Supporting Information

Detection, Quantification, and Simplified Wastewater Surveillance Model of SARS-CoV-2 RNA in the Tijuana River

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Supplemental Materials and Methods

Samples were analyzed for SARS-CoV-2 (accession number: MN908947.3) nucleocapsid (N) gene via N1 region with starting position 28287 and N2 region with starting position 29164, as well as PMMoV (accession number: NC_003630.1) with starting position 1878. The US CDC has stated that these two primers for SARS-CoV-2 provide useful information for epidemiology and surveillance, and they have been widely used for wastewater surveillance. In addition, an in silico BLAST (performed on March 28, 2022) using the primer set from N1 on the nr database in NCBI did not yield hits on any genes or genomes except for those associated with SARS-CoV-2, even when allowing up to two total mismatches.

The Taqman® Fast Virus 1-step master mix includes AmpliTaq® Fast DNA Polymerase, Thermostable MMLV enzyme, dNTPs (dATP, dGTP, dCTP, and dTTP), RNaseOUT™ Recombinant Ribonuclease Inhibitor, ROX™ dye (passive reference), and buffer components to control several common RT-PCR inhibitors. Results were analyzed via the ThermoFisher Design and Analysis software (v1.5.2). For RT-qPCR, the threshold was set at 0.09 for both nCoV-N1 and nCoV-N2 and 0.06 for PMMoV.

The LOD for RT-qPCR was determined for each assay using the pooled data from standards analyzed on all plates via Microsoft Excel, Version 2202. For nCoV-N1 and nCoV-N2, the LOD was 8 and 8.5 copies/reaction at 95% probability, respectively. For PMMoV the LOD was 5 copies/reaction at 95% probability. For SARS-CoV-2 plasmids, the DNA concentration range measured was 4 - 400,000 gc/rxn where the lowest dilution 4gc/rxn resulted in a standard deviation of 0.67 for all detected Cq values determined with N1 assay and 1.02 for all detected Cq values determined with N2 assay. For PMMoV gBlocks, the range measured was 1.56 – 1,560,000 gc/rxn where the lowest dilution 1.56gc/rxn resulted in a standard deviation of 1.3 for all detected Cq values.

Intra-assay repeatability for samples was checked by calculating the standard deviation of Cq values for technical replicates. The standard deviations were below 0.1 Cq units for most samples and were below 0.5 for all samples for the N1 and PMMoV assays. Only for the N2 assay, the standard deviation of the Cq values for technique replicates was greater than 0.5 for samples from Radio Club collected on 7/31/2020, 8/6/2020, and 3/14/2021. However, the Cq values of N2 for these three samples were close to and below the threshold associated with a 95% probability of detection.

Sample concentrations were determined via Microsoft Excel, Version 2202 using the Cq values obtained, based on the values of the standard curves analyzed on the same plate as the samples (to factor out any variability between plates). Visual outliers which corresponded to likely errors in pipetting or in the preparation of dilutions, such as wells that were unreliable due to human error were not included in the compiling of the standard curve for each assay. The plasmids and gBlocks are double stranded but the sample target RNA is single stranded, so the concentrations of the standards were divided by two prior to the completion of the regression to adjust for the offset of amplification of RNA in our samples compared to our standards.

A conversion quantified the number of copies per liter of sample using subsequent volumes that were filtered, extracted, reverse transcribed, and analyzed.

\[
N = \frac{1}{C\%} \times \frac{1}{P\%} \times \frac{1}{D\%} \times \frac{1}{K\%} \times \frac{G}{V} \times \frac{1000mL}{L}
\]
Where C\% is the conversion factor of cDNA to RNA which was assumed to be 100% for one-step RT-qPCR kits, P\% is the percent of extracted DNA used in qPCR in which 2 µL of sample was used in each well/50 µL extracted, D\% is dilution of RNA sample which was either never diluted (100\%) or diluted at 10\%, R\% is the nucleic acid recovery during the extraction process and assumed to be 100\%, G is the concentration in copies/reaction unit as determined by Cq Values, and V is the volume of sample (mL) filtered through the 0.45 µm membrane. It should be noted that recovery was not measured using matrix spikes in this study, but it has previously been reported for murine hepatitis virus (MHV, a surrogate of SARS-CoV-2) to be 65\% using the adsorption-extraction method with MgCl$_2$ addition, and 27\% using the adsorption-extraction method with acidification but no MgCl$_2$ addition$^3$. As such, the concentrations reported here may be underestimates of the true concentrations.

The Qubit 4 Fluorometer was used with the Qubit RNA XR Assay - Extended Range kit for quantification of RNA in extracted samples, and the Qubit 1X dsDNA HS Assay Kit was used for quantification of DNA in gBlock standards. The purity and yield of gBlocks and plasmids were quantified and verified by the manufacturer (IDT).

To reduce the possibility of cross-contamination, master mixes were prepared separately from samples and standards, in an AirClean 600 PCR Workstation. A biosafety cabinet was used to add samples and standards to the plate after the master mix was already aliquoted. Different sets of pipettes were also used in each of these stations to further reduce the possibility of nucleic acid contamination in the PCR workstations. Gloves were replaced after handling standards as an additional precaution. Spaces used for processing samples were disinfected before and after with 10% bleach, 70% ethanol, and UV light. All equipment used to concentrate samples, such as beakers, graduated cylinders, and filtration devices were autoclaved using the Hirayama Hiclave HVA-100.

2 Supplemental Discussion

To assess the impact of differences in the fecal strength of Tijuana River samples$^1$, concentrations of SARS-CoV-2 were normalized by PMMoV (Figure S1). However, normalizing to PMMoV in this setting was not consistent from sample to sample, potentially suggesting that there is less impact of non-fecal sources of pollution and no need to normalize especially since it is a smaller sewer-shed.
3 Supplemental Figures

**Figure S1.** Changes in accumulating shedding COVID-19 cases with time and SARS-CoV-2 concentrations normalized to respective PMMoV concentrations at Tijuana River Radio Club site.
Figure S2. RT-qPCR Probability of Detection for nCoV-N1, nCoV-N2, and PMMoV.
4 Supplemental Tables

**Table S1.** Physical and chemical water quality parameters measured at the Boca Rio and Radio Club sampling sites

| Date     | DO (mg/L) | pH  | Cond. (mS/cm) | TDS (g/L) | Turb. (NTU) | COD (mg/L) | DOC (mg/L) | TN (mg/L) |
|----------|-----------|-----|---------------|-----------|-------------|------------|------------|-----------|
| **Boca Rio** |           |     |               |           |             |            |            |           |
| 31 Jul 2020 | 19.63     | 8.71 | 36.18         | 18.45     | 3.48        | NM^a       | 2.30       | 0.26      |
| 6 Aug 2020  | NA^b      | 7.76 | 38.05         | 19.05     | 7.22        | NM^a       | 6.88       | 1.59      |
| 28 Aug 2020 | 10.51     | 8.01 | 98.87         | 29.00     | 4.26        | NM^a       | 3.45       | 0.43      |
| 10 Nov 2020 | NA^b      | 7.16 | 44.97         | 22.53     | 11.62       | NM^a       | 7.03       | 3.94      |
| 30 Dec 2020 | 10.35     | 7.90 | 51.67         | 25.83     | 4.92        | NM^a       | 1.50       | 0.42      |
| 27 Jan 2021 | 9.72      | 7.99 | 55.10         | 26.53     | 7.28        | NM^a       | 2.53       | 1.10      |
| 14 Mar 2021 | 10.35     | 8.13 | 57.30         | 29.60     | 2.58        | NM^a       | 1.71       | 0.50      |
| 5 May 2021  | 5.54      | 7.98 | 56.80         | 29.60     | 7.65        | NM^a       | 2.98       | 0.84      |
| **Arithmetic Average** | 11.02 | 7.96 | 54.87         | 25.08     | 6.13        | NM^a       | 3.55       | 1.13      |
| **Standard Deviation** | 4.63 | 0.43 | 19.59         | 4.56      | 2.92        | NM^a       | 2.20       | 1.22      |
| **Radio Club** |         |     |               |           |             |            |            |           |
| 31 Jul 2020 | 0.49      | 7.92 | 2.44          | 1.22      | 59.05       | 256        | 16.56      | 32.20     |
| 6 Aug 2020  | NA^b      | 7.59 | 2.65          | 1.32      | 45.55       | 201        | 18.00      | 31.43     |
| 28 Aug 2020 | 3.75      | 7.96 | 4.97          | 1.49      | 90.00       | 234        | 35.53      | 9.33      |
| 10 Nov 2020 | NA^b      | 7.45 | 2.87          | 1.44      | 29.57       | 180        | 39.35      | 61.14     |
| 30 Dec 2020 | 6.01      | 7.91 | 2.97          | 1.49      | 59.47       | 144        | 38.80      | 55.99     |
| 27 Jan 2021 | 8.22      | 7.92 | 3.00          | 1.58      | 45.47       | 159        | 23.28      | 23.71     |
| 14 Mar 2021 | 6.01      | 7.71 | 2.79          | 1.46      | 23.87       | 113        | 18.47      | 56.26     |
| 5 May 2021  | 8.08      | 8.69 | 2.75          | 1.46      | 47.63       | 243        | 55.38      | 17.41     |
| **Arithmetic Average** | 5.43 | 7.89 | 3.05          | 1.43      | 50.08       | 191        | 30.67      | 35.93     |
| **Standard Deviation** | 2.92 | 0.37 | 0.79          | 0.11      | 20.42       | 51         | 13.83      | 19.59     |

Note: Dissolved oxygen (DO); electrical conductivity (Cond.); turbidity (Turb.); chemical oxygen demand (COD); dissolved organic carbon (DOC); total nitrogen (TN).

^a Not measurable (NM) due to high chlorine interference.

^b Not available (NA) due to inavailability of DO meter.
**Table S2.** Collection times and field conditions during sampling dates of Tijuana River

| Sample Collection Date | Collection Time at Radio Club | Collection Time at Boca Rio | 24-h Average Flow Rate at International Boundary (m³/h) | Instantaneous Flow Rate at International Boundary² (m³/h) | 72-h Precipitation Accumulated (mm) | Antecedent Dry Daysᵇ (d) | Mean Lower Low Water Tide Levelᶜ (m) | Field Notes |
|------------------------|-------------------------------|-------------------------------|---------------------------------------------------|---------------------------------------------------|---------------------------------|---------------------|-------------------------------------|-------------|
| 31 Jul 2020            | 9:20                          | 10:14                         | 1.15E+03                                          | 6.1E+02                                          | 0.0                             | 32                  | 1.1                                 |             |
| 6 Aug 2020             | 10:05                         | 11:05                         | 3.72E+02                                          | 1.1E+01                                          | 0.0                             | 38                  | 1.2                                 |             |
| 28 Aug 2020            | 12:20                         | 13:20                         | 2.90E+02                                          | 0.00E+00                                         | 0.0                             | 60                  | 0.8                                 | Stagnant flow |
| 10 Nov 2020            | 9:40                          | 10:40                         | 8.68E+02                                          | 3.6E+02                                          | 7.6                             | 2                   | 0.6                                 |             |
| 30 Dec 2020            | 10:16                         | 11:12                         | 4.94E+03                                          | 5.8E+03                                          | 14.0                            | 3                   | 1.3                                 |             |
| 27 Jan 2021            | 10:05                         | 11:10                         | 1.10E+04                                          | 9.4E+03                                          | 19.6                            | 2                   | 0.9                                 |             |
| 14 Mar 2021            | 10:20                         | 11:28                         | 8.32E+03                                          | 6.2E+03                                          | 48.2                            | 2                   | 1.5                                 |             |
| 5 May 2021             | 10:01                         | 10:33                         | 0.00E+00                                          | 0.00E+00                                         | 0.0                             | 51                  | 0.3                                 | Stagnant flow; Abnormal green color |

ᵃ Instantaneous flow rate measurements were obtained from the International Boundary Water Commission at the time of collection at Radio Club to the nearest 15-minute interval.

ᵇ Dry days were observed as 2.5 mm or less of precipitation, if any, following San Diego Water Board guidelines.

c Mean lower low water tide levels were obtained from the National Oceanic and Atmospheric Administration Imperial Beach, California station located 2.5 km northwest of Boca Rio.
| Sample Collection Date | Effective Volume Filtered (mL) | Fraction of RNA Analyzed | Equivalent Volume Analyzed (mL)<sup>b</sup> |
|------------------------|-------------------------------|--------------------------|---------------------------------------------|
| Boca Rio               |                               |                          |                                             |
| 31 Jul 2020            | 198                           | 2µL/50µL = 0.04          | 7.9                                         |
| 6 Aug 2020             | 198                           | 2µL/50µL = 0.04          | 7.9                                         |
| 28 Aug 2020            | 495<sup>a</sup>              | 2µL/50µL = 0.04          | 19.8                                        |
| 10 Nov 2020            | 297                           | 2µL/50µL = 0.04          | 11.9                                        |
| 30 Dec 2020            | 198                           | 2µL/50µL = 0.04          | 7.9                                         |
| 27 Jan 2021            | 396                           | 2µL/50µL = 0.04          | 15.8                                        |
| 14 Mar 2021            | 495                           | 2µL/50µL = 0.04          | 19.8                                        |
| 5 May 2021             | 495                           | 2µL/50µL = 0.04          | 19.8                                        |
| Radio Club             |                               |                          |                                             |
| 31 Jul 2020            | 48.6                          | 2µL/50µL = 0.04          | 1.9                                         |
| 6 Aug 2020             | 49.5                          | 2µL/50µL = 0.04          | 2.0                                         |
| 28 Aug 2020            | 99.0<sup>a</sup>             | 2µL/50µL = 0.04          | 4.0                                         |
| 10 Nov 2020            | 49.5                          | 2µL/50µL = 0.04          | 2.0                                         |
| 30 Dec 2020            | 39.6                          | 2µL/50µL = 0.04          | 1.6                                         |
| 27 Jan 2021            | 69.3                          | 2µL/50µL = 0.04          | 2.8                                         |
| 14 Mar 2021            | 69.3                          | 2µL/50µL = 0.04          | 2.8                                         |
| 5 May 2021             | 99.0                          | 2µL/50µL = 0.04          | 4.0                                         |

<sup>a</sup> Samples were pre-filtered using 8µm filter

<sup>b</sup> Equivalent Volume Analyzed = Equivalent Volume Filtered * Fraction of RNA Analyzed
| Assay   | Sequence (5'-3')                                                                 | Amplicon Length | Accession Number (Start Position) | Reference(s) |
|---------|---------------------------------------------------------------------------------|-----------------|-----------------------------------|---------------|
| nCoV-N1 | F: GACCCCAAAATCAGCGAAAT R: TCTGGTTACTGCCAGTTGAATCTG [FAM]-ACCCCGCATTAAGTTGGTGAGCC-[BHQ1] | 72 bp           | MN908947.3 (28287)                | 4             |
| nCoV-N2 | F: TTACAAACATTTGGCGCAGAAA R: GCGCGACATTCCGAAGAA [FAM]-ACAATTTGCCCCAGCGCTTCAG-[BHQ1] | 67 bp           | MN908947.3 (29164)                | 4             |
| PMMoV   | F: GAGTGGTTTGACCTTAACGTTTGA R: TTGTCGGTTGCAATGCAAGT [FAM]-CCTACCGAGCGTAATG-[MGB] | 68 bp           | NC_003630.1 (1878)                | 5,6           |

*Primers were purified by standard desalting; probes were HPLC purified*
Table S5. Slope, y-intercept, and efficiency of nCoV-N1, nCoV-N2, and PMMoV assays using TaqMan Fast Virus One Step RT-qPCR

| Parameter   | nCoV-N1 | nCov-N2 | PMMoV |
|-------------|---------|---------|-------|
| slope       | -3.20   | -3.28   | -3.41 |
| intercept   | 37.27   | 39.15   | 37.28 |
| efficiency  | 105%    | 102%    | 96%   |
| $R^2$       | 0.9557  | 0.9559  | 0.9779 |
Table S6. MIQE checklist: Essential and Desirable Information

| ITEM TO CHECK                                      | IMPORTANCE | CHECKLIST                                      |
|---------------------------------------------------|------------|------------------------------------------------|
| **EXPERIMENTAL DESIGN**                           |            |                                                |
| Definition of experimental and control groups     | E          | Included in Materials and Methods              |
| Number within each group                          | E          | Included in Materials and Methods              |
| Assay carried out by core lab or investigator's lab? | D          | Included in Materials and Methods              |
| Acknowledgement of authors’ contributions         | D          | Included in Acknowledgements                   |
| **SAMPLE**                                        |            |                                                |
| Description                                       | E          | Included in Materials and Methods/SI           |
| Volume/mass of sample processed                   | D          | Included in Materials and Methods/SI           |
| Microdissection or macrodissection                | E          | Not Applicable                                 |
| Processing procedure                              | E          | Included in Materials and Methods              |
| If frozen - how and how quickly?                  | E          | Included in Materials and Methods              |
| If fixed - with what, how quickly?                | E          | Not Applicable                                 |
| Sample storage conditions and duration (especially for FFPE samples) | E          | Included in Materials and Methods              |
| **NUCLEIC ACID EXTRACTION**                       |            |                                                |
| Procedure and/or instrumentation                 | E          | Included in Materials and Methods              |
| Name of kit and details of any modifications      | E          | Included in Materials and Methods              |
| Source of additional reagents used                | D          | Not Applicable                                 |
| Details of DNase or RNase treatment               | E          | Not Applicable                                 |
| Contamination assessment (DNA or RNA)             | E          | Included in Materials and Methods              |
| Nucleic acid quantification                       | E          | Included in SI                                 |
| Instrument and method                             | E          | Included in SI                                 |
| Purity (A260/A280)                                | D          | Included in SI                                 |
| Yield                                             | D          | Included in SI                                 |
| RNA integrity method/instrument                    | E          | Not Determined                                 |
| RIN/RQI or Cq of 3’ and 5’ transcripts            | E          | Not Determined                                 |
| Electrophoresis traces                            | D          | Not Determined                                 |
| Inhibition testing (Cq dilutions, spike or other) | E          | Included in Materials and Methods              |
| **REVERSE TRANSCRIPTION**                         |            |                                                |
| Complete reaction conditions                      | E          | Included in Materials and Methods              |
| Amount of RNA and reaction volume                 | E          | Included in Materials and Methods              |
| Priming oligonucleotide (if using GSP) and concentration | E          | Not Applicable                                 |
| Reverse transcriptase and concentration            | E          | Included in Materials and Methods              |
| Temperature and time                               | E          | Included in Materials and Methods              |
| Manufacturer of reagents and catalogue numbers | Included in Materials and Methods |
|-----------------------------------------------|----------------------------------|
| Cqs with and without RT                        | D*                               |
| Storage conditions of cDNA                     | D                                |
| **qPCR TARGET INFORMATION**                   |                                  |
| Gene symbol                                    | E                                |
| Sequence accession number                      | E                                |
| Location of amplicon                           | D                                |
| Amplicon length                                | E                                |
| *In silico* specificity screen (BLAST, etc)    | E                                |
| Pseudogenes, retropseudogenes or other homologs? | D                                |
| Sequence alignment                             | D                                |
| Secondary structure analysis of amplicon       | D                                |
| Location of each primer by exon or intron (if applicable) | E  |
| What splice variants are targeted?             | E                                |
| **qPCR OLIGONUCLEOTIDES**                     |                                  |
| Primer sequences                               | E                                |
| RTPrimerDB Identification Number               | D                                |
| Probe sequences                                | D**                              |
| Location and identity of any modifications     | E                                |
| Manufacturer of oligonucleotides               | D                                |
| Purification method                            | D                                |
| **qPCR PROTOCOL**                              |                                  |
| Complete reaction conditions                   | E                                |
| Reaction volume and amount of cDNA/DNA         | E                                |
| Primer, (probe), Mg++ and dNTP concentrations  | E                                |
| Polymerase identity and concentration          | E                                |
| Buffer/kit identity and manufacturer           | E                                |
| Exact chemical constitution of the buffer      | D                                |
| Additives (SYBR Green I, DMSO, etc.)           | E                                |
| Manufacturer of plates/tubes and catalog number| D                                |
| Complete thermocycling parameters              | E                                |
| Reaction setup (manual/robotic)                | D                                |
| Manufacturer of qPCR instrument               | E                                |
| **qPCR VALIDATION**                            |                                  |
| Evidence of optimisation (from gradients)      | D                                |
| Specificity (gel, sequence, melt, or digest)   | E                                |
| Feature                                                | Requirement | Description                                    |
|--------------------------------------------------------|-------------|------------------------------------------------|
| For SYBR Green I, Cq of the NTC                         | E           | Not Applicable                                 |
| Standard curves with slope and y-intercept             | E           | Included in SI                                 |
| PCR efficiency calculated from slope                   | E           | Included in SI                                 |
| Confidence interval for PCR efficiency or standard error| D           | Not Determined                                 |
| R² of standard curve                                   | E           | Included in SI                                 |
| Linear dynamic range                                   | E           | Included in SI                                 |
| Cq variation at lower limit                            | E           | Included in SI                                 |
| Confidence intervals throughout range                  | D           | Not Determined                                 |
| Evidence for limit of detection                        | E           | Included in SI                                 |
| If multiplex, efficiency and LOD of each assay.        | E           | Not Applicable                                 |

**DATA ANALYSIS**

| Feature                                                | Requirement | Description                                    |
|--------------------------------------------------------|-------------|------------------------------------------------|
| qPCR analysis program (source, version)                | E           | Included in SI                                 |
| Cq method determination                                | E           | Included in SI                                 |
| Outlier identification and disposition                 | E           | Included in SI                                 |
| Results of NTCs                                        | E           | Included in Materials and Methods              |
| Justification of number and choice of reference genes  | E           | Not Applicable                                 |
| Description of normalisation method                    | E           | Included in SI                                 |
| Number and concordance of biological replicates        | D           | Not Determined                                 |
| Number and stage (RT or qPCR) of technical replicates  | E           | Included in Materials and Methods              |
| Repeatability (intra-assay variation)                  | E           | Included in SI                                 |
| Reproducibility (inter-assay variation, %CV)           | D           | Not Determined                                 |
| Power analysis                                         | D           | Not Determined                                 |
| Statistical methods for result significance            | E           | Not Applicable                                 |
| Software (source, version)                             | E           | Included in SI                                 |
| Cq or raw data submission using RDML                   | D           | Not Provided                                   |
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