Supporting Information

The Impact of Sampling Type, Frequency and Scale of Collection System on SARS-CoV-2 Quantification Fidelity

Andrea D. George¹,², Devrim Kaya², Blythe A. Layton¹, Kestrel Bailey¹, Scott Mansell¹, Christine Kelly², Kenneth J. Williamson¹, Tyler S. Radniecki²*

*Corresponding author: tyler.radniecki@oregonstate.edu

¹Department of Research & Innovation, Clean Water Services, Hillsboro, OR, 97123

²School of Chemical, Biological, and Environmental Engineering, Oregon State University, Corvallis, OR, 97331

21 pages
6 Equations
7 Figures
5 Tables
**Equation S1. Mean Absolute Error (MAE)**

\[
\frac{\sum_{i=1}^{n} |\hat{y}_i - y_i|}{n}
\]

Where \( \hat{y}_i = \) grab sample SARS-CoV-2 concentration (log-transformed)

\( y_i = \) composite sample SARS-CoV-2 concentration (log-transformed)

\( n = \) number of grab samples collected

Please note that the interpretation of the MAE requires the recognition of the use of log-transformed values. The log-transformation of the concentration establishes the metric as one of relative rather than absolute error (as the name may suggest), since log rules dictate that there is a division of the grab by the composite within the MAE calculation. Thus, the resulting MAE is a percent error not an absolute error.

**Equation S2. Root Mean Square Log Error (RMSLE)**

\[
\sqrt{\frac{\sum_{i=1}^{n} (\hat{y}_i - y_i)^2}{n}}
\]

Where \( \hat{y}_i = \) grab sample SARS-CoV-2 concentration (log-transformed)

\( y_i = \) composite sample SARS-CoV-2 concentration (log-transformed)

\( n = \) number of grab samples collected

Please note that the interpretation of the RMSLE requires the recognition of the use of log-transformed values. The log-transformation of the concentration establishes the metric as one of relative rather than absolute error (as the name may suggest), since log rules dictate that there is a division of the grab by the composite within the RMSLE calculation. Thus, the resulting RMSLE is a percent error not an absolute error. This metric penalizes underestimation more than overestimation, which can be seen by comparing two arithmetic values equidistant from the mean (above and below), log-transforming those values, and calculating RMSLE for each.
Equation S3. Percent Non-Detects

\[ \frac{n_{negative}}{n_{tot}} \times 100 \]

Where \( n_{negative} \) = number of grab samples collected that were negative for SARS-CoV-2

\( n_{tot} \) = total number of grab samples collected

Equation S4. Percent Grabs Below Composite

\[ \frac{n_{gbc}}{n_{tot}} \times 100 \]

Where \( n_{gbc} \) = number of grab samples with a SARS-CoV-2 concentration less than that of the associated composite

\( n_{tot} \) = total number of grab samples collected

Equation S5. Geometric Mean of N1 and N2 in Log10 gene copies per liter

\[
\frac{\log_{10}(N1)_1 + \log_{10}(N1)_2 + \log_{10}(N2)_1 + \log_{10}(N2)_2}{n}
\]

Where \((N1)_1\) and \((N1)_2\) = N1 measurements in two wells, calculated in gc/L from Equation S6

\((N2)_1\) and \((N2)_2\) = N2 measurements in two wells, calculated in gc/L from Equation S6

\( n \) = number of measurements of N1 and N2

Equation S6. Conversion of copies/reaction to copies/L

\[
\frac{\text{copies}}{\text{rxn}} \times \frac{1}{\text{Template Volume} (\mu L)} \times \frac{1}{\text{Elution Volume} (\mu L)} \times \frac{1}{\text{Lysate Volume} (\mu L)} \times \frac{1}{\text{Shield Volume} (\mu L)} \times \frac{1}{\text{Volume Filtered} (ml)} \times 1000 \times \frac{mL}{L} = \frac{\text{copies}}{L}
\]
Concentration Method Recovery using Bovine Coronavirus

**Bovine coronavirus (BCoV) stock solution preparation**

BCoV solution was prepared from freeze-dried Calf Guard cattle vaccine (Bovine Rotavirus-Coronavirus Vaccine from Zoetis, NJ, USA) after rehydrating in 3 mL of sterile diluent provided with the vaccine. Aliquots (100 μL) of stock solution were stored at -20 ºC. Each aliquot was used for a maximum of two freeze-thaw cycles. To determine the stock concentration, 10 μL BCoV stock was added to 390 μL PBS. From this mixture, RNA was extracted from 200 μL as described in the main text. The extracted BCoV RNA was serially diluted (1:10) in nuclease-free water for six dilutions and run in duplicate using a previously published BCoV assay by following the one-step RT-ddPCR procedure as described in the text. Stock concentration of BCoV was around 2.3 x 10^6 gc/μL.

**Bovine coronavirus (BCoV) process recovery control**

Similar to other studies, an attenuated vaccine strain of BCoV, was selected as a process recovery control due to its morphological and structural similarity to SARS-CoV-2. BCoV solution was prepared from freeze-dried Calf Guard cattle vaccine (Bovine Rotavirus-Coronavirus Vaccine from Zoetis, NJ, USA) as described above.

To determine process recovery efficiency, 5 μL of BCoV was added to 25 mL of separate wastewater samples (n=8) not collected for the purposes of this study, just prior to filtration. Samples were processed following the methods described in the main text. BCoV was quantified in the samples using the One-Step RT-ddPCR Advanced Kit for Probes on the QX-200 ddPCR system (Bio-Rad, Hercules, CA) using previously published primers and probes. All other assay and thermal cycling conditions are described in the main text. The BCoV recovery was calculated by dividing the quantity measured in wastewater samples to the quantity added to each wastewater sample prior to concentration. The mean BCoV recovery was 57 (± 4) %. Non-spiked wastewater samples (n=4) were also quantified for BCoV to assess background concentration. No BCoV was detected in non-spiked samples.

**N1 and N2 LOD Determination**

The limit of blank (LOB) for N1 was found to be 2.0 copies per reaction and 4.2 copies per reaction for N2. Since both LOB values are below the 3-droplet threshold, this further justifies our choice of threshold for calling positive reactions. No LOB reactions met the positive threshold for N1, but 4 non-target reactions (n=104) had 3 or more droplets in N2, generating a false positive rate of 4%. The predicted limits of detection (LOD) based on the LOB results were 4 copies per reaction for N1 and 12 copies per reaction for N2. Greater than 95% of test reactions at the predicted LOD value need to amplify above the LOB to validate an LOD estimate. The
LOD of N2 was confirmed to be 12 copies per reaction, as 97% of the test reactions (n = 60) at that concentration had gene copies above the N2 LOB. The N1 LOD appears to lie somewhere between 4 and 12 copies per reaction: at 12 copies per reaction, all 60 reactions amplified above the N1 LOB, but at 4 copies per reaction, 13 reactions (n=60) amplified below the N1 LOB (22%). Using a parametric method as an imperfect estimate, though the test reaction data are not normally distributed, yields an N1 LOD estimated at 8 copies per reaction.

**Figure S1.** The decay in Log10-transformed concentration of SARS-CoV-2 RNA in frozen aliquots of the same WWTP influent sample over 57 days. Points and error bars denote the mean and standard error, respectively, among 12 measurements (triplicate biological replicates analyzed in duplicate, and N targets combined). Samples were analyzed as described in the main text.
Figure S2. The N1 target concentrations (log$_{10}$ copies per reaction) plotted against the N2 target concentration (log$_{10}$ copies per reaction) in each sample and control reaction (n=412). Non-detects were replaced with 0.1 copies per reaction.
Figure S3. The 5-min grab samples collected in the first 2 h of sample collection plotted with a solid line indicating the (a) 5-min sampling frequency composite, (b) 10-min sampling frequency composite, and (c) 15-min sampling frequency composite. The error bars on the grab samples and the shaded range on the composite lines denote standard error. Non-detects are represented by open markers.
Figure S4. The 15-min grab samples collected in 8 h of sample collection plotted with a solid line indicating the (a) 15-min sampling frequency composite, (b) 30-min sampling frequency composite, and (c) 1-h sampling frequency composite. The error bars on the grab samples and the shaded range on the composite lines denote standard error. Non-detects are represented by open markers.
Figure S5. The fluorescence plots of (a) no-template control, (b) negative control (contains RP), and (c) positive controls used to show example positive and negative results.
Figure S6. The two technical replicates for every sample ($n = 268$) plotted against each other in $\log_{10}$ gc/L to show intra-experiment repeatability. The black line is the 1:1 line, and the equation gives the linear model fit to the data.

$y = 0.45 + 0.89 \cdot x$, $r^2 = 0.82$
Figure S7. The boxplot exhibiting the distribution of viral concentrations (log_{10} gene copies/liter) of the grab samples at ultra-low-, low-, medium-, and high-flow sites.
**Table S1.** The storage time, temperature, volume concentrated, and Total Suspended Solids or Chemical Oxygen Demand for each of the raw wastewater samples collected from the four sites. Total Suspended Solids values were approximate and should only be used for comparative purposes because samples were frozen before analysis. Chemical Oxygen Demand tests were conducted on hourly grab high-flow samples (not frozen) and an asterisk added (*) to denote that these samples have a COD value but no TSS value.

| LOCATION       | HOUR | TEMP STORED (°C) | DAYS STORED | VOLUME FILTERED (mL) | TOTAL SUSPENDED SOLIDS/ CHEMICAL OXYGEN DEMAND* |
|----------------|------|------------------|-------------|----------------------|--------------------------------------------------|
| Ultra-Low Flow | 9:10 | -20              | 50          | 30                   | 227                                              |
| Ultra-Low Flow | 9:15 | -20              | 9           | 30                   | 450                                              |
| Ultra-Low Flow | 9:20 | -20              | 50          | 35                   | 168                                              |
| Ultra-Low Flow | 9:25 | -20              | 50          | 10                   | 567                                              |
| Ultra-Low Flow | 9:30 | -20              | 9           | 30                   | 253                                              |
| Ultra-Low Flow | 9:35 | -20              | 50          | 50                   | 78                                               |
| Ultra-Low Flow | 9:40 | -20              | 50          | 35                   | 210                                              |
| Ultra-Low Flow | 9:45 | -20              | 9           | 40                   | 149                                              |
| Ultra-Low Flow | 9:50 | -20              | 50          | 30                   | 210                                              |
| Ultra-Low Flow | 9:55 | -20              | 50          | 35                   | 150                                              |
| Ultra-Low Flow | 10:00| -20              | 9           | 30                   | 562                                              |
| Ultra-Low Flow | 10:05| -20              | 50          | 30                   | 307                                              |
| Ultra-Low Flow | 10:10| -20              | 50          | 40                   | 82                                               |
| Ultra-Low Flow | 10:15| -20              | 9           | 30                   | 213                                              |
| Ultra-Low Flow | 10:20| -20              | 50          | 10                   | 375                                              |
| Ultra-Low Flow | 10:25| -20              | 50          | 30                   | 287                                              |
| Ultra-Low Flow | 10:30| -20              | 9           | 30                   | 188                                              |
| Ultra-Low Flow | 10:35| -20              | 50          | 30                   | 393                                              |
| Ultra-Low Flow | 10:40| -20              | 50          | 20                   | 570                                              |
| Ultra-Low Flow | 10:45| -20              | 9           | 35                   | 80                                               |
| Ultra-Low Flow | 10:50| -20              | 50          | 15                   | 335                                              |
| Ultra-Low Flow | 10:55| -20              | 50          | 30                   | 143                                              |
| Ultra-Low Flow | 11:00| -20              | 9           | 30                   | 96                                               |
| Ultra-Low Flow | 11:05| -20              | 50          | 40                   | 84                                               |
| Ultra-Low Flow | 11:30| -20              | 24          | 40                   | 93                                               |
| Ultra-Low Flow | 11:45| -20              | 24          | 30                   | 237                                              |
| Ultra-Low Flow | 12:00| -20              | 24          | 50                   | 74                                               |
| Ultra-Low Flow | 12:15| -20              | 11          | 30                   | 159                                              |
| Ultra-Low Flow | 12:30| -20              | 11          | 30                   | 49                                               |
| Ultra-Low Flow | 12:45| -20              | 11          | 30                   | 284                                              |
| Ultra-Low Flow | 13:00| -20              | 11          | 45                   | 71                                               |
| Ultra-Low Flow | 13:15| -20              | 24          | 30                   | 198                                              |
| Time      | Flow  | Temp | Flow | Temp | Flow |
|-----------|-------|------|------|------|------|
| 13:45     | Ultra-Low Flow | -20  | 24   | 50   | 46   |
| 14:00     | Ultra-Low Flow | -20  | 24   | 40   | 140  |
| 14:15     | Ultra-Low Flow | -20  | 24   | 35   | 245  |
| 14:30     | Ultra-Low Flow | -20  | 24   | 50   | 144  |
| 14:45     | Ultra-Low Flow | -20  | 31   | 35   | 190  |
| 15:00     | Ultra-Low Flow | -20  | 31   | 30   | 565  |
| 15:15     | Ultra-Low Flow | -20  | 31   | 40   | 142  |
| 15:30     | Ultra-Low Flow | -20  | 31   | 50   | 1260 |
| 15:45     | Ultra-Low Flow | -20  | 31   | 35   | 212  |
| 16:00     | Ultra-Low Flow | -20  | 31   | 30   | 565  |
| 16:15     | Ultra-Low Flow | -20  | 31   | 40   | 142  |
| 16:30     | Ultra-Low Flow | -20  | 31   | 50   | 1260 |
| 16:45     | Ultra-Low Flow | -20  | 31   | 40   | 278  |
| 17:00     | Ultra-Low Flow | -20  | 31   | 50   | 138  |
| 17:15     | Ultra-Low Flow | -20  | 31   | 40   | 610  |
| 17:30     | Ultra-Low Flow | -20  | 31   | 50   | 60   |
| 18:00     | Ultra-Low Flow | -20  | 31   | 50   | 60   |
| 18:15     | Low-Flow | -20  | 12   | 20   | 1345 |
| 18:30     | Low-Flow | -20  | 12   | 20   | 820  |
| 18:45     | Low-Flow | -20  | 12   | 20   | 687  |
| 19:00     | Low-Flow | -20  | 12   | 20   | 1473 |
| 19:15     | Low-Flow | -20  | 12   | 20   | 1353 |
| 19:30     | Low-Flow | -20  | 12   | 10   |      |
| 19:45     | Low-Flow | -20  | 12   | 30   |      |
| 20:00     | Low-Flow | -20  | 12   | 20   |      |
| 20:15     | Low-Flow | -20  | 12   | 30   | 685  |
| 20:30     | Low-Flow | -20  | 12   | 30   | 657  |
| 20:45     | Low-Flow | -20  | 12   | 30   | 900  |
| 21:00     | Low-Flow | -20  | 12   | 30   | 1632 |
| 21:15     | Low-Flow | -20  | 12   | 20   | 1877 |
| 21:30     | Low-Flow | -20  | 12   | 20   | 768  |
| 21:45     | Low-Flow | -20  | 12   | 30   | 1117 |
| 22:00     | Low-Flow | -20  | 12   | 20   | 681  |
| 22:15     | Low-Flow | -20  | 12   | 20   | 3230 |
| 22:30     | Low-Flow | -20  | 12   | 30   | 723  |
| 22:45     | Low-Flow | -20  | 12   | 30   | 1337 |
| 23:00     | Low-Flow | -20  | 12   | 20   | 5082 |
| 23:15     | Low-Flow | -20  | 12   | 20   | 2533 |
| 23:30     | Low-Flow | -20  | 5    | 30   | 2063 |
| 23:45     | Low-Flow | Composite | -20 | 5 | 30 | 2063 |
| 00:00     | Med-Flow | -20  | 15   | 40   | 740  |
| 00:15     | Med-Flow | -20  | 15   | 40   | 500  |
| 00:30     | Med-Flow | -20  | 15   | 30   | 3557 |
| 00:45     | Med-Flow | -20  | 15   | 30   | 600  |
| 01:00     | Med-Flow | -20  | 15   | 35   | 1753 |
| 01:15     | Med-Flow | -20  | 15   | 40   | 303  |
| 01:30     | Med-Flow | -20  | 15   | 25   |      |
| Med-Flow | 8     | -20   | 15 | 40 | 393 |
| Med-Flow | 9     | -20   | 15 | 40 | 623 |
| Med-Flow | 10    | -20   | 15 | 40 | 693 |
| Med-Flow | 11    | -20   | 15 | 40 | 480 |
| Med-Flow | 12    | -20   | 15 | 45 | 277 |
| Med-Flow | 13    | -20   | 15 | 50 | 106 |
| Med-Flow | 14    | -20   | 15 | 50 | 90  |
| Med-Flow | 15    | -20   | 15 | 50 | 73  |
| Med-Flow | 16    | -20   | 15 | 50 | 73  |
| Med-Flow | 17    | -20   | 15 | 40 | 163 |
| Med-Flow | 18    | -20   | 15 | 50 | 213 |
| Med-Flow | 19    | -20   | 15 | 40 | 387 |
| Med-Flow | 20    | -20   | 15 | 40 | 683 |
| Med-Flow | 21    | -20   | 15 | 40 | 581 |
| Med-Flow | 22    | -20   | 15 | 40 | 737 |
| Med-Flow | 23    | -20   | 15 | 30 | 857 |
| Med-Flow | 24    | -20   | 15 | 40 | 287 |
| Med-Flow Composite | -20 | 1 | 40 | 471 |
| High-Flow | 1    | -20   | 8  | 50 | 1200* |
| High-Flow | 2    | -20   | 8  | 50 | 1090* |
| High-Flow | 3    | -20   | 8  | 50 | 1010* |
| High-Flow | 4    | -20   | 8  | 50 | 1080* |
| High-Flow | 5    | -20   | 8  | 30 | 1210* |
| High-Flow | 6    | -20   | 8  | 50 | 1120* |
| High-Flow | 7    | -20   | 8  | 50 | 907* |
| High-Flow | 8    | -20   | 8  | 50 | 896* |
| High-Flow | 9    | -20   | 8  | 50 | 1050* |
| High-Flow | 10   | -20   | 8  | 50 | 1340* |
| High-Flow | 11   | -20   | 8  | 50 | 1330* |
| High-Flow | 12   | -20   | 8  | 50 | 1220* |
| High-Flow | 13   | -20   | 8  | 50 | 1190* |
| High-Flow | 14   | -20   | 8  | 50 | 1140* |
| High-Flow | 15   | -20   | 8  | 50 | 1270* |
| High-Flow | 16   | -20   | 8  | 50 | 1220* |
| High-Flow | 17   | -20   | 8  | 50 | 1090* |
| High-Flow | 18   | -20   | 8  | 50 | 774* |
| High-Flow | 19   | -20   | 8  | 50 | 859* |
| High-Flow | 20   | -20   | 8  | 50 | 670* |
| High-Flow | 21   | -20   | 8  | 50 | 634* |
| High-Flow | 22   | -20   | 8  | 50 | 838* |
| High-Flow | 23   | -20   | 8  | 50 | 713* |
| High-Flow | 24   | -20   | 8  | 50 | 627* |
Table S2. The decay-corrected data and error metrics based on the decay constant calculated from the experiment in Figure S1.

| Site                | Number of Grab Samples (n) | Total Sampling Time (h) | Avg Dry Weather Flowrate (GPM) | Sampling Frequency | Composite Concentration ($\log_{10}$ g/L) | Maximum Grab Concentration ($\log_{10}$ g/L) | Minimum Grab Concentration ($\log_{10}$ g/L) | Percent Non-Detects | Percent Grabs Below Composite | MAE | RMSLE |
|---------------------|----------------------------|-------------------------|--------------------------------|-------------------|------------------------------------------|----------------------------------------------|----------------------------------------------|----------------------|----------------------------------|------|-------|
| Ultra-Low-Flow***   | 32                         | 8                       | 13** 15-min                      | 5.87 ± 0.08 * 7.21 ± 0.02 | 3.56 ± 0.04                          | 40.6%                                        | 93.8%                                        | 1.81                 | 1.89                             |
| Low-Flow            | 21                         | 24                      | 111 1-h                          | 4.79 ± 0.03 5.60 ± 0.01 | 3.75 ± 0.05                          | 47.6%                                        | 76.2%                                        | 0.68                 | 0.76                             |
| Medium-Flow         | 24                         | 24                      | 700 1-h                          | 3.92 ± 0.10 4.74 ± 0.02 | 3.64 ± 0.05                          | 37.5%                                        | 70.8%                                        | 0.26                 | 0.31                             |
| High-Flow           | 24                         | 24                      | 2430 1-h                         | 3.99 ± 0.13 4.51 ± 0.04 | 3.52 ± 0.06                          | 0.0%                                         | 58.3%                                        | 0.24                 | 0.29                             |

| Site                | Number of Grab Samples (n) | Total Sampling Time (h) | Avg Dry Weather Flowrate (GPM) | Sampling Frequency | Composite Concentration ($\log_{10}$ g/L) | Maximum Grab Concentration ($\log_{10}$ g/L) | Minimum Grab Concentration ($\log_{10}$ g/L) | Percent Non-Detects | Percent Grabs Below Composite | MAE | RMSLE |
|---------------------|----------------------------|-------------------------|--------------------------------|-------------------|------------------------------------------|----------------------------------------------|----------------------------------------------|----------------------|----------------------------------|------|-------|
| Ultra-Low-Flow      | 24                         | 2                       | 13** 5-min                       | 5.97 ± 0.09 7.21 ± 0.02 | 3.61 ± 0.05                          | 25.0%                                        | 87.5%                                        | 1.59                 | 1.69                             |
| Ultra-Low-Flow      | 12                         | 2                       | 13** 10-min                      | 6.17 ± 0.15 7.21 ± 0.02 | 3.61 ± 0.05                          | 16.7%                                        | 91.7%                                        | 1.72                 | 1.86                             |
| Ultra-Low-Flow      | 8                          | 2                       | 13** 15-min                      | 6.31 ± 0.20 7.21 ± 0.02 | 3.56 ± 0.04                          | 12.5%                                        | 87.5%                                        | 2.03                 | 2.10                             |

| Site                | Number of Grab Samples (n) | Total Sampling Time (h) | Avg Dry Weather Flowrate (GPM) | Sampling Frequency | Composite Concentration ($\log_{10}$ g/L) | Maximum Grab Concentration ($\log_{10}$ g/L) | Minimum Grab Concentration ($\log_{10}$ g/L) | Percent Non-Detects | Percent Grabs Below Composite | MAE | RMSLE |
|---------------------|----------------------------|-------------------------|--------------------------------|-------------------|------------------------------------------|----------------------------------------------|----------------------------------------------|----------------------|----------------------------------|------|-------|
| Ultra-Low-Flow***   | 32                         | 8                       | 13** 15-min                      | 5.87 ± 0.08 7.21 ± 0.02 | 3.56 ± 0.04                          | 40.6%                                        | 93.8%                                        | 1.81                 | 1.89                             |
| Ultra-Low-Flow      | 17                         | 8                       | 13** 30-min                      | 6.03 ± 0.12 7.21 ± 0.02 | 3.61 ± 0.05                          | 41.2%                                        | 88.2%                                        | 1.89                 | 2.01                             |
| Ultra-Low-Flow      | 9                          | 8                       | 13** 1-h                         | 6.28 ± 0.20 7.21 ± 0.02 | 3.73 ± 0.05                          | 33.3%                                        | 88.9%                                        | 1.84                 | 2.03                             |

*These composites were created digitally using the respective grab samples.  
**This flow was estimated using the number of residents and a 67 GPD/resident flow estimate based on previous wastewater data in the region.  
***This series is shown twice for comparison purposes.
Table S3. The oligonucleotide primers and probes used in the study, along with the number of bases and references for each sequence\(^3,4\).

| Target     | Oligonucleotide | Sequence and label (5' → 3')                  | Size (# of bases) | Reference            |
|------------|-----------------|------------------------------------------------|-------------------|----------------------|
| 2019-nCoV N1-F | GAC CCC AAA ATC AGC GAA AT | Lu et al. 2020 |
| 2019-nCoV N1-R | TCT GGT TAC TGC CAG TTG AAT CTG | 72 | Lu et al. 2020 |
| SARS-CoV-2  | 2019-nCoV N1- P | FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1 | Lu et al. 2020 |
| 2019-nCoV N2-F | TTA CAA ACA TTG GCC GCA AA | Lu et al. 2020 |
| 2019-nCoV N2-R | GCG CGA CAT TCC GAA GAA | 67 | Lu et al. 2020 |
| 2019-nCoV N2- P | FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1 | Lu et al. 2020 |
| RP (RNAseP)  | RP-F            | AGATTTGGACCTGCGAGCG                               |                  | Lu et al. 2020 |
|             | RP-R            | GACGGCTGTCTCCACAAGT                                | 65               | Lu et al. 2020 |
|             | RP-P            | FAM-TCCTGACCTGAAGGCTCTGCG CG-BHQ-1                 |                  | Lu et al. 2020 |
| BCoV        | BcoV-F          | CTGGAAGTTGAGTGCAGTT                              | Decaro et al. 2008 |
|             | BcoV-R          | ATTATCGGCTAACATACATC                             | 85               | Decaro et al. 2008 |
|             | BcoV-P          | FAM/CCTTCATAT/ZEN/CTATA CA                       | Decaro et al. 2008 |
|             |                 | CATCAAGTTGT- IABkFQ                              |                  |                      |

FAM: 6-carboxyfluorescein; IABkFQ: Iowa Black® FQ Quencher 1; BHQ1: Black Hole Quencher 1

Table S4. The sample-specific limit of detection of each sample analyzed in this study. This was calculated using the empirical N1 and N2 LOD determined in the “N1 and N2 LOD Determination” section above (N1: 8 copies/reaction, N2: 12 copies/reaction).

| LOCATION     | TIME OR HOUR | SAMPLE-SPECIFIC LOD (LOG\(_{10}\) GENE COPIES/LITER) |
|--------------|--------------|--------------------------------------------------------|
| Ultra-Low Flow | 9:10         | 3.99                                                   |
| Ultra-Low Flow | 9:15         | 3.99                                                   |
| Ultra-Low Flow | 9:20         | 3.92                                                   |
| Ultra-Low Flow | 9:25         | 4.47                                                   |
| Time  | Flow Rate |
|-------|-----------|
| 9:30  | 3.99      |
| 9:35  | 3.77      |
| 9:40  | 3.92      |
| 9:45  | 3.87      |
| 9:50  | 3.99      |
| 9:55  | 3.92      |
| 10:00 | 3.99      |
| 10:05 | 3.99      |
| 10:10 | 3.87      |
| 10:15 | 3.99      |
| 10:20 | 4.47      |
| 10:25 | 3.99      |
| 10:30 | 3.99      |
| 10:35 | 3.99      |
| 10:40 | 4.17      |
| 10:45 | 3.92      |
| 10:50 | 4.29      |
| 10:55 | 3.99      |
| 11:00 | 3.99      |
| 11:05 | 3.87      |
| 11:10 | 3.87      |
| 11:15 | 3.99      |
| 11:20 | 3.99      |
| 11:25 | 3.99      |
| 11:30 | 3.99      |
| 11:45 | 3.99      |
| 12:00 | 3.77      |
| 12:15 | 3.99      |
| 12:30 | 3.99      |
| 12:45 | 3.99      |
| 13:00 | 3.82      |
| 13:15 | 3.99      |
| 13:45 | 3.77      |
| 14:00 | 3.87      |
| 14:15 | 3.92      |
| 14:30 | 3.77      |
| 14:45 | 3.92      |
| 15:00 | 3.99      |
| 15:15 | 3.87      |
| 15:30 | 3.99      |
| 16:15 | 3.87      |
| 16:30 | 3.99      |
| 16:45 | 3.92      |
| Flow Type     | Time  | Value |
|--------------|-------|-------|
| Ultra-Low Flow | 17:00 | 3.82  |
| Ultra-Low Flow | 17:15 | 3.87  |
| Ultra-Low Flow | 17:30 | 3.77  |
| Ultra-Low Flow | 17:45 | 4.17  |
| Ultra-Low Flow | 18:00 | 3.77  |
| Low-Flow 1    |       | 4.17  |
| Low-Flow 2    |       | 4.17  |
| Low-Flow 3    |       | 4.17  |
| Low-Flow 4    |       | 4.17  |
| Low-Flow 5    |       | 4.17  |
| Low-Flow 7    |       | 4.47  |
| Low-Flow 8    |       | 3.99  |
| Low-Flow 9    |       | 4.17  |
| Low-Flow 10   |       | 3.99  |
| Low-Flow 11   |       | 3.99  |
| Low-Flow 12   |       | 3.99  |
| Low-Flow 13   |       | 3.99  |
| Low-Flow 14   |       | 4.17  |
| Low-Flow 15   |       | 4.17  |
| Low-Flow 16   |       | 3.99  |
| Low-Flow 17   |       | 4.17  |
| Low-Flow 18   |       | 4.17  |
| Low-Flow 20   |       | 3.99  |
| Low-Flow 22   |       | 3.99  |
| Low-Flow 23   |       | 4.17  |
| Low-Flow 24   |       | 4.17  |
| Low-Flow 1     |       | 4.17  |
| Low-Flow 2     |       | 3.87  |
| Low-Flow 3     |       | 3.99  |
| Med-Flow 1     |       | 3.87  |
| Med-Flow 2     |       | 3.87  |
| Med-Flow 3     |       | 3.99  |
| Med-Flow 4     |       | 3.99  |
| Med-Flow 5     |       | 3.92  |
| Med-Flow 6     |       | 3.87  |
| Med-Flow 7     |       | 4.07  |
| Med-Flow 8     |       | 3.87  |
| Med-Flow 9     |       | 3.87  |
| Med-Flow 10    |       | 3.87  |
| Med-Flow 11    |       | 3.87  |
| Med-Flow 12    |       | 3.82  |
| Flow Type | Number | Value |
|-----------|--------|-------|
| Med-Flow  | 13     | 3.77  |
| Med-Flow  | 14     | 3.77  |
| Med-Flow  | 15     | 3.77  |
| Med-Flow  | 16     | 3.77  |
| Med-Flow  | 17     | 3.87  |
| Med-Flow  | 18     | 3.77  |
| Med-Flow  | 19     | 3.87  |
| Med-Flow  | 20     | 3.87  |
| Med-Flow  | 21     | 3.87  |
| Med-Flow  | 22     | 3.87  |
| Med-Flow  | 23     | 3.99  |
| Med-Flow  | 24     | 3.87  |
| Med-Flow  | Composite | 3.87 |
| High-Flow | 1      | 3.77  |
| High-Flow | 2      | 3.77  |
| High-Flow | 3      | 3.77  |
| High-Flow | 4      | 3.77  |
| High-Flow | 5      | 3.99  |
| High-Flow | 6      | 3.77  |
| High-Flow | 7      | 3.77  |
| High-Flow | 8      | 3.77  |
| High-Flow | 9      | 3.77  |
| High-Flow | 10     | 3.77  |
| High-Flow | 11     | 3.77  |
| High-Flow | 12     | 3.77  |
| High-Flow | 13     | 3.77  |
| High-Flow | 14     | 3.77  |
| High-Flow | 15     | 3.77  |
| High-Flow | 16     | 3.77  |
| High-Flow | 17     | 3.77  |
| High-Flow | 18     | 3.77  |
| High-Flow | 19     | 3.77  |
| High-Flow | 20     | 3.77  |
| High-Flow | 21     | 3.77  |
| High-Flow | 22     | 3.77  |
| High-Flow | 23     | 3.77  |
| High-Flow | 24     | 3.77  |
| High-Flow | Composite | 3.77 |
Table S5. The completed dMIQE checklist for the analysis of samples for this paper.

| ITEM TO CHECK | PROVIDED | COMMENT |
|---------------|----------|---------|
| 1. SPECIMEN   |          |         |
| Detailed description of specimen type and numbers | Y        | Included in the Methods section and SI |
| Sampling procedure (including time to storage) | Y        | Included in the Methods section |
| Sample aliquotation, storage conditions and duration | Y        | Included in the Methods section |
| 2. NUCLEIC ACID EXTRACTION |          |         |
| Description of extraction method including amount of sample processed | Y        | Included in the Methods section |
| Volume of solvent used to elute/resuspend extract | Y        | Included in the Methods section |
| Number of extraction replicates | Y        | Included in the Methods section |
| Extraction blanks included? | Y        | Included in the Methods section |
| 3. NUCLEIC ACID ASSESSMENT AND STORAGE |          |         |
| Method to evaluate quality of nucleic acids | N        | Not done |
| Method to evaluate quantity of nucleic acids (including molecular weight and calculations when using mass) | N        | N/A |
| Storage conditions: temperature, concentration, duration, buffer, aliquots | Y        | Included in the Methods section |
| Clear description of dilution steps used to prepare working DNA solution | N        | No dilutions were made. |
| 4. NUCLEIC ACID MODIFICATION |          |         |
| Template modification (digestion, sonication, pre-amplification, bisulphite etc.) | N        | N/A |
| Details of repurification following modification if performed | N        | N/A |
| 5. REVERSE TRANSCRIPTION |          |         |
| cDNA priming method and concentration | N        | N/A |
| One or two step protocol (include reaction details for two step) | Y        | One step and Included in Methods section |
| Amount of RNA added per reaction | Y        | Included in the Methods section |
| Detailed reaction components and conditions | Y        | Included in the Methods section |
| Estimated copies measured with and without addition of RT* | N        | Not done |
| 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        | N/A, since it is one step RT |
| 6. dPCR OLIGONUCLEOTIDES DESIGN AND TARGET INFORMATION |          |         |
| Sequence accession number or official gene symbol | Y        | Included in the Methods section |
| Method (software) used for design and in silico verification | Y        | Included in the Methods section |
| Location of amplicon | Y        | As reported in methods of Lu et al. 2020, and in Graham et al., 2021 |
| Amplicon length | Y        | Included in the SI and also Lu et al. 2020, and in Graham et al., 2021 |
| Primer and probe sequences (or amplicon context sequence)** | Y        | Included in SI, also in methods of Lu et al. 2020 and Decaro et al., 2008 |
| Location and identity of any modifications | N        | N/A |
| Manufacturer of oligonucleotides | Y        | Included in the Methods section |
| 7. dPCR PROTOCOL |          |         |
| Manufacturer of dPCR instrument and instrument model | Y        | Included in the Methods section |
| Buffer/kit manufacturer with catalogue and lot number | Y        | Included in the Methods section |
| Primer and probe concentration | Y        | Included in the Methods section |
| Pre-reaction volume and composition (incl. amount of template and if restriction enzyme added) | Y        | Included in the Methods section |
| Template treatment (initial heating or chemical denaturation) | N        | N/A |
| Polymerase identity and concentration, Mg++ and dNTP concentrations*** | N        | N/A |
| Complete thermocycling parameters | Y        | Included in the Methods section |
| 8. ASSAY VALIDATION |          |         |
| Details of optimisation performed | N        | Not performed, optimized commerical assays were utilized. |
| Analytical specificity (vs. related sequences) and limit of blank (LOB) | Y        | Included in SI |
| Analytical sensitivity/LoD and how this was evaluated | Y        | Included in SI |
| Testing for inhibitors (from biological matrix/extraction) | N        | Not done |
9. DATA ANALYSIS

| Description of dPCR experimental design | Y | Included in the Methods section |
| Comprehensive details negative and positive of controls (whether applied for QC or for estimation of error) | Y | Included in the Methods section |
| Partition classification method (thresholding) | Y | Included in the Methods section |
| Examples of positive and negative experimental results (including fluorescence plots in supplemental material) | Y | Included in SI |
| Description of technical replication | Y | Included in the Methods section |
| Reproducibility (intra-experiment variation) | Y | Included in SI |
| Reproducibility (inter-experiment/user/lab etc. variation) | N | Not performed |
| Number of partitions measured (average and standard deviation) | Y | Included in the Methods section |
| Partition volume | N | Not estimated |
| Copies per partition (K or equivalent) (average and standard deviation) | Y | Included in the Methods section |
| dPCR analysis program (source, version) | Y | Included in the Methods section |
| Description of normalization method | Y | Included in SI |
| Statistical methods used for analysis | Y | Included in the Methods section |
| Data transparency | raw data available on request |

References

(1) 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.

(2) M. Pecson, B.; Darby, E.; N. Haas, C.; M. Amha, Y.; Bartolo, M.; Danielson, R.; Dearborn, Y.; Giovanni, G. D.; Ferguson, C.; Fevig, S.; Gaddis, E.; Gray, D.; Lukasik, G.; Mull, B.; Olivas, L.; Olivieri, A.; Qu, Y.; Consortium, S.-C.-2 I. Reproducibility and Sensitivity of 36 Methods to Quantify the SARS-CoV-2 Genetic Signal in Raw Wastewater: Findings from an Interlaboratory Methods Evaluation in the U.S. *Environ. Sci. Water Res. Technol.* 2021, 7 (3), 504–520. https://doi.org/10.1039/D0EW00946F.

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

(4) Lu, X.; Wang, L.; Sakthivel, S. K.; Whitaker, B.; Murray, J.; Kamili, S.; Lynch, B.; Malapati, L.; Burke, S. A.; Harcourt, J.; Tamin, A.; Thornburg, N. J.; Villanueva, J. M.; Lindstrom, S. US CDC Real-Time Reverse Transcription PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2 - Volume 26, Number 8—August 2020 - Emerging Infectious Diseases Journal - CDC. *2020*. https://doi.org/10.3201/eid2608.201246.

(5) dMIQE Group; Huggett, J. F. The Digital MIQE Guidelines Update: Minimum Information for Publication of Quantitative Digital PCR Experiments for 2020. *Clin. Chem.* 2020, 66 (8), 1012–1029. https://doi.org/10.1093/clinchem/hvaa125.