Quantification of the SARS-CoV-2 using electronegative membrane filtration and dPCR V.2

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This protocol was developed in an effort to serve as a timely and efficient method for the surveillance of the SARS-CoV-2 in primary influent wastewater samples. This process describes viral concentration via electronegative adsorption with pH adjustment, RNA extraction using the MP Biomedicals FastPrep-24 and the Qiagen AllPrep PowerViral DNA/RNA kits with the Qiacube Connect, and quantification of the N1 and N2 genes in SARS-CoV-2 using the GT Digital SARS-CoV-2 Wastewater Surveillance for QIAcuity.

To compile the entire process from beginning to the end some sections were taken from the AllPrep PowerViral DNA/RNA Kit (Qiagen) and the GT Digital SARSCoV- 2 Wastewater Surveillance for QIAcuity v1.0 (GT Molecular) handbooks.

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Equipment:

MP Biomedicals™ FastPrep -24™ Classic Instrument
Benchtop homogenizer
Fisher Scientific **12079310**

QIAcube Connect
Automated nucleic acid extraction
Qiagen **9002864**

QIAcuity One, 5-plex
dPCR
Qiagen 911021

Reagents:

Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445** In 2 steps

Hydrochloric acid **Sigma Aldrich** Step 6

Zymo DNA/RNA Shield **Fisher Scientific Catalog #50-125-1706** In 2 steps

2-Mercaptoethanol **Sigma Aldrich**

Qiagen AllPrep PowerViral DNA/RNA Extraction Kit **Qiagen Catalog #28000-50** In 2 steps

Qiagen QIAcuity One-Step Viral RT-PCR Kit **Qiagen Catalog #1123145** Step 22

GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity **GT Molecular** Step 22

Consumables:

50 mL conical tubes, sterile, nuclease-free
Pall Microfunnel Filter Funnel with GN-6 Membrane, 100 mL, 0.45 m, sterile (Fischer Scientific, Catalog #: NC0553687)
Qiagen Sample Tubes (Qiagen, Catalog #: 990381)
Qiagen Rotor Adapters (Qiagen, Catalog #: 990394)
Qiagen QIAcuity Filter Tips, 1000 microL (Qiagen, Catalog #: 990352)
PCR tubes, RNase free, low-binding
Qiagen QIAcuity Nanoplate 26k 24-well (Qiagen, Catalog #: 250002)

In addition to the materials listed above, this protocol requires access to typical microbiology equipment including pipettes, sterile, nuclease-free, filter barrier pipette tips, microcentrifuge, a Class II biosafety cabinet, a PCR cabinet, a vacuum pump, vacuum flask and stopper, vortexes, and standard PPE.

Prior to processing wastewater samples for the detection of the SARS-CoV-2, work

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with your local biosafety committee to establish biosafety protocols, and review the CDC Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19) available at https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html#environmental

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Sample Collection 1d

1 Composite primary influent samples are collected over 24 hours with several samples taken each hour.

2 **100 mL** sub-samples are collected in 2 x 50 mL sterile conical tubes. The samples are stored at **4 °C** until further processing.

Concentration of Viral Fraction 1h

3

These steps should be performed in a Class II Biosafety Cabinet, which has been cleaned with RNase Away and water.

4 Thaw an aliquot of $BCoV^{working}$ (Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**) **On ice**
(see section Preparation of Bovilis Coronavirus).

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5 Add **62 µL** of BCoV^{working} to each **50 mL** wastewater sample and invert to mix.

6 Adjust the pH to 3.5 using Hydrochloric acid **Sigma Aldrich**, mixing the sample between titrations.

7 Setup the filtration apparatus by attaching tubing to the vacuum port of the vacuum flask and to the vacuum pump. Place a microfunnel on top of the vacuum flask stopper.

8 Use a 25 mL serological pipette to transfer **25 mL** of wastewater sample into the microfunnel apparatus and turn on the vacuum pump. Once the entire sample has passed through the filter, remove the upper section of the microfunnel; the filter should remain on the lower portion of the microfunnel.

9 Submerge two forceps in 70% ethanol and sterilize using a lit flame. While leaving the vacuum on to hold the filter in place, use both forceps to gently roll the filter tightly enough to fit inside a 2 mL tube. Place the rolled filter into a Glass Bead tube, a consumable included in the Qiagen AllPrep PowerViral DNA/RNA Extraction Kit **Qiagen Catalog #28000-50** .

10 Add **500 µL** of 1x Zymo DNA/RNA Shield **Fisher Scientific Catalog #50-125-1706** to the Glass Bead tube and vortex to mix.

Nucleic Acid Extraction 2h

11

Proper aseptic technique should be used when working with RNA. Always wear powder-free latex, vinyl, or nitrile gloves while handling reagents, tubes, and RNA samples to prevent RNase contamination from the surface of the skin or from the environment. Change gloves frequently and keep tubes closed. During the procedure work quickly and keep everything on cold blocks when possible, to avoid degradation of RNA by endogenous or residual RNAses. Clean working surfaces and

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pipettes with RNase away.

The first step should be performed in the Class II Biosafety Cabinet decontaminated with UV light and RNase away. After the lysis solutions have been applied in step 9, the additional steps can take place outside of the Biosafety Cabinet.

12 Warm PM1 from the Qiagen AllPrep PowerViral DNA/RNA Extraction Kit **Qiagen Catalog #28000-50** at **55 °C** for 5-10 minutes before use. Add **6 µL** 2-mercaptoethanol **Sigma Aldrich Catalog #M6250** and **600 µL** PM1 to the Glass Bead tube.

Also add these reagents to an empty Glass Bead with **500 µL** of Zymo DNA/RNA Shield **Fisher Scientific Catalog #50-125-1706** to serve as the extraction control.

13 Place the Glass Bead tubes in balanced positions in the FastPrep-24 Instrument and run 4 cycles of 20 seconds each at 4.5 m/s. Ensure that the spoke plate has been rotated so that the tube lids are held down.

MP Biomedicals™ FastPrep -24™ Classic Instrument
Benchtop homogenizer
Fisher Scientific **12079310**

14 Centrifuge the Glass Bead tubes for 1 minute at **16000 x g** .

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15 Place the spin columns and elution tubes in their appropriate locations on the Qiagen QIAcube rotor adapter. Transfer the supernatant from the Glass Bead tubes into the center tube of the rotor adapter.

16 Place the QIAcube rotor adapters in the QIAcube centrifuge and follow the instructions on the QIAcube control tablet to set up the shaker rack, reagents, and tips. When setting up the reagents, shake to mix the PM5 buffer. Set the elution volume to **100 µL** . Start the extraction run.

QIAcube Connect
Automated nucleic acid extraction
Qiagen **9002864**

17 When the extraction is completed, cap the elution tubes and begin the dPCR steps or store at **-80 °C** if the dPCR run will occur in the following days.

18 Discard the used pipette tips and wipe the waste drawer and QIAcube workspace with 70% ethanol. After each run, remove the plastic tube holder and the reagent tray before running 2 cycles of UV decontamination.

Detection and Quantification of SARS-CoV-2 4h

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Technical notes:

The dPCR instrument is sensitive to microbubbles. To prevent errors in quantification and imaging, ensure that pipettes only reach the first stop when preparing this reaction.

The preparation of the dPCR plate should be done in a PCR workstation previously decontaminated by UV light and has been cleaned with RNase away and water.

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20 Thaw GT-Molecular controls and assay solutions on ice. If necessary, also thaw the extracted RNA on ice. Once thawed, vortex to mix.

21 Dilute **1 µL** extracted RNA with **99 µL** RNase-free water for PMMoV analysis.

22 Prepare master mixes for PMMoV and N1-N2-BCoV assays. Allow for one extra sample. Vortex to mix.

N1-N2-BCoV Master Mix Components	Volume/sample (microL)
Qiagen 4x One-Step Viral RT-PCR Master Mix	10
Qiagen 100x Multiplex Reverse Transcription Mix	0.4
GT Molecular N1-N2-BCoV Assay Solution	2.0
RNase/DNase free water	7.6

N1-N2-BCoV Master Mix

PMMoV Master Mix Components	Volume/sample (microL)
Qiagen 4x One-Step Viral RT-PCR Master Mix	10
Qiagen 100x Multiplex Reverse Transcription Mix	0.4
GT Molecular PMMoV Assay Solution	2.0
RNase/DNase free water	7.6

PMMoV Master Mix

GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity **GT Molecular**

Qiagen QIAcuity One-Step Viral RT-PCR Kit **Qiagen Catalog #1123145**

23 Pipette **20 µL** of the appropriate master mix (N1-N2-BCoV or PMMoV) into the wells of a PCR strip tube.

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24 Add **20 µL** of extracted RNA sample or positive control to the PCR strip tube following the planned layout. Use the 1:100 diluted samples for the wells being used for the PMMoV assay. After transferring, pipette gently to mix. Keep the PCR strips on ice while loading.

25 For the non-template control: pipette **20 µL** of molecular grade water into a PCR tube in place of adding extracted RNA.

26 Place a Qiagen QIAcuity 26k 24-well Nanoplate onto the Nanoplate protection tray. If the tray is not used, dust can collect on the bottom side of the plate and interfere with the imaging step. Occasionally wipe the tray with 70% ethanol to clean dust.

27 Using a multichannel pipette, transfer **39 µL** of solution from the PCR strips to their respective location on the Nanoplate. Be careful to not transfer air bubbles during this step.

Do not push the pipette beyond the first stop during this step.

28 Carefully seal the Nanoplate with a Nanoplate seal and the roller provided with the QIAcuity Instrument.

29 Place the sealed plate in the plate drawer inside the QIAcuity instrument.

QIAcuity One, 5-plex
dPCR
Qiagen **911021**

30 Setup the plate by selecting "New Plate". Name the plate and choose the plate type "Nanoplate 26k 24-well".

31 In the dPCR Parameters section under the Priming tab, select the Qiagen Standard Priming

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Profile.

32 Under the Cycling tab create the cycling conditions shown below. These are the conditions recommended by the GT Molecular Wastewater Surveillance Guide.

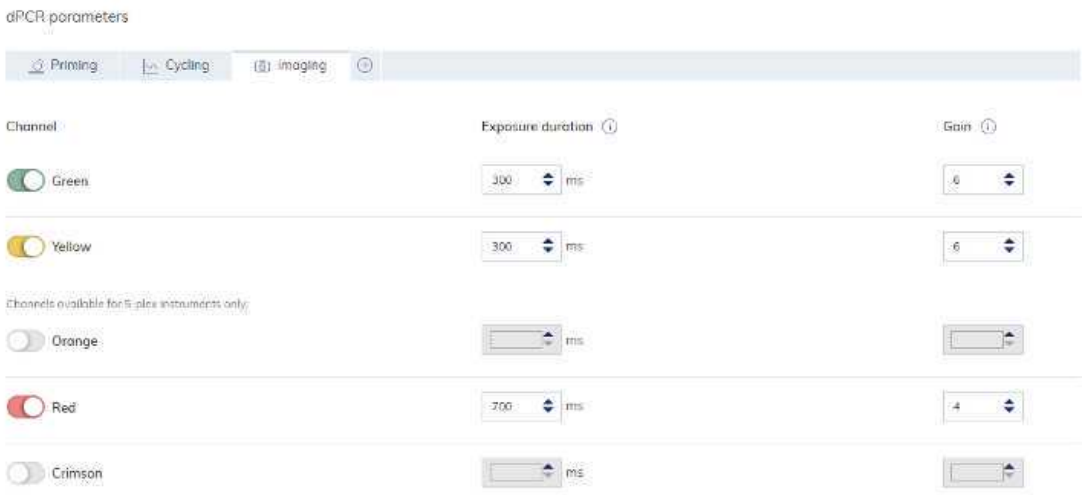
Cycling profile

Start (room temperature)

1 x	50.0 °C	30 min
1 x	95.0 °C	2 min
45 x	95.0 °C	10 s
	55.0 °C	30 s

End

33 In the Imaging tab create the conditions shown below. These are GT Molecular recommended.



Navigate to "Reaction mixes". Create reaction mixes named "N1-N2-BCoV Triplex Assay" and

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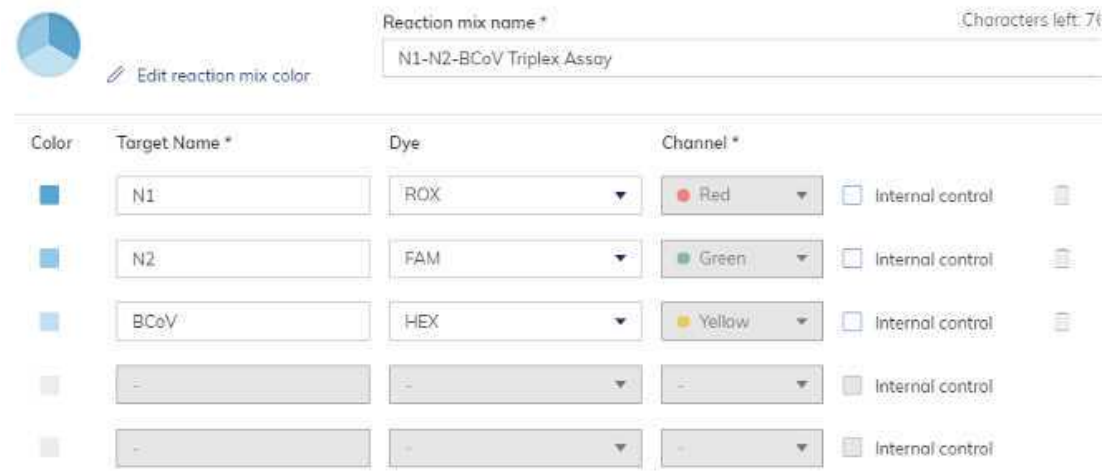
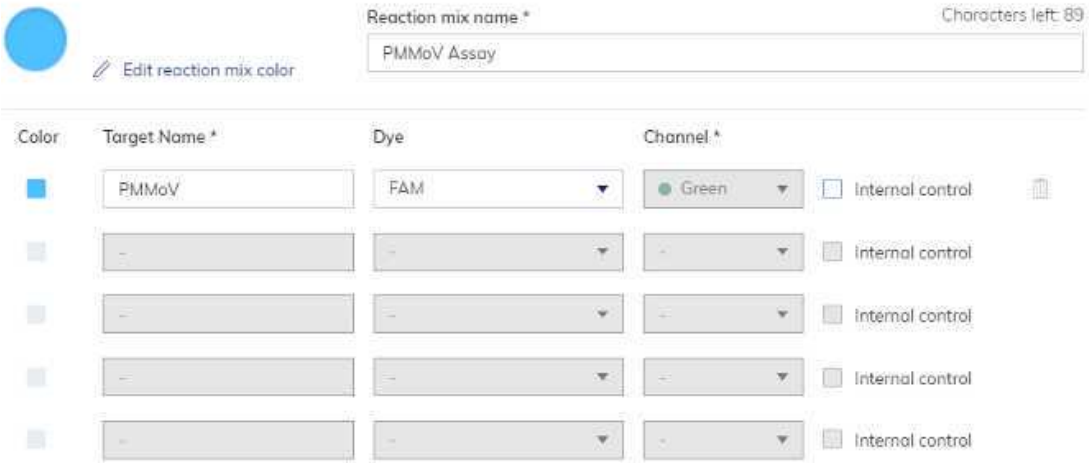
34 "PMMoV Assay" that contain the following details.





35 Navigate to "Samples and controls". Add samples names that are being quantified on this run. Extraction controls should be added as samples. Under the "Controls" tab create both a "N1-N2-BCoV Positive Control" and a "PMMoV Positive Control". Under the Non Template Controls" tab create a "N1-N2-BCoV dPCRNeg" and a "PMMoV dPCRNeg".

36 Navigate to "Plate Layout". Assign reaction mixes, samples and controls to their wells. Save plate and exit the setup.

37 On the QIAcuity tablet, select the plate and run the reaction.

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Analysis and Interpretations 15m

38 When the QIAcuity run is complete, ensure the image transfer is marked as complete in the QIAcuity Software Suite before inspecting the data.

39 Open the plate results by selecting "Analysis". Select all the wells and targets before selecting "Show results".

40 In the 1D Scatterplot tab verify that the automatic threshold is accurately placed between the negative and positive partitions. If needed, adjust the threshold placement to the accurate position.

41 Use the "Export to CSV" button in the List tab to export the data.

Index: Preparation of Bovilis Coronavirus 1h

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The Bovilis Coronavirus (BCoV) is used as internal process control and is added to each sample prior to filtration and extraction.

It is necessary to quantify BCoV for each new batch of $BCoV^{working}$ is made from a new tube of $BCoV^{ND}$.

BCoV should not be vortexed at any point in the preparation or spiking of samples.

43 Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445** is lyophilized when received. Reconstitute the virus in **5 mL** pre-chilled molecular grade water and swirl to mix.

44 Aliquot **100 µL** stock in sterile 1.5 mL tubes and label each tube $BCoV^{ND}$ (non-diluted). Store $BCoV^{ND}$ at **-80 °C**

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45 Dilute **60 µL** BCoVND with **540 µL** pre-chilled molecular grade water and pipette to mix. Label this tube BCoV^{INT} (intermediate).

46 Dilute **500 µL** BCoV^{INT} with **49.5 mL** pre-chilled molecular grade water and invert to mix. Aliquot **1 mL** of BCoV^{working} into sterile 1.5 mL tubes and store aliquots at **-80 °C** .

47 To quantify the BCoV spike, extract an aliquot of BCoV^{working} in triplicate.

47.1 Add **100 µL** of BCoV^{working} working into three 1.5 mL tubes.

47.2 To each tube add **6 µL** 2-mercaptoethanol and **600 µL** PM1 from the Qiagen AllPrep PowerViral DNA/RNA Kit and invert to mix.

47.3 Microcentrifuge the tubes at **13000 x g** for 1 minute.

47.4 Add the supernatant to the center column of a rotor adapter and continue the extraction in the Qiagen QIAcube.

48 Quantify the extracted BCoV^{working} RNA by analyzing the extraction triplicates using the same dPCR steps beginning in the Detection and Quantification section .

49 To calculate BCoV^{working} concentration, use the average measured concentration in copies/microL of the 3 replicates analyzed by dPCR.

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$BCoV^M$ is the average measured concentration in copies/ µL of the three replicates reported by the QIAcuity® dPCR system in step 20.

V^R is the reaction volume for QIACuity® of 40µL.

D^{Ext} is the extraction dilution factor that compensates for the dilution of the BCov template during RNA extraction in Step 17. The dilution factor for this protocol is 2.

V^T is the template volume of extracted BCoV RNA added to each reaction in µL.

$$[BCoV^{working}] = \frac{BCoV^M * V^R}{V^T} * D^{Ext}$$

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