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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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We use this protocol and it's working.

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Abstract

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).

Materials

Equipment:

Equipment	
DynaMag-2	NAME
Magnet	TYPE
Invitrogen	BRAND
12321D	SKU
https://www.thermofisher.com/order/catalog/product/12321D#/12321D ^{LINK}	

Equipment	
DynaMag-15	NAME
15 mL magnetic rack	TYPE
Invitrogen	BRAND
12301D	SKU
https://www.thermofisher.com/order/catalog/product/12301D ^{LINK}	

Equipment

QIAcube Connect

NAME

Automated nucleic acid extraction

TYPE

Qiagen

BRAND

9002864

SKU

<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/instruments-equipment/qiacube-connect/>

LINK

Equipment

QIAcuity One, 5-plex

NAME

dPCR

TYPE

Qiagen

BRAND

911021

SKU

<https://www.qiagen.com/us/products/instruments-and-automation/pcr-instruments/qiacuity-digital-pcr-system/?catno=911021>

LINK

Reagents:

☒ Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**

☒ Hydrochloric acid **Merck MilliporeSigma (Sigma-Aldrich)**

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

☒ 2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)**

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

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

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

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)

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.

Protocol materials

 Bovilis Coronavirus Calf Vaccine **Merck Animal Health Catalog #16445**

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

 2-Mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich)**

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

 Hydrochloric acid **Merck MilliporeSigma (Sigma-Aldrich)**

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

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

 2-mercaptoethanol **Merck MilliporeSigma (Sigma-Aldrich) Catalog #M6250**



Safety warnings

- ⚠ 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

Note

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). 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 magnetic rack for 1 minute to allow the magnetic particles to separate from the supernatant. Discard the supernatant.
- 13 Add  200 μ L  Zymo DNA/RNA Shield **Fisher 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

14

Safety information

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 Add  6 μL  2-mercaptoethanol **Merck MilliporeSigma (Sigma-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  2-mercaptoethanol **Merck MilliporeSigma (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.



Equipment

QIAcube Connect

NAME

Automated nucleic acid extraction

TYPE

Qiagen

BRAND

9002864

SKU

<https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/instruments-equipment/qiacube-connect/>

LINK

- 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

Note

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.

Safety information

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

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.
- Note**
- 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 Instrument.
 - 33 Place the sealed plate in the plate drawer inside the QIAcuity instrument.

Equipment

QIAcuity One, 5-plex

NAME

dPCR

TYPE

Qiagen

BRAND

911021

SKU

<https://www.qiagen.com/us/products/instruments-and-automation/pcr-instruments/qiacuity-digital-pcr-system/?catno=911021>

LINK

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)

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

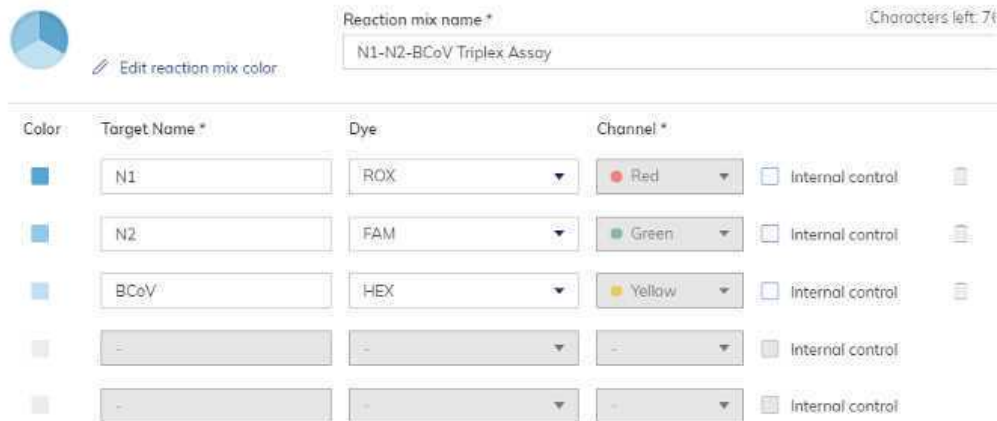
End

- 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.

Priming
Cycling
Imaging
+

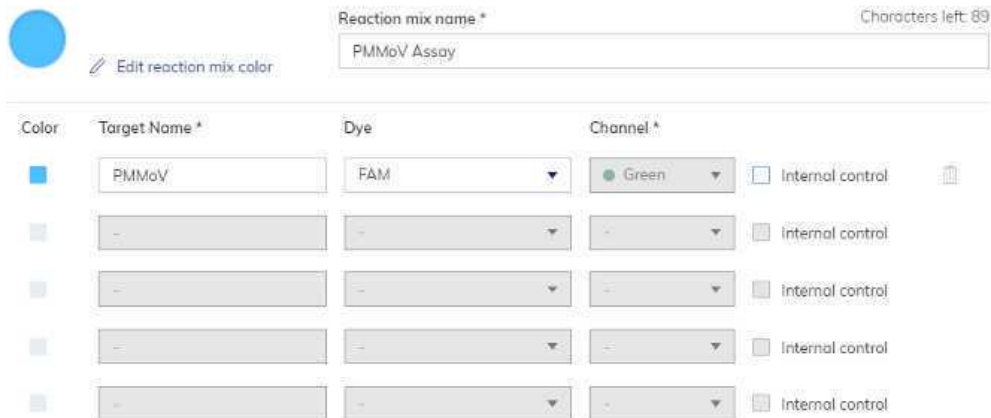
Channel	Exposure duration ⓘ	Gain ⓘ
☒ Green	300 ms	3
☒ Yellow	300 ms	6
Channels available for 5-plex instruments only:		
☐ Orange		
☒ Red	200 ms	3
☐ Crimson		

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



The screenshot shows the 'Reaction mixes' interface for the 'N1-N2-BCoV Triplex Assay'. At the top, there is a color selection icon (a circle divided into three segments) and a text input field for the 'Reaction mix name *' containing 'N1-N2-BCoV Triplex Assay'. Below this is a table with columns: Color, Target Name *, Dye, Channel *, and Internal control. The table contains five rows. The first three rows are for the targets N1, N2, and BCoV, each with a specific dye (ROX, FAM, HEX) and channel (Red, Green, Yellow). The last two rows are for internal controls, each with a greyed-out target name, dye, and channel, and an 'Internal control' checkbox.

Color	Target Name *	Dye	Channel *	Internal control
	N1	ROX	Red	☐
	N2	FAM	Green	☐
	BCoV	HEX	Yellow	☐
	-	-	-	☐
	-	-	-	☐



The screenshot shows the 'Reaction mixes' interface for the 'PMMoV Assay'. At the top, there is a color selection icon (a solid blue circle) and a text input field for the 'Reaction mix name *' containing 'PMMoV Assay'. Below this is a table with columns: Color, Target Name *, Dye, Channel *, and Internal control. The table contains five rows. The first row is for the target PMMoV, with dye FAM and channel Green. The last four rows are for internal controls, each with a greyed-out target name, dye, and channel, and an 'Internal control' checkbox.

Color	Target Name *	Dye	Channel *	Internal control
	PMMoV	FAM	Green	☐
	-	-	-	☐
	-	-	-	☐
	-	-	-	☐
	-	-	-	☐

- 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.

Index: Preparation of Bovilis Coronavirus

1h

46

Note

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 BCoVND.

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 BCoVND (non-diluted). Store BCoVND at  -80 °C



- 49 Dilute  60 μL BCoVND 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.
 - 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 40 μL .

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.