

40 Introduction

41 Communities around the world have quickly adopted wastewater-based surveillance (WBS) of
42 SARS-CoV-2 viral fragments to detect and infer prevalence of COVID-19 disease burden in communities
43 (D'Aoust et al., 2021; Kitajima et al., 2020; Medema et al., 2020). Because it does not require testing of
44 individual sensitivity and includes all individuals, symptomatic or asymptomatic, that contribute to the
45 sewershed, WBS has several advantages over more costly and invasive clinical testing. WBS utilizes
46 reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) based techniques
47 targeting various regions of the SARS-CoV-2 genome, including the popular US-Centers for Disease
48 Control (CDC) N1, N2 (nucleocapsid) and E (envelope), to test for the presence of SARS-CoV-2 in
49 wastewater (Hamouda et al., 2021; Kitajima et al., 2020; Kumblathan et al., 2021).

50 Continuous emergence of Variants of Concern (VOCs) and Variants of Interest (VOIs) has
51 presented additional challenges to the surveillance of SARS-CoV-2 and has been an area where WBS can
52 provide more timely information quicker than clinical testing. In addition to clinical tracking of
53 abundance of SARS-CoV-2 infections, RT-qPCR methods have been used to detect and monitor
54 prevalence of emerging VOCs in wastewater (Carcereny et al., 2021; Graber et al., 2021; Lee et al., 2021;
55 Peterson et al., 2022; Wurtzer et al., 2021). This information provides an integrated estimation of the
56 relative abundance of variants within populations. There have been several examples of RT-qPCR assays
57 for identification and quantifications of VOCs wastewater. The mutations N501Y and $\Delta 69-70$ del in the
58 spike protein or the presence of mutation D3L in the N gene have been targeted by RT-qPCR assays to
59 monitor for the presence of VOCs in wastewaters across Canada (Peterson et al., 2022). Using an allele
60 specific RT-qPCR, with an artificial mismatch, three mutations, HV69/70del, Y144del and A570D, were
61 targeted to discriminate and quantify the Alpha from VOC relative to the wild type in wastewater of 19
62 communities in the USA (Lee et al, 2021). Another assay targets mutations $\Delta 69-70$ in the S gene to
63 detect the Alpha and Beta variants respectively (Yaniv et al., 2021). An assay based on mutation D3L of

64 the gene, was applied for detection and quantification of relative proportion of the Alpha VOC (Graber
65 et al., 2021). Those authors were able to effectively trace the increase of the proportion of Alpha variant
66 in wastewater of Ottawa, Canada, in almost real time. The trends in incidence of Alpha were similar to
67 that of the number of clinical cases in the community. These various studies demonstrate that WBS can
68 rapidly provide information on incidence and prevalence of SARS-CoV-2 to authorities with sensitivity
69 and lineage specificity. RT-qPCR assays are specific and can be deployed rapidly across existing WBS
70 networks. The challenges with using RT-qPCR assays in surveillance of VOCs are the amount of time
71 needed to develop and validate new assays each time a new VOC emerges as well as increasing the
72 number assays being conducted to continually monitor for various VOCs and VOIs.

73 The objective of this study was to develop a multiplex RT-qPCR assay that could be used to
74 simultaneously screen for several VOCs, as well as a marker of the total SARS-CoV-2 present in the
75 sample so that an estimation of the relative proportion of VOCs can be reliably determined. The N gene
76 region was selected for several reasons, including that this region is targeted by the N1 and N2 assays,
77 developed by the US CDC, which are commonly used for monitoring wastewater (Lu et al., 2020). There
78 is evidence to suggest that assays developed for the N-region of the SARS-CoV-2 genome have greater
79 sensitivity for measuring various VOCs of SARS-CoV-2 in wastewater than assays targeting than does the
80 S-(Yaniv et al., 2021) or the envelope regions (Pérez-Cataluña et al., 2021). The N-gene was selected as
81 the target region as the target of the assay since it is also rich in mutations unique to VOCs (Kiryanov et
82 al., 2022). Specifically, the 121-basepair region in the N-gene open reading frame (ORF) (nucleotides
83 28,837 to 28,958) that includes single or multiple nucleotide variants for each VOC was selected (Table
84 1). According to GISAID data summarized by nextrain.org (Hadfield et al., 2018), the nucleotide 28880-
85 28882 (AA N:203) have one of the highest entropy levels in the SARS-CoV-2 genome, at 0.972, with
86 nucleotides 28883-28885 (AA N:204) also having high entropy level of 0.711 (nextstrain.org; accessed
87 April 1, 2022). The non-synonymous functional polymorphisms present in this region include R203M,

88 present in Delta, which increases the spread of the virus (Syed et al., 2021); R203K and G204R, (both
89 present in Alpha, Gamma Lambda, and Omicron, which are related to increased infectivity, fitness and
90 virulence (Johnson et al., 2021; Lee et al., 2021; Wu et al., 2021); and T205I, present in Beta and Mu
91 variants that were predicted to have reduced antigenicity and greater affinity for HLA-I (Antonio et al.,
92 2021). The presence of multiple mutations in a single region allowed for the design of a single amplicon,
93 multiple probe assay, that can distinguish among several VOCs. A Universal probe, that targets an area
94 of the amplicon without mutations allows for the quantification of the total SARS-CoV-2 signal,
95 regardless of which variants are present in wastewater. This allows for the measurement of the total
96 SARS-CoV-2 signal as well as providing estimations of the relative abundance of individual VOCs.

97 Prior to being incorporated into a wastewater surveillance program, the N200 assay was
98 validated using several synthetic standards as well as samples of wastewaters. As an example of the
99 utility of this assay, between November and January 2021, samples from six wastewater treatment
100 facilities in Ontario, Canada, were examined with the N200 assay. During this period the N200 assay was
101 utilized to test for the presence and increased frequency of the R203K/G204R mutation (present in the
102 Omicron VOC), while comparing directly to the decrease in frequency of the R203M mutation (present
103 in the Delta VOC), relative to a VOC-agnostic Universal marker, all within a single RT-qPCR reaction.

104 **Materials and Methods**

105 **Target selection**

106 To select an appropriate RT-qPCR target for testing wastewater, mutations that were indicative
107 of each VOC, encompassing amino acids N:202-205 were identified. Mutations selected for each VOC
108 and their known prevalence are presented in Table 1. The template sequence used for designs were
109 retrieved from accession numbers provided by TWIST Bioscience (South San Francisco, CA, USA) for
110 synthetic controls 14 (Alpha), 16 (Beta), 17 (Gamma) and 23 (Delta) and sequences were aligned using
111 MAFFT (Katoh et al., 2002).

112 Table 1. Amino acid mutations and corresponding nucleotide mutations present in the targeted
113 region of the SARS-CoV-2 nucleocapsid genome (AA 199-214).

| Amino acid mutation(s) | Nucleotide mutation(s) | Variant(s) of concern with mutation(s) | Mutation Prevalence (# of GISAID sequences) |
|--------------------------------|------------------------|--|---|
| S202S (synonymous mutation) | A28877T G28878C | Gamma | 98.0% and 97% respectively (6,925,348) |
| R203K | G28881A G28882A | Alpha Gamma Omicron | 97.8% (1,144,688) 94.5% (120,031) 98.5% (295,436) |
| R203M | A28880T | Delta | 98.7% (3,984,837) |
| G204R | G28883C | Alpha Gamma Omicron | 90.8% (1,144,688) 94.9% (120,031) 98.6% (295,496) |
| T205I | C28886T | Beta | 96% (40,518) |

114 ^A Mutation prevalence was obtained from outbreak.info on January 14, 2022.

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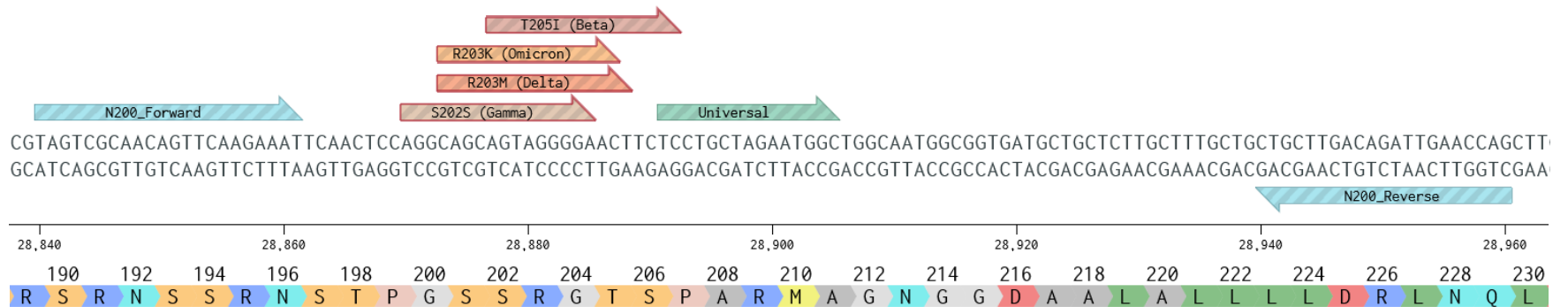
116 **Assay design**

117 Candidate forward and reverse primers that encompassed an amplicon 121 base pairs
118 long containing all the desired mutations were designed with a target predicted T_m around 61°C
119 and 40-60% GC content. Candidate primers were screened for hairpins, homodimers and
120 heterodimers *in silico* using OligoAnalyzer by integrated DNA technologies (IDT;

121 RRID:SCR_001363) with the “qPCR” parameter selected. Candidate primers that passed were
122 then screened against NCBI’s non-redundant nucleotide database using BLAST (Altschul et al.,
123 1990) and Primer-BLAST (Ye et al., 2012) to maximize mismatches with as much non-target
124 genetic material as possible (human, microbes, food, etc.).

125 Five probes were designed to be selective for each of the mutations (Table 1, 2), as well
126 as a universal probe for the “Universal” probe complementary to a nearby, highly conserved
127 (bp 28891-28905, mutation rates <0.87% according to covidcg.org) region of the amplicon to
128 detect total SARS-CoV-2 (Table 2, Figure 1). Probes were designed with the mutated base(s) at
129 their center using PrimerExpress v3.0.1 with a GC content of 40-60%. BHQplus technology (LCG,
130 Biosearch Technologies, Inc., California, USA) was used to increase the T_m of each probe, to have
131 a predicted T_m of 69°C, which enabled the design of short probes approximately 15 nucleotides
132 long. Probes were then tested for hairpins, homodimers, and heterodimers. Candidate probes
133 were also screened for % identity to non-target sequences using NCBI’s BLAST tool (Agarwala et
134 al., 2016).

135



136 Figure 1. N200 assay amplicon with the location of the N200 forward and reverse primers and all probes are shown on a portion of the N gene of
137 the SARS-CoV-2 genome. The sequence, nucleotide numbers, and amino acid displayed are based on the SARS-CoV-2 reference genome
138 (Wuhan; NC_045512.2; figure generated using Benchling.com).

139 **Assay Validation**

140 Initial tests of the N200 assay were performed on synthetic templates. TWIST synthetic
141 RNA controls 2, 14, 16, 17, and 23 (TWIST Bioscience) were employed to generate standard
142 curves (sequences representative in lieu of Wuhan-Hu-1 (Genbank MN908957.3), as well as the
143 VOCs, Alpha, Beta, Gamma, and Delta VOCs, respectively). The N200 standard curves was
144 assessed for linearity and efficiency by use of each of the probes in singleplex. After validation
145 with synthetic templates assays were tested with wastewater RNA extracts to ensure successful
146 amplification. Temperature gradients and primer/probe concentration gradients were
147 performed to determine the optimal assay conditions in singleplex.

148 Cross-reactivity of probes was assessed by use of synthetic RNA controls. Each probe, in
149 singleplex, was tested for reactivity with TWIST RNA controls 2, 14, 16, 17 and 23 at a
150 concentration of 500 copies per reaction. Specificity was also determined in a triplex assay
151 iteration (Universal, R203M, and R203K probes) by use of digital PCR (dPCR) at multiple
152 concentrations of TWIST RNA controls (see dPCR section for additional details).

153 The N200 assay was validated in multiplex by comparing the efficiency, R^2 and y -
154 intercept of standard curves, as well as the estimated concentration and proportion of variants
155 tested in wastewater relative to singleplex. Sensitivity of the N200 assay with the Universal,
156 R203K, and R203M probes were determined by use of a 12-point standard curve (TWIST
157 synthetic controls) with 15-replicates each. The limit of detection (LOD) was defined as the
158 standard concentration at which >95% of the replicates were detected. This was performed by
159 two independent labs (at the University of Waterloo and the Canadian National Microbiology
160 Laboratory – Winnipeg) using the same suppliers of primers and probes and standards.

161

162 **Wastewater sample collection, concentration, and extraction**

163 Wastewater from the Regions of Peel (wastewater treatment plant influents GE Booth
164 and Clarkson), York (access points Humber Air Management Facility (AMF) and Warden) and
165 Waterloo (wastewater treatment plant influents Kitchener and Waterloo) Ontario, Canada. At
166 each site wastewater was collected by plant operators using 24-hour cooled composite
167 samplers (combined sample of three grabs at Warden) 3-5 times a week between November
168 28th, 2021, and January 4th, 2022. Samples were aliquoted into 250 mL HPDE bottles (Systems
169 Plus, Baden, Canada) and transported on ice to the University of Waterloo.

170 To concentrate RNA, 250 mL samples were well-mixed by inversion, 40 mL aliquots
171 poured into 50 mL conical tubes with 4 g PEG-8000 and 0.9 g NaCl, then mixed on an orbital
172 shaker at low speed at 4°C for 2 h before being stored at 4°C overnight (Wu et al., 2020).
173 Samples were then centrifuged at 12,000 x g at 4°C for 90 minutes with no brake. The
174 supernatant was discarded, and the remaining sample was centrifuged again at 12,000 x g at
175 4°C for 5 min with no brake. The remaining supernatant was decanted and pipetted out and the
176 wet weight of the resulting pellet recorded. Up to 250 mg of the pellet was used for extraction
177 of RNA by use of the RNeasy PowerMicrobiome Kit (Qiagen, Germantown, MD) as per
178 manufacturers instructions and included the addition of 100 µL of TRIzol (Thermo Fisher,
179 Mississauga, Canada) to the pellet before bead-beating. Total RNA was eluted in 100 µL of
180 RNase-free water.

181 **RT-qPCR**

182 One-step RT-qPCR was performed using TaqPath 1-Step Master Mix (Thermo Fisher,
 183 Mississauga, Canada). The primer and probe sequences and final working concentrations are
 184 presented in Table 2. RT-qPCR reactions were run in triplicate using 5 µL RNA template and a
 185 final reaction volume of 20 µL. Cycling was performed on an OPUS or CFX 96 Touch qPCR
 186 thermocycler (BioRad, Hercules, CA) as follows: RT at 50°C for 15 min, 95°C for 2 min, 45 cycles
 187 of 95°C for 3 sec followed by 57°C for 30 sec. All qPCR plates were run with controls including
 188 no template controls (NTCs), a positive control (wastewater sample with previously determined
 189 amount of Delta variant) and a standard curve based on a homologous synthetic RNA (i.e., EDX,
 190 TWIST).

191 Table 2. Forward (F) and Reverse (R) primer and probe designs for the N200 VOC assay. The
 192 reporter and quencher moieties, and final working concentrations are included for each
 193 oligonucleotide.

| | Variant targeted | Oligo Name | Sequence (5' to 3') | Reporter/Quencher | Working concentration (nM) |
|---------|------------------|--------------|------------------------|-------------------------------|----------------------------|
| Primers | | N200 Forward | TAGTCGCAACAGTTCAAGAAAT | N/A | 500 |
| | | N200 Reverse | CTGGTTCAATCTGTCAAGCAG | N/A | 500 |
| Probes | All | Universal | TCCTGCTAGAATGGC | FAM/BHQ-1 plus | 100 |
| | Omicron/Alpha | R203K | CAGCAGTAAACGAAC | Quasar670/BHQ-2 plus | 400 |
| | Beta | T205I | AGTAGGGGAATTTCTT | Cal Fluor Red 610 /BHQ-2 plus | 100 |
| | Gamma | S202S | AGGCAGCTCTAAACGA | Quasar670/BHQ-2 plus | 100 |
| | Delta | R203M | CAGCAGTATGGGAACT | Cal Fluor Gold 540/BHQ-1 plus | 400 |

194

195 Digital PCR (dPCR)

196 For precise quantification, commercial standards were analyzed via singleplex one-step
 197 RT-dPCR (QIAcuity, Qiagen, Hilden, Germany). QIAcuity One-Step Viral RT-PCR Kit (Qiagen)
 198 together with CDC_N1 probe and primers (Lu et al., 2020) were used to determine absolute
 199 copy number (cp) of RNA in the following SARS-CoV-2 genomic RNA standards: TWIST

200 Bioscience Controls 2, 14, 16, 17, 23. Testing for specificity of probes was also conducted using
201 the N200 assay in triplex (Universal, R203M, and R203K probes) with two concentrations of
202 TWIST Bioscience Controls 14, and 23. Reactions consisted of 10 μ L of RNA template, forward
203 and reverse primers (working concentration of 500 nM each), probe (working concentration of
204 125 nM for N1, or 100, 400 and 400 nM for the Universal, R203M and R203K probes
205 respectively), 10 μ L of supermix, 0.4 μ L of reverse transcriptase, and 14.6 μ L of PCR-grade water
206 to make up the balance of the 40 μ L reaction. All reactions were performed in triplicate using
207 24 well x 26,000 channel plates. Cycling was performed on the QIAcuity as follows: RT at 50°C
208 for 30 min, 95°C for 2 min, with 40 cycles at 95°C for 3 sec then annealing at 55°C or 57°C (for
209 N1 and N200 assays respectively) for 30 sec. The N200 assay was also tested on RT-dPCR in
210 triplex (Universal, R203M, and R203K probes) using the TWIST Bioscience Controls 23 and 48 at
211 multiple concentrations to determine the specificity of the assay.

212 **Data Analysis**

213 Samples were quantified (copies/well) by use of a 7-point standard curve with the
214 relevant TWIST standard. Samples were then corrected for elution volume and volume of
215 wastewater extracted and reported as copies/mL wastewater (Quantity). The proportion of
216 total Quantity associated with each VOC in a sample was determined by use of the equation
217 mutation specific for the VOC (Equation 1).

$$218 \quad \% \text{ Variant} = (\text{Quantity of target mutation} / \text{Quantity of SARS-CoV-2 (Universal)}) * 100 \quad (1)$$

219 **Results**

220 **N200 assay conditions and performance**

221 The N200 assay performed well with all probes in singleplex. When tested with a serial
222 dilution of TWIST standards, the singleplex standard curves were linear, had an efficiency near
223 100%, and the slopes and y-intercepts were comparable between each of the probes (Table 3).
224 The N200 assay performed similarly when tested in singleplex, duplex and triplex (Table 3). All
225 probes targeting mutations were found to be highly specific with little to no cross-reactivity for
226 non-target templates using qPCR (in singleplex; Table 4). Using RT-qPCR, this was demonstrated
227 by the lack of amplification on all VOC TWIST templates without the target mutation (Table 4).
228 Some cross-reactivity was observed between the T205I probe and the Wuhan variant control
229 (TWIST Control 2; Table 4), but this accounted for less than 5% of the signal observed on the
230 homologous template. Positive amplification with similar sensitivity was seen on of all TWIST
231 templates tested was observed with the Universal probe (Table 4). High target specificity of
232 each probe was confirmed by RT-dPCR, where in the presence of a high concentration of non-
233 target template, almost no amplification was detected (Table 5). Copy numbers reported by the
234 mutation probes (R203M, R203K) were comparable to the copy numbers reported by the
235 Universal probe in the RT-dPCR reactions at multiple concentrations, which further suggests
236 that each probe has similar sensitivities and efficiencies (Table 5). The LOD (95% detection) was
237 determined to be 4-6 copies per reaction for the Universal, R203K, and R203M probes
238 respectively when tested in two separate laboratories.

239 Table 3. Performance metrics of the N200 VOC assay for each of the probes in singleplex, duplex or
 240 triplex.

| | Assay | Slope | y-intercept | Efficiency | R ² |
|--------------------------|---------------------------------|--------|-------------|------------|----------------|
| Universal | Singleplex | -3.347 | 40.236 | 98.9 | 0.990 |
| | Duplex (with R203M) | -3.366 | 39.50 | 98.2 | 0.991 |
| | Duplex (with R203K) | -3.299 | 38.793 | 101.0 | 0.990 |
| | Duplex (with S202S) | -3.311 | 40.207 | 100.5 | 0.990 |
| | Duplex (with T205I) | -3.461 | 39.773 | 94.5 | 0.990 |
| | Triplex (with R203M, R203K) | -3.443 | 39.661 | 95.2 | 0.995 |
| R203K (Omicron/Alpha) | Singleplex | -3.429 | 38.834 | 95.7 | 0.998 |
| | Duplex (with Universal) | -3.334 | 39.585 | 99.5 | 0.991 |
| | Triplex (with Universal, R203M) | -3.464 | 39.902 | 94.4 | 0.995 |
| T205I (Beta) | Singleplex | -3.396 | 39.951 | 97.0 | 0.997 |
| | Duplex (with Universal) | -3.533 | 39.952 | 91.9 | 0.994 |
| S202S (Gamma) | Singleplex | -3.249 | 40.363 | 103.1 | 0.998 |
| | Duplex (with Universal) | -3.352 | 39.891 | 98.8 | 0.988 |
| R203M (Delta) | Singleplex | -3.541 | 40.365 | 91.6 | 0.998 |
| | Duplex (with Universal) | -3.387 | 39.513 | 97.4 | 0.990 |
| | Triplex (with Universal, R203K) | -3.442 | 39.124 | 95.2 | 0.995 |

241

242 Table 4. Cycle Threshold (CT) value (mean) of N200 assay performed in singleplex with probes targeting
 243 a mutation (R203K; T205I; S202S; R203M) or a common region (Universal) of the SARS-CoV-2 genome.
 244 Individual probes were performed on each of five synthetic templates synthesized by TWIST Bioscience
 245 (San Francisco, USA), controls for the original Wuhan strain and for each VOC. ND = not detected

| | Universal (All) | R203K (Omicron/Alpha) | T205I (Beta) | S202S (Gamma) | R203M (Delta) |
|------------------|--------------------|--------------------------|-----------------|------------------|------------------|
| Twist 2 (Wuhan) | 29.52 | ND | 35.90 | ND | ND |
| Twist 14 (Alpha) | 29.73 | 30.1 | ND | ND | ND |
| Twist 16 (Beta) | 29.49 | ND | 30.05 | ND | ND |
| Twist 17 (Gamma) | 29.40 | ND | ND | 27.69 | ND |
| Twist 23 (Delta) | 30.44 | ND | ND | ND | 29.16 |

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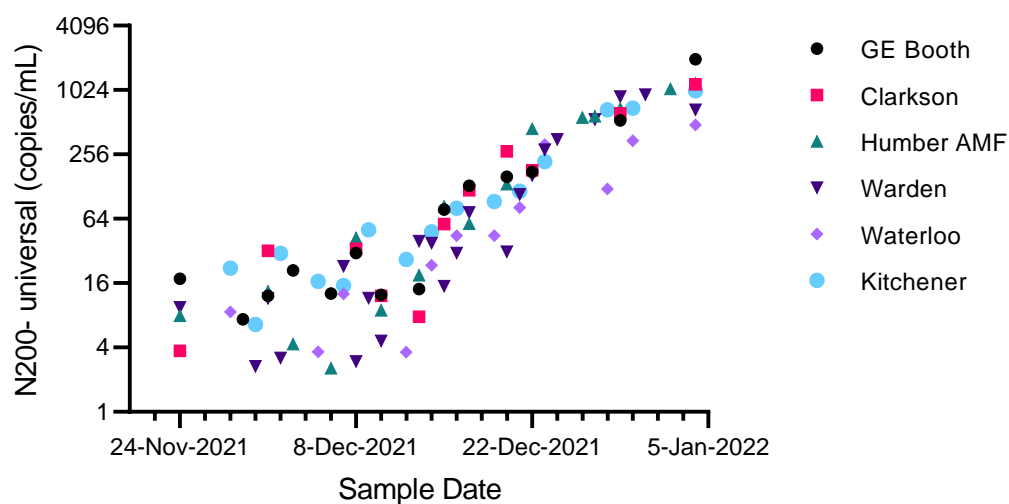
247 Table 5. Gene copies/ μL of RNA (mean \pm SD) detected by the Universal, R203M, or R203K probes in the
248 N200 assay (triplexed) when tested against TWIST controls for variants of SARS-CoV-2 using RT-dPCR.

| | Dilution from stock ($\sim 1 \times 10^4$ copies/ μL) | Universal (All) | R203K (Omicron/Alpha) | R203M (Delta) |
|-----------------------|---|--------------------|--------------------------|------------------|
| Twist 23 (Delta) | 5x | 2769 \pm 173 | 0 \pm 0 | 2767 \pm 173 |
| | 500x | 25 \pm 2 | 0 \pm 0 | 25 \pm 2 |
| | 5000x | 3 \pm 1 | 0 \pm 0 | 3 \pm 1 |
| Twist 48 (Omicron) | 5x | 824 \pm 12 | 820 \pm 22 | 2 \pm 2 |
| | 50x | 78 \pm 3 | 77 \pm 3 | 0 \pm 0 |
| | 500x | 5 \pm 0 | 5 \pm 0 | 0 \pm 0 |

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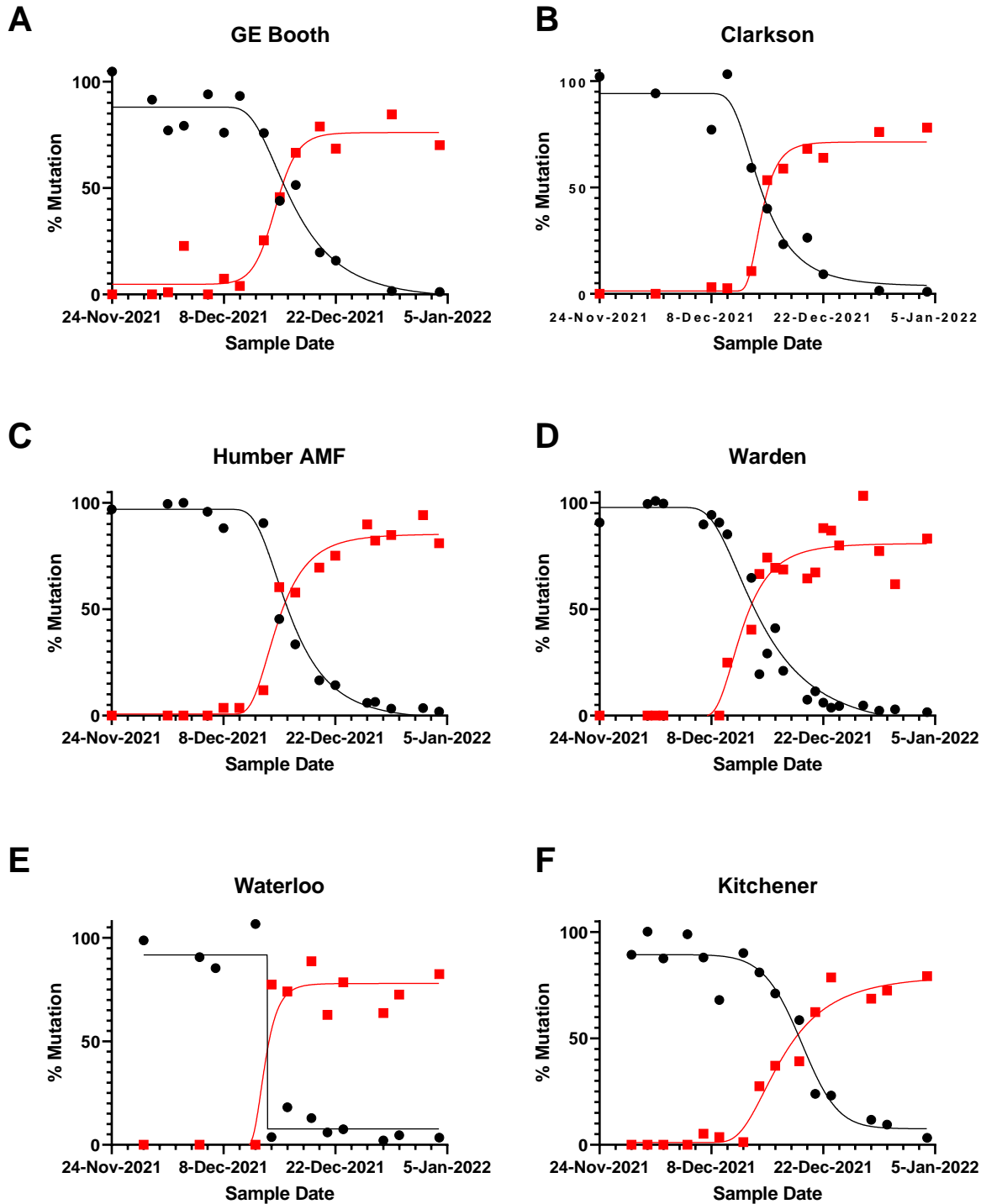
250 **Performance of N200 assay with samples of wastewater**

251 The triplex N200 assay was applied to wastewater samples throughout December 2021
252 and January 2022 to monitor for community transmission of the Omicron VOC, which would be
253 signified by decrease in the proportion of the R203M (Delta) mutation and an increase in the
254 R203K mutation (Omicron). The total SARS-CoV-2 (Universal) signal in samples during this
255 period started out low, but rapidly increased over the sampling period (Figure 2). During this
256 time, we observed a rapid transition in relative abundance of mutations from being dominated
257 by R203M (Delta) to being dominated by R203K (Omicron) at all monitoring locations (Figure 3).



258

259 Figure 2. SARS-CoV-2 signal measured in municipal wastewater using the N200 assay with the Universal
260 probe in a multiplexed reaction (Log₂ scale – copies/mL). Samples from the Peel (GE Booth and
261 Clarkson), York (Humber AMF and Warden) and Waterloo (Waterloo and Kitchener) Regions were
262 collected during the period encompassing both the introduction of Omicron into these communities and
263 the concomitant reduction of the endemic Delta VOC.
264



265

266 Figure 3. Application of the N200 triplex assay to monitor the relative abundance (%) of the mutations
267 R203M (black circles- presumed Delta) or R203K/G204R (red squares – presumed Omicron) in municipal
268 wastewater effluent. Effluent was collected from the Region of Peel (A, B), York Region (C, D) and
269 Waterloo Region (E, F) using composite (A-B, D-F) and grab (C) sampling. Lines drawn through points are
270 a 5-point sigmoidal curve calculated using GraphPad PRISM (v 9.3.1).

271 Discussion

272 This study presents an RT-qPCR approach for detection of SARS-CoV-2 and VOCs
273 Omicron/Alpha, Beta, Gamma and Delta in a multiplexed assay with a single amplicon. The
274 specificity and sensitivity of the N200 assay allow for simultaneous detection and quantification
275 of mutations associated with VOCs. These individual assays were multiplexed and validated in
276 duplex and triplex reactions, which allowed for rapid, cost-effective detection and quantitation
277 of mutations associated with VOCs. Relative amounts of these mutations are then compared to
278 a non-mutated region of the same amplicon to estimate the prevalence of current VOCs in a
279 wastewater sample. This assay was able to track the initial presence and then the transition
280 from the R203M mutation (i.e., Delta) as it was replaced by the R203K/G204R mutation (i.e.,
281 Omicron/Alpha) in multiple communities in south-central Ontario in December 2021. This is
282 representative of the transition from the Delta variant to the Omicron variant observed in
283 clinical cases at this time in these communities.

284 In addition to R203M (Delta) and R203K/G204R (Omicron/Alpha) targets, probes
285 targeting the T205I (Beta) mutation and the silent mutation in the amino acid S202 (Gamma),
286 allow for surveillance of known VOCs using a single assay. Having multiplexed, variant specific
287 probes that are compared to a Universal reference within the same amplicon is beneficial for
288 improved consistency and accuracy of measuring relative prevalence of variants. As the gene
289 targets are all in the same 121 bp amplicon, there is minimal concern about the dynamics of
290 different regions of the SARS-CoV-2 genome in either the wastewater system itself, or in the
291 extraction process. Because the reactions occur together in a multiplexed assay there is
292 reduced variability and replication compared to running assays separately. This approach saves

293 resources and time, as fewer reactions are run per sample. Critically, this assay also saves
294 sample volume, which is often limited, allowing for additional assays to be run on the same
295 extract or reducing the need for multiple extractions to be performed. Ultimately it makes the
296 process more efficient, reduces turnaround time and improves quality.

297 The combined use of Universal and VOC-specific probes in the N200 assay to estimate
298 prevalence of VOCs in wastewater is advantageous for several reasons. First, since when
299 multiplexed, the mutation and comparator are being assessed within the same amplicon as well
300 the same reaction, this method provides increased precision of VOCs prevalence estimates.
301 Additional confidence in prevalence of VOCs is provided by the fact that the mutation and
302 Universal signals can be quantified using the same standard. In comparison, other VOC qPCR
303 assays estimate prevalence by comparing a mutant allele to a genetic marker of SARS-CoV-2 in
304 another amplicon, /genetic locus such as the CDC_N1 (Vogels et al., 2021; Wolfe et al., 2021),
305 or by comparing the frequencies of the mutant and “wild-type” (e.g., ancestral/endemic allele)
306 at the same locus (Chan et al., 2022; Graber et al., 2021; Lee et al., 2021; Peterson et al., 2022).
307 While these assays can produce accurate data (from both analytical and clinical perspectives),
308 there are additional controls needed to account for variations in efficiencies of variation (inter-
309 assay variability and matching standard quantities), that are eliminated by the N200 assay.
310 Estimations of VOC prevalence are important during wastewater surveillance SARS-CoV-2 in
311 that they can provide useful information to public health units in a timely manner.

312 During analyses of wastewaters through late 2021 and early 2022, a rapid increase in
313 the SARS-CoV-2 signal (Universal probe) as well as a quick change over of the dominant variant
314 from Delta (R203M) to Omicron (R203K/G204R) was observed at all sites monitored. The assay

315 first detected the R203K mutation in a sample collected from GE Booth (Peel Region) on
316 December 3rd, 2021. While it is possible that this early detection of the R203K/G204R mutation
317 could have been a variant other than Omicron, within a week of this detection, the
318 R203K/G204R mutation was consistently detected in the wastewater at this site while R203M
319 decreased in proportion. The use of two mutation targets provided confidence in the data as it
320 was being collected and reported because both a decrease in the proportion of the R203M
321 mutation (Delta) and an increase in the proportion of the R203K/G204R mutations (Omicron)
322 occurred simultaneously. This data was reported to public health partners within a week of
323 samples being collected and this was immediately disseminated to the public by the Public
324 Health Unit as part of a weekly Epi-Report ([https://data.peelregion.ca/documents/profile-of-](https://data.peelregion.ca/documents/profile-of-covid-19-cases/explore)
325 [covid-19-cases/explore](https://data.peelregion.ca/documents/profile-of-covid-19-cases/explore)). Rapid emergence of Omicron presented a major challenge for clinical
326 testing and sequencing capacity. This was also confounded by the holiday season in Ontario.
327 The wastewater data in general, quickly became important to Public Health Units as the clinical
328 testing became less reliable because of changes to testing eligibility in Ontario. They were able
329 to confirm the rapid replacement of Delta with the Omicron variant using wastewater that was
330 independent of clinical testing (Arts et al., 2022).

331 The N200 multiplex qPCR was developed to improve estimates of the proportion of
332 various variants in wastewater, while also making the assay more efficient to use in terms of
333 reduced use of extracts, reagents, and time. The assay was validated using synthetic templates
334 and was successfully applied to monitor for the emergence of the Omicron variant in several
335 communities in Ontario. Although, this assay is one among several now available for detection
336 of VOCs in wastewater, the unique design, with a single amplicon, multiple target mutations,

337 and universal comparator targeting a highly mutable area of the N-gene make it very useful in
338 wastewater-based surveillance of SARS-CoV-2.

339

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350 **References**

- 351 Agarwala, R., Barrett, T., Beck, J., Benson, D.A., Bollin, C., Bolton, E., Bourexis, D., Brister, J.R.,
352 Bryant, S.H., Canese, K., Charowhas, C., Clark, K., Dicuccio, M., Dondoshansky, I., Federhen,
353 S., Feolo, M., Funk, K., Geer, L.Y., Gorelenkov, V., Hoepfner, M., Holmes, B., Johnson, M.,
354 Khotomlianski, V., Kimchi, A., Kimelman, M., Kitts, P., Klimke, W., Krasnov, S., Kuznetsov,
355 A., Landrum, M.J., Landsman, D., Lee, J.M., Lipman, D.J., Lu, Z., Madden, T.L., Madej, T.,
356 Marchler-Bauer, A., Karsch-Mizrachi, I., Murphy, T., Orris, R., Ostell, J., O'sullivan, C.,
357 Panchenko, A., Phan, L., Preuss, D., Pruitt, K.D., Rodarmer, K., Rubinstein, W., Sayers, E.,
358 Schneider, V., Schuler, G.D., Sherry, S.T., Sirotkin, K., Siyan, K., Slotta, D., Soboleva, A.,
359 Soussov, V., Starchenko, G., Tatusova, T.A., Todorov, K., Trawick, B.W., Vakatov, D., Wang,
360 Y., Ward, M., Wilbur, W.J., Yaschenko, E., Zbicz, K., 2016. Database resources of the
361 National Center for Biotechnology Information. *Nucleic Acids Res.* 44, D7.
362 <https://doi.org/10.1093/NAR/GKV1290>
- 363 Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search
364 tool. *J. Mol. Biol.* 215, 403–410. [https://doi.org/10.1016/S0022-2836\(05\)80360-2](https://doi.org/10.1016/S0022-2836(05)80360-2)
- 365 Antonio, M., Pretti, M, Galvani, R.G., Farias, A.S., Boroni, M., Antônio, M., Pretti, Marques, Lima,
366 M., Martins, B., 2021. New SARS-CoV-2 lineages could evade CD8+ T-cells response.
367 *bioRxiv* 2021.03.09.434584. <https://doi.org/10.1101/2021.03.09.434584>
- 368 Arts, E., Brown, S., Bulir, D., Charles, T.C., Degroot, C.T., Delatolla, R., Desaulniers, J.-P.,
369 Edwards, E.A., Fuzzen, M., Gilbride, K., Gilchrist, J., Goodridge, L., Graber, T.E., Habash, M.,
370 2022. Community Surveillance of Omicron in Ontario: Wastewater-based Epidemiology
371 Comes of Age. <https://doi.org/10.21203/RS.3.RS-1439969/V2>

372 Carcereny, A., Martínez-Velázquez, A., Bosch, A., Allende, A., Truchado, P., Cascales, J.,
373 Romalde, J.L., Lois, M., Polo, D., Sánchez, G., Pérez-Cataluña, A., Díaz-Reolid, A., Antón, A.,
374 Gregori, J., Garcia-Cehic, D., Quer, J., Palau, M., Ruano, C.G., Pintó, R.M., Guix, S., 2021.
375 Monitoring Emergence of the SARS-CoV-2 B.1.1.7 Variant through the Spanish National
376 SARS-CoV-2 Wastewater Surveillance System (VATar COVID-19). *Environ. Sci. Technol.* 55,
377 11756–11766. <https://doi.org/10.1021/acs.est.1c03589>

378 Chan, C.T.-M., Leung, J.S.-L., Lee, L.-K., Lo, H.W.-H., Wong, E.Y.-K., Wong, D.S.-H., Ng, T.T.-L.,
379 Lao, H.-Y., Lu, K.K., Jim, S.H.-C., Yau, M.C.-Y., Lam, J.Y.-W., Ho, A.Y.-M., Luk, K.S., Yip, K.-T.,
380 Que, T.-L., To, K.K.-W., Siu, G.K.-H., 2022. A low-cost TaqMan minor groove binder probe-
381 based one-step RT-qPCR assay for rapid identification of N501Y variants of SARS-CoV-2. *J.*
382 *Virol. Methods* 299, 114333. <https://doi.org/10.1016/j.jviromet.2021.114333>

383 D’Aoust, P.M., Graber, T.E., Mercier, E., Montpetit, D., Alexandrov, I., Neault, N., Baig, A.T.,
384 Mayne, J., Zhang, X., Alain, T., Servos, M.R., Srikanthan, N., MacKenzie, M., Figeys, D.,
385 Manuel, D., Jüni, P., MacKenzie, A.E., Delatolla, R., 2021. Catching a resurgence: Increase in
386 SARS-CoV-2 viral RNA identified in wastewater 48 h before COVID-19 clinical tests and 96 h
387 before hospitalizations. *Sci. Total Environ.* 770.
388 <https://doi.org/10.1016/j.scitotenv.2021.145319>

389 Graber, T.E., Mercier, É., Bhatnagar, K., Fuzzen, M., D’Aoust, P.M., Hoang, H.D., Tian, X., Towhid,
390 S.T., Plaza-Diaz, J., Eid, W., Alain, T., Butler, A., Goodridge, L., Servos, M., Delatolla, R.,
391 2021. Near real-time determination of B.1.1.7 in proportion to total SARS-CoV-2 viral load
392 in wastewater using an allele-specific primer extension PCR strategy. *Water Res.* 205,
393 117681. <https://doi.org/10.1016/J.WATRES.2021.117681>

- 394 Hadfield, J., Megill, C., Bell, S.M., Huddleston, J., Potter, B., Callender, C., Sagulenko, P.,
395 Bedford, T., Neher, R.A., 2018. NextStrain: Real-time tracking of pathogen evolution.
396 *Bioinformatics* 34, 4121–4123. <https://doi.org/10.1093/bioinformatics/bty407>
- 397 Hamouda, M., Mustafa, F., Maraqa, M., Rizvi, T., Aly Hassan, A., 2021. Wastewater surveillance
398 for SARS-CoV-2: Lessons learnt from recent studies to define future applications. *Sci. Total*
399 *Environ.* 759, 143493. <https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.143493>
- 400 Johnson, B.A., Zhou, Y., Lokugamage, K.G., Vu, M.N., Bopp, N., Crocquet-Valdes, P.A.,
401 Schindewolf, C., Liu, Y., Scharton, D., Plante, J.A., Xie, X., Aguilar, P., Weaver, S.C., Shi, P.-Y.,
402 Walker, D.H., Routh, A.L., Plante, K.S., Menachery, V.D., 2021. Nucleocapsid mutations in
403 SARS-CoV-2 augment replication and pathogenesis. *bioRxiv* 2021.10.14.464390.
404 <https://doi.org/10.1101/2021.10.14.464390>
- 405 Katoh, K., Misawa, K., Kuma, K.I., Miyata, T., 2002. MAFFT: a novel method for rapid multiple
406 sequence alignment based on fast Fourier transform. *Nucleic Acids Res.* 30, 3059–3066.
407 <https://doi.org/10.1093/NAR/GKF436>
- 408 Kiryanov, S.A., Levina, T.A., Konopleva, M. V., Suslov, A.P., 2022. Identification of Hotspot
409 Mutations in the N Gene of SARS-CoV-2 in Russian Clinical Samples That May Affect the
410 Detection by Reverse Transcription-PCR. *Diagnostics* 2022, Vol. 12, Page 147 12, 147.
411 <https://doi.org/10.3390/DIAGNOSTICS12010147>
- 412 Kitajima, M., Ahmed, W., Bibby, K., Carducci, A., Gerba, C.P., Hamilton, K.A., Haramoto, E., Rose,
413 J.B., 2020. SARS-CoV-2 in wastewater: State of the knowledge and research needs. *Sci.*
414 *Total Environ.* 739, 139076.
415 <https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.139076>

- 416 Kumblathan, T., Liu, Y., Uppal, G.K., Hrudey, S.E., Li, X.-F., 2021. Wastewater-Based
417 Epidemiology for Community Monitoring of SARS-CoV-2: Progress and Challenges. ACS
418 Environ. Au 1, 18–31. <https://doi.org/10.1021/acsenvironau.1c00015>
- 419 Lee, W.L., Imakaev, M., Armas, F., McElroy, K.A., Gu, X., Duvallet, C., Chandra, F., Chen, H.,
420 Leifels, M., Mendola, S., Floyd-O’Sullivan, R., Powell, M.M., Wilson, S.T., Berge, K.L.J., Lim,
421 C.Y.J., Wu, F., Xiao, A., Moniz, K., Ghaeli, N., Matus, M., Thompson, J., Alm, E.J., 2021.
422 Quantitative SARS-CoV-2 Alpha Variant B.1.1.7 Tracking in Wastewater by Allele-Specific
423 RT-qPCR. Environ. Sci. Technol. Lett. 8, 675–682.
424 <https://doi.org/10.1021/acs.estlett.1c00375>
- 425 Lu, X., Wang, L., Sakthivel, S.K., Whitaker, B., Murray, J., Kamili, S., Lynch, B., Malapati, L., Burke,
426 S.A., Harcourt, J., Tamin, A., Thornburg, N.J., Villanueva, J.M., Lindstrom, S., 2020. US CDC
427 Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory
428 Syndrome Coronavirus 2. Emerg. Infect. Dis. 26, 1654–1665.
429 <https://doi.org/10.3201/eid2608.201246>
- 430 Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-
431 Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the
432 Early Stage of the Epidemic in The Netherlands. Environ. Sci. Technol. Lett. 7, 511–516.
433 <https://doi.org/10.1021/acs.estlett.0c00357>
- 434 Pérez-Cataluña, A., Cuevas-Ferrando, E., Randazzo, W., Falcó, I., Allende, A., Sánchez, G., 2021.
435 Comparing analytical methods to detect SARS-CoV-2 in wastewater. Sci. Total Environ. 758,
436 143870. <https://doi.org/10.1016/J.SCITOTENV.2020.143870>
- 437 Peterson, S.W., Lidder, R., Daigle, J., Wonitowy, Q., Dueck, C., Nagasawa, A., Mulvey, M.R.,

438 Mangat, C.S., 2022. RT-qPCR detection of SARS-CoV-2 mutations S 69–70 del, S N501Y and
439 N D3L associated with variants of concern in Canadian wastewater samples. *Sci. Total*
440 *Environ.* 810, 151283. <https://doi.org/10.1016/J.SCITOTENV.2021.151283>

441 Syed, A.M., Taha, T.Y., Tabata, T., Chen, I.P., Ciling, A., Khalid, M.M., Sreekumar, B., Chen, P.-Y.,
442 Hayashi, J.M., Soczek, K.M., Ott, M., Doudna, J.A., 2021. Rapid assessment of SARS-CoV-2
443 evolved variants using virus-like particles. *Science* eabl6184.
444 <https://doi.org/10.1126/science.abl6184>

445 Vogels, C.B.F., Breban, M.I., Ott, I.M., Alpert, T., Petrone, M.E., Watkins, A.E., Kalinich, C.C.,
446 Earnest, R., Rothman, J.E., de Jesus, J.G., Claro, I.M., Ferreira, G.M., Crispim, M.A.E., Singh,
447 L., Tegally, H., Anyaneji, U.J., Hodcroft, E.B., Mason, C.E., Khullar, G., Metti, J., Dudley, J.T.,
448 MacKay, M.J., Nash, M., Wang, J., Liu, C., Hui, P., Murphy, S., Neal, C., Laszlo, E., Landry,
449 M.L., Muyombwe, A., Downing, R., Razeq, J., de Oliveira, T., Faria, N.R., Sabino, E.C., Neher,
450 R.A., Fauver, J.R., Grubaugh, N.D., da Silva Sales, F.C., Ramundo, M.S., Candido, D.S., Silva,
451 C.A.M., de Pinho, M.C., Coletti, T. de M., Andrade, P. dos S., de Souza, L.M., Rocha, E.C.,
452 Gomes Jardim, A.C., Manuli, E., Gaburo, N., Granato, C., Levi, J.E., Costa, S., de Souza,
453 W.M., Salum, M.A., Pereira, R., de Souza, A., Matkin, L.E., Nogueira, M.L., Levin, A.S.,
454 Mayaud, P., Alexander, N., Souza, R., Acosta, A.L., Prete, C., Quick, J., Brady, O., Messina, J.,
455 Kraemer, M., Gouveia, N. da C., Oliva, I., de Souza, M., Lazari, C., Alencar, C.S., Thézé, J.,
456 Buss, L., Araujo, L., Cunha, M.S., Loman, N.J., Pybus, O.G., Aguiar, R.S., Wilkinson, E.,
457 Msomi, N., Iranzadeh, A., Fonseca, V., Doolabh, D., San, E.J., Mlisana, K., von Gottberg, A.,
458 Walaza, S., Allam, M., Ismail, A., Mohale, T., Glass, A.J., Engelbrecht, S., van Zyl, G., Preiser,
459 W., Petruccione, F., Sigal, A., Hardie, D., Marais, G., Hsiao, M., Korsman, S., Davies, M.A.,

460 Tyers, L., Mudau, I., York, D., Maslo, C., Goedhals, D., Abrahams, S., Laguda-Akingba, O.,
461 Alisoltani-Dehkordi, A., Godzik, A., Wibmer, C.K., Sewell, B.T., Lourenço, J., Kosakovsky
462 Pond, S.L., Weaver, S., Giovanetti, M., Alcantara, L.C.J., Martin, D., Bhiman, J.N.,
463 Williamson, C., 2021. Multiplex qPCR discriminates variants of concern to enhance global
464 surveillance of SARS-CoV-2. *PLoS Biol.* 19, e3001236.
465 <https://doi.org/10.1371/journal.pbio.3001236>

466 Wu, F., Zhang, J., Xiao, A., Gu, X., Lee, W.L., Armas, F., Kauffman, K., Hanage, W., Matus, M.,
467 Ghaeli, N., Endo, N., Duvallet, C., Poyet, M., Moniz, K., Washburne, A.D., Erickson, T.B.,
468 Chai, P.R., Thompson, J., Alm, E.J., 2020. SARS-CoV-2 Titers in Wastewater Are Higher than
469 Expected from Clinically Confirmed Cases. *mSystems* 5.
470 <https://doi.org/10.1128/msystems.00614-20>

471 Wu, H., Xing, N., Meng, K., Fu, B., Xue, W., Dong, P., Tang, W., Xiao, Y., Liu, G., Luo, H., Zhu, W.,
472 Lin, X., Meng, G., Zhu, Z., 2021. Nucleocapsid mutations R203K/G204R increase the
473 infectivity, fitness, and virulence of SARS-CoV-2. *Cell Host Microbe* 29, 1788-1801.e6.
474 <https://doi.org/10.1016/j.chom.2021.11.005>

475 Wurtzer, S., Waldman, P., Levert, M., Mouchel, J.M., Gorgé, O., Boni, M., Maday, Y.,
476 consortium, O., Marechal, V., Moulin, L., 2021. Monitoring the propagation of SARS CoV2
477 variants by tracking identified mutation in wastewater using specific RT-qPCR. *medRxiv*
478 2021.03.10.21253291. <https://doi.org/10.1101/2021.03.10.21253291>

479 Yaniv, K., Ozer, E., Shagan, M., Lakkakula, S., Plotkin, N., Bhandarkar, N.S., Kushmaro, A., 2021.
480 Direct RT-qPCR assay for SARS-CoV-2 variants of concern (Alpha, B.1.1.7 and Beta, B.1.351)
481 detection and quantification in wastewater. *Environ. Res.* 201, 111653.

482 <https://doi.org/10.1016/j.envres.2021.111653>

483 Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST:
484 a tool to design target-specific primers for polymerase chain reaction. BMC Bioinformatics
485 13, 134. <https://doi.org/10.1186/1471-2105-13-134/FIGURES/5>

486