May 27, 2022

Quantification of the SARS-CoV-2 using Nanotrap Particles®, the QIAcuity™ Digital PCR System, and GT-Digital SARS-CoV-2 Wastewater Surveillance Assay

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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 Nanotrap particles, RNA extraction using 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 as well as the Ceres Nano Manual Nanotrap Wastewater Protocol using Qiagen AllPrep PowerViral DNA/RNA Kit protocol (Ceres Nano).

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SARS-CoV-2, Wastewater, Wastewater Based Epidemiology

protocol ,

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Equipment:

DynaMag-2
Magnet
Invitrogen **12321D**

DynaMag-15
15 mL magnetic rack
Invitrogen **12301D**

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**

Zymo DNA/RNA Shield **Fisher Scientific Catalog #50-125-1706** Step 13

2-Mercaptoethanol **Sigma Aldrich**

Qiagen AllPrep PowerViral DNA/RNA Extraction Kit **Qiagen Catalog #28000-50** Step 15

Qiagen QIAcuity One-Step Viral RT-PCR Kit **Qiagen Catalog #1123145** Step 26

GT-Digital SARS-CoV-2 Wastewater Surveillance Assay for QIAcuity **GT Molecular** Step 26

Consumables:

50 mL conical tubes, sterile, nuclease-free
15 mL conical tubes, sterile, nuclease-free
Ceres Nano Nanotrap Enhancement Reagent 2 (Ceres Nano, Catalog #: 10112-10)
Ceres Nano Nanotrap Magnetic Particles, in solution (Ceres Nano, Catalog#: 44202)
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)

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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, vortexes, and standard PPE.

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

:

Sample Collection 1d

1 Composite primary influent samples are collected (grab samples, flow composite, or time composite)

2 **50 mL** sub-samples are collected in 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

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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). Pipette up and down to mix the aliquot and briefly centrifuge.

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

6 Using a serological pipette transfer **10 mL** of the wastewater sample into a 15 mL conical tube. Repeat this for a duplicate.

7 Add **100 µL** of Nanotrap Enhancement Reagent 2 to each 10 mL wastewater sample and vortex for a few seconds.

8 Add **150 µL** of Nanotrap Magnetic Virus Particles and invert 2-3 times to mix.

9 Incubate the samples for 10 minutes at room temperature.

10 Place the sample tubes in a 15 mL magnetic rack. Wait 1 minute. Remove the supernatant using a serological pipette. The magnetic particles form a pellet on the sides of the tube so it is essential to hold the tip of the pipette in the center of the tube while removing the supernatant. Transfer the supernatant to a waste container.

11 Add **1 mL** molecular grade water to each 15 mL tube and remove from magnetic rack. Invert the sample to collect the Nanotrap Magnetic Particles in the water.

12 Transfer the sample using a pipet to a 1.5 mL tube. Place the 1.5 mL tubes on a 1.5 mL

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magnetic rack for 1 minute to allow the magnetic particles to separate from the supernatant. Discard the supernatant.

13 Zymo DNA/RNA Shield **Fisher**

Add **200 µL** **Scientific Catalog #50-125-1706** to each sample and vortex to mix. If extraction is delayed, store samples up to 3 days at **4 °C** .

Nucleic Acid Extraction 2h

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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 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.

15 Warm PM1 from the Qiagen AllPrep PowerViral DNA/RNA Extraction Kit **Qiagen Catalog #28000-50** at **55 °C** for 5-10 minutes before use.

16 2-mercaptoethanol **Sigma**

Add **6 µL** **Aldrich Catalog #M6250** and **600 µL** PM1 to each 1.5 mL tube containing the concentrated sample and Nanotrap Magnetic Virus Particles. Vortex to mix.

17 Prepare an extraction control by adding **6 µL**

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2-mercaptoethanol **Sigma Aldrich Catalog #M6250** , **600 µL** PM1, and **200 µL** RNA Shield to an empty tube. Also add **100 µL** Nanotrap Enhancement Reagent 2 and **150 µL** Nanotrap Magnetic Virus Particles.

18 Incubate the samples including the extraction control for 10 minutes at room temperature.

19 Place the 1.5 mL sample tubes on the magnetic rack and wait 1 minute before transferring the supernatant into the center tube of the rotor adapter.

20 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**

21 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.

22 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

23

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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.

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

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

26 Prepare master mixes for PMMoV and N1-N2-BCoV assays. Allow for one extra sample. Vortex to mix. Briefly centrifuge the tubes to collect the master mix at the bottom of the tube.

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

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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**

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

28 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.

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

30 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.

31 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.

32 Carefully seal the Nanoplate with a Nanoplate seal and the roller provided with the QIAcuity

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Instrument.

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

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

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

35 In the dPCR Parameters section under the Priming tab, select the Qiagen Standard Priming Profile.

36 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

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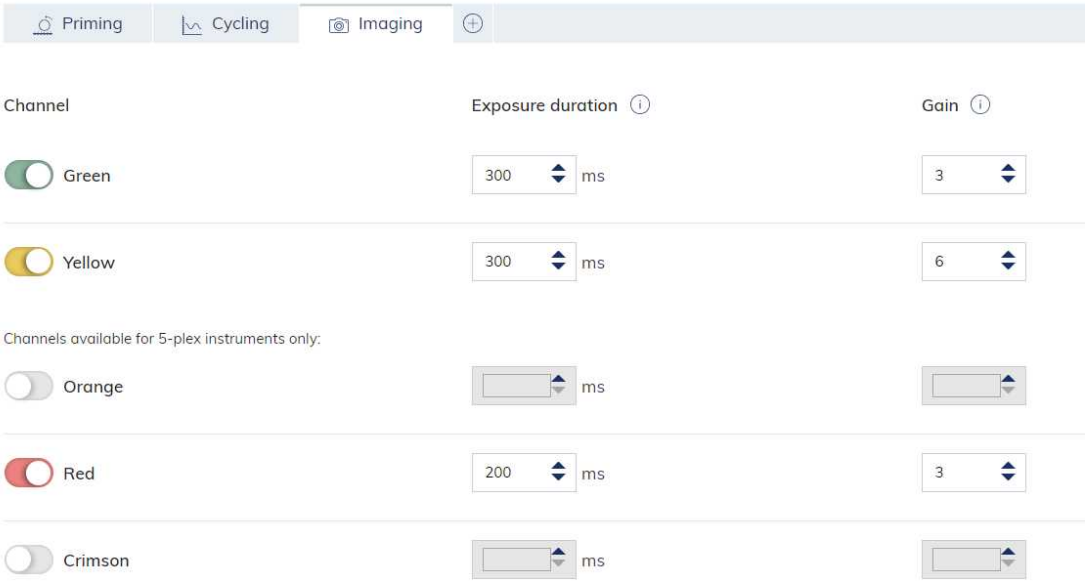
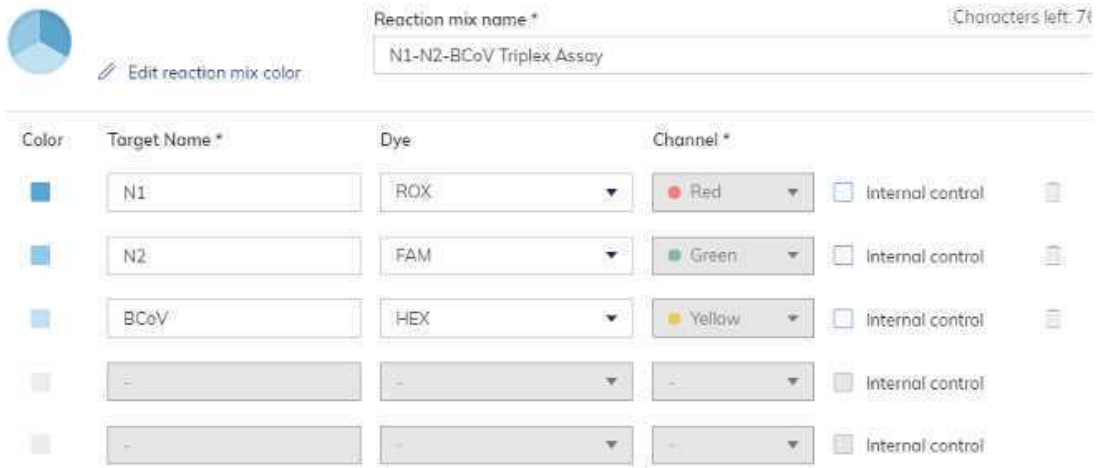
37 In the Imaging tab create the conditions shown below. These settings are different from the GT Molecular recommended settings, but provides less frequent saturation errors.



38 Navigate to "Reaction mixes". Create reaction mixes named "N1-N2-BCoV Triplex Assay" and "PMMoV Assay" that contain the following details.



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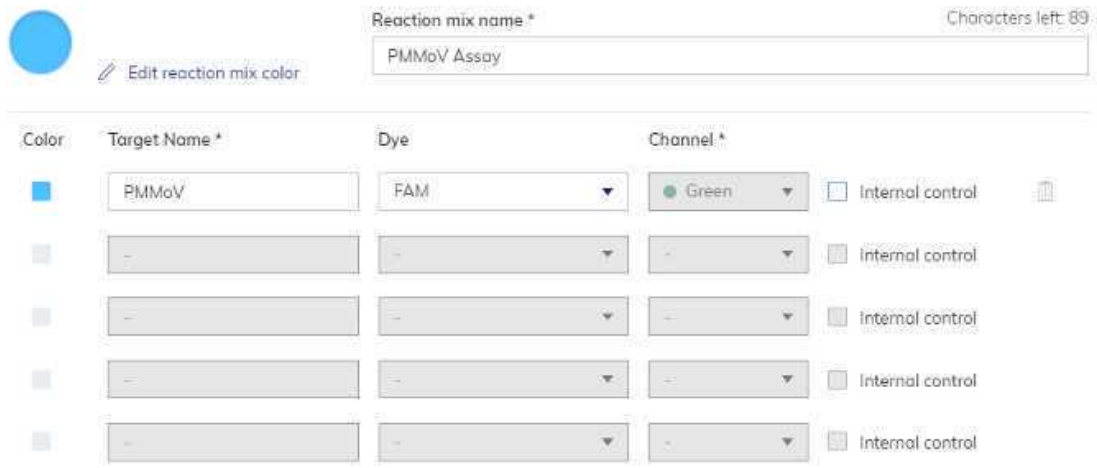

39 Navigate to "Samples and controls". Add samples names that are being quantified on this run. Extraction controls should be added as samples. Under the "Controls" tab create both a "N1-N2-BCoV Positive Control" and a "PMMoV Positive Control". Under the Non Template Controls" tab create a "N1-N2-BCoV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

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

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

Analysis and Interpretations 15m

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

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

44 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.

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

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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.

47 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.

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

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

50 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** .

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

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

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

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

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

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

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

$BCoV^{M}$ 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.

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

Index: Imaging Errors

54 The QIAcuity instrument may show an error when viewing results that a "channel has reached saturation". To resolve this error, reduce the imaging gain and exposure duration and re-image the plate.

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