*PMMoV is performed as a simplex assay, so it uses 5.5 µL water to compensate for no HEX primers/probes.

Table S3 Mastercycler amplification settings.

Step	Cycle #	Temp °C	Time (min)
Reverse Transcription	1	50	60
Enzyme Activation	1	95	10
Denaturing	40	94	0.5
Annealing/Extension	40	55*	1
Enzyme Deactivation	1	98	10
Droplet Stabilization	1	4	30

*PMMoV annealing and extention was performed at 60°C.

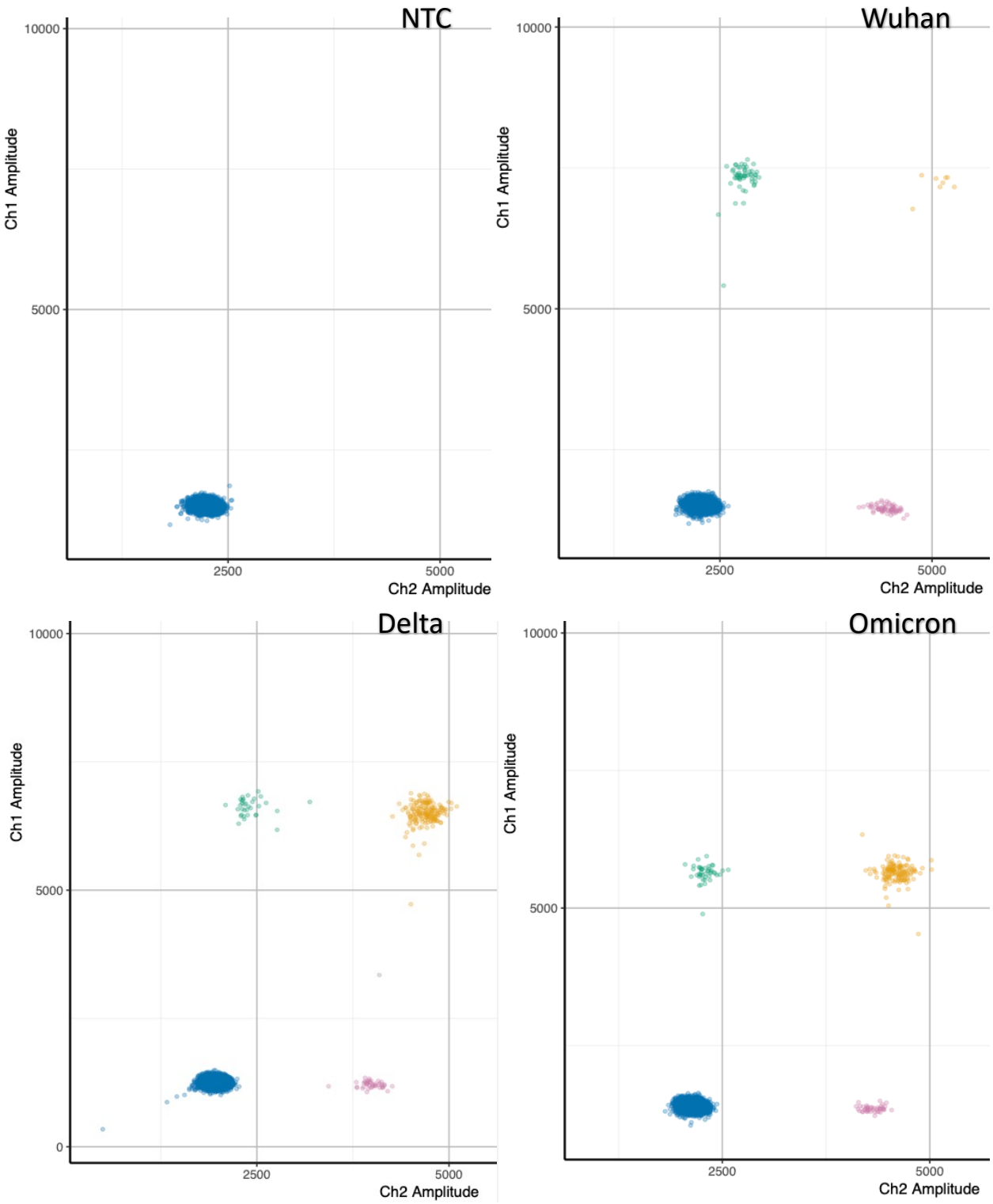

Figure S1 Representative plots of no template controls (upper left), and standard reactions. This includes the classic Wuhan standard (Bio-Rad) diluted 1:8 in RNase-free water (upper right), residual Delta clinical sample diluted 1:50 in RNase-free water (lower left), and Omicron clinical sample diluted 1:50 in RNase-free water (lower right).

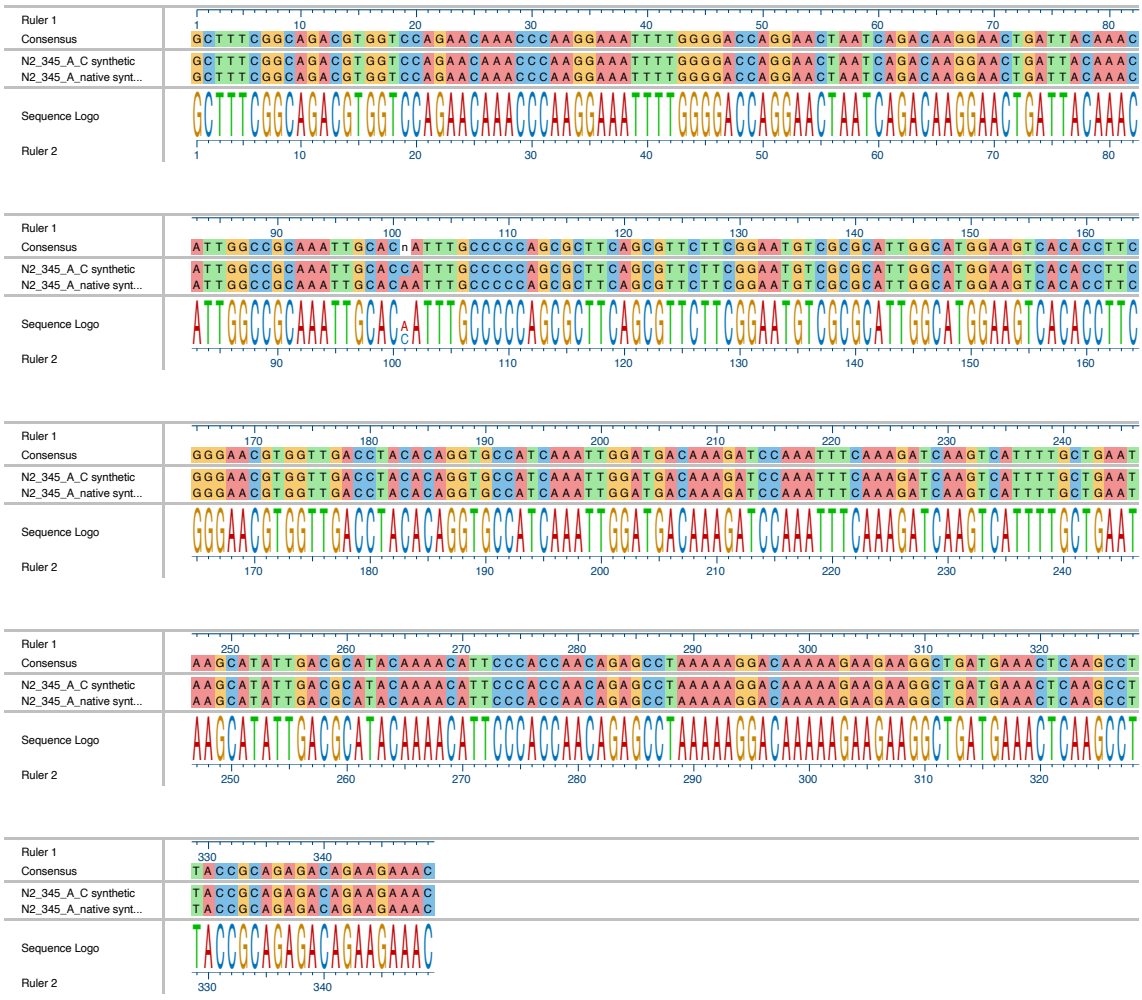

Figure S2 Original N2 probe binding site region from a clinical strain isolated February 2020 aligned with the synthesized DNA template with an A:C change at the on 5' end of probe. Alignment performed using DNASTAR (version 17.3.3) of SARS-CoV-2 N gene, N gene sequence available in GenBank (accession number MT081066.1).

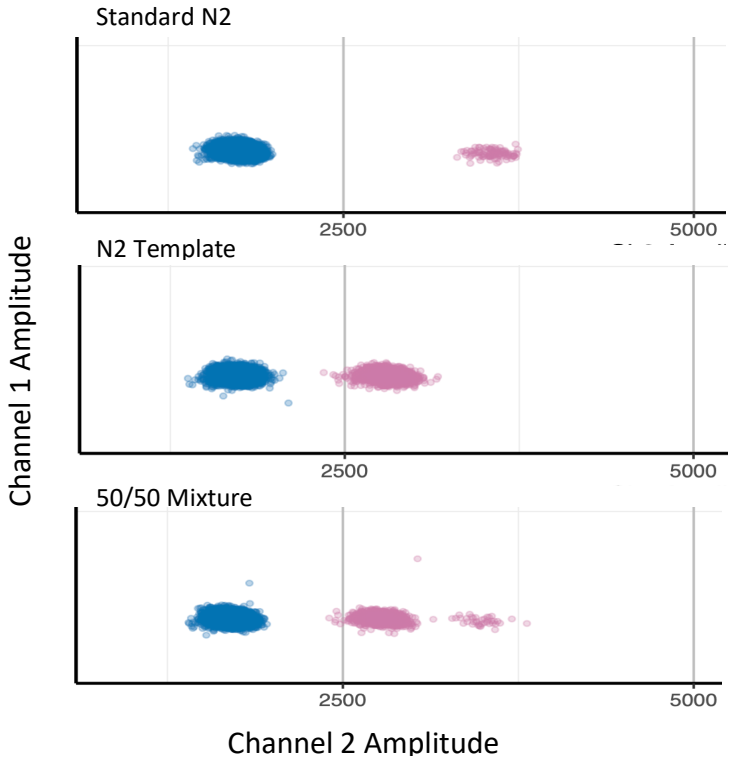

Figure S3 N2 multiplex ddPCR results of 1:8 diluted Exact Diagnostics SARS- CoV-2 standards (Bio-Rad) (top image), 1:100,000 diluted (1 ng/ul) 350bp linear synthesized DNA N2 sequence which is identical to the Exact Diagnostics sequence with the exception of a A:C mutation on the third base pair (Twist Bioscience) (middle image), and a 50/50 mixture of both samples (bottom image). Channel 1 amplitude above baseline (i.e., N1 signal, not shown).

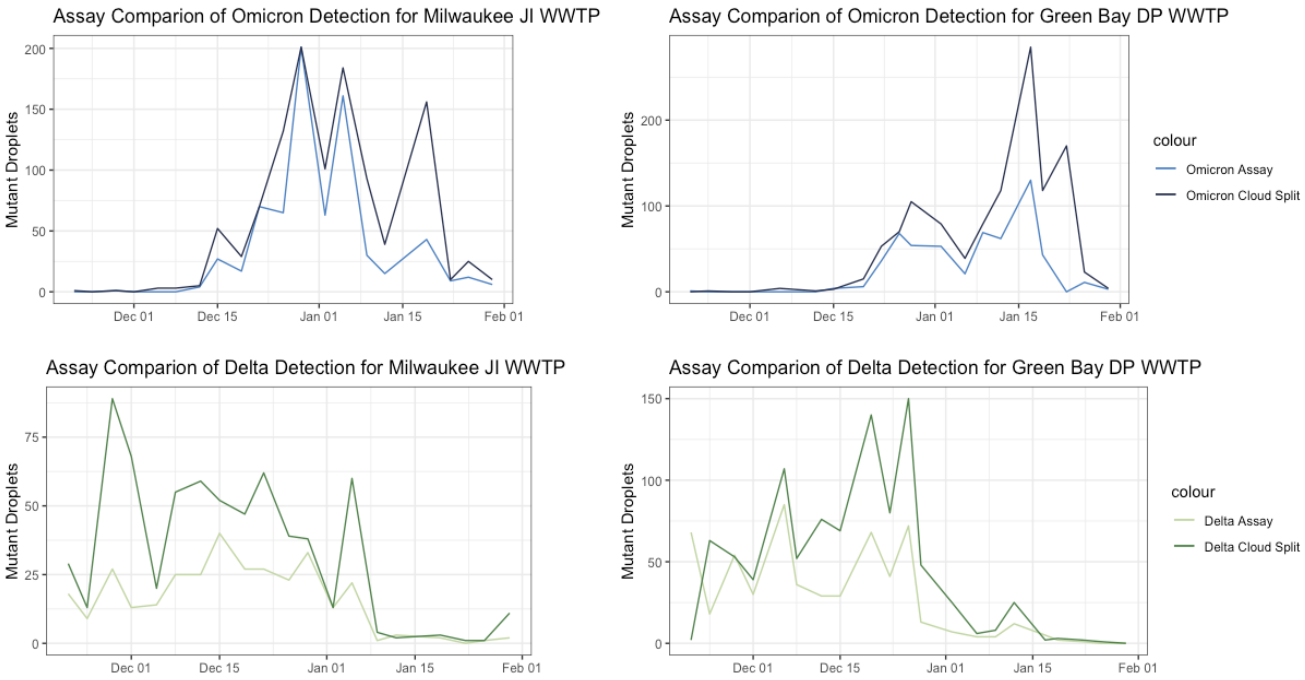

Figure S4 Comparison of the quantification of Omicron and Delta mutant droplets using the TaqMan SARS-CoV-2 Mutation panels (ThermoFisher) and N1 probe cloud split methods. Shapiro-Wilk normality test and Spearman's rank correlation analysis performed in R-Studio version 1.4.0013.

References

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3. Kirill M. here: A Simpler Way to Find Your Files. R package version 1.0.1. 2020; Available from: https://CRAN.R-project.org/package=here

4. CDC. 2019-Novel Coronavirus (2019-nCoV) Real-time rRT-PCR Panel Primers and Probe. Division of Viral Diseases, National Center for Immunization and Respiratory Diseases, Centers for Disease Control and Prevention. 2020 May 29;

5. Applied Biosystems. TaqMan SARS-CoV-2 Mutation Panel. Thermo Fisher Scientific.