Electronic Supplementary Material (ESI) for Environmental Science: Water Research & Technology.
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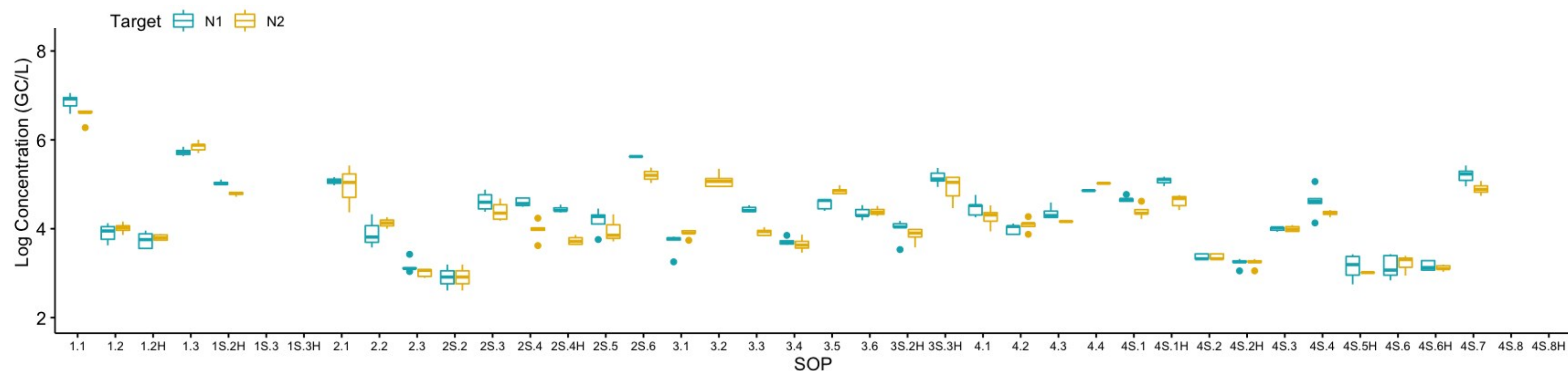
Electronic Supplementary Information

The uncorrected SARS-CoV-2 concentrations, recovery efficiency, and frequency of non-detects at Plant 1 are presented in Figure ESI-1.



	1.1	1.2	1.2H	1.3	1S.2H	1S.3	1S.3H	2.1	2.2	2.3	2S.2	2S.3	2S.4	2S.4H	2S.5	2S.6	3.1	3.2	3.3	3.4
N1						3/3	3/3	1/3			2/5	1/5				4/5		X		
N2		1/5				3/3	3/3		1/3		2/5			1/5	2/5	3/5				1/5
Average OC43 Recovery %	71	38	10	1.9	340	0.01	1.0	5.3	19	2.6	0.25	3.6	0.94	0.17	6.8	410	4.8	3.9	13	1.5

	3.5	3.6	3S.2H	3S.3H	4.1	4.2	4.3	4.4	4S.1	4S.1H	4S.2	4S.2H	4S.3	4S.4	4S.5H	4S.6	4S.6H	4S.7	4S.8	4S.8H
N1							1/5								4/8				3/3	3/3
N2							3/5						1/5	1/5	7/8				3/3	3/3
Average OC43 Recovery %	5.4	6.2	40	190	4.3	3.7	4.5	76	78	65	2.2	1.4	36	0.51	0.85	2.8	0.96	5.7	0.03	0.03

Figure ESI-1. Uncorrected SARS-CoV-2 concentrations (N1 and N2) at Plant 1. The total number of non-detects (ND) (combined for SARS-CoV-2 N1 and N2 targets) out of total number of sample replicates processed by each SOP and the average OC43 recovery are shown in the table below the box plot (a blank cell indicates no non-detects).

The average log-difference between the measured SARS-CoV-2 concentrations and the theoretical LOD for each SOP is shown in Table ESI-1.

Table ESI-1. Average log-difference between the measured SARS-CoV-2 concentrations for the two WWTP samples and the theoretical LOD

SOP	Average log-difference between measured SARS-CoV-2 concentrations and theoretical LOD	
	Plant 1 (Hyperion)	Plant 2 (JWPCP)
1.1	2.6	2.5
1.2	1.2	--
1.2H	1.2	--
1.3	3.1	2.8
1S.2H	1.9	1.4
1S.3	0 (below limit of detection)	0 (below limit of detection)
1S.3H	0 (below limit of detection)	0 (below limit of detection)
2.1	1.2	0 (below limit of detection)
2.2	0.4	0.4
2.3	1.0	0.5
2S.1	0 (below limit of detection)	0 (below limit of detection)
2S.2	0.2	0 (below limit of detection)
2S.3	1.5	1.1
2S.4	1.7	--
2S.4H	1.4	--
2S.5	0.8	0.2
2S.6	0.4	0 (below limit of detection)
3.1	0.9	0.6
3.2	2.7	2.5
3.3	1.6	1.0
3.4	0.9	0.4
3.5	1.7	0.9
3.6	1.4	0.7
3S.1	1.4	1.7
3S.2H	1.4	0.6
3S.3H	1.9	1.2
4.1	1.0	0.6
4.2	0.5	0.6
4.3	0.5	0.4
4.4	2.0	1.3
4S.1	2.4	--
4S.1H	2.6	--
4S.2	0.8	--
4S.2H	0.7	--
4S.3	0.7	0 (below limit of detection)
4S.4	1.8	0.8
4S.5H	0.2	0.5
4S.6	0.2	--
4S.6H	0.1	--
4S.7	1.9	0.3
4S.8	0 (below limit of detection)	0 (below limit of detection)
4S.8H	0 (below limit of detection)	0 (below limit of detection)

The uncorrected SARS-CoV-2 concentrations (N1 target) and OC43 recovery efficiency for the SOPs that evaluated the impact of heat pasteurization are shown in Figure ESI-2.

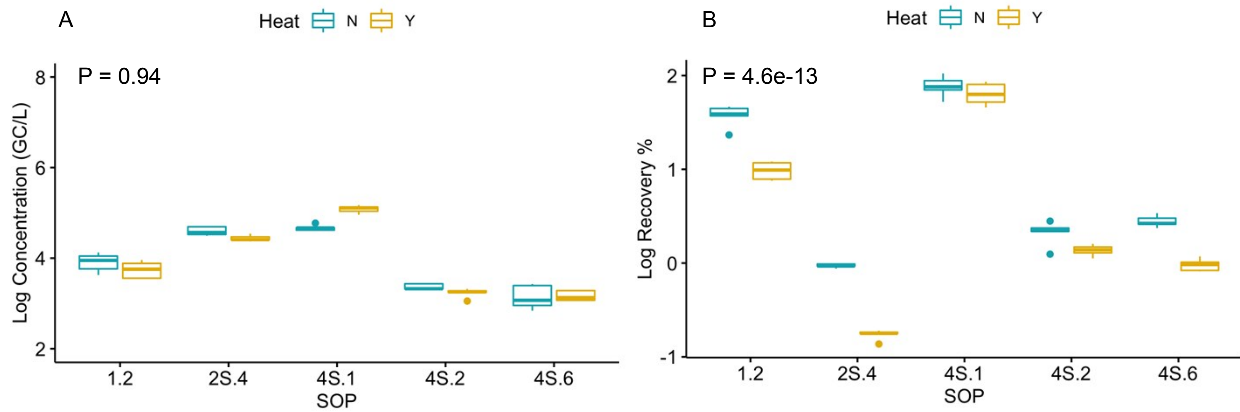


Figure ESI-2. Impact of heat pasteurization on A) the uncorrected log-transformed SARS-CoV-2 (N1 target) concentrations and B) the OC43 recovery efficiency at Plant 1. Five sample replicates for each SOP, with and without heat pasteurization, were performed.

Standard Operating Procedures (SOPs)

Labs were not required to share their SOPs publicly in order to participate in the study, though full SOPs were shared with the project team. Nearly all of the labs agreed to share their detailed SOPs for this publication and are provided in the Electronic Supplementary Information. The SOPs provided here cover each of the eight major method groups described in the manuscript.

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Standard Operating Procedure

Collection and Analysis Protocols for SARS-CoV-2 RT-qPCR Analysis from Wastewater

(Revised 6-5-20)

Collection:

1. For each influent sample, pipette 5 mL of influent into a 15 mL conical tube and store it at -80ºC. Nothing is added to the samples these are just raw influent archives. Each sampling day there will be two samples, one from SJC and one from JW, so a total of two, 5mL influent samples per day will get archived.
2. Each sampling day, a total of six tubes of each sample will be preserved with DNA/RNA shield (Zymo Research Cat. R1100-250). So for two samples a total of 12 tubes will be prepared each day.
    a. The ratio of DNA/RNA shield to sample is 250 µL of sample to 750µL of RNA shield (1x) for each tube. First pipette the RNA shield into a sterile 2 mL screw-capped cryotubes then add the sample. Mix by inverting a couple of times.
    b. Store the cryotubes containing the influent mixed with the DNA/RNA shield in the -80ºC revco freezer.
3. We will extract three tubes per sample leaving the other three in the freezer as back-ups.

RNA Extraction:

1. For each sample three replicate extractions will be performed (i.e. three for the SJCe influent and three for the JWPCP influent). The Quick-RNA Fecal/Soil microbe microprep kit (Zymo Research Cat. R2040) is used for the RNA extractions.
2. For each extraction, take the tubes out of the -80ºC freezer and thaw them in the BSC (class II B2) in Virology B lab.
3. Two mock extractions will be performed with each batch. One with extraction control RNA (*in vitro* transcribed AdV IPC RNA) and one without the extraction control RNA.
    a. The first will have 240µL of DEPC water and 750µL of RNA shield. The other 10µL will be carrier RNA (Qiagen Cat. 1068337).
    b. The second will have 235µL of DEPC water and 750µL of RNA shield. The remaining volume will be the carrier RNA (10µL) and the AdV IPC extraction control RNA (5µL).
4. For each tube, place the entire volume (about 1 mL) into a bead bashing tube from the Zymo Quick-RNA fecal/soil kit (cat. R2040). There should be eight tubes in total.
5. Bead beat on the FastPrep24 homogenizer for 40 seconds at 6m/s.
6. To get rid of the foam after bead beating, spin the samples down in the refrigerated microcentrifuge in the Virology B lab set at 4ºC. Spin at >12,000 x g for 5-7 minutes. Note the Zymo protocol says to spin for one minute, this was found to be insufficient to get rid of the foam.
7. After centrifugation, remove the supernatant and place in a clean microfuge tube then add 5 µL of the *in vitro* transcribed AdV IPC RNA (approximately 10^4 copies/µL, usually a -7 dilution) to each sample tube and to the one mock extraction tube containing 230µL of DEPC water. This can be added directly after the sample is transferred to the new tube.
    a. The remaining mock extraction tube does not get the AdV IPC RNA and serves as another negative extraction control.

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8. To the two mock extraction tubes (one with extraction control RNA and one without) add 10 µL of carrier RNA diluted to 1ug/ul (Qiagen cat. 1068337).

9. Proceed with the Zymo extraction as stated in the instructions.
   a. Note that in step 4 of the Zymo protocol it states to transfer 400µL of supernatant to a new tube. The extraction volume we have is more than 400µL (usually around 700µL). We pipette a fixed volume of 700µL and place it in a sterile 2 mL tube then add an equal volume of RNA binding buffer (700µL) from the Zymo kit as stated in the Zymo kit's instruction notes.
   b. The total volume is processed through one Zymo-Spin IIICG Column as stated in step 5 of the Zymo protocol (around 1.4mL). This will require multiple spins to get the entire volume through. Collect and save each of the supernatants from the spins. Combine all of the supernatants in a sterile 15 mL conical tube and add one volume of ethanol (approximately 1.4 mL) as stated in step 6 of the protocol.
   c. The total volume of supernatant with ethanol is processed through a single new Zymo-spin IIICG Column in step 7 (about 2.8 mL). This will require multiple spins to get the entire volume through the column.
   d. Proceed with the Zymo protocol as stated from step 8 on.
   e. Add an additional centrifuge spin (2 minutes, >12,000 x g) for step 17 is done to ensure the column is dry prior to the final elution step. This equates to two, two-minute spins for this step. This was added as we found that a single spin was insufficient to completely dry the column.
   f. Elute with 15µL of the supplied RNase-free, DNA-free water as stated in the protocol.
   g. Store the eluted RNA at -80ºC until analyzed. We recommend performing analysis as soon as possible on the RNA extracts to limit any degradation. RNA should be analyzed within one week of extraction and definitely not past three weeks.

RT-qPCR analysis:

1. The SARS-CoV-2 N1 and N2 RT-qPCRs along with the extraction control will be analyzed on the same 96-well PCR plate (three separate master mixes are required). All three RT-qPCRs are performed with the same thermocycling conditions.
   a. This RT-qPCR uses the iTaq Universal Probes One-step RT-qPCR reaction mix (BioRad Cat. 1725140).
2. Create a PCR log sheet report and update the mastermix tables for the appropriate number of samples to be analyzed (see Table 1) including the standard curves for N1, N2, and the AdV IPC extraction control RNA.
   a. Use *in vitro* transcribed (IVT) RNA standards for the N1 and N2 standard curves. IVT standards should be regenerated every three weeks. Currently, we are using the MAXIscript kit for IVT (Invitrogen cat. AM1320) with Turbo DNase (Ambion/Life technologies cat. AM1907) to eliminate the DNA template after transcription.
3. Wipe down the reagent preparation cabinet and the sample addition PCR cabinet along with any equipment that will be used with RNase displace (Fisher cat. 04-355-137) or equivalent before starting. Wash your gloves with RNase displace before beginning any work and as necessary thereafter.
4. Take an aliquot of all three primer/probe sets (N1, N2, AdV IPC) out of the freezer and place into the reagent preparation PCR cabinet. Note that the N1 and N2 primer/probe sets come premixed from IDT at 6.7µM concentrations. The IPC primers and probe stock solutions are all 20 µM.

5. Place a tube of iTaq Universal Probes One-step RT-qPCR reaction mix and RT enzyme (BioRad cat. 1725140) into a 4°C cooler rack.
6. Set up the RT-qPCR according to the specifications in the PCR log sheet and aliquot into a 96-well PCR plate. This is all done in the reagent preparation cabinet.
7. Take one pre-aliquoted tube of N1 and N2 *in vitro* transcribed RNA out of the -80°C and place them in the sample preparation PCR cabinet. Dilute each RNA 10-fold out to the 10^{-12} dilution (i.e. -12) with 50% glycerol/DEPC water. These will be used for the standard curves.
8. Take the samples out of the -80°C freezer and place in a rack in the sample preparation PCR cabinet.
9. Move the 96-well PCR plate with the mastermix to the sample preparation cabinet and add the appropriate amount of sample and/or standards to the designated wells (as outlined in the PCR log sheet, see Table 1). Seal the plate with PCR sealing foil, use a plate spinner to collect the volume in the bottom of each well.
10. Perform the RT-qPCR on the LightCycler480 using the cycling conditions outlined in Table 2.
11. Analyze the data using the LightCycler480 software and ensure that the run meets the efficiency and detection limit QA criteria for the assay.
    a. Samples with a Cp greater than 40 cycles are considered negative.
12. Include the LC480 RT-qPCR data analysis report in the PCR log sheet report along with the analyst's write-up of the results.
    a. Calculate and report the concentrations in copies per milliliter of raw wastewater or copies per liter of raw wastewater.

Table 1. RT-qPCR mastermix set up.

Covid N1 Master mix.	Initial Conc.	1X Vol. (ul)	Vol in Mstr. Mix(ul) &	Final Conc.	Product Info/Reagent Lot#
			# of rxn's		
Reagents		25.00	30.0		
DEPC H2O		5.01	150.2		Nuclease free BioRad 64332648
PCR Buffer	2X BioRad iTaq Universal Probes	12.50	375.0	0.91X	64301314
MgCl2(mM)	1	in buffer	-	-	
mM dNTP's (no dUTP)	10	in buffer	-	-	
BSA (non-acytlated)	50 mg/ml Ambion		-	-	
nCov N1 Mix	6.7	1.87	56.1	0.50	0000507469 aliq. 4/26/20
Enzyme mix	BioRad RT	0.625	18.8	0.91X	64332646
Template	RNA Extracted	5.00	-	5.00	5/7/2020

Covid N2 Master mix	Initial Conc.	1X Vol. (ul)	Vol in Mstr. Mix(ul) &	Final Conc.	Product Info/Reagent Lot#
			# of rxn's		
Reagents		25.00	30.0		
DEPC H2O		5.01	150.2		Nuclease free BioRad 64332648
PCR Buffer	2X BioRad iTaq Universal Probes	12.50	375.0	0.91X	64301314
MgCl2(mM)	1	in buffer	-	-	
mM dNTP's (no dUTP)	10	in buffer	-	-	
BSA (non-acytlated)	50 mg/ml Ambion		-	-	
nCov N2 Mix	6.7	1.87	56.1	0.50	0000507469 aliq. 4/26/20
Enzyme mix	BioRad RT	0.625	18.8	0.91X	64332646
Template	RNA Extracted	5.00	-	5.00	5/7/2020

SPC Master mix.	Initial Conc.	1X Vol. (ul)	Vol in Mstr. Mix(ul) & # of rxn's	Final Conc.	Product Info/Reagent Lot#
Reagents		**25.00**	**16.0**		
DEPC H2O		9.83	157.2	9.83	Nuclease free BioRad 64332648
PCR Buffer	2X BioRad iTaq Universal Probes	12.50	200.0	1X	64341907
MgCl2(mM)	1	in buffer	-	-	
mM dNTP's (no dUTP)	10	in buffer	-	-	
Sal IPC Pr	20	0.25	4.0	0.20	3-050220
Adv Hex RT F	20	0.40	6.4	0.32	1-050220
Adv Hex RT R	20	0.40	6.4	0.32	2-050220
RT	BioRad RT	0.625	10.0	1x	64332646
Template	Transcribed RNA	1.00			4/18/2020

Table 2. RT-qPCR cycling conditions.

	Temp. (^{o}C)	Time	
Reverse transcription	50	15 min	
Inactivate & Denature	95	2 min	
Denature	95	3 sec	45 cycles
Anneal/Acquire/Extend (FAM)	55	30 sec	

Interlaboratory and Methods Assessment of the SARS-CoV-2 Genetic Signal in Wastewater (WRF 5089)

Participating Laboratory Qualifications and Quality Assurance

Promega Corporation
2800 Woods Hollow Road
Madison, WI 53711

Laboratory Organization and responsibility

Laboratory QA Manager: Vince Cannistraro

Key Individuals responsible for production of ensuring production of valid measurements and routine assessment of QC data:

Subhanjan Mondal, Ph.D.

Nathan Feirer, Ph.D.

Vince Cannistraro is responsible for internal audits and reviews implementation of the laboratory QA plan and requirements.

Nathan Feirer reports to Subhanjan Mondal and are both in the R&D Group of Promega Corporation, directed by Jim Cali.

Personnel

Subhanjan Mondal: – Senior Research Scientist.

-Experience – 8+ years of Industrial experience. 4+ years of postdoctoral experience.

-Education – B.S. in Human Biology, M.S. in Biotechnology and Ph.D. in Biochemistry.

-Training – Trained on the analytical methods, experimental design, data analysis and planning. Extensive experience with assay development, nucleic acid purification and qPCR analysis.

CV attached below.

Nathan Feirer – Research Scientist.

-Experience – 2 years of Industrial experience. 2+ years of postdoctoral experience.

-Education – B.S. in Natural Sciences, Ph.D. in Microbiology.

-Training – Trained on the analytical methods, experimental design, data analysis and planning. Extensive experience with assay development, nucleic acid purification and qPCR analysis.

CV attached below.

Vincent Cannistraro – Senior Quality Assurance Scientist / Custom Assay Services (CAS) QA Reviewer.

-Experience – Over 11 years of Industrial experience. 8+ years of experience as a Product QA reviewer.

-Education – B.S. in Biology, M.S. in Biochemistry and Ph.D. in Biochemistry.

-Training – Trained on the review of data and data reports generated from CAS.

Training: Promega has a documented training program. The training program document describes the processes for hiring, orientation, training, and performance management necessary to demonstrate employee competence. Promega maintains electronic or hard copy records that demonstrates employee training and competency.

Facility

Arrangement and size of labs: Laboratory space includes BSL1 labs.

Relevant for nucleic acid amplification: laboratory space for pre-amplification process (sample processing, viral concentration and nucleic acid extraction) are separated from post-amplification process RT-qPCR. Amplification tubes are discarded in biohazard waste after qPCR is completed.

Wastewater sample processing and decontamination: Immediately upon being received, wastewater samples are pasteurized by incubating at 60°C for 60 minutes in a circulating water bath. Subsequent sample processing is detailed below. Any wastewater sample remaining is decontaminated by adding bleach, incubating at room temp for >30 minutes. After bleach decontamination, material is disposed of down laboratory sink alongside continuously running water.

Laboratory Reagent Water System: Water purified through a reverse osmosis system is dispensed in cleaned pyrex glass bottles in a laminar flow hood and autoclaved as per internal SOP000763 "Autoclave Guidelines". This is the laboratory reagent water that is used.

The primary goal of the Promega Corporation Waste Management and Minimization Plan SOP21745 is to protect the health of employees, the environment and the community by reducing the quantity and the toxicity of waste generated at Promega Corp. It is critical that waste generated at Promega Corp. be disposed of in a manner that is in compliance with all federal, state and local regulations.

Field Sampling procedure

Promega receives wastewater samples from the Madison Metropolitan Sewage District (MMSD, 1610 Moorland Rd, Madison WI 53713). These samples consist of influent (Raw Wastewater) that has been degritted and filtered to remove floatable/settleable solids. Samples are collected in 1 liter Nalgene bottles (Fisher# 2187-0032) by MMSD staff and directly transferred at ambient temperature to Promega staff without prior storage. Time of sample collection is ~7:30 am. The samples are sealed within two layers of ziploc plastic bags to contain spillage and transported to the Promega lab in an insulated carry case. The sample collection and transport are otherwise performed under ambient conditions.

Information about collected samples (e.g. collection temperature and rainfall events on days prior to collection) is recorded in an electronic notebook at Promega (Probook). Date and time of sample

collection and time from sample collection to pasteurization are recorded on a printed label that is attached to the bottle.

Laboratory test sample handling procedures

Sample processing/handling (or assay workflow) involves five steps:

1) Sample pasteurization

2) Direct Viral Nucleic Acid Capture and Extraction

3) Reverse transcription and qPCR

4) Data analysis

Also included in the workflow is determination of recovery by spiking MS2 phage in the sample and analysis % of MS2 phage recovered after the sample handling and processing.

1) **Sample Pasteurization**

Samples are pasteurized by heating at 60°C for 60 minutes in a water bath. 100ul of the sample before and after pasteurization is inoculated on an LB agar plate to examine the difference in bacterial viability. The pasteurized sample is held at 4°C and further processed within 48 hours or are frozen at –20°C for later processing and analysis. Frozen samples are thawed at 4°C prior to use.

Prior to sample concentration the 40 ml of pasteurized sample is spiked with41X10^5 PFU (plaque forming units) of MS2 phage. MS2 genetic material will copurify with the SARS-CoV-2 genetic material and indicate the % recovery of SARS-CoV-2.

2) **Direct Viral Nucleic Acid Capture and Extraction**

- Take 40ml of pasteurized (or unpasteurized) in a 50 ml conical tube.
- To it add 12 ml of Binding Buffer 1 and 1 ml of Binding Buffer 2. Close the cap and mix well.
- Divide the mixture equally into two conical tubes containing 26 ml each. To each of the tubes add 24 ml of isopropanol. Mix well.
- Assemble a column stack by nesting a PureYield™ Midi Clearing Column (**blue**) into the top of a PureYield™ Midi Binding Column (**white**). Place the assembled column stack onto the vacuum manifold as shown in Figure 1.

Figure1. The blue clearing column inserted into the white binding column. The two columns sit on a vacuum manifold port.

- Pour the mixture onto the PureYield™ Midi Clearing Column Apply vacuum. The sample will pass through the clearing membrane in the PureYield™ Midi Clearing Column, and the Nucleic acid will bind to the binding membrane in the PureYield™ Binding Column. The PureYield™ Midi Clearing Column can hold 20 ml at a time, keep adding the sample at regular intervals and continue the vacuum until all the liquid has passed through both columns.
- After all the sample has passed, switch off the vacuum to slowly release the vacuum from the filtration device before proceeding. Remove the PureYield™ Midi Clearing Column, leaving the PureYield™ Midi Binding Column on the vacuum manifold.
- Add 5 ml Inhibitor Removal Wash to the PureYield™ Midi Binding Column and pass it through the column by switching on the vacuum.
- Add 20 ml of RNA Wash Solution (RWA) to the PureYield™ Midi Binding Column and pass it through the column by vacuum. Then Pass another 10 ml of RNA Wash Solution (RWA) to the PureYield™ Midi Binding Column and pass it through the column by vacuum.

Figure 2. PureYield™ Midiprep Columns are labeled with 5, 10 and 20ml fill levels.

- Elution of the Nucleic acid can be done by vacuum using the Eluator™ Vacuum Elution Device (Cat.# A1071), resulting in better nucleic acid recovery and yield. Place a 1.5ml microcentrifuge tube in the base of the Eluator™ Vacuum Elution Device, securing the tube cap in the open position, as shown (Figure 3, Panel A). Assemble the Eluator™ Device and insert the DNA binding column into it, making sure the column is fully seated on the collar.

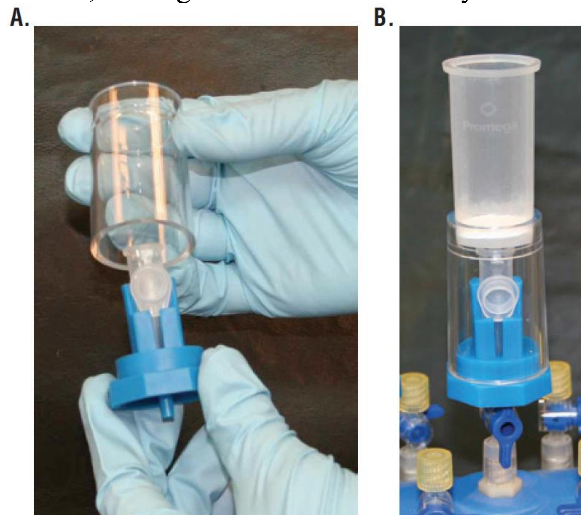

Figure 3. Elution by vacuum. Panel A. A 1.5ml microcentrifuge tube is placed in the base of the Eluator™ Vacuum Elution Device and the microcentrifuge tube cap is locked as shown. Panel B. The final Eluator™ Vacuum Elution Device assembly, including the binding column, ready for use on a vacuum manifold.

- Place the Eluator™ Device assembly onto a vacuum manifold (Figure 3, Panel B). Add 500µl of pre-heated Nuclease-Free Water to the PureYield™ Midi Binding Column. Apply maximum vacuum for 1 minute or until all liquid has passed through the column. Repeat the process by adding another 500ul of pre-heated Nuclease-Free Water to the PureYield™ Midi Binding Column to collect a total of 1ml of Total Nucleic acid.
- Concentration and further clean-up of the extracted material is done using the PureYield Mini Column as described in Figure 4. Briefly, to the 1 ml nucleic acid extracted using the PureYield Midi Column add 400ul of Binding Buffer 1 and 100ul of Binding Buffer 2. Mix well and divide the contents into two 1.5ml tube containing 750ul each. To each tube add 750ul of isopropanol. Mix well and pass the entire volume through a PureYield Mini Column, 750ul at a time. Centrifuge at 10,000g for 1 minute each time. Pass 300ul of Inhibitor Removal Wash through the PureYield™ Mini Column by centrifugation. Then pass 500ul of RNA wash Solution (RWA) twice through the PureYield™ Mini Column.
- To elute the Nucleic acid, add 20ul of pre-heated Nuclease Free Water to the PureYield Mini Column. Let it soak for 1 minute. Elute by spinning at 10,000g for 1 minute. Repeat elution with another 20ul of Nuclease Free water, to collect a total of 40ul.

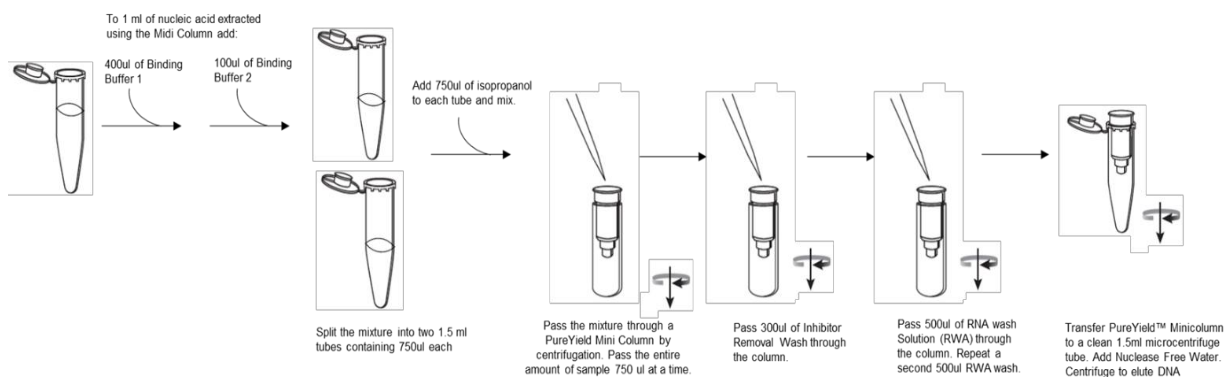

Figure 4. Scheme for clean-up and concentration of the Total Nucleic acid by the PureYield Mini Column.

This method allows for 40 ml of wastewater viral nucleic acid material to be simultaneously extracted and concentrated (1000x).

3) Reverse Transcription and qPCR

a) Description of the qPCR Assay

The qPCR assay for detection of SARS-CoV-2 genetic signal includes

20X N1 Primer/ Probe/IAC mix
20X N2 Primer/Probe/IAC Mix
20X E Primer/Probe/IAC Mix
GoTaq® WW Master Mix
GoScript® Enzyme Mix
Positive Control RNA (1pg/ul)
Quantification Standard dsDNA (2 x 10^6 copies/ul)
Water (Negative control)

Name	Sequence
2019-nCoV_N1-F	5’-GACCCCAAAATCAGCGAAAT-3’
2019-nCoV_N1-R	5’-TCTGGTTACTGCCAGTTGAATCTG-3’
2019-nCoV_N1-P	5’-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3’
2019-nCoV_N2-F	5’-TTACAAACATTGGCCGCAAA-3’
2019-nCoV_N2-R	5’-GCGCGACATTCCGAAGAA-3’
2019-nCoV_N2-P	5’-FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1-3’
E_Sarbeco_F	5’-ACAGGTACGTTAATAGTTAATAGCGT -3’
E_Sarbeco_R	5’-ATATTGCAGCAGTACGCACACA-3’
E_Sarbeco_P1	5’-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ-3’
PMMoV FP	5’- GAGTGGTTTGACCTTAACGTTGA-3’
PMMoV RP	5’-TTGTCGGTTGCAATGCAAGT-3’
PMMoV P	5’-Quasar 670- CCTACCGAAGCAAATG-BHQ2-3’
MS2 F2:	5’ AAATATCGTTCGAACTGGACTC 3’
MS2 R2:	5’ CACCTTGATCTATCGATGTGAC 3’
MS2 P2:	5' FAM– AAACAGTGGCACTACCCCTCTCCGT –BHQ1 3

Three different primer/ probes set are designed, each as a 20X mixture detecting either the N1 or N2 fragment of the Nucleocapsid (N) gene or the Envelope protein (E) gene of SARS-CoV-2. The target

genes are detected using Fluorescein dye-labelled probe. All the three 20X Primer/Probe mix also contain two additional controls: 1) a process control and 2) an internal amplification control.

The process control is designed to detect Pepper mild mottle virus (PMMoV), an abundant RNA virus found in wastewater samples. The primers and Quasar® 670 dye-labeled probe to amplify a 68bp region of PMMoV genome.

The 20X Primer/Probe /IAC Mix also contains primers, probe and template for an internal amplification control (IAC). The CAL Fluor® 560 dye-labeled probe detects the IAC, a novel DNA template that is included in every amplification reaction. The amplify a 435 bp product. Amplification performance of the IAC is used to detect PCR inhibitors (ex: Taq polymerase inhibitors) in the sample. This is the longest target in the SARS-CoV-2 RT-qPCR assay, making the IAC more sensitive to inhibitors than the other targets in the multiplex reaction.

The 20X Primer/Probe/IAC Mix also includes a reference dye (CXR) which has similar spectral properties as ROX.

GoTaq® WW Mastermix and the GoScript® Enzyme Mix, which are tolerant to a diverse range of PCR inhibitors, are used.

A Quantitation Standard SARS-CoV-2 dsDNA containing portions N and E genes, is used in the assay as a positive quantitation control. The stock concentration of this quantitative standard is at 2×10^6 copies/ ul. The quantitative standard was formulated internally at Promega Corporation

b. The qPCR assay for detection of MS2 phage for spike recovery

For detection and quantification of MS2 phage RNA a separate 20X Primer/Probe/IAC mix is utilized. This mix includes primers and a FAM-dye-labeled probe for detection of MS2 genetic material. It also includes the process control primers and probe for detection of PMMoV and the primers, probe and template for the previously mentioned internal amplification control (IAC) and the reference dye CXR.

The MS2 phage genetic material is quantified using a linearized dsDNA standard. The dsDNA standard is at 2×10^6 copies/ul. An in-vitro transcribed RNA is also included as a positive control.

c. qPCR Reaction Protocol

Note: To avoid contamination of samples with external sources of DNA and RNA template, perform all steps with filtered pipette tips.

i. Assemble qPCR Standard Curves using Quantitation Standard SARS-CoV-2 dsDNA.

- Starting Quantitation Standard SARS-CoV-2 dsDNA:(2 x10^6 GU/µL)
- Dilute Quantification Standard dsDNA 100-fold by mixing 2uL in 198uL nuclease-free water to obtain a concentration of 2 x 10^4 GU/uL
- Perform subsequent serial 1:10 dilutions in low-bind 0.5 mL tubes: 5µL DNA + 45µL nuclease-free water to obtain the following standard curve tubes (2 x 10^3-2x10^0 copies/ul).

SARS-CoV-2 Quantitation Standard dsDNA Concentration (GU/µL)	Copies/ well (5µL Sample Volume / reaction)	RNA Equivalent: GU/ well (5µL Sample Volume / reaction)
2 x 10^4	1 x 10^5	2 x 10^5
2 x 10^3	1 x 10^4	2 x 10^4
2 x 10^2	1 x 10^3	2 x 10^3
2 x 10^1	1 x 10^2	2 x 10^2
2 x 10^0	10	20

ii. Assemble qPCR Standard curve for MS2 phage.

- Starting MS2 Quantification dsDNA concentration:(2 x10^6 GU/µL)
- Dilute Quantification Standard dsDNA 100-fold by mixing 2uL in 198uL nuclease-free water to obtain a concentration of 2 x 10^4 GU/uL
- Perform subsequent 1:10 dilutions in low-bind 0.5 mL tubes (e.g., Eppendorf Cat. #:022431005), (5µL DNA + 45µL nuclease-free water) to obtain following standard curve tubes.

MS2 Quantitation Standard dsDNA Concentration (GU/µL)	Copies/ well (5µL Sample Volume / reaction)	RNA Equivalent: GU/ well (5µL Sample Volume / reaction)
2 x 10^4	1 x 10^5	2 x 10^5
2 x 10^3	1 x 10^4	2 x 10^4
2 x 10^2	1 x 10^3	2 x 10^3
2 x 10^1	1 x 10^2	2 x 10^2
2 x 10^0	10	20

b. Assemble qPCR Reaction Master mix

Note: It is recommended to do RT-qPCR in three technical replicates. If increased statistical power is desired, increase number of technical replicates accordingly

Determine the number of reactions wells needed (n)

Reaction Mixture Worksheet (20µL final reaction volume)

	1X	X n
GoTaq WW Master Mix (2X)	10µL	
GoScript Enzyme Mix (50X)	0.4µL	
20X Prime/Probe/IAC Mix	1µL	
Nuclease-free Water	3.6µL	

Prepare reaction mixture >10% excess than what is required.

- Aliquot 15µL of RT-qPCR Reaction Mastermix into wells of 96-well qPCR plate
- Pipette 5µL of each RNA sample or standard curve dsDNA into corresponding wells (See sample plate layout on next page)
- For No Template Control (NTC) use 5ul of Nuclease Free water (Promega # P119A).

- For MS2 positive RNA control use in-vitro transcribed RNA.
- Spin (~300 RCF x 1 minute) to ensure all liquid is collected at bottom of wells

Example Plate Layout

	SARS-CoV-2 quantification standard dsDNA / N1			SARS-CoV-2 quantification standard dsDNA/ N2			MS2 Phage quantification standard dsDNA					
	1	2	3	4	5	6	7	8	9	10	11	12
A	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5	2 x 10^5			
B	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4	2 x 10^4			
C	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3	2 x 10^3			
D	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2	2 x 10^2			
E	20	20	20	20	20	20	20	20	20			
F	NTC	NTC	NTC	NTC	NTC	NTC	MS2/RNA	MS2/RNA	MS2/RNA			
G	Sample/N1	Sample/N1	Sample/N1	Sample/N2	Sample/N2	Sample/N2	Seeded/MS2	Seeded/MS2	Seeded/MS2			
H							Sample/MS2	Sample/MS2	Sample/MS2			

PCR cycling program / Instrument Settings

PCR condition			
Step	**Temperature**	**Time**	**cycles**
Rev Transcriptase	45	15 min	1
RT inactivation/ GoTaq activation	95	2 min	1
Denaturation	95	3 sec	40
Annealing/Ext	62	30 sec	

Collect data from following fluorescence channels at end of 62°C Annealing/Extension step

Fluorophores	***Target***
FAM	N1, N2, E (SARS-CoV-2), MS2
HEX/JOE	Internal amplification Control
ROX	Reference Dye
Cy5	PMMoV

After the completion of the RT-qPCR run, the PCR plate should be disposed into biohazard waste.

4) Data Analysis and Interpretation

a. Evaluate qPCR assay standard curve (FAM)

- Common qPCR analysis software packages perform a linear regression to the standard dilution series data and calculate the equation for the line of best fit (the standard curve). The equation is in the form of $y = mx + b$, where x = log concentration and y = Cq/Ct. The R^2 value is a measure of the fit of the data points to the regressed line. The slope (m in the equation) is an indication of the PCR efficiency. A slope of –3.3 indicates 100% PCR efficiency (i.e., the number of copies of amplification product is doubled at each cycle). The Y-intercept (b in the equation) is defined as the y value (Cq) when x (log concentration) equals 0.
- In general, the standard curve for each PCR target has an average slope (m) in the range of –3.1 to –3.6 (corresponding to a qPCR efficiency of >90%) and an R2 value >0.990. We recommend tracking and evaluating Y-intercept values for any significant changes from run to run.

b. Analyze Internal Amplification Control (IAC) signal (HEX)

- Amplification performance for the IAC can be used to evaluate overall performance of the SARS-CoV-2 qPCR amplification reaction and/or detect qPCR inhibitors (Inhibitors of Taq DNA polymerase) in the DNA sample.
- Depending on the qPCR instrument and analysis software used, the IAC Ct should fall in the range: 20-25
- If the IAC Ct is shifted >1 Ct compared to NTC well, PCR inhibitors are present in experimental sample. Repeat purification if necessary
- If IAC fails to amplify, no conclusions can be made about absence of SARS-CoV-2 genetic material from a sample. Results may be considered invalid.

c. Analyze Process Control signal, PMMoV (Cy5)

- All wastewater samples should exhibit fluorescence growth curves for PMMoV reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the Pepper mild mottle virus genetic material, an abundant RNA virus present in wastewater samples. Failure to detect PMMoV in wastewater samples may indicate:
    a. Improper extraction of nucleic acid from clinical materials resulting in loss of RNA and/or RNA degradation.
    b. Inhibition of Reverse transcriptase and/or Taq DNA polymerase by inhibitors in the sample.
    c. Absence of sufficient nucleic acid due to poor collection/ pasteurization of sample leading to loss of sample integrity.
    d. Improper assay set up and execution.
    e. Reagent or equipment malfunction.

- If no signal is obtained from the PMMoV reaction (Cy5-channel) , interpret as follows:
  a. If the 2019-nCoV N1 or N2 or E are positive even in the absence of a positive PMMoV signal, the result should be considered valid. It is possible, that some samples may fail to exhibit PMMoV growth curves due to low viral (PMMoV) load.
- If all 2019-nCoV markers, PMMoV (process control) and Internal Amplification control (IAC) are all negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.
- Including both PMMoV and a dsDNA based Internal amplification control (IAC) helps dissect inhibition of two critical component of the RT-qPCR, reverse transcriptase and Taq polymerase.

d. No Template Control (NTC)

The NTC consists of using nuclease-free water (Promega# P119) in the RT-qPCR reactions instead of RNA. The NTC reactions for all primer and probe sets should not exhibit fluorescence growth curves that cross the threshold line. If any of the NTC reactions exhibit a growth curve that crosses the cycle threshold before the lowest concentration standard on the standard curve, sample contamination may have occurred. Invalidate the run and repeat the assay with strict adherence to the guidelines. NTC samples will show amplification curves with only Internal Amplification Control (IAC) in the HEX channel.

Amplification from any other channel (FAM or Cy5) would indicate contamination of reagents/NTC reaction.

e. Analyze qPCR signal for sample replicates

- If a sample yields no detectable amplification for SARS-CoV-2 but exhibits IAC amplification (Ct: 20-25), and PMMoV amplification (Ct: 20-40) the sample can be categorized as *SARS-CoV-2* negative.

SARS-CoV-2 N1 (FAM)	SARS-CoV-2 N2 (FAM)	SARS-CoV-2 E (FAM)	NTC (FAM)	PMMoV Process control (Cy5)	Internal Amplification control (HEX)	Result interpretation
Any one or more is positive			-	+/-	+/-	SARS-CoV-2 Detected
-	-	-	-	+	+	SARS-CoV-2 Not Detected
Any one or more is positive			+	+/-	+/-	Invalid (False Positive)
-	-	-	-	+/-	-	Invalid (False Negative)

f. Spike recovery

Spike recovery is performed by spiking (seeding) approximately1x10^6 PFU MS2 phage to 40ml of the wastewater sample. The main assumption of the spike recovery analysis is that the MS2 count in the wastewater matrix is very low compared to the amount of virus used for spiking.

1mL of MS2 spiked wastewater input is purified using the PureYield Minicolumn as descrbed above (Fig. 4). This will be used to quantify the amount of virus seeded (α) in copies/ml of influent. 5ul of total nucleic acid used in the RT-qPCR represents 125ul of wastewater influent.

40mL of wastewater is processed using the procedure mention above, resulting in a 40uL elution of purified viral nucleic acid. This will be used to quantify the recovered amount of virus (β) in copies/ml of influent. 5ul of total nucleic acid used in the RT-qPCR represents 5 ml of wastewater influent.

% recovery (r) = (β/α) x 100%

g. SARS-CoV-2 quantification

Quantitative Standard SARS-CoV-2 dsDNA containing portions of N and E genes at 2x 10^6/ul will be titrated using serial log dilutions in a reference matrix (nuclease Free water) in a 5-log standard curve. The standard curve will be used to derive concentration of sample based on the Ct values.

Limit of detection (LOD) and Limit of quantification (LOQ) will be analyzed.

Equipment

Promega has procedures to describe the process involved for equipment and calibrations using Promega's Electronic Resource Planning (ERP) system. The ERP system is used to inventory equipment, update equipment information, enter calibration information, complete calibrations, enter repair/pm information and generate reports. Calibration, repair, Metrology preventive maintenance and historical entries in the ERP will be made by the Metrology staff.

List of equipment used, and internal instrument ID Is listed:

<u>Centrifuges</u>

- Beckman Coulter Allegra X-22R – Promega#: 106493
- Beckman Coulter J2-21 – Promega#: 106510

<u>Heat Block</u>

- Fisher Dry Bath –- Promega #: 105817

<u>Water Bath</u>

- Fisher Isotemp 3013 – Promega#: 105774

QPCR Thermocyclers

- Stratagene MX3005P – Promega#: 109790

Pipettes

- Rainin Pipet-Lite XLS (1-10uL) -- Promega#: 117507
- Rainin Pipet-Lite XLS (2-20uL) -- Promega#: 117588
- Rainin Pipet-Lite XLS (20-200uL) -- Promega#: 11777
- Rainin Pipet-Lite XLS (200-1000uL) -- Promega#: 117667
- Rainin E4-XLS (0.5-10uL) -- Promega#: 119012
- Rainin E4-XLS (10-100uL) -- Promega#: 119011
- Rainin (1-10uL) -- Promega#: 106386
- Rainin (2-20uL) -- Promega#: 106367
- Rainin (10-100uL) -- Promega#: 106366

Refrigerators/Freezers

- -20°C Freezer – Promega#: 105635
- -20°C Freezer – Promega#: 111093
- -20°C Freezer – Promega#: 106279
- 4°C Refrigerator – Promega#: 105641
- 4°C Refrigerator – Promega#: 115150

Sample Collection Bottles

- 1L Nalgene Wide-Mouth Polypropylene Bottle -- Fisher 2187-0032

Supplies

Supply	Catalog #	Storage Condition
96-well PCR Plate-Mx3000P	Agilent -- 401333	RT
96-well Plate Optical Caps	Agilent -- 41425	RT
PureYield Midi Kit	Promega – A245A	RT
PureYield Mini Kit	Promega – A166B	RT
qPCR Reagents	Promega -A6120	-20°C
Primers and Probes	IDT and LGC BioSearch Technologies	-20°C

Analytical Procedures

Analytical methods suitable for intended use will be performed as per Promega's QSOP14.006

Quality Control Checks

The laboratory's Quality control checks for the SARS-CoV-2 assay includes:

- Positive quantitative control: Quantitative Standard SARS-CoV-2 dsDNA containing portions of N and E genes
- Negative Control: Nuclease Free Water
- Process Control Virus: Pepper Mild Mottle Virus (PMMoV)
- Internal Amplification Control: primers, probe and template for an internal amplification control (IAC), a novel DNA template that is included in every amplification reaction.
- Matrix Spike Recovery: MS2 phage

Per Promega's Custom Assay Services (CAS) program, the CAS Quality Assurance reviewer reviews the data report for the criteria listed a reviewing checklist. The checklist includes, but is not limited to confirmation the data report meets the deliverables in the quote or statement of work.

Data Reduction, verification and reporting

qPCR fluorescence signal curves are used to plot Ct values using the MxPro software linked to the Stratagene Mx3005P instrument. The instrument generates data files in Microsoft Excel. Data is saved in the network server. Microsoft Excel is used for data analysis and calculations.

Processed samples (extracted total nucleic acid) will be analyzed by two analysts (Subhanjan Mondal and Nathan Feirer) independently by RT-qPCR for verification.

Corrective actions

For CAS orders, if an unexpected result is observed for Level 2 the CAS Research Manager or the R&D scientist on the project will discuss the unexpected result with the customer and decide path forward.

The decision will be recorded and communicated by email for customer approval.

Any resulting changes will also be communicated to QA. Any actions or decisions will be documented by the Project Manager or CAS Marketing Manager and included in the project record.

Any resulting data from the discussion will also be documented in the project record.

Recordkeeping

Scientists create and document experiments or ideas, notebooks and raw data files in ProBook. ProBook is a system that contains electronic laboratory experiments, notebooks, raw data files, as well as electronic copies of paper laboratory notebooks, electronic copies of paper raw data files and electronic Table of Contents (eTOCs).

In addition to ProBook, Data Reports are generated as part of Custom Assay Services (CAS). The data report completed at the end of each project milestone, summarizes the work performed and demonstrates the meeting of requirements outlined in the Quotation or SOW.

Prototype Wastewater Total Nucleic Acid Extraction Kit

Direct Capture of Nucleic Acid from Wastewater

Introduction

In December 2019, an outbreak of a new severe acute respiratory syndrome coronavirus (SARS-CoV-2) was detected in Wuhan, China. The outbreak quickly became a global pandemic (COVID-19), and by June 2020, over 10 million cases with over half a million deaths were reported. Early in the COVID-19 pandemic, scientific studies demonstrated that the genetic material of SARS-CoV-2, an enveloped RNA virus, could frequently be detected in the feces of infected individuals and thereafter was also detected in wastewater. This finding mobilized the global water sector to investigate if wastewater-based epidemiology (WBE) using the genetic signal of SARS-CoV-2 could be used to track the spread of COVID-19 in communities. In the past, WBE was critical in identifying the community prevalence of poliovirus in support of the World Health Organization's (WHO) program to eradicate poliomyelitis. . Detection of SARS-CoV-2 in wastewater has the potential to provide an integrated, community-level indication of the presence or prevalence of COVID-19.

The process of detecting viral genetic signatures in wastewater samples involves collection of wastewater influent at specific points in the sewage system, either as a grab sample or as a 24-hour composite sample. This is followed by optional heat pasteurization and concentration of viral matter. The viral matter and/or its genetic signature is present at a low concentration in wastewater, making sample concentration a prerequisite for sensitive detection and utility in WBE. Concentration of viral matter can be performed using a variety of methods such as charged membrane filtration, centrifugal ultrafiltration and flocculation/precipitation using skimmed milk, or polyethylene glycol (PEG)/NaCl. Most of the viral concentration methods currently being used were originally developed to concentrate viral matter with the objective of culturing the virus for downstream applications, though they have been also used for direct PCR based detection. Several of the methods described above may work efficiently with several virus types, but they have proven to be inconsistent for SARS-CoV-2 RNA recovery leading to significant analytical variability. In addition, these viral concentration methods are labor intensive and time consuming.

To address these issues, we have developed a convenient method to capture and concentrate total nucleic acids (TNA) from a large volume of wastewater using silica based PureYield® columns. The method uses a short protocol that minimizes the need for specialized laboratory equipment. In a first step total nucleic acid from a large volume sample (e.g. 40 ml of wastewater) is captured on a GFA/silica column (PureYield Midi Column). and then eluted in 1ml. In a second step the material is purified and concentrated using the PureYield Mini column. This method achieves consistent recovery rates and significant reduction in PCR inhibitors.

The total nucleic acid extracted using this kit may be analyzed for SARS-CoV-2 targets using the SARS-CoV-2 RT-qPCR kit for Wastewater (# CS317402, CS317409) or other methods

Kit components

Contains sufficient materials and reagents for 20 extractions

1 × 270ml Binding Buffer 1
1 × 50ml Binding Buffer 2
1 × 10ml Proteinase
1 × 85.3ml Inhibitor Removal Wash
2 × 206ml RNA Wash Solution (RWA)
1 × 150ml Nuclease-Free Water
4 × 5 each PureYield™ Midi Binding Columns (Clear)
2 × 10 PureYield™ Minicolumns
2 × 10 PureYield™ Collection Tubes
2 × 25 1.5ml Elution tubes

Storage Conditions: Store all components at room temperature.

BEFORE YOU BEGIN:

Inhibitor Removal Wash: Add 57ml of isopropanol to the Inhibitor Removal Wash bottle and mark on the bottle "plus isopropanaol". The reagent is stable at 15–30°C when tightly capped.

RNA Wash Solution (RWA): Add 350ml of 95% ethanol to each bottle containing 206ml concentrated RNA Wash Solution (RWA) and mark the bottle "plus ethanol". The reagent is stable at 15–30°C when tightly capped.

Materials to Be Supplied by the User

- Isopropanol
- Ethanol, 95%
- Tabletop centrifuge (capable of 3,000 x g)
- Swinging bucket rotor
- 50ml disposable plastic screw-cap tubes (e.g., Corning or Falcon™ brand)
- 1.5ml microcentrifuge tube
- High-speed microcentrifuge tube centrifuge (capable of at least 10,000 × *g*).
- Vacuum manifold (e.g., Vac-Man® Laboratory Vacuum Manifold [Promega Cat.# A7231])
- Eluator™ Vacuum Elution Device (Promega Cat.# A1071)
- Vacuum pump, single or double stage, producing pressure of approximately 650mm Hg (25.6 inches Hg, 12.57 psi, 86.7 kPa). For example: Welch Vacuum Pump (Model 2522B-01 for North America electrical, Cat.# A6720; Model 2522C-02 for Europe electrical, Cat.# A6722; Model 2522C-05 for Japan electrical, Cat.# A6724)

Direct Viral Nucleic Acid Capture and Extraction

1. Dispense 40ml of optionally pasteurized* wastewater into a 50ml conical tube
   *Note: pasteurize by incubation at 60°C for 1 hour.
2. **A Protease step was found to increase the nucleic acid recovery after the WRF Interlaboratory Method Assessment. The protease step was not performed for the WRF Interlaboratory Method Assessment.** To perform the proteolytic digestion, add 0.5 ml of Protease solution, mix well and incubate for 30 minutes.
3. Clarify sample by centrifugation at 3000xg for 10 minutes. **It is important to remove solids to avoid clogging the PureYield™ Midi Binding Column.**
4. Carefully decant the supernatant into two clean 50 ml conical tubes, 20mL each. Discard the 50ml conical tube containing the pellet into an appropriate biohazard waste container.
5. To each tube containing 20 ml of the clarified supernatant, add 6ml of Binding Buffer 1 and 0.5ml of Binding Buffer 2. Mix well by inversion.
6. Add 24ml of isopropanol to each tube. Mix well by inversion.

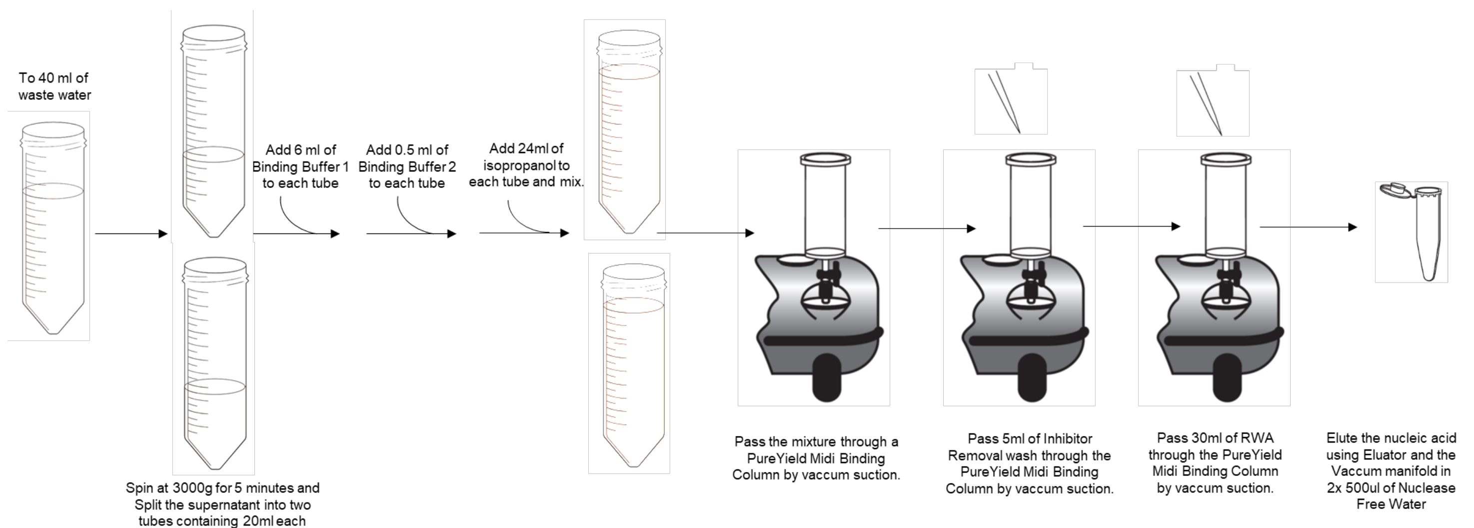

Figure 1. Scheme for direct capture of nucleic acid from wastewater using PureYield Midi Column.

If different sample volumes are tested the following scheme may be used:

Sample volume (ml)	Alk Protease (ml)	Binding Buffer 1 (ml)	Binding Buffer 2 (ml)	Isopropanol (ml)
10	0.125	3	0.25	12
20	0.25	6	0.5	24
40	0.5	12	1	48
80	1	24	2	96

Using lower sample volume can reduce the sensitivity of your assay.

7. Remove the vacuum port cap and attach the PureYield™ Midi Column onto the vacuum manifold by pressing the nozzle gently into the vacuum port fitting as shown in Figure 2.

Figure 2. The PureYield™ Midi Binding Column is attached to a Vac Man vacuum manifold port.

8. Pour the mixture from step 6 into the PureYield™ Midi Binding Column, turn on the pump and apply vacuum to capture TNA on the column. The content of two tubes is added stepwise in 20mL increments to a single column.

Note: Empty the blue Vac-Man® Laboratory Vacuum Manifold as needed.

Note: If the flow rate is <2 ml/ minutes or the pressure reading on the vacuum pump is lower than 10 inches Hg/250mm Hg, there may be a leak in the tubing connections or in the Vac-Man® Laboratory Vacuum Manifold. Consider replacing your existing vacuum manifold.

9. Pass 5ml Inhibitor Removal Wash through the PureYield™ Midi Binding Column by vacuum.
10. Pass a total of 30ml of RNA Wash Solution (RWA) through the PureYield™ Midi Binding Column in two steps20mL plus 10mL by vacuum.
11. Pre-heat 1.2ml per sample of Nuclease–Free Water to 60°C
12. Vacuum elution TNA can be performed using the Eluator™ Vacuum Elution Device (Promega Cat.# A1071), Place a 1.5ml microcentrifuge tube in the base of the Eluator™ Vacuum Elution Device, securing the tube cap in the open position, as shown (Figure 3, Panel A). Assemble the Eluator™ Device and insert the PureYield™ Midi Binding Column into the top of the Eluator Device, making sure the column is fully seated on the collar as shown in Figure 3, Panel B.

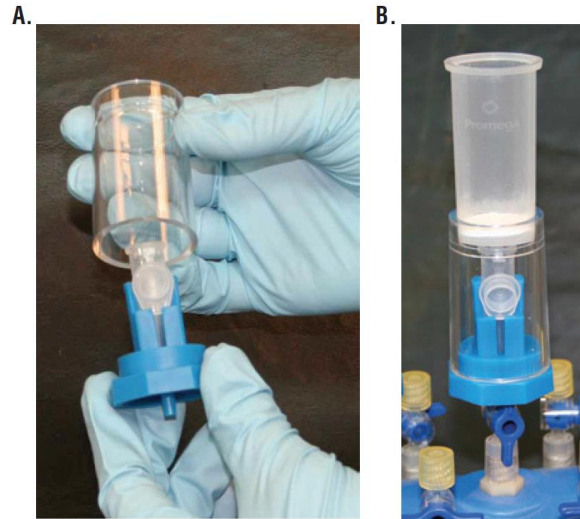

Figure 3. Elution by vacuum. **Panel A.** A 1.5ml microcentrifuge tube is placed in the base of the Eluator™ Vacuum Elution Device with the microcentrifuge tube cap is locked as shown. **Panel B.** The final Eluator™ Vacuum Elution Device assembly, including the binding column, ready for use on a vacuum manifold.

13. Place the Eluator™ Device assembly onto a vacuum manifold (Figure 3, Panel B). Add 500µl of pre-heated (60°C) Nuclease-Free Water to the PureYield™ Midi Binding Column. Apply maximum vacuum for 1 minute or until all liquid has passed through the column. Repeat the process by adding another 500µl of pre-heated Nuclease-Free Water to the PureYield™ Midi Binding Column to collect a total of 1ml of TNA solution.

Concentration and further purification are performed with a Pure Yield Mini Column as follows.

14. Add 400µl of Binding Buffer 1 and 100µl of Binding Buffer 2 to 1ml of liquid eluted in step 13. Mix well by inversion and divide the contents into two 1.5ml tubes containing 750µl each. To each tube add 750µl of isopropanol and mix well.

15. Put the PureYield Mini column into a collection tube. Pass the entire volume of the Mixture through a PureYield Mini Column, 750µl at a time using a MicroCentrifuge set at 10,000g for 1 minute. Pass 300µl of Inhibitor Removal Wash through the PureYield™ Mini Column by centrifugation.

16. Pass 500µl of RNA wash Solution (RWA) twice through the PureYield™ Mini Column.

17. To elute the TNA, transfer the PureYield™ Mini Column to a clean 1.5mL microcentrifuge tube and add 20µl of pre-heated (60°C) Nuclease-Free Water to the column. Let the water soak into the column filter for approximately 1 minute. Elute by spinning at 10,000g for 1 minute. Repeat elution with another 20µl of pre-heated Nuclease Free water, to collect a total of 40µl.

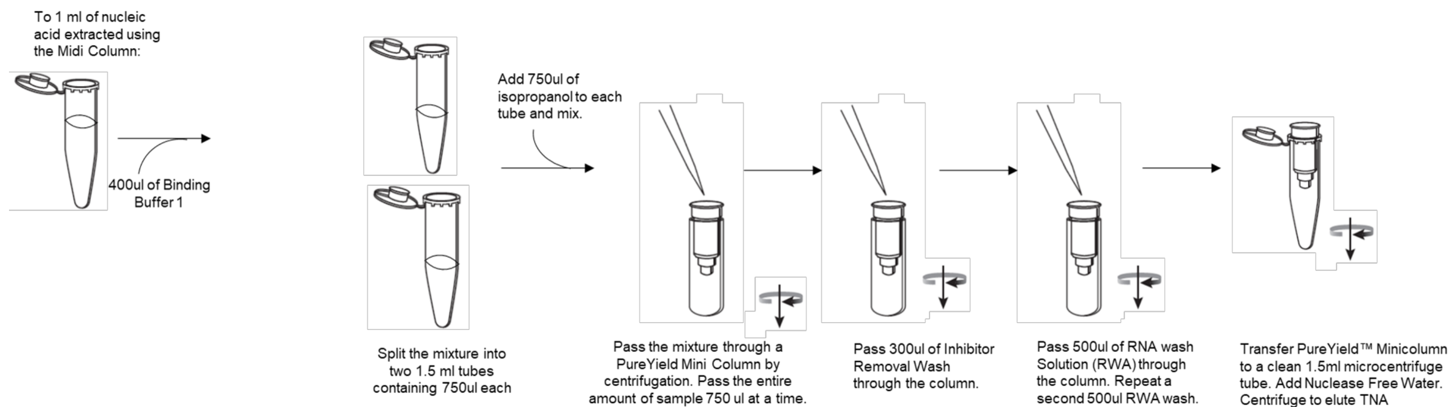

Figure 4. Scheme for clean-up and concentration of the TNA by the PureYield Mini Column.

Store sample at ≤ 20°C until further analysis. TNA purified using this method can be directly used for RT-qPCR.

SOP for wastewater-based monitoring of SARS-CoV-2

UC Berkeley WBE team

Direct RNA extraction via Sewage, Salt, Silica and SARS-CoV-2 (4S): A rapid, economical, and kit-free method for direct-capture of wastewater RNA

PLEASE NOTE: for the most up-to-date protocol, visit:
https://www.protocols.io/view/v-4-direct-wastewater-rna-capture-and-purification-bpdfmi3n

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This rapid and economical method employs common laboratory reagents to extract and directly capture RNA from wastewater using salt-based lysis and silica mediated nucleic acid capture to recover high quality wastewater RNA.

Sample volumes as low as 40 mL have proven sufficient to detect low- abundance viruses such as SARS-CoV-2 present in Bay Area wastewater. This method can be used to detect SARS-CoV-2 RNA in wastewater volumes ranging from 40 mL to 1 L. Further, the method enriches all wastewater RNAs without bias, enabling the simultaneous purification and detection of viral RNAs of interest alongside wastewater human fecal load markers suitable for “fecal load” normalization. Below is a protocol of the steps needed to extract RNA from 40mL of wastewater.

Required reagents and equipment:

Reagents:

- NaCl
- EDTA
- Tris(hydroxymethyl)aminomethane (TRIS), pH 7.2
- Ethanol

Equipment:

- Silica column (Zymo III-P, cat #C1040-5)
- 5uM filter (Durapore SVLP/PVDF)
- Vacuum manifold
- Microcentrifuge capable of 10,000xg speed

Sample preparation for 40 mL wastewater (Thawing, Lysis & Filtration)

1. If frozen, thaw wastewater sample at 37 C in a waterbath until it reaches room temperature.
2. Add 9.35 grams of NaCl (final concentration 4 M), EDTA to a final concentration of 1 mM and Tris (pH 7.2) to a final concentration of 10mM.
3. Agitate sample until all NaCl dissolves. Vortex or shake for 15 seconds.
4. Heat inactivation at 75 C for 45 min (recommended). Unpublished analyses have shown that this step will not affect SARS-CoV-2 detection.
5. Filter lysed sample through a 5 μM PVDF filter via vacuum or syringe filtration.

Proceed to RNA extraction

Direct RNA extraction (RNA Binding, Washing, Eluting)

1. Add 1 volume (40 mL) of 70% ethanol to lysed and filtered sample.
    a. Agitate sample to mix ethanol and wastewater lysate.
2. Pass the 80 mL lysed and ethanol treated sample through Zymo III-P silica column using a vacuum manifold.
3. Pass 5 mL of 4SWB1 through the column to wash.
4. Pass 10 mL of 4SWB2 through the column to wash.
5. Detach the reservoir from the column, and centrifuge the column at 10000xg for 2 minutes to remove residual 4SWB2.
6. Add 200 uL of elution buffer (Commercial column elution buffer, e.g. ZymoPURE, or pH 8 TE buffer) preheated to 50 C.
    a. Incubate columns with elution buffer for 10 minutes.
7. Place columns into LoBind 1.5 mL microfuge tubes, and spin at 10,000xg for 5 minutes to elute bound RNA.

The eluted RNA is now present in the spun-through eluate. Proceed with downstream RNA analysis via qRT-PCR, sequencing or other.

4S buffer recipes

4SLB – Lysis buffer	Original molarity or concentration	Final molarity or concentration	Volume per litre
NaCl	Dry salt	4M	232 grams
Tris pH 7.2	1M	10mM	10mL
EDTA	0.5M	1mM	2mL
Water			Add to 1L

4SWB1 – Wash buffer #1	Original molarity or concentration	Final molarity or concentration	Volume per litre
NaCl	5M	1.5M	300mL
Tris pH 7.2	1M	10mM	10mL
Ethanol	100%	20%	200mL
Water			490mL

4SWB2 - Wash buffer #2	Original molarity or concentration	Final molarity or concentration	Volume per litre
NaCl	5M	100mM	20mL
Ethanol	100%	80%	800mL
Tris pH 7.2	1M	10mM	10mL
Water			170mL

Quantitative Reverse Transcription PCR

- Materials and reagents
- TaqMan FastVirus mastermix
- Primers and probes
- DEPC-treated PCR water
- Cleaning: bleach, ethanol, RNase away
- Strip tubes
- Plates
- Plate seals

Equipment

- QuantStudio 3

Mastermix recipe:

Reagent	Volume per reaction (ul)
4x Taqman FastVirus mastermix	5
Primer/probe mix (IDT ROU SARS-CoV-2)	1.5
Nuclease-free H2O	8.5
Total	15

Plate preparation

1. Make mastermix in 5 mL LoBind tube.
2. Add 15 ul of mastermix per well.
3. To three NTC wells, add 5 ul DEPC-treated PCR water.
4. In strip tubes, prepare serial 1:10 dilutions for standard curves using DEPC-treated PCR water. Design standard curve concentrations to cover expected range of samples.
5. Pipet 5 ul of each standard dilution into triplicate wells.
6. Thoroughly clean workspace with bleach, ethanol, and RNaseaway before continuing.
7. Pipet 5 ul of each sample into triplicate wells.
8. Seal plate.

9. Spin down the plate.

Analysis

1. Set up plate in QuantStudio software.
2. Run plate (see conditions below).
3. Interpret data using QuantStudio software and manually account for outliers within triplicates.

SARS-CoV-2 N1 cycling conditions

Incubation	25	2:00
RT step	50	15:00
Polymerase activation	95	2:00
#cycles: 45	95	0:03
	55	0:30
	-	**-**
Final Extension	NONE	
Melt Curve	NA	

SOP for SARS-CoV-2 Detection in Wastewater by QIAamp Viral RNA Kit
(for Inter-Lab comparison only)

Jiang Lab
University of California, Irvine
Version: September 2020

1. Upon sample arrival in the lab, place one sample in 60 °C incubator for 90 mins (labeled pasteurized) and leave the second sample un-pasteurized in 4 °C refrigerator
2. Centrifuge both samples at 4,000×g for 30 min to remove large debris
3. Add 280 µL of supernatant from step 2 to 1120 µL viral RNA lysis buffer from QIAamp Viral RNA Kit
4. Perform RNA extraction using QIAamp Viral RNA Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocols
5. Quantify RNA using nano-drop
6. Use RNA (8-12 µL) from previous step for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol
7. Proceed directly with PCR and ddPCR or store at -20 °C until analysis
8. Perform ddPCR using N1, N2 primers and probes reported by CDC
9. Prepare ddPCR reaction following the instruction from Bio-Rad (Hercules CA, USA) for QX200 Droplet Digital PCR System
10. Assemble the ddPCR reaction mixture according to Table 1
11. ddPCR thermocycling conditions is set for: 95 °C for 10 min (DNA polymerase activation), followed by 40 cycles of 94 °C for 30 s (denaturation) and 55 °C for 60 s (annealing), and 98 °C for 10 min followed by an infinite 4 °C hold
12. Analyze the ddPCR data using QuantaSoft Analysis Pro software v.1.0.596 (Bio-Rad, Hercules CA, USA) to calculate the concentration of the target.

Table 1. ddPCR mixture for SARS-CoV-2 detection

Component	**Volume per Reaction (µl)**	
	N1	**N2**
Primers/probe (FAM)	1	1
	500nM/125nM	500nM/125nM
Sample	2 (Variable)	
2x ddPCR Supermix for Probes (No dUTP)	10	
RNase-/DNase-free water	7 (Variable)	
Total volume	20	

Standard Operating Procedure for Processing and Enumerating Enterovirus, Adenovirus, Norovirus, SARS-CoV-2, and the Corresponding Matrix Spikes in Raw Wastewater

EPA Method 1615 along with the following addendum shall be used to process and analyze the virus sample by both culture and molecular methods. Many modifications have been made to EPA Method 1615, including revisions to the concentration/purification steps, addition of a culture and molecular procedure for adenovirus; a molecular procedure for SARS-CoV-2 and betacoronavirus OC43 (enveloped viruses); and revisions to the matrix spike organism, method, and frequency. The addendum lists the method modifications and clarifies certain steps in EPA Method 1615. Follow <u>all</u> of the steps in EPA Method 1615 unless otherwise specified by the addendum.

Refer to EPA Method 1602 for the list of required equipment, supplies, and reagents for the matrix spike assays.

In this addendum, dashed lines (------) are used to highlight sections with large additions and/or sections that are highly modified.

<u>Addendum to EPA Method 1615:</u>

1.1	Method has been modified to also include culture and/or molecular procedures for adenovirus, SARS-CoV-2, betacoronavirus OC43 (matrix spike), MS2 (matrix spike); and PhiX174 (matrix spike). The method has been modified specifically for use with raw wastewater.
2.0	Significant modifications have been made.
6.1	Not applicable. The sample will not be filtered.
6.2	Not applicable - WWTP will do the sample collection.
6.3.1	Not applicable. Matrix spike sample is same as field sample.
6.3.6	Not applicable. The sample will not be filtered.
6.3.7	Not applicable. Matrix spike sample is same as field sample.
6.3.8	Not applicable. The sample will not be filtered.
6.4	Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615.
10.0 & 11.0	Concentration and Purification has been modified for analysis of raw wastewater.
12.0	Addition of Adenovirus and SARS-CoV-2/betacoronavirus OC43 analysis
13.0	Modification from two-step RT-qPCR procedure to a one-step RT-qPCR procedure. Some primer sets have been modified or added.

ADDITION TO SECTION 6: In addition to the equipment/supplies specified in 1615, the following equipment/supplies will be needed:

Equipment and Supplies for Concentration/Purification and Nucleic Acid Extraction:

1. Aerosol/filter barrier micropipette tips, 20, 200, 1000 μL (E&K Cat # EK 3016 and EK 3025, or equivalent)
2. Micropipettes
3. Vortex Mixer
4. Waterbath at 56 °C
5. Refrigerated large capacity swing bucket centrifuge (Beckman X-14R or equivalent) for 250 or larger conical bottom tubes.
6. High speed refrigerated centrifuge (Beckman JE or equivalent) capable of running 13,500xg using 50 or 15 mL tubes
7. pH Meter
8. Balance
9. Transfer Pipets, disposable (Fisher brand 13-711-9BM, or equivalent)
10. Centrifuge Tubes, 15, 50, 250, 500 mL including 15 mL tubes rated for high speed centrifugation (13500xg) (VWR 21008- 242, or equivalent)
11. 1, 2, 3 and/or 4-L Sterile beakers with sterile stir magnetic bars
12. Microcentrifuge Tubes, 1.5 mL
13. Micro Tubes, screw cap, 1.5 mL (VWR 89004-294, or equivalent)
14. 0.2 uM sterilizing filters (Sigma-Millipore Cat# F-9768 for filtering up to 250 ml)
15. Centrifugal concentrator: Vivaspin 20 (30,000 MWCO PES) Sartorius Cat# VS022 or equivalent
16. DNA/RNA purification kit (Zymo Quick-DNA/RNA Viral Kit # D7020 or equivalent

Equipment and Supplies for MS2 and PhiX174 Culture

Refer to Section 6 of EPA Method 1602 (attached) for the equipment and supplies needed for the MS2 and PhiX174 culture assays. Use 1X TSA for plaque assay

Equipment and Supplies for MS2 and PhiX174 Molecular

No additional equipment or supplies are required for the MS2 and PhiX174 molecular assays

Equipment and Supplies for Adenovirus Culture

No additional equipment or supplies are required for the adenovirus culture assays

Equipment and Supplies for Adenovirus Molecular

No additional equipment or supplies are required for the adenovirus molecular assays

Equipment and Supplies for SARS-CoV-2 Molecular

No additional equipment or supplies are required for the SARS-CoV-2 molecular assays

Equipment and Supplies for OC43 Culture

Equipment and Supplies for OC43 Molecular

No additional equipment or supplies are required for the OC43 molecular

CONTINUE TO SECTION 7.0 AFTER COMPLETING SECTION 6 (W/ ADDITIONS)

7.2.4 Not applicable. Matrix spike will be MS2 and PhiX174 for the enteric viruses ,rather than Sabin Poliovirus 3, and OC43 for enveloped viruses,.

7.3 Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615

ADDITION TO SECTION 7: In addition to the reagents, media, and standards specified in 1615, the following reagents and standards will be needed:

Reagents and Standards for Concentration/Purification and Nucleic Acid Extraction:

1. Purity of Reagents: ACS reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The microbiological growth media must be of microbiological grade.
2. Chloroform:Butanol: Add equal parts chloroform and butanol together. Shake to mix. Store for three months refrigerated. **Note: do not use chloroform:butanol reagent with the enveloped virus procedure.**
3. Phosphate Buffered Saline (PBS): Add 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 to 1000mL of molecular grade water. Mix until dissolved. Adjust the pH to 7.3. Sterilize by autoclaving. Store refrigerated for 3 months.
4. Molecular Grade Water (MGW, VWR 82021-434 or equivalent)
5. Sodium Chloride (BDH9286-2.5KG)
6. Polyethylene Glycol MW 8000 (VWR 97061-098, or equivalent)
7. Chloroform (Sigma 496189-1L, or equivalent)
8. Butanol (Sigma B7906-500 mL, or equivalent)

9. Sodium phosphate dibasic (Na2HPO4.7H2O) (BDH9296-500G)
10. Trisodium citrate dihydrate (BDH9288-500G)
11. BBL Beef Extract Desiccated Powder (BD 211520)

12. 5% BSA (5g of albumin/bovine crystalline (United States Biochemical cat# 10856 or equivalent)
13. 0.2% BSA in PBS (Dulbecco's Phosphate buffer saline, without CaCl2 and MgCl2)
14.

Reagents and Standards for Matrix Spike (MS2 and PhiX174)

1. Refer to Section 7 of EPA Method 1602 (attached) for the reagents and standards needed for enumeration of the matrix spikes MS2 and PhiX174.
2. Prepare MS2 and PhiX174 spiking suspensions

a. MS2 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this solution if is less concentrated than 10^8PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**
b. PhiX174 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this solution if is less concentrated than 10^8 PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**
c. **The laboratory must perform a molecular assay of the MS2 and PhiX174 spiking suspensions every time a sample is spiked with these matrix spikes to determine the concentration (as genome copies per liter) of the stock.**

3. Primers, probes, and standards for molecular assay:
PhiX174 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**: 4242-4250**
Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'
Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'
Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'
MS2 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**:4242-4250)**
Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'
Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'
Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'

Reagents and Standards for Adenovirus Culture Assay

Adenovirus stock.

1. Adenovirus stock; Adenovirus serotype 10 (AdV10; ATCC 1504) stored at -70°C. for use as a positive control in the culture assay

Cell culture media.

1. DMEM (Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
   a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL

Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    a. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    b. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

A549 cell culture.

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
    a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
    b. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.

5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.
6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 (~3×10^5 per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.

15. If cells used for bioassay are not confluent within 1-4 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

Reagents and Standards for Adenovirus Molecular Assay

1. Human Adenovirus Primers/Probe (Integrated DNA Technologies or equivalent) (Ko *et al*., J. Virol Methods, 2005; 127: 148-153)

Adenovirus

Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'
Probe: 5'-**CTGACACGGGCACTCTTCGC**-3'

2. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
3. ABI real-time PCR Machine (or equivalent)
4. strip tubes and caps or 96 well plates and optical film
5. Standards for preparing the standard curve.

Reagents and Standards for OC43 Matrix Spike

OC43 stock.

1. OC43 (Betacoronavirus 1 (ATCC® VR-1558™) stored at -70°C for use as a positive control in the molecular assay

Cell culture media.

1. DMEM Media **(**Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity)

and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    i. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    ii. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

HRT cell culture (HRT-18G (ATCC CRL-11663)).

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
    ii. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.
5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.

6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 (~3×10^5 per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.
15. If cells used for bioassay are not 80-90% confluent within 1-3 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

OC43 Primers/Probe for Molecular Assay.

1. OC43 (Integrated DNA Technologies or equivalent):
   Forward Primer: 5′- CGATGAGGCTATTCCGACTAGGT -3′
   Reverse Primer: 5′- CCTTCCTGAGCCTTCAATATAGTAACC -3′
   Probe: 5′- TCCGCCTGGCACGGTACTCCCT -3′

Reagents and Standards for Enveloped Virus Molecular Assay:

1. Coronavirus SARS-CoV - Primers/Probe (2019-nCoV CDC EUA Kit, 500 rxn Cat# 1000660 from Integrated DNA Technologies or equivalent)
2. **Zymo Quick-DNA/RNA Viral Kit #** D7020) or equivalent.
3. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
4. ABI real-time PCR Machine (or equivalent)
5. strip tubes and caps/ 96 well plates and optical film
6. Standard for preparing the standard curve: Plasmid linearized by Cel serially diluted

CONTINUE TO SECTION 8.0 AFTER COMPLETING SECTION 7 (W/ ADDITIONS)

8.0 The QA/QC requirements for this project are summarized in the following table:

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Demonstration of capability for this study	Three field sub-samples with blind spikes (poliovirus) and three field sub-samples without blind spikes; both shall include matrix spikes (MS2 and PhiX174)	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (Do not need to run adenovirus or norovirus assays)	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	Appendix D of RFQ 4952
QC sample set-Enteric viruses	One positive control (poliovirus in reagent water)	Modified 1615 (PEG/chloroform extraction,	Once initially and then once per month for the	To demonstrate ongoing control of the analytical system and verify	Appendix D of RFQ 4952

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
	and one negative control	culture, and molecular) (For positive control, do not need to run adenovirus or norovirus assays)	duration of the study	continuing method performance	
Matrix spike Enteric viruses	MS2 and PhiX174 in field sample	Modified 1615 (PEG/chloroform extraction, culture, and molecular) Verify the concentration of the spiking suspension in terms of both PFU/L and GC/L every time a matrix spike is performed	Every other field sample from each WWTP during full monitoring campaign; every field sample during pre-campaign testing	To determine the effect of the matrix on virus recoveries	Appendix D of RFQ 4952
QC sample set – Enveloped Viruses	One positive control (OC43 in reagent water) and one negative control	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	To Be Determined
Matrix Spike Enveloped Viruses	OC43 in field sample	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay Verify the concentration of the spiking suspension (GC/L) every time a matrix spike is performed	Every field sample from each WWTP during campaign testing	To determine the effect of the matrix on enveloped virus recovery	To Be Determined
Positive Control: Enterovirus Culture Assay	3 culture test vessels with poliovirus in buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Positive Control: Adenovirus Culture Assay	3 culture test vessels with adenovirus in buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Negative Control: Enterovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Negative Control: Adenovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: MS2 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: PhiX174 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* CN-13)	Every *E. coli* CN-13 culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: Molecular Assay	1 sample with PCR grade water	(rt)-qPCR	One per every fourth sample on a plate	To verify the lack of contamination	EPA 1615 Section 13.4.4.2
Inhibition Control (Positive Control): Molecular Assay – Enteric Viruses	1 test sample with hepatitis G armored RNA (compare to hepatitis G in reagent water)	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	EPA 1615 Section 13.6.2
Inhibition Control (Positive Control): Molecular Assay, Enveloped Viruses	TaqMan™ Internal Controls run with OC43 assay	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	To Be Determined

8.3.1 **PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952. One QC sample set will be processed initially and then one QC sample set per month thereafter.**

8.4 One QC sample set (poliovirus and OC43 betacoronavirus in reagent water and a negative control) should be conducted per month during the duration of this monitoring campaign (and one set initially). This QC sample set should be processed and analyzed using the modified 1615 method for enteric viruses and the enveloped virus method for coronaviruses.

8.4.1.1 Sample volume is 1100 mL

8.4.1.2-8.4.1.4 Skip these steps (filtration has been eliminated from the sample prep)

8.4.1.5 Process and analyze the negative QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure, Sections 10 and 11, below), enterovirus culture assay, adenovirus culture assay, MS2 culture assay, PhiX174 culture, and molecular assay procedures (enterovirus, adenovirus, norovirus, MS2, and PhiX174).

8.4.2 Sample volume is 1100 mL

8.4.2.2 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.5 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.6 Enteric Viruses: Process and analyze the positive QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure; Sections 10 and 11, below), enterovirus culture assay, and enterovirus molecular assay.

8.4.2.7 Enveloped Viruses: Process and analyze the positive QC sample by adding 1ml of 10^6 GCMPN/ml OC43 virus to 1100 ml of laboratory deionized water and process (see the modified concentration/purification procedure; Sections 10 and 11, below), OC43 molecular assay.

8.4.3 **Laboratory performance for the positive QC samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952**

8.5 PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952.

8.6.1 Enteric viruses: Run a matrix spike for every other field sample from each site (during the pre-campaign testing, a matrix spike will be run with every field sample).

8.6.1.1 Enveloped viruses: Run a matrix spike for every field sample from each site.

8.6.2 **Replace with the following steps:**

8.6.2 REPLACEMENT: To the samples that will include a matrix spike, complete the following steps:

1. **Spike 10^8 PFU of MS2 to 1100 mL of the field sample** (the same sample that is used for measuring the native virus). This number can be achieved by adding approximately 0.1 mL of a MS2 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the MS2 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of MS2 in the field sample of 10^8 PFU/L).
2. To this same sample, **spike 10^8 PFU of PhiX174**. This number can be achieved by adding approximately 0.1 mL of a PhiX174 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the PhiX174 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of PhiX174 in the field sample of 10^8 PFU/L).
3. Spike **10^7 GCMPN/ml of OC43** to 1100 ml of the field sample (the same sample that has been spiked with MS2 and PhiX174 and is used for measuring the native virus). This number can be achieved by adding approximately 1ml of an OC43 spiking suspension with a concentration of approximately 10^7 GCMPN/ml (may adjust the volume of the OC43 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of OC43 in the field sample of 10^7 GCMPN/L).
4. Process and analyze the sample using the sample procedures that are used for the samples without the matrix spike with the exception that this sample will also

be analyzed for MS2 and Phix174 using both culture and molecular procedures; and, for OC43 use molecular procedures only.

5. Laboratories are to report PFU in all dilutions where there are countable plaques (zero to 300 PFU per plate for male-specific (F^+) coliphage and zero to 100 PFU per plate for somatic coliphage. For too numerous to count plates, please enter TNTC in the comment section of the report associated with that dilution. Make the final calculation based on all dilutions with countable plaques (refer to equation in section 13.1.5 from EPA 1602).
6. For enveloped viruses, report the concentration as determined from rt-qPCR standard curve.

CONTINUE TO SECTION 8.6.3

8.6.3 **Laboratory performance for the MS samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952.**

9.1 Replace this step with the following:
A 4 L raw wastewater sample will be collected by the wastewater treatment facility staff and will be delivered to the lab. The sample should be labeled with a unique sample number. The facility staff should record the unique sample number, facility name, sampler's name, location at sampling site, date, and time on a sample data sheet

9.2. Procedure is same only the 4 L sample will not be filtered so there is no cartridge housing or intake/discharge module. The sample should be shipped cold (1-10 °C) not frozen.

9.3.2 Labs will receive a 4 L sample (using a portion for measuring virus, a portion for protozoa, and a portion for phage). The samples will not be filtered, so the lab will not receive a cartridge filter.

10 and 11 **Skip these steps. Instead complete the following modified concentration/purification procedure:**

MODIFIED CONCENTRATION/PURIFICATION PROCEDURE (to replace Section 10 and 11 of EPA 1615)

1. Transfer 1100 ml of raw sewage into sterile beaker. <u>Record this volume (designated Total Sample Volume, TSV) on the virus data sheet.</u>
2. <u>After spiking the virus matrix spikes,</u> incubate for 30 min at Room temperature.
3. Add 1.43 g Sodium phosphate dibasic, 1.32 g Trisodium citrate dihydrate and 11 g beef extract to the sample and stir to dissolve.
4. Stir for 30 minutes at room temperature. Record pH (should be ambient 7.2-7.5). Remove 100 ml aliquot and process the aliquot according to the following steps:
    a. In duplicate, load 15-20 mL of the aliquot into ultrafiltration concentrator-Vivaspin 20 (pre-soaked in PBS plus 0.2% BSA for 2hr to 24hr). Freeze the remaining aliquot at -80°C for backup and archival purposes.

   b. Centrifuge the filter at 4000-5000 x g for 30 minutes or until the concentrate is reduced to less than 1 mL.
   c. Record initial and final volume. Resuspend concentrate and transfer to sterile 1.5 mL sterile tube. Store at 4 +/- 3° C for up to 24 hours or at -80°C until extraction. Proceed to SECTION 13 (Molecular Analysis).

5. Slowly adjust pH of the remaining sample (approximately 1 L) for enteric virus analysis to 9.5 +/- 0.1 using 1-3 N NaOH. Stir at room temperature for an additional 60 minutes, confirm pH is still in range after 30 minutes.
6. Transfer into 250- or 500-mL centrifuge tubes. Centrifuge at 3,000 x g for 20 minutes (brake no more than 20%).
7. Decant or pipet supernatant into 1 L container and record the volume. Discard pellets.
8. Check pH of supernatant using pH meter and adjust to 7.2+/- 0.2 using 1-3 N HCL.
9. Add 52 g of Sodium chloride (NaCl) and mix/stir to dissolve then add 120 g PEG 8000and Mix by inversion or stirring for minimum 2 hours/ or up to 20 hours at 4 +/- 3° C
10. If necessary, transfer to appropriate centrifuge tube. Centrifuge at a minimum of 4,000 x g for 45 mins at 4°C (Brake at no more than 20%).
11. Carefully decant and discard the supernatant. Invert tube over a paper towel for up to 30 seconds to ensure liquid removal from tube.
12. Resuspend the pellet in 1-2 mL of cold PBS. Mix with pipet tip. Avoid making bubbles. Do not vortex.
13. <u>Quantitatively</u> Transfer (using additional 0.5 mL cold PBS) the re-dissolved pellet to a 15 mL chemical resistant centrifuge tube
14. <u>Record the re-dissolved pellet final volume resulting from the previous step (designated Final Concentrated Sample Volume, FCSV) on the virus data sheet.</u>
15. Add 4 mL (or twice the volume of the transferred volume if it is greater than 2.5 mL) of chloroform/butanol solution. Vortex and let stand for 5 minutes.
16. Centrifuge at minimum of 13500 x g for 15 minutes at 4°C
17. Using a micropipette, transfer the top layer (the aqueous phase) to a sterile 15mL centrifuge tube (approximately 2 mL). Chloroform will be on the bottom. Avoid transferring chloroform, go slowly and do not slant tube while transferring.
18. Record the weight of the 15 mL centrifuge tube before and after transfer. Record both and calculate saved volume. Record this volume (designated FCSV recovered after extraction) on the virus data sheet.
19. The pellet/concentrate can be held for up to 18 hours in refrigerator or frozen at -80°C for longer storage. Freezing and thawing can lead to virus losses so avoid multiple freeze/thaw cycles.
20. A portion of the pellet/concentrate will proceed to nucleic acid extraction, a portion of the pellet/concentrate will be diluted and then proceed to the culture assays, and the remaining portion of the pellet/concentrate will be frozen for backup/archival purposes.
    a. 0.4 mL of the pellet shall be diluted to a total volume of 4 mL using cold PBS.

i. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the enterovirus culture assay (called subsample 1)
ii. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the adenovirus culture assay (called subsample 1B)
iii. 0.3 mL of the 4 mL re-dissolved pellet/concentrate will be used for the MS2 and PhiX174 culture assay (called subsample 1C)
iv. The rest of the 4 mL re-dissolved pellet/concentrate will be frozen at -70°C for backup and archival purposes

b. 1 mL of the pellet will be used for nucleic acid extraction (called subsample 2)
c. The remaining pellet will use frozen at -70°C for backup and archival purposes (called subsample 3)

Proceed to Section 12 (with the addition of adenovirus, MS2, and PhiX174 culture) and Section 13 (with the addition of adenovirus, SARS-CoV-2, OC43, MS2, PhiX174 molecular). Section 12 and Section 13 may be completed in parallel.

SUBSAMPLE 1 SHALL PROCEED TO SECTION 12.0 (culture analysis of enterovirus)

12.1.2.2 **The inoculum volume is 0.1 mL**. Record the Inoculum Volume on the virus data sheet. The number of cell culture **replicates should be 10**.

12.1.2.3 The laboratories should prepare **10- and 100-fold dilutions for every analysis** of the raw wastewater samples.

12.1.2.3.1 The 10-fold dilution is prepared by diluting 0.3 mL of subsample 1 (from step 17.a.i; Section 10 and 11, above) to a volume of 3.0 mL with cold PBS.

12.1.2.3.2 The 100-fold dilution is prepared by diluting 0.3 mL of the 10-fold dilution to a volume of 3.0 mL with cold PBS

12.1.2.4 **The lab must perform these negative controls**

12.1.2.5 **The lab must perform these positive controls**

12.1.4 **Lab must perform a second passage for all negative flasks; not positives. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.**

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

12.1.4.3 Skip this step

12.2.5 The Volume of Original Water Sample Assayed (D) is calculated by the following equation:

$$D = S \times \frac{TSV}{FCSV}$$

12.2.9 The QC samples are the negative and positive controls

ADDITION to Section 12: CULTURE ANALYSIS ADENOVIRUS (SUBSAMPLE 1B)

The adenovirus culture assay involves an MPN approach similar to what is specified in 1615 for enterovirus, with 10 replicates at each dilution and three dilutions (undiluted, 10-fold, and 100-

fold). **Lab must perform a second passage for all negative flasks; not positive flasks. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.** Run negative and positive controls (3 vessels as positive control and 3 vessels as negative controls) with each culture assay.

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

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ADDITION to Section 12: CULTURE ANALYSIS MS2 and PHIX174 (SUBSAMPLE 1C)
(Adapted from EPA Method 1602, Section 12)
<u>Only complete this step for samples that contain matrix spikes of MS2 and PhiX174 (every other virus sample from a given site should contain these matrix spikes)</u>

1. **Four dilutions** of concentrate will be analyzed in **duplicate** for each coliphage type. As a result, nine double-agar layer plates will be required for each coliphage type: two plates per dilution (0.1, 0.01, 0.001, 0.0001) and one method blank plate.
2. Dilutions:
   a. Four dilutions will be needed: 0.1, 0.01, 0.001, 0.0001. **Note: Subsample 1C (prepared in step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) is already 0.1 dilution.** Additional dilutions (beyond 0.0001) may be necessary. PBS (or PBW) is used as the diluent and is used to prepare the negative control.
   b. To prepare the dilutions, aseptically add 0.9 mL of PBS (or PBW) into each of four (or more) sterile dilution tubes. Label them as “0.01,” “0.001,” “0.0001,” “method blank,” etc.
   c. Add 0.1 mL of the subsample 1C (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) to the tube of PBS (or PBW) labeled “0.01.”
   d. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
   e. Add 0.1 mL of the well-mixed 0.01 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.001".
   f. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
   g. Add 0.1 mL of the well-mixed 0.001 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.0001.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
   h. Add 0.1 mL of PBS (or PBW) the tube labeled “method blank.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
3. Agar preparation:
   a. Place TSA molten agar with respective antibiotics in a 48-53°C water bath. The agar should remain molten in the water bath until ready for use. 18 tubes are necessary to enumerate four dilution volumes in duplicate for each phage. The 18

tubes also include an additional method blank tube for each phage type. Nine of the top agar tubes should contain nalidixic acid for growth of *E. coli* CN-13; the other nine should contain ampicillin/streptomycin for growth of *E. coli* F_{amp}.

b. As a precaution against contamination, disinfect a workspace near the water bath with a 1:100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.
c. Assemble plates and label so that the following information is identifiable:
    i. Dilution of stock filtrate or method blank
    ii. Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
    iii. Coliphage type (somatic for the *E. coli* CN-13 bacterial host or male-specific for the *E. coli* F_{amp} bacterial host)
    iv. Date
    v. Time

4. Please note: The following steps are critical. To ensure viability of bacterial host and coliphage, do not add bacterial host and concentrate (re-dissolved pellet) until ready to plate.
5. Preparation of plates for enumeration of somatic coliphage:
    a. With the agar still in the water bath, aseptically inoculate tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN-13.
    b. Immediately add 100 µL (0.1 mL) of subsample 1c (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above).
    c. Add 12-15 mL molten agar
    d. Mix the inoculum by rolling the tube briefly in palm of hand.
    e. Pour contents into one of the two petri plates marked “undiluted, *E. coli* CN-13, somatic."
    f. Duplicate analysis—Repeat Sections 5a through 5d for the duplicate.
    g. Repeat Sections 5a through 5e for each dilution volume.
6. Preparation of plates for enumeration of male-specific (F^+) coliphage—Repeat Section 5 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}
7. Preparation of somatic coliphage method blank:
    a. With the agar still in the water bath, aseptically inoculate a tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN- 13.
    b. Immediately add 100 µL (0.1 mL) of TSB from the “method blank” dilution tube. Add 12-15 mL molten agar.
    Mix the inoculum by rolling the tube briefly in palm of hand.
    c. Pour contents into a petri plate marked “method blank, *E. coli* CN-13, somatic."
8. Preparation of the male-specific (F^+) coliphage method blank—Repeat Section 7 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}.
9. After the agar hardens, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0°C.
10. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 14 to 24 hours of incubation are plaques. Count the number of

plaques on each plate. Please note: The use of a light box to evaluate results is recommended. Record the results in the virus sheet.

SUBSAMPLE 2 SHALL PROCEED TO SECTION 13 (Molecular Analysis)

13.1 – 13.3 **Replace these steps with the following modified nucleic acid extraction procedure:**

MODIFIED NUCLEIC ACID EXTRACTION PROCEDURE (replace 13.1 – 13.3) or

1. **Follow manufacturer instructions in commercial kit (Zymo Quick-DNA/RNA Viral Kit #** D7020) or equivalent.
2. Add 2 parts of Viral DNA/RNA Buffer to 1 part of sample and mix well (DNA/RNA Shield step is not necessary).
3. Transfer 700 µl of the mixture into one IIC-XL Column in a collection tube and centrifuge @ 10,000 x g for 1-2 minutes. Transfer the column into a new collection tube.
4. Reload the column with another 700 µl of the mixture and repeat centrifuge. Repeat until all of the mixture has been passed through the column.
5. Add 500 µl Viral Wash Buffer to the column, centrifuge for 30 seconds @ 10,000 x g and discard the flow-through. Repeat this step.
6. Add 500 µl ethanol (95-100%) to the column and centrifuge for 1 minute @ 10,000 x g to ensure complete removal of the wash buffer. Carefully, transfer the column into a 1.5 mL nuclease-free tube.
7. Add DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 seconds @ 10,000 x g.
8. The final extraction concentrate volume is 0.2 mL (enteric viruses) and 0.05 mL (0.1 mL in total) (coronaviruses)
9. Refrigerate at 4°C for a maximum of 24 hours or <-20°C for up to one week until PCR analysis. Indefinite storage @ -80°C.
10. Extract RNA from poliovirus, MS2, PHIX174, and OC43 stocks following the protocol above to calculate percent recoveries.

CONTINUE TO SECTION 13.4 of EPA 1615 for Molecular Enumeration of Enterovirus, Norovirus, Adenovirus, OC43, SARS-CoV-2, MS2, and PhiX174

13.4, 13.5 **These two steps may be completed as a one-step reaction (i.e., reverse transcription should be completed in the same tube as qPCR); Each assay will be completed in triplicate**. The test sample requires 36 plate wells/tubes (i.e., 3 replicates x 12 qPCR assay). The 12 assays are enterovirus, norovirus GIA, norovirus GIB, norovirus GII, hepatitis G, adenovirus, MS2, and PhiX174, SARS-Cov-2 (N1, N2 & E), and OC43. 5-6 µL of the concentrate from nucleic acid extraction will be used in each of plate wells/tubes. Note: the assays for MS2 and PhiX174 should only be run if the sample contains these matrix spikes (every other virus sample from each site should contain these matrix spikes)

- For RT-qPCR 1-step assays: Refer run conditions for each primers/probe is specified in Tables 5 though 11
- For qPCR assays: refer to run conditions specified in Tables 10, and 12
- SARS-CoV-2 CDC N gene RT-qPCR (One step) assays**:** Run condition is specified in Table 14
- WHO E gene RT-qPCR (One step) assay: Run condition is specified in Table 15

Confirm (move if necessary) the threshold line lies within the exponential phase of the fluorescence curves and above any background signal.

13.5.6 Use the following equation to calculate genome copies per liter in the original sample rather than Eq. 5 in EPA 1615:

$$GC_{original\ sample\ per\ L} = \frac{GC_{assay} \times V_{nucleic\ acid\ extract} \times FCSV \times DF}{V_{assay} \times V_{pellet\ for\ molecular} \times TSV}$$

Where,
$GC_{original\ sample\ per\ L}$ = Calculate genome copies per liter in the original sample
GC_{assay} = Genome copies measured from the qPCR assay
$V_{nucleic\ acid\ extract}$ = Volume of the concentrate after nucleic acid extraction (should be 0.2 mL)
FCSV = Final concentrated sample volume (before chloroform extraction) (should be 2.0 mL)
DF = dilution factor = the reciprocal of any dilution performed to compensate for inhibition (e.g., 10 and 100 for the 1:10 and 1:100 dilutions)
V_{assay} = volume of the nucleic acid extraction used for each (rt)qPCR assay (should be 0.006 mL)
$V_{pellet\ for\ molecular}$ = volume of the re-dissolved pellet/concentration used for nucleic acid extraction (should be 1 mL)
TSV = total sample volume (should be 1 L)

13.5.7 skip

13.6 **The laboratory must use hepatitis G armored RNA as an inhibition control in all assays.** The laboratory may option 1, 2, or 3 of 1615 as long as the inhibition control is run for all assays.

13.7 Armored RNA containing the enterovirus, norovirus GI, and norovirus GII sequences, (Armored RNA 1615 from Asuragen) will be used for preparing the standard curves. For OC43 assay, standard Curve is prepared from OC43 virus serially diluted from 10^6 GCMPN /5 uL to 10^1 GCMPN / 5uL; for WHO & CDC SARS-CoV2 assay: standards are plasmids or gene strands made by IDT

Note: No RNA extraction is necessary for the armored RNA

However, due to variability in stock Armored RNA Gene copies (up to ±20%), it is highly recommended to determine the GC-MPN/ml titer of the stock armored RNA using the following steps;

13.7.1 Prepare 10-fold serial dilution (in TSM buffer)

13.7.2 Use the highest three dilutions (10, 100,1000) and 10 replicates per dilution.

13.7.3 Perform RT-qPCR and qPCR (section 13.5) using primers and probe.

13.7.4 Obtain GC-MPN/ml for Armored RNA using EPA most probable Number Calculator or equivalent. Change the calculator's "Number of Dilutions" to 3, the "Number of Tubes per dilution" to 10, and the "Dilution Type" to Standard 10-Fold Serial. For each stock, input the number of positive replicates from the highest dilution giving at least one positive replicate and from the next two lower dilutions.

13.8 **For this project, a full standard curve will be required for every sample. It is not acceptable to use stored standard curves and calibrators.**

14.0 While the information in this section provides good perspective, **the acceptance criteria given in Appendix D of the RFQ will be used for this study instead of the acceptance criteria in EPA 1615 (for both matrix spikes and positive QC samples).**

16, Table 2 Not applicable

16, Revised Table 4

Table 4: Primers and TaqMan® Probes for Virus Detection by RT-qPCR

Virus Group	Primer/Probe Name/Sequence (1,2,3,4**)
Enterovirus	
	EntF: CCTCCGGCCCCTGAATG
	EntR: ACCGGATGGCCAATCCAA
	EntP: 6FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA
Norovirus GIA	
	NorGIAF (JJVMF): CCA TGT TCC GTT GGA TGC**
	NorGIAR: TCCTTAGACGCCATCATCAT
	NorGIAP (RING1): FAM-AGA TYG CGI TCI CCT GTC CA-BHQ **
Norovirus GIB	
	NorGIBF: CGCTGGATGCGNTTCCAT
	NorGIBR: CCTTAGACGCCATCATCATTTAC
	NorGIBP: 6FAM-TGGACAGGAGAYCGCRATCT-TAMRA
Norovirus GII	
	NorGIIF: ATGTTCAGRTGGATGAGRTTCTCWGA
	NorGIIR: TCGACGCCATCTTCATTCACA
	NorGIIP: 6FAM-AGCACGTGGGAGGGCGATCG-TAMRA

Virus Group	Primer/Probe Name/Sequence [1,2,3,4**]
Hepatitis G	
	HepF: CGGCCAAAAGGTGGTGGATG
	HepR: CGACGAGCCTGACGTCGGG
	HepP: 6FAM-AGGTCCCTCTGGCGCTTGTGGCGAG-TAMRA
OC43	OC43F: CGATGAGGCTATTCCGACTAGGT
	OC43R: CCTTCCTGAGCCTTCAATATAGTAACC
	OC43P : TCCGCCTGGCACGGTACTCCCT
SARS-CoV-2	2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606
	2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'
	N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
	N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3'
N1-Pdouble: FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	
	N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'
	N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'
	N2-P: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'
N2-Pdouble: FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF	

SARS CoV-2 WHO Primers/Probes:

E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA
E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ

(1) Primers and probes are designated by the first three letters of the virus name followed by F, R, or P for forward, reverse, and probe. GIA, GIB, or GII are also added to the norovirus designations.
(2) All primer and probe sequences are 5' to 3'
(3) Degenerate bases in primers and probes are as follows: N equals a mixture of all four nucleotides; R equals A + G; Y equals T + C; W equals A + T; and I equal inosine.
(4) ** NorG1A forward primer and NorGIA probe differ from EPA 1615 recommended NorG1A primer/probe. These new sequences are more efficient in one-step RT PCR. Reference: Vincent R. Hill • Bonnie Mull •Narayanan Jothikumar • Karen Ferdinand •Jan Vinje´Food Environ Virol (2010) 2:218–224: Detection of GI and GII Noroviruses in Ground Water Using Ultrafiltration and TaqMan Real-time RT-PCR:

ADDITION to Table 4: Primers and probes for Adenovirus, MS2, PhiX174, Coronavirus OC43, and SARS-CoV-2.

4.1 PhiX174 Primer and Probes

Sequence	Reference
Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):424-4250
Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'	
Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'	

PhiX174

Target sequence length: 123 bp

ACAAAGTTTGGATTGCTACTGACCG**CTCTCGTGCTCGTCGCTGCGTTGA**GGCTTGCGTTTATGGTACGCTGGACTTT
GTGGGATACCCTCGCTTTCCTGCTCCTGTTGAGTTTATTGCTGCCG

Total length with pUCIDT-Amp Plasmid- 2875 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc. is measured on nanodrop and copy number determined using the following formula:

PhiX 174 number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

4.2 MS2 Primer and Probes

Sequence	Reference
Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):4242 – 4250.
Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'	
Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'	

MS2 RNA sequence for Standard curve;
-Length 3559 bp
-Concentration: 10 A260 Unit or Conc provided on the tube
(each 1 OD_{260} Unit = 40µg/ml ssRNA)
number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 800ng/ul* 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 4.16E+11 copies

4.3 Adenovirus Primer and Probes

Sequence	Reference
Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'	Ko *et al*., J. Virol Methods, 2005, 127: 148-153
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'	
Probe: 5'-6FAM- **CTGACACGGGCACTCTTCGC** -BHQ-3'	

Standard Curve Target sequence: 118 bp

AACTTTCTCTCTTAATAGACGCCCCACTTAATG**CTGACACGGGCACTCTTCGC**CTTCAAAGTGCTGCACCTCTTGGAC TAGTGGACAAAACACTAAAAGTTTTGTTTTCTAGCCCCCT

Total length with pUCIDT-Amp Plasmid - 2870 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

4.4 Coronavirus OC43 Primers and Probe

Sequence	Reference
Forward Primer: 5'- CGATGAGGCTATTCCGACTAGGT-3 '	Dare, R.K. et al. J Infect. Diseases, 2007, 196: 1321-8
Reverse Primer: 5'-CCTTCCTGAGCCTTCAATATAGTAACC -3'	
Probe: 5'-6FAM- TCCGCCTGGCACGGTACTCCCT -BHQ-3'	

Standard Curve use OC43 virus serially diluted from 10^{6} GCMPN /5 uL to 10^{1} GCMPN / 5uL

4.5 SARS -oV-2 Primers and Probes

Sequence	Reference
2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'	2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606
N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	
N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3' N1-Pdouble: FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	
N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'	
N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'	
N2-P: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	

N2-Pdouble: FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF	

Standards for preparing the standard curve: Positive Control provided by IDT Kit is 2E+05 GC/ul or 1E+06/5ul is serially diluted to 1E+01 and used as standard curve.

4.6 WHO SARS-CoV-2 Primers and Probe

Sequence	Reference
E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT	WHO Protocol: Diagnostic detection of 2019-nCoV by real-time RT-PCR on Jan 17, 2020
E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA	
E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	

Standard curve Target sequence for the E gene:
114 bp
ACAGGTACGT TAATAGTTAA TAGCGTACTT CTTTTTCTTG CTTTCGTGGT
ATTCTTGCTA GTTACACTAG CCATCCTTAC TGCGCTTCGA TTGTGTGCGT
ACTGCTGCAA TATT

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

QPCR MASTER MIX TABLES
The Following Tables provide the reaction volume for each RT qPCR/qPCR reactions.

Table 5 -Enterovirus RT-qPCR reaction

Master Mix - Enterovirus	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.25	4.25
10 uM EntF	0.75	0.75
10 uM EntR	2.25	2.25
10 uM EntP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 6 -Norovirus GIA RTqPCR reaction

Master Mix - Norovirus GIA	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.75	4.75
10 uM NorV GIA F(JJVMF)	1.25	1.25
10 uM NorV GIA R	1.25	1.25
10 uM NorV GIA P (RING1)	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 7 -Norovirus GIB RTqPCR reaction

Master Mix – Norovirus GIB	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GIB F	1.25	1.25
10 uM NorV GIB R	2.25	2.25
10 uM NorV GIB P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 8 -Norovirus GII RTqPCR reaction

Master Mix - Norovirus GII	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GII F	1.25	1.25
10 uM NorV GII R	2.25	2.25
10 uM NorV GII P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 9 -Hepatitis G RTqPCR Inhibition Assay reaction

Master Mix - Hepatitis G	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
HepG	2	2.5
PCR grade water	1.75	2.25
10 uM HepF	1.25	1.25
10 uM HepR	1.25	1.25
10 uM HepP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Notes
-depending on the instrument used, a longer cDNA extension of 35-50 minutes (instead of 15 min) may be required.

-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 10 - PhiX174 qPCR reaction

Master Mix - PhiX174	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2X qPCR MM (1)	12.5	12.5
PCR grade water	3.5	4.5
10 uM PhiX174F	1.25	1.25
10 uM PhiX174R	1.25	1.25
10 uM PhiX174P	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

PhiX174 Thermal cycler qPCR program: 5 min at 94°C and then 40 amplification cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
Or
95°C for 2 min, followed by 40 cycles with a 95°C denaturation for 10 s, 55°C annealing for 30 s, and 72°C elongation for 15 s (Adenovirus assay conditions)
-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Table 11 - MS2 phage RTqPCR Assay reaction

Master Mix - MS2 phage	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.5	4.5
10 uM MS2	1.25	1.25
10 uM MS2	1.25	1.25
10 uM MS2	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

MS2 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.

Or
1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence). (All other RT-qPCR cycling conditions)

Table 12 – Adenovirus qPCR reaction

Master Mix - Adenovirus	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2x qPCR MM [1]	12.5	12.5
PCR grade water	4	5
10 uM AdvF	1	1
10 uM AdvR	1	1
10 uM AdvP	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

Adenovirus Thermal cycler qPCR:
Hot-start denaturation step at 95°C for 2 min, followed by 40 cycles with a 95°C denaturation for 10 s, 55°C annealing for 30 s, and 72°C elongation for 15 s

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-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Table 13 – Coronavirus OC43 RTqPCR Assay reaction

Master Mix – OC43	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.25	4.25
10 uM OC43F	1.25	1.25
10 uM OC43R	1.875	1.875
10 uM OC43P	0.125	0.125
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

Final conc: OC43 Forward: 500nM; OC43 reverse: 750nM; OC43probe: 50nM

OC43 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
Or
1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Table 14 – SARS-CoV-2 CDC RTqPCR Assay reaction

Master Mix – CoV-2	**CoV-n1**	**CoV-2 n2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	10	10
PCR grade water	3.5	3.5
Combined Primer/Probe Mix n1	1.5	
Combined Primer/Probe Mix n2		1.5
Sample Volume	5	5
Total Reaction Vol	20	20

SARS-CoV-2 RT-PCR (One step): 15 min at 50°C, 2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

Master Mix - E2	# Rxns:	x
Reagent	**Volume per rxn (uL)**	**Volume per MM**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	10	12.5
PCR grade water	4.5	5.0
10 uM E2 F	0.8	1.0
10 uM E2R	0.8	1.0
10 uM E2 P	0.4	0.5
Total MM	15	20
Sample Volume	5	5
Total reaction Vol	20	25

Final conc. Forward Primer 400 nM; Reverse Primer 400nM; and Probe 200 nM

E2 RT-PCR (One step): 10 min at 50°C, 3 min at 95°C, and then 45 cycles including denaturation at 95°C for 15 s and annealing and elongation at 58°C for 30 s, followed by fluorescence measurement.

Standard Operating Procedure for Processing and Enumerating Enterovirus, Adenovirus, Norovirus, SARS-CoV-2, and the Corresponding Matrix Spikes in Raw Wastewater

EPA Method 1615 along with the following addendum shall be used to process and analyze the virus sample by both culture and molecular methods. Many modifications have been made to EPA Method 1615, including revisions to the concentration/purification steps, addition of a culture and molecular procedure for adenovirus; a molecular procedure for SARS-CoV-2 and betacoronavirus OC43 (enveloped viruses); and revisions to the matrix spike organism, method, and frequency. The addendum lists the method modifications and clarifies certain steps in EPA Method 1615. Follow all of the steps in EPA Method 1615 unless otherwise specified by the addendum.

Refer to EPA Method 1602 for the list of required equipment, supplies, and reagents for the matrix spike assays.

In this addendum, dashed lines (------) are used to highlight sections with large additions and/or sections that are highly modified.

Addendum to EPA Method 1615:

1.1	Method has been modified to also include culture and/or molecular procedures for adenovirus, SARS-CoV-2, betacoronavirus OC43 (matrix spike), MS2 (matrix spike); and PhiX174 (matrix spike). The method has been modified specifically for use with raw wastewater.
2.0	Significant modifications have been made.
6.1	Not applicable. The sample will not be filtered.
6.2	Not applicable - WWTP will do the sample collection.
6.3.1	Not applicable. Matrix spike sample is same as field sample.
6.3.6	Not applicable. The sample will not be filtered.
6.3.7	Not applicable. Matrix spike sample is same as field sample.
6.3.8	Not applicable. The sample will not be filtered.
6.4	Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615.
10.0 & 11.0	Concentration and Purification has been modified for analysis of raw wastewater.
12.0	Addition of Adenovirus and SARS-CoV-2/betacoronavirus OC43 analysis
13.0	Modification from two-step RT-qPCR procedure to a one-step RT-qPCR procedure. Some primer sets have been modified or added.

ADDITION TO SECTION 6: In addition to the equipment/supplies specified in 1615, the following equipment/supplies will be needed:

Equipment and Supplies for Concentration/Purification and Nucleic Acid Extraction:

1. Aerosol/filter barrier micropipette tips, 20, 200, 1000 µL (E&K Cat # EK 3016 and EK 3025, or equivalent)
2. Micropipettes
3. Vortex Mixer
4. Waterbath at 56 °C
5. Refrigerated large capacity swing bucket centrifuge (Beckman X-14R or equivalent) for 250 or larger conical bottom tubes.
6. High speed refrigerated centrifuge (Beckman JE or equivalent) capable of running 13,500xg using 50 or 15 mL tubes
7. pH Meter
8. Balance
9. Transfer Pipets, disposable (Fisher brand 13-711-9BM, or equivalent)
10. Centrifuge Tubes, 15, 50, 250, 500 mL including 15 mL tubes rated for high speed centrifugation (13500xg) (VWR 21008- 242, or equivalent)
11. 1, 2, 3 and/or 4-L Sterile beakers with sterile stir magnetic bars
12. Microcentrifuge Tubes, 1.5 mL
13. Micro Tubes, screw cap, 1.5 mL (VWR 89004-294, or equivalent)
14. 0.2 uM sterilizing filters (Sigma-Millipore Cat# F-9768 for filtering up to 250 ml)
15. Centrifugal concentrator: Vivaspin 20 (30,000 MWCO PES) Sartorius Cat# VS022 or equivalent
16. DNA/RNA purification kit (Zymo Quick-DNA/RNA Viral Kit # D7020 or equivalent

Equipment and Supplies for MS2 and PhiX174 Culture

Refer to Section 6 of EPA Method 1602 (attached) for the equipment and supplies needed for the MS2 and PhiX174 culture assays. Use 1X TSA for plaque assay

Equipment and Supplies for MS2 and PhiX174 Molecular

No additional equipment or supplies are required for the MS2 and PhiX174 molecular assays

Equipment and Supplies for Adenovirus Culture

No additional equipment or supplies are required for the adenovirus culture assays

Equipment and Supplies for Adenovirus Molecular

No additional equipment or supplies are required for the adenovirus molecular assays

Equipment and Supplies for SARS-CoV-2 Molecular

No additional equipment or supplies are required for the SARS-CoV-2 molecular assays

Equipment and Supplies for OC43 Culture

Equipment and Supplies for OC43 Molecular

No additional equipment or supplies are required for the OC43 molecular

CONTINUE TO SECTION 7.0 AFTER COMPLETING SECTION 6 (W/ ADDITIONS)

7.2.4 Not applicable. Matrix spike will be MS2 and PhiX174 for the enteric viruses ,rather than Sabin Poliovirus 3, and OC43 for enveloped viruses,.

7.3 Not applicable. The sample will not be processed by the filtration/elution/organic flocculation method in 1615

ADDITION TO SECTION 7: In addition to the reagents, media, and standards specified in 1615, the following reagents and standards will be needed:

Reagents and Standards for Concentration/Purification and Nucleic Acid Extraction:

1. Purity of Reagents: ACS reagent grade chemicals shall be used in all tests. Unless otherwise indicated, reagents shall conform to the specifications of the Committee on Analytical Reagents of the American Chemical Society. The microbiological growth media must be of microbiological grade.
2. Chloroform:Butanol: Add equal parts chloroform and butanol together. Shake to mix. Store for three months refrigerated. **Note: do not use chloroform:butanol reagent with the enveloped virus procedure.**
3. Phosphate Buffered Saline (PBS): Add 8g NaCl, 0.2g KCl, 1.15g Na_2HPO_4 to 1000mL of molecular grade water. Mix until dissolved. Adjust the pH to 7.3. Sterilize by autoclaving. Store refrigerated for 3 months.
4. Molecular Grade Water (MGW, VWR 82021-434 or equivalent)
5. Sodium Chloride (BDH9286-2.5KG)
6. Polyethylene Glycol MW 8000 (VWR 97061-098, or equivalent)
7. Chloroform (Sigma 496189-1L, or equivalent)
8. Butanol (Sigma B7906-500 mL, or equivalent)

9. Sodium phosphate dibasic (Na2HPO4.7H2O) (BDH9296-500G)
10. Trisodium citrate dihydrate (BDH9288-500G)
11. BBL Beef Extract Desiccated Powder (BD 211520)

12. 5% BSA (5g of albumin/bovine crystalline (United States Biochemical cat# 10856 or equivalent)
13. 0.2% BSA in PBS (Dulbecco's Phosphate buffer saline, without CaCl2 and MgCl2)
14.

Reagents and Standards for Matrix Spike (MS2 and PhiX174)

1. Refer to Section 7 of EPA Method 1602 (attached) for the reagents and standards needed for enumeration of the matrix spikes MS2 and PhiX174.
2. Prepare MS2 and PhiX174 spiking suspensions

a. MS2 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this solution if is less concentrated than 10^8PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**
b. PhiX174 spiking suspension: **Prepare a 10^9 PFU/mL stock virus suspension**. Measure and record the exact concentration. Repeat preparation of this solution if is less concentrated than 10^8 PFU/mL. **Verify the concentration of the stock every time a matrix spike is performed.**
c. The laboratory must perform a molecular assay of the MS2 and PhiX174 spiking suspensions every time a sample is spiked with these matrix spikes to determine the concentration (as genome copies per liter) of the stock.

3. Primers, probes, and standards for molecular assay:
PhiX174 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**: 4242-4250**
Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'
Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'
Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'
MS2 Primer and Probes (Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14)**:4242-4250)**
Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'
Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'
Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'

Reagents and Standards for Adenovirus Culture Assay

Adenovirus stock.

1. Adenovirus stock; Adenovirus serotype 10 (AdV10; ATCC 1504) stored at -70°C. for use as a positive control in the culture assay

Cell culture media.

1. DMEM (Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
   a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL

Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    a. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    b. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

A549 cell culture.

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
   a. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
   b. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.

5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.
6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 (~3×10^5 per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.

15. If cells used for bioassay are not confluent within 1-4 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

Reagents and Standards for Adenovirus Molecular Assay

1. Human Adenovirus Primers/Probe (Integrated DNA Technologies or equivalent) (Ko *et al*., J. Virol Methods, 2005; 127: 148-153)

Adenovirus
Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'
Probe: 5'-**CTGACACGGGCACTCTTCGC**-3'

2. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
3. ABI real-time PCR Machine (or equivalent)
4. strip tubes and caps or 96 well plates and optical film
5. Standards for preparing the standard curve.

Reagents and Standards for OC43 Matrix Spike

OC43 stock.

1. OC43 (Betacoronavirus 1 (ATCC® VR-1558™) stored at -70°C for use as a positive control in the molecular assay

Cell culture media.

1. DMEM Media **(**Corning cat. no. 10-013-CV or equivalent)
2. Fetal Bovine Serum (FBS) (Corning cat. no. 35-010-CV or equivalent)
3. Antibiotic; Penicillin (10,000 IU) and Streptomycin (10,000 µg/ml); 100X (Corning cat. no. 30-002-CI or equivalent)
4. Antimycotic Amphotericin B (25 µg/mL Amphotericin) 100X (Corning cat. No. 30-003-CF); Antimycotic can be part of the 100X Antibiotic Solution above.
5. Kanamycin (Invitrogen cat. no. 759788 or equivalent) or Gentamycin Sulfate (Corning 30-005-CR or equivalent)
6. Dulbecco's phosphate buffered saline (PBSA) (Sigma cat. no. D8537 or equivalent)
7. Trypsin-EDTA solution (Sigma cat. no. T4049 or equivalent). For long term storage, thaw upon receipt in the laboratory and dispense 5 mL aliquots to sterile conical tubes and store at –20°C until expiration date.
8. Trypan Blue, 0.4% (Invitrogen cat. no. 15250-061).
9. Growth medium preparation in Biological Safety Cabinet:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity)

and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.

10. Maintenance medium preparation in Biological Safety Cabinet:
    i. Maintenance Medium (2% FBS in DMEM): To each 1L sterile bottle of DMEM add 20 mL FBS, 10.2 ml Antibiotic, 10.2 mL Antimycotic, and 10.2 Kanamycin or Gentamycin. For checking of sterility, aseptically transfer 5 mL of the growth medium to a sterile tube and incubate in the CO_2 incubator at 37 ± 1.0°C for a minimum of 2-3 days. Look for contamination (turbidity) and discard if contamination is evident. Store media at 4°C for up to 6 months or earliest expiration date of reagents.
    ii. Optionally, maintenance medium consists of a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.

HRT cell culture (HRT-18G (ATCC CRL-11663)).

1. Warm all reagents to 37°C prior to use in this procedure.
2. Growth media and maintenance media are as described for BGM cells, briefly:
    i. Growth Medium (10% FBS in DMEM): To each 1L sterile bottle of DMEM add 115 mL FBS, 11.52 ml Antibiotic, 11.5mL Antimycotic, and 11.5 mL Kanamycin or Gentamycin.
    ii. Maintenance Medium consists of 2% FBS in DMEM or a 50/50 mixture of DMEM and Leibovitz L-15 medium with L-glutamine (Sigma-Aldrich, Cat. No. L4386), 1 mL/L of 7.5% sodium bicarbonate, 10 mL/L of antibiotic-antimycotic solution, and 20 mL/L of fetal bovine serum and may be used for incubation of inoculated flasks at 37 ± 1.0°C without CO2.
3. Turn on UV light and blower in biosafety cabinet (BSC) for a minimum of ½ hr before working in hood. Be sure that tube racks that will be used are placed in the BSC while the UV light is on. Immediately prior to working in hood switch the UV light off. If the UV light is left on, exposed skin will burn. NOTE: BEFORE PLACING REAGENTS IN BIOSAFETY CABINET, SPRAY ALL CONTAINERS AROUND THE CAP WITH 70% ETHANOL SOLUTION PRIOR TO OPENING THROUGHOUT THE ENTIRE PROCEDURE. USE GLOVES THAT HAVE BEEN SPRAYED WITH 70% ETHANOL SOLUTION THROUGHOUT PROCEDURE.
4. Remove the cells to be split from the incubator and spray the cap and neck of the flask with 70% ethanol before placing the flask in the BSC. Place pre-wrapped sterile pipettes in the BSC. Remove the PBSA and the trypsin-EDTA solution from 37°C and spray the outside and cap of each vial with 70% ethanol. Place the tubes in a rack in the BSC and loosen caps.
5. Remove the spent growth medium from the culture flask. Tilt the flask away from the cell monolayer to avoid disturbing it. Place spent media in waste beaker located in the BSC.

6. Aseptically add pre-warmed PBSA to flask. Do not put the pipet down or touch the tip to anything. Gently tilt flask to rinse residual growth medium from cells. Using the same pipette or a new pipette, remove the PBSA from flask without disturbing cells and discard in waste beaker. Repeat once more. This removes any media that contains serum, a trypsin inhibitor. If the pipette is put down or the tip touches any object use a new sterile pipette to remove PBS rinse from culture flask.

Flask Size	Amount of PBS to Add (mL)	Amount of Growth Medium (mL)
25	5-10	10-15
75	10-15	20-30
225	15-25	60-100
6-well plate	1 mL per well	3-5

7. Immediately add enough pre-warmed trypsin-EDTA solution to cover the bottom of the flask (1- 12 mL) cap and place in incubator for 1-5 min. Check flask to see if the cells have lifted from the flask surface after 1 min. Rock the flask to dislodge the cells from the flask surface. If there are still cells attached to the flask put back in incubator for an additional 1 minute and recheck. Continue checking frequently until all cells have lifted from the flask surface. Place 25 mL and 10 mL sterile pipettes in the BSC. In addition, place a sterile 15 mL conical tube and if needed a new cell culture flask into the BSC and loosen the caps. NOTE: It is important to minimize the time the cells are exposed to the trypsin-EDTA solution.
8. Remove growth media from 37°C and spray with 70% ethanol solution and place in BSC. Be sure to loosen the cap.
9. When all cells have been lifted from the flask surface, spray around the cap of the flask with 70% ethanol solution and place in the BSC. Using an appropriate size pipette, aseptically add pre-warmed growth media (equivalent to the amount of trypsin in the flask) and rinse bottom of flask with cell suspension several times.
10. If enumeration is necessary, transfer the entire content to a sterile tube and tightly cap the tube. Concentrate the cells by centrifugation (400 x *g*, 2 min or 175 x *g*, 6 min) and discard supernatant. Re-suspend cells in 10 mL of pre-warmed growth media. Be sure to break up clumps by vigorously pipetting suspension up and down in tube.
11. Allow the suspension to settle for 1 min and remove a 50 µL aliquot with a sterile pipette tip and place in 1.5 mL Eppendorf tube containing 50 µL Trypan Blue. Flick tube to mix and perform a hemocytometer count to determine the concentration of viable cells.
12. Transfer an appropriate amount of cells to the culture flask containing pre-warmed growth media

Flask Size	Seeding Density	Days until Confluent
75	0.75×10^6	3-4
225	1×10^6	4-7
6-well plate	1×10^6 (~3×10^5 per well)	1-2

13. Place flask in incubator at 37.0 ± 1°C under 5% CO_2. Place well plates into a Ziploc™ bag before placing into incubator.
14. If cells are not confluent within the expected time frame, replace media. Aseptically remove old media using a sterile pipette. Replace with pre-warmed maintenance media. Place flask back in incubator until cells are confluent.
15. If cells used for bioassay are not 80-90% confluent within 1-3 days the flasks should be discarded. An older monolayer will not optimally support an infection.
16. Autoclave media waste and any media that have had contact with cells or serum before discarding.
17. Discard cells after they have been passaged a total of 200 times.

OC43 Primers/Probe for Molecular Assay.

1. OC43 (Integrated DNA Technologies or equivalent):
   Forward Primer: 5'- CGATGAGGCTATTCCGACTAGGT -3'
   Reverse Primer: 5'- CCTTCCTGAGCCTTCAATATAGTAACC -3'
   Probe: 5'- TCCGCCTGGCACGGTACTCCCT -3'

Reagents and Standards for Enveloped Virus Molecular Assay:

1. Coronavirus SARS-CoV - Primers/Probe (2019-nCoV CDC EUA Kit, 500 rxn Cat# 1000660 from Integrated DNA Technologies or equivalent)
2. **Zymo Quick-DNA/RNA Viral Kit #** D7020) or equivalent.
3. QuantaBio qScript™ XLT One-Step RT-qPCR ToughMix® or equivalent
4. ABI real-time PCR Machine (or equivalent)
5. strip tubes and caps/ 96 well plates and optical film
6. Standard for preparing the standard curve: Plasmid linearized by Cel serially diluted

CONTINUE TO SECTION 8.0 AFTER COMPLETING SECTION 7 (W/ ADDITIONS)

8.0 The QA/QC requirements for this project are summarized in the following table:

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Demonstration of capability for this study	Three field sub-samples with blind spikes (poliovirus) and three field sub-samples without blind spikes; both shall include matrix spikes (MS2 and PhiX174)	Modified 1615 (PEG/chloroform extraction, culture, and molecular) (Do not need to run adenovirus or norovirus assays)	Once (during pre-campaign testing)	To demonstrate acceptable method performance using the modified method	Appendix D of RFQ 4952
QC sample set-Enteric viruses	One positive control (poliovirus in reagent water)	Modified 1615 (PEG/chloroform extraction,	Once initially and then once per month for the	To demonstrate ongoing control of the analytical system and verify	Appendix D of RFQ 4952

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
	and one negative control	culture, and molecular) (For positive control, do not need to run adenovirus or norovirus assays)	duration of the study	continuing method performance	
Matrix spike Enteric viruses	MS2 and PhiX174 in field sample	Modified 1615 (PEG/chloroform extraction, culture, and molecular) Verify the concentration of the spiking suspension in terms of both PFU/L and GC/L every time a matrix spike is performed	Every other field sample from each WWTP during full monitoring campaign; every field sample during pre-campaign testing	To determine the effect of the matrix on virus recoveries	Appendix D of RFQ 4952
QC sample set – Enveloped Viruses	One positive control (OC43 in reagent water) and one negative control	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay	Once initially and then once per month for the duration of the study	To demonstrate ongoing control of the analytical system and verify continuing method performance	To Be Determined
Matrix Spike Enveloped Viruses	OC43 in field sample	Direct incubation in beef extract Amicon filter Concentration, RNA extraction, molecular assay Verify the concentration of the spiking suspension (GC/L) every time a matrix spike is performed	Every field sample from each WWTP during campaign testing	To determine the effect of the matrix on enveloped virus recovery	To Be Determined
Positive Control: Enterovirus Culture Assay	3 culture test vessels with poliovirus in buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Positive Control: Adenovirus Culture Assay	3 culture test vessels with adenovirus in buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify the continued sensitivity of the cell cultures to virus infection	EPA 1615 Section 12.1.2.5.2
Negative Control: Enterovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with BGMK cells	Every BGMK culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2

QC Sample	Description	Method	Frequency/Timing	Purpose	Acceptance Criteria
Negative Control: Adenovirus Culture Assay	3 culture test vessels with buffer	MPN culture assay with A549 cells	Every A549 culture assay	To verify that external factors aren't harming the cells	EPA 1615 Section 12.1.2.4.2
Negative Control: MS2 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* F_{amp})	Every *E. coli* F_{amp} culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: PhiX174 Culture Assay	1 culture test vessel with buffer	PFU culture assay (with *E. coli* CN-13)	Every *E. coli* CN-13 culture assay	To verify that external factors aren't preventing the growth of *E. coli*	If *E. coli* fails to grow, troubleshoot and repeat assay
Negative Control: Molecular Assay	1 sample with PCR grade water	(rt)-qPCR	One per every fourth sample on a plate	To verify the lack of contamination	EPA 1615 Section 13.4.4.2
Inhibition Control (Positive Control): Molecular Assay – Enteric Viruses	1 test sample with hepatitis G armored RNA (compare to hepatitis G in reagent water)	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	EPA 1615 Section 13.6.2
Inhibition Control (Positive Control): Molecular Assay, Enveloped Viruses	TaqMan™ Internal Controls run with OC43 assay	rt-qPCR	Every molecular assay (one per field sample)	To identify any matrix inhibition	To Be Determined

8.3.1 **PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952. One QC sample set will be processed initially and then one QC sample set per month thereafter.**

8.4 One QC sample set (poliovirus and OC43 betacoronavirus in reagent water and a negative control) should be conducted per month during the duration of this monitoring campaign (and one set initially). This QC sample set should be processed and analyzed using the modified 1615 method for enteric viruses and the enveloped virus method for coronaviruses.

8.4.1.1 Sample volume is 1100 mL

8.4.1.2-8.4.1.4 Skip these steps (filtration has been eliminated from the sample prep)

8.4.1.5 Process and analyze the negative QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure, Sections 10 and 11, below), enterovirus culture assay, adenovirus culture assay, MS2 culture assay, PhiX174 culture, and molecular assay procedures (enterovirus, adenovirus, norovirus, MS2, and PhiX174).

8.4.2 Sample volume is 1100 mL

8.4.2.2 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.5 Skip this step (filtration has been eliminated from the sample prep)

8.4.2.6 Enteric Viruses: Process and analyze the positive QC sample using the PEG flocculation/chloroform extraction procedure (see the modified concentration/purification procedure; Sections 10 and 11, below), enterovirus culture assay, and enterovirus molecular assay.

8.4.2.7 Enveloped Viruses: Process and analyze the positive QC sample by adding 1ml of 10^6 GCMPN/ml OC43 virus to 1100 ml of laboratory deionized water and process (see the modified concentration/purification procedure; Sections 10 and 11, below), OC43 molecular assay.

8.4.3 **Laboratory performance for the positive QC samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952**

8.5 PT and PE samples not required. Once contracted, the labs will perform the pre-campaign testing that will include some demonstration of capability testing. The scope of this testing is described in RFQ 4952.

8.6.1 Enteric viruses: Run a matrix spike for every other field sample from each site (during the pre-campaign testing, a matrix spike will be run with every field sample).

8.6.1.1 Enveloped viruses: Run a matrix spike for every field sample from each site.

8.6.2 **Replace with the following steps:**

8.6.2 REPLACEMENT: To the samples that will include a matrix spike, complete the following steps:

1. **Spike 10^8 PFU of MS2 to 1100 mL of the field sample** (the same sample that is used for measuring the native virus). This number can be achieved by adding approximately 0.1 mL of a MS2 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the MS2 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of MS2 in the field sample of 10^8 PFU/L).
2. To this same sample, **spike 10^8 PFU of PhiX174**. This number can be achieved by adding approximately 0.1 mL of a PhiX174 spiking suspension with a concentration of approximately 10^9 PFU/mL (may adjust the volume of the PhiX174 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of PhiX174 in the field sample of 10^8 PFU/L).
3. Spike **10^7 GCMPN/ml of OC43** to 1100 ml of the field sample (the same sample that has been spiked with MS2 and PhiX174 and is used for measuring the native virus). This number can be achieved by adding approximately 1ml of an OC43 spiking suspension with a concentration of approximately 10^7 GCMPN/ml (may adjust the volume of the OC43 spiking suspension added based on the actual concentration of the spiking suspension to achieve a minimum concentration of OC43 in the field sample of 10^7 GCMPN/L).
4. Process and analyze the sample using the sample procedures that are used for the samples without the matrix spike with the exception that this sample will also

be analyzed for MS2 and Phix174 using both culture and molecular procedures; and, for OC43 use molecular procedures only.

5. Laboratories are to report PFU in all dilutions where there are countable plaques (zero to 300 PFU per plate for male-specific (F^+) coliphage and zero to 100 PFU per plate for somatic coliphage. For too numerous to count plates, please enter TNTC in the comment section of the report associated with that dilution. Make the final calculation based on all dilutions with countable plaques (refer to equation in section 13.1.5 from EPA 1602).
6. For enveloped viruses, report the concentration as determined from rt-qPCR standard curve.

CONTINUE TO SECTION 8.6.3

8.6.3 **Laboratory performance for the MS samples conducted for this study will be compared to the acceptance criteria in Appendix D of RFQ 4952.**

9.1 Replace this step with the following:
A 4 L raw wastewater sample will be collected by the wastewater treatment facility staff and will be delivered to the lab. The sample should be labeled with a unique sample number. The facility staff should record the unique sample number, facility name, sampler's name, location at sampling site, date, and time on a sample data sheet

9.2. Procedure is same only the 4 L sample will not be filtered so there is no cartridge housing or intake/discharge module. The sample should be shipped cold (1-10 °C) not frozen.

9.3.2 Labs will receive a 4 L sample (using a portion for measuring virus, a portion for protozoa, and a portion for phage). The samples will not be filtered, so the lab will not receive a cartridge filter.

10 and 11 **Skip these steps. Instead complete the following modified concentration/purification procedure:**

MODIFIED CONCENTRATION/PURIFICATION PROCEDURE (to replace Section 10 and 11 of EPA 1615)

1. Transfer 1100 ml of raw sewage into sterile beaker. Record this volume (designated Total Sample Volume, TSV) on the virus data sheet.
2. After spiking the virus matrix spikes, incubate for 30 min at Room temperature.
3. Add 1.43 g Sodium phosphate dibasic, 1.32 g Trisodium citrate dihydrate and 11 g beef extract to the sample and stir to dissolve.
4. Stir for 30 minutes at room temperature. Record pH (should be ambient 7.2-7.5). Remove 100 ml aliquot and process the aliquot according to the following steps:
    a. In duplicate, load 15-20 mL of the aliquot into ultrafiltration concentrator-Vivaspin 20 (pre-soaked in PBS plus 0.2% BSA for 2hr to 24hr). Freeze the remaining aliquot at -80°C for backup and archival purposes.

  b. Centrifuge the filter at 4000-5000 x g for 30 minutes or until the concentrate is reduced to less than 1 mL.
  c. Record initial and final volume. Resuspend concentrate and transfer to sterile 1.5 mL sterile tube. Store at 4 +/- 3° C for up to 24 hours or at -80°C until extraction. Proceed to SECTION 13 (Molecular Analysis).

5. Slowly adjust pH of the remaining sample (approximately 1 L) for enteric virus analysis to 9.5 +/- 0.1 using 1-3 N NaOH. Stir at room temperature for an additional 60 minutes, confirm pH is still in range after 30 minutes.
6. Transfer into 250- or 500-mL centrifuge tubes. Centrifuge at 3,000 x g for 20 minutes (brake no more than 20%).
7. Decant or pipet supernatant into 1 L container and record the volume. Discard pellets.
8. Check pH of supernatant using pH meter and adjust to 7.2+/- 0.2 using 1-3 N HCL.
9. Add 52 g of Sodium chloride (NaCl) and mix/stir to dissolve then add 120 g PEG 8000and Mix by inversion or stirring for minimum 2 hours/ or up to 20 hours at 4 +/- 3° C
10. If necessary, transfer to appropriate centrifuge tube. Centrifuge at a minimum of 4,000 x g for 45 mins at 4°C (Brake at no more than 20%).
11. Carefully decant and discard the supernatant. Invert tube over a paper towel for up to 30 seconds to ensure liquid removal from tube.
12. Resuspend the pellet in 1-2 mL of cold PBS. Mix with pipet tip. Avoid making bubbles. Do not vortex.
13. Quantitatively Transfer (using additional 0.5 mL cold PBS) the re-dissolved pellet to a 15 mL chemical resistant centrifuge tube
14. Record the re-dissolved pellet final volume resulting from the previous step (designated Final Concentrated Sample Volume, FCSV) on the virus data sheet.
15. Add 4 mL (or twice the volume of the transferred volume if it is greater than 2.5 mL) of chloroform/butanol solution. Vortex and let stand for 5 minutes.
16. Centrifuge at minimum of 13500 x g for 15 minutes at 4°C
17. Using a micropipette, transfer the top layer (the aqueous phase) to a sterile 15mL centrifuge tube (approximately 2 mL). Chloroform will be on the bottom. Avoid transferring chloroform, go slowly and do not slant tube while transferring.
18. Record the weight of the 15 mL centrifuge tube before and after transfer. Record both and calculate saved volume. Record this volume (designated FCSV recovered after extraction) on the virus data sheet.
19. The pellet/concentrate can be held for up to 18 hours in refrigerator or frozen at -80°C for longer storage. Freezing and thawing can lead to virus losses so avoid multiple freeze/thaw cycles.
20. A portion of the pellet/concentrate will proceed to nucleic acid extraction, a portion of the pellet/concentrate will be diluted and then proceed to the culture assays, and the remaining portion of the pellet/concentrate will be frozen for backup/archival purposes.
    a. 0.4 mL of the pellet shall be diluted to a total volume of 4 mL using cold PBS.

i. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the enterovirus culture assay (called subsample 1)
ii. 1.35 mL of the 4 mL re-dissolved pellet/concentrate will be used for the adenovirus culture assay (called subsample 1B)
iii. 0.3 mL of the 4 mL re-dissolved pellet/concentrate will be used for the MS2 and PhiX174 culture assay (called subsample 1C)
iv. The rest of the 4 mL re-dissolved pellet/concentrate will be frozen at -70°C for backup and archival purposes

b. 1 mL of the pellet will be used for nucleic acid extraction (called subsample 2)
c. The remaining pellet will use frozen at -70°C for backup and archival purposes (called subsample 3)

Proceed to Section 12 (with the addition of adenovirus, MS2, and PhiX174 culture) and Section 13 (with the addition of adenovirus, SARS-CoV-2, OC43, MS2, PhiX174 molecular). Section 12 and Section 13 may be completed in parallel.

SUBSAMPLE 1 SHALL PROCEED TO SECTION 12.0 (culture analysis of enterovirus)

12.1.2.2 **The inoculum volume is 0.1 mL**. Record the Inoculum Volume on the virus data sheet. The number of cell culture **replicates should be 10**.

12.1.2.3 The laboratories should prepare **10- and 100-fold dilutions for every analysis** of the raw wastewater samples.

12.1.2.3.1 The 10-fold dilution is prepared by diluting 0.3 mL of subsample 1 (from step 17.a.i; Section 10 and 11, above) to a volume of 3.0 mL with cold PBS.

12.1.2.3.2 The 100-fold dilution is prepared by diluting 0.3 mL of the 10-fold dilution to a volume of 3.0 mL with cold PBS

12.1.2.4 **The lab must perform these negative controls**

12.1.2.5 **The lab must perform these positive controls**

12.1.4 **Lab must perform a second passage for all negative flasks; not positives. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.**

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

12.1.4.3 Skip this step

12.2.5 The Volume of Original Water Sample Assayed (D) is calculated by the following equation:

$$D = S \times \frac{TSV}{FCSV}$$

12.2.9 The QC samples are the negative and positive controls

ADDITION to Section 12: CULTURE ANALYSIS ADENOVIRUS (SUBSAMPLE 1B)

The adenovirus culture assay involves an MPN approach similar to what is specified in 1615 for enterovirus, with 10 replicates at each dilution and three dilutions (undiluted, 10-fold, and 100-

fold). **Lab must perform a second passage for all negative flasks; not positive flasks. However, if positive flasks are suspected to be due to contamination or cell toxicity, conduct a second passage.** Run negative and positive controls (3 vessels as positive control and 3 vessels as negative controls) with each culture assay.

Note: Freeze all negative flasks and suspect positives prior to performing the second passage

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ADDITION to Section 12: CULTURE ANALYSIS MS2 and PHIX174 (SUBSAMPLE 1C)
(Adapted from EPA Method 1602, Section 12)
<u>Only complete this step for samples that contain matrix spikes of MS2 and PhiX174 (every other virus sample from a given site should contain these matrix spikes)</u>

1. **Four dilutions** of concentrate will be analyzed in **duplicate** for each coliphage type. As a result, nine double-agar layer plates will be required for each coliphage type: two plates per dilution (0.1, 0.01, 0.001, 0.0001) and one method blank plate.
2. Dilutions:
    a. Four dilutions will be needed: 0.1, 0.01, 0.001, 0.0001. **Note: Subsample 1C (prepared in step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) is already 0.1 dilution.** Additional dilutions (beyond 0.0001) may be necessary. PBS (or PBW) is used as the diluent and is used to prepare the negative control.
    b. To prepare the dilutions, aseptically add 0.9 mL of PBS (or PBW) into each of four (or more) sterile dilution tubes. Label them as “0.01,” “0.001,” “0.0001,” “method blank,” etc.
    c. Add 0.1 mL of the subsample 1C (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above) to the tube of PBS (or PBW) labeled “0.01.”
    d. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
    e. Add 0.1 mL of the well-mixed 0.01 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.001".
    f. Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
    g. Add 0.1 mL of the well-mixed 0.001 dilution to a tube with 0.9 mL of PBS (or PBW) labeled “0.0001.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
    h. Add 0.1 mL of PBS (or PBW) the tube labeled “method blank.” Cap the tube and vortex for 5 seconds on a medium-high setting (if available) or until well-mixed.
3. Agar preparation:
    a. Place TSA molten agar with respective antibiotics in a 48-53°C water bath. The agar should remain molten in the water bath until ready for use. 18 tubes are necessary to enumerate four dilution volumes in duplicate for each phage. The 18

tubes also include an additional method blank tube for each phage type. Nine of the top agar tubes should contain nalidixic acid for growth of *E. coli* CN-13; the other nine should contain ampicillin/streptomycin for growth of *E. coli* F_{amp}.

b. As a precaution against contamination, disinfect a workspace near the water bath with a 1:100 dilution of household bleach and allow to dry. If workspace can be corroded by bleach use an ethanol solution of 70% or greater.

c. Assemble plates and label so that the following information is identifiable:
   i. Dilution of stock filtrate or method blank
   ii. Bacterial host (*E. coli* CN-13 or *E. coli* F_{amp})
   iii. Coliphage type (somatic for the *E. coli* CN-13 bacterial host or male-specific for the *E. coli* F_{amp} bacterial host)
   iv. Date
   v. Time

4. Please note: The following steps are critical. To ensure viability of bacterial host and coliphage, do not add bacterial host and concentrate (re-dissolved pellet) until ready to plate.

5. Preparation of plates for enumeration of somatic coliphage:
   a. With the agar still in the water bath, aseptically inoculate tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN-13.
   b. Immediately add 100 µL (0.1 mL) of subsample 1c (from step 17.a.iii of the modified concentration/purification procedure; section 10 and 11, above).
   c. Add 12-15 mL molten agar
   d. Mix the inoculum by rolling the tube briefly in palm of hand.
   e. Pour contents into one of the two petri plates marked “undiluted, *E. coli* CN-13, somatic."
   f. Duplicate analysis—Repeat Sections 5a through 5d for the duplicate.
   g. Repeat Sections 5a through 5e for each dilution volume.

6. Preparation of plates for enumeration of male-specific (F^+) coliphage—Repeat Section 5 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}

7. Preparation of somatic coliphage method blank:
   a. With the agar still in the water bath, aseptically inoculate a tube containing nalidixic acid with 100 µL of log-phase *E. coli* CN- 13.
   b. Immediately add 100 µL (0.1 mL) of TSB from the “method blank” dilution tube. Add 12-15 mL molten agar.
   Mix the inoculum by rolling the tube briefly in palm of hand.
   c. Pour contents into a petri plate marked “method blank, *E. coli* CN-13, somatic."

8. Preparation of the male-specific (F^+) coliphage method blank—Repeat Section 7 using agar containing ampicillin/streptomycin and log-phase *E. coli* F_{amp}.

9. After the agar hardens, invert the plates and incubate for 16 to 24 hours at 36°C ± 1.0°C.

10. Circular zones of clearing (typically 1 to 10 mm in diameter) in lawn of host bacteria after 14 to 24 hours of incubation are plaques. Count the number of

plaques on each plate. Please note: The use of a light box to evaluate results is recommended. Record the results in the virus sheet.

SUBSAMPLE 2 SHALL PROCEED TO SECTION 13 (Molecular Analysis)

13.1 – 13.3 **Replace these steps with the following modified nucleic acid extraction procedure:**

MODIFIED NUCLEIC ACID EXTRACTION PROCEDURE (replace 13.1 – 13.3) or

1. **Follow manufacturer instructions in commercial kit (Zymo Quick-DNA/RNA Viral Kit #** D7020) or equivalent.
2. Add 2 parts of Viral DNA/RNA Buffer to 1 part of sample and mix well (DNA/RNA Shield step is not necessary).
3. Transfer 700 µl of the mixture into one IIC-XL Column in a collection tube and centrifuge @ 10,000 x g for 1-2 minutes. Transfer the column into a new collection tube.
4. Reload the column with another 700 µl of the mixture and repeat centrifuge. Repeat until all of the mixture has been passed through the column.
5. Add 500 µl Viral Wash Buffer to the column, centrifuge for 30 seconds @ 10,000 x g and discard the flow-through. Repeat this step.
6. Add 500 µl ethanol (95-100%) to the column and centrifuge for 1 minute @ 10,000 x g to ensure complete removal of the wash buffer. Carefully, transfer the column into a 1.5 mL nuclease-free tube.
7. Add DNase/RNase-Free Water directly to the column matrix and centrifuge for 30 seconds @ 10,000 x g.
8. The final extraction concentrate volume is 0.2 mL (enteric viruses) and 0.05 mL (0.1 mL in total) (coronaviruses)
9. Refrigerate at 4°C for a maximum of 24 hours or <-20°C for up to one week until PCR analysis. Indefinite storage @ -80°C.
10. Extract RNA from poliovirus, MS2, PHIX174, and OC43 stocks following the protocol above to calculate percent recoveries.

CONTINUE TO SECTION 13.4 of EPA 1615 for Molecular Enumeration of Enterovirus, Norovirus, Adenovirus, OC43, SARS-CoV-2, MS2, and PhiX174

13.4, 13.5 **These two steps may be completed as a one-step reaction (i.e., reverse transcription should be completed in the same tube as qPCR); Each assay will be completed in triplicate**. The test sample requires 36 plate wells/tubes (i.e., 3 replicates x 12 qPCR assay). The 12 assays are enterovirus, norovirus GIA, norovirus GIB, norovirus GII, hepatitis G, adenovirus, MS2, and PhiX174, SARS-Cov-2 (N1, N2 & E), and OC43. 5-6 µL of the concentrate from nucleic acid extraction will be used in each of plate wells/tubes. Note: the assays for MS2 and PhiX174 should only be run if the sample contains these matrix spikes (every other virus sample from each site should contain these matrix spikes)

- For RT-qPCR 1-step assays: Refer run conditions for each primers/probe is specified in Tables 5 though 11

- For qPCR assays: refer to run conditions specified in Tables 10, and 12

- SARS-CoV-2 CDC N gene RT-qPCR (One step) assays**:** Run condition is specified in Table 14

- WHO E gene RT-qPCR (One step) assay: Run condition is specified in Table 15

Confirm (move if necessary) the threshold line lies within the exponential phase of the fluorescence curves and above any background signal.

13.5.6 Use the following equation to calculate genome copies per liter in the original sample rather than Eq. 5 in EPA 1615:

$$GC_{original\ sample\ per\ L} = \frac{GC_{assay} \times V_{nucleic\ acid\ extract} \times FCSV \times DF}{V_{assay} \times V_{pellet\ for\ molecular} \times TSV}$$

Where,
$GC_{\text{original sample per L}}$ = Calculate genome copies per liter in the original sample
GC_{assay} = Genome copies measured from the qPCR assay
$V_{\text{nucleic acid extract}}$ = Volume of the concentrate after nucleic acid extraction (should be 0.2 mL)
FCSV = Final concentrated sample volume (before chloroform extraction) (should be 2.0 mL)
DF = dilution factor = the reciprocal of any dilution performed to compensate for inhibition (e.g., 10 and 100 for the 1:10 and 1:100 dilutions)
V_{assay} = volume of the nucleic acid extraction used for each (rt)qPCR assay (should be 0.006 mL)
$V_{\text{pellet for molecular}}$ = volume of the re-dissolved pellet/concentration used for nucleic acid extraction (should be 1 mL)
TSV = total sample volume (should be 1 L)

13.5.7 skip

13.6 **The laboratory must use hepatitis G armored RNA as an inhibition control in all assays.** The laboratory may option 1, 2, or 3 of 1615 as long as the inhibition control is run for all assays.

13.7 Armored RNA containing the enterovirus, norovirus GI, and norovirus GII sequences, (Armored RNA 1615 from Asuragen) will be used for preparing the standard curves. For OC43 assay, standard Curve is prepared from OC43 virus serially diluted from 10^6 GCMPN /5 uL to 10^1 GCMPN / 5uL; for WHO & CDC SARS-CoV2 assay: standards are plasmids or gene strands made by IDT

Note: No RNA extraction is necessary for the armored RNA

However, due to variability in stock Armored RNA Gene copies (up to ±20%), it is highly recommended to determine the GC-MPN/ml titer of the stock armored RNA using the following steps;

13.7.1 Prepare 10-fold serial dilution (in TSM buffer)

13.7.2 Use the highest three dilutions (10, 100,1000) and 10 replicates per dilution.

13.7.3 Perform RT-qPCR and qPCR (section 13.5) using primers and probe.

13.7.4 Obtain GC-MPN/ml for Armored RNA using EPA most probable Number Calculator or equivalent. Change the calculator's "Number of Dilutions" to 3, the "Number of Tubes per dilution" to 10, and the "Dilution Type" to Standard 10-Fold Serial. For each stock, input the number of positive replicates from the highest dilution giving at least one positive replicate and from the next two lower dilutions.

13.8 **For this project, a full standard curve will be required for every sample. It is not acceptable to use stored standard curves and calibrators.**

14.0 While the information in this section provides good perspective, **the acceptance criteria given in Appendix D of the RFQ will be used for this study instead of the acceptance criteria in EPA 1615 (for both matrix spikes and positive QC samples).**

16, Table 2 Not applicable

16, Revised Table 4

Table 4: Primers and TaqMan® Probes for Virus Detection by RT-qPCR

Virus Group	Primer/Probe Name/Sequence (1,2,3,4**)
Enterovirus	
	EntF: CCTCCGGCCCCTGAATG
	EntR: ACCGGATGGCCAATCCAA
	EntP: 6FAM-CGGAACCGACTACTTTGGGTGTCCGT-TAMRA
Norovirus GIA	
	NorGIAF (JJVMF): CCA TGT TCC GTT GGA TGC**
	NorGIAR: TCCTTAGACGCCATCATCAT
	NorGIAP (RING1): FAM-AGA TYG CGI TCI CCT GTC CA-BHQ **
Norovirus GIB	
	NorGIBF: CGCTGGATGCGNTTCCAT
	NorGIBR: CCTTAGACGCCATCATCATTTAC
	NorGIBP: 6FAM-TGGACAGGAGAYCGCRATCT-TAMRA
Norovirus GII	
	NorGIIF: ATGTTCAGRTGGATGAGRTTCTCWGA
	NorGIIR: TCGACGCCATCTTCATTCACA
	NorGIIP: 6FAM-AGCACGTGGGAGGGCGATCG-TAMRA

Virus Group	Primer/Probe Name/Sequence (1,2,3,4**)
Hepatitis G	
	HepF: CGGCCAAAAGGTGGTGGATG
	HepR: CGACGAGCCTGACGTCGGG
	HepP: 6FAM-AGGTCCCTCTGGCGCTTGTGGCGAG-TAMRA
OC43	OC43F: CGATGAGGCTATTCCGACTAGGT
	OC43R: CCTTCCTGAGCCTTCAATATAGTAACC
	OC43P : TCCGCCTGGCACGGTACTCCCT

SARS-CoV-2 2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606

2019-nCoV_N1-F: 5’-GAC CCC AAA ATC AGC GAA AT-3’
N1-R: 5’-TCT GGT TAC TGC CAG TTG AAT CTG-3’
N1-P: 5’-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3’
N1-Pdouble: FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ

N2-F: 5’-TTA CAA ACA TTG GCC GCA AA-3’
N2-R: 5’-GCG CGA CAT TCC GAA GAA-3’
N2-P: 5’-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3’
N2-Pdouble: FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF

SARS CoV-2 WHO Primers/Probes:

E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT
E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA
E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ

(1) Primers and probes are designated by the first three letters of the virus name followed by F, R, or P for forward, reverse, and probe. GIA, GIB, or GII are also added to the norovirus designations.
(2) All primer and probe sequences are 5’ to 3’
(3) Degenerate bases in primers and probes are as follows: N equals a mixture of all four nucleotides; R equals A + G; Y equals T + C; W equals A + T; and I equal inosine.
(4) ** NorG1A forward primer and NorGIA probe differ from EPA 1615 recommended NorG1A primer/probe. These new sequences are more efficient in one-step RT PCR. Reference: Vincent R. Hill • Bonnie Mull •Narayanan Jothikumar • Karen Ferdinand •Jan Vinje´Food Environ Virol (2010) 2:218–224: Detection of GI and GII Noroviruses in Ground Water Using Ultrafiltration and TaqMan Real-time RT-PCR:

ADDITION to Table 4: Primers and probes for Adenovirus, MS2, PhiX174, Coronavirus OC43, and SARS-CoV-2.

4.1 PhiX174 Primer and Probes

Sequence	Reference
Forward: 5'-ACAAAGTTTGGATTGCTACTGACC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):424-4250
Reverse: 5'-CGGCAGCAATAAACTCAACAGG-3'	
Probe: 5'- FAM/CTCTCGTGCTCGTCGCTGCGTTGA/BHQ-3'	

PhiX174

Target sequence length: 123 bp

ACAAAGTTTGGATTGCTACTGACCG**CTCTCGTGCTCGTCGCTGCGTTGA**GGCTTGCGTTTATGGTACGCTGGACTTT GTGGGATACCCTCGCTTTCCTGCTCCTGTTGAGTTTATTGCTGCCG

Total length with pUCIDT-Amp Plasmid- 2875 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc. is measured on nanodrop and copy number determined using the following formula:

PhiX 174 number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

4.2 MS2 Primer and Probes

Sequence	Reference
Forward: 5'-GTCCATACCTTAGATGCGTTAGC-3'	Turgeon, N. et al. 2014; Appl Environ Microbiol 80(14):4242 – 4250.
Reverse: 5'-CCGTTAGCGAAGTTGCTTGG-3'	
Probe: 5'-FAM/ACGTCGCCAGTTCCGCCATTGTCG/BHQ-3'	

MS2 RNA sequence for Standard curve;
-Length 3559 bp
-Concentration: 10 A260 Unit or Conc provided on the tube
(each 1 OD_{260} Unit = 40µg/ml ssRNA)
number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 800ng/ul* 6.022×10^{23}) / (length * 1×10^{9} * 650/2)
= 4.16E+11 copies

4.3 Adenovirus Primer and Probes

Sequence	Reference
Forward Primer: 5'-AACTTTCTCTCTTAATAGACGCC-3'	Ko *et al*., J. Virol Methods, 2005, 127: 148-153
Reverse Primer: 5'-AGGGGGCTAGAAAACAAAA-3'	
Probe: 5'-6FAM- **CTGACACGGGCACTCTTCGC** -BHQ-3'	

Standard Curve Target sequence: 118 bp

AACTTTCTCTCTTAATAGACGCCCCACTTAATG**CTGACACGGGCACTCTTCGC**CTTCAAAGTGCTGCACCTCTTGGAC
TAGTGGACAAAACACTAAAAGTTTTGTTTTCTAGCCCCCT

Total length with pUCIDT-Amp Plasmid - 2870 bp
For Standard curve: linearize with PvuI enzyme

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

4.4 Coronavirus OC43 Primers and Probe

Sequence	Reference
Forward Primer: 5'- CGATGAGGCTATTCCGACTAGGT-3 '	Dare, R.K. et al. J Infect. Diseases, 2007, 196: 1321-8
Reverse Primer: 5'-CCTTCCTGAGCCTTCAATATAGTAACC -3'	
Probe: 5'-6FAM- TCCGCCTGGCACGGTACTCCCT -BHQ-3'	

Standard Curve use OC43 virus serially diluted from 10^{6} GCMPN /5 uL to 10^{1} GCMPN / 5uL

4.5 SARS -oV-2 Primers and Probes

Sequence	Reference
2019-nCoV_N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3'	2019-nCoV CDC EUA Kit, 500 rxn IDTCat# 10006606
N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'	
N1-P: 5'-FAM-ACC CCG CAT TAC GTT TGG TGG ACC-BHQ1-3' N1-Pdouble: FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	
N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'	
N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'	
N2-P: 5'-FAM-ACA ATT TGC CCC CAG CGC TTC AG-BHQ1-3'	

N2-Pdouble: FAM-ACA ATT TGC /ZEN/ CCC CAG CGC TTC AG-3IABkF	

Standards for preparing the standard curve: Positive Control provided by IDT Kit is 2E+05 GC/ul or 1E+06/5ul is serially diluted to 1E+01 and used as standard curve.

4.6 WHO SARS-CoV-2 Primers and Probe

Sequence	Reference
E_Sarbeco_F1: ACAGGTACGTTAATAGTTAATAGCGT	WHO Protocol: Diagnostic detection of 2019-nCoV by real-time RT-PCR on Jan 17, 2020
E_Sarbeco_R2: ATATTGCAGCAGTACGCACACA	
E_Sarbeco_P1: FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ	

Standard curve Target sequence for the E gene:
114 bp
ACAGGTACGT TAATAGTTAA TAGCGTACTT CTTTTTCTTG CTTTCGTGGT
ATTCTTGCTA GTTACACTAG CCATCCTTAC TGCGCTTCGA TTGTGTGCGT
ACTGCTGCAA TATT

Plasmid is linearized with PVUI High Fidelity Enzyme from NEB and PCR cleaned Using QIAGEN PCR Cleanup Kit, Linear DNA conc is measured on nanodrop and copy number determined using the following formula:

number of copies = (amount * 6.022×10^{23}) / (length * 1×10^{9} * 650)

QPCR MASTER MIX TABLES
The Following Tables provide the reaction volume for each RT qPCR/qPCR reactions.

Table 5 -Enterovirus RT-qPCR reaction

Master Mix - Enterovirus	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.25	4.25
10 uM EntF	0.75	0.75
10 uM EntR	2.25	2.25
10 uM EntP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 6 -Norovirus GIA RTqPCR reaction

Master Mix - Norovirus GIA	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.75	4.75
10 uM NorV GIA F(JJVMF)	1.25	1.25
10 uM NorV GIA R	1.25	1.25
10 uM NorV GIA P (RING1)	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 7 -Norovirus GIB RTqPCR reaction

Master Mix – Norovirus GIB	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GIB F	1.25	1.25
10 uM NorV GIB R	2.25	2.25
10 uM NorV GIB P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 8 -Norovirus GII RTqPCR reaction

Master Mix - Norovirus GII	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	2.375	3.375
10 uM NorV GII F	1.25	1.25
10 uM NorV GII R	2.25	2.25
10 uM NorV GII P	0.625	0.625
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).
Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 9 -Hepatitis G RTqPCR Inhibition Assay reaction

Master Mix - Hepatitis G	**Option 1**	**Option 2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
HepG	2	2.5
PCR grade water	1.75	2.25
10 uM HepF	1.25	1.25
10 uM HepR	1.25	1.25
10 uM HepP	0.25	0.25
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

For 1-step: set up the thermocycler program and run with a setting of 1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Notes
-depending on the instrument used, a longer cDNA extension of 35-50 minutes (instead of 15 min) may be required.

-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

Table 10 - PhiX174 qPCR reaction

Master Mix - PhiX174	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2X qPCR MM (1)	12.5	12.5
PCR grade water	3.5	4.5
10 uM PhiX174F	1.25	1.25
10 uM PhiX174R	1.25	1.25
10 uM PhiX174P	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

PhiX174 Thermal cycler qPCR program: 5 min at 94°C and then 40 amplification cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
Or
95°C for 2 min, followed by 40 cycles with a 95°C denaturation for 10 s, 55°C annealing for 30 s, and 72°C elongation for 15 s (Adenovirus assay conditions)
-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Table 11 - MS2 phage RTqPCR Assay reaction

Master Mix - MS2 phage	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.5	4.5
10 uM MS2	1.25	1.25
10 uM MS2	1.25	1.25
10 uM MS2	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

MS2 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.

Or
1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence). (All other RT-qPCR cycling conditions)

Table 12 – Adenovirus qPCR reaction

Master Mix - Adenovirus	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
2x qPCR MM [1]	12.5	12.5
PCR grade water	4	5
10 uM AdvF	1	1
10 uM AdvR	1	1
10 uM AdvP	0.5	0.5
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

Adenovirus Thermal cycler qPCR:
Hot-start denaturation step at 95°C for 2 min, followed by 40 cycles with a 95°C denaturation for 10 s, 55°C annealing for 30 s, and 72°C elongation for 15 s

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-Use Option 1 if standards are prepared to correspond to 6 ul per reaction- Use Option 2 if standards are prepared to correspond to 5 ul per reaction volume.

1. **qPCR master mixes: Roche 480 Probe Master or Quantabio PerfeCTa® qPCR ToughMix**

Table 13 – Coronavirus OC43 RTqPCR Assay reaction

Master Mix – OC43	Option 1	Option 2
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	12.5	12.5
PCR grade water	3.25	4.25
10 uM OC43F	1.25	1.25
10 uM OC43R	1.875	1.875
10 uM OC43P	0.125	0.125
Total MM	19	20
Sample Volume	6	5
Total reaction Vol	25	25

Final conc: OC43 Forward: 500nM; OC43 reverse: 750nM; OC43probe: 50nM

OC43 _RT-PCR (One step): 15 min at 50°C, 5 min at 95°C, and then 40 cycles including denaturation at 94°C for 15 s and annealing and elongation at 60°C for 60 s, followed by fluorescence measurement.
Or
1 cycle hold at 50 °C for 10 min, 1 cycle hold at 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Table 14 – SARS-CoV-2 CDC RTqPCR Assay reaction

Master Mix – CoV-2	**CoV-n1**	**CoV-2 n2**
Reagent	**Volume per rxn (uL)**	**Volume per rxn (uL)**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	10	10
PCR grade water	3.5	3.5
Combined Primer/Probe Mix n1	1.5	
Combined Primer/Probe Mix n2		1.5
Sample Volume	5	5
Total Reaction Vol	20	20

SARS-CoV-2 RT-PCR (One step): 15 min at 50°C, 2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

Master Mix - E2	# Rxns:	x
Reagent	**Volume per rxn (uL)**	**Volume per MM**
Quanta Bio qScript XLT 1-**Step** RT-qPCR ToughMix	10	12.5
PCR grade water	4.5	5.0
10 uM E2 F	0.8	1.0
10 uM E2R	0.8	1.0
10 uM E2 P	0.4	0.5
Total MM	15	20
Sample Volume	5	5
Total reaction Vol	20	25

Final conc. Forward Primer 400 nM; Reverse Primer 400nM; and Probe 200 nM

E2 RT-PCR (One step): 10 min at 50°C, 3 min at 95°C, and then 45 cycles including denaturation at 95°C for 15 s and annealing and elongation at 58°C for 30 s, followed by fluorescence measurement.

Concentration of Municipal Wastewater Samples for Viral Analysis

Nichole Brinkman, USEPA/ORD

07/19/2020

Scope and Applicability

The purpose of this protocol is to outline the steps necessary to concentrate untreated municipal wastewater samples for microbial analysis. The scope of this protocol applies to small volumes (e.g., 250 ml) of untreated municipal wastewater and for detection of a wide range of microorganisms but has been specifically designed for analysis of SARS-CoV-2. As such, additional biosafety measures are required for processes that could generate aerosols from wastewater samples.

Summary of Method

Upon receipt in the lab, samples will be stored at 4°C for no more than 72 hours from the start of sample collection (holding time subject to change as more data become available). Each sample will be amended with PBS and an human coronavirus (OC43) matrix spike and a small volume (0.2 ml) will be removed for direct nucleic acid extraction and analysis of the matrix spike and endogenous viruses of human fecal origin. The sample concentration steps consist of centrifugation, membrane filtration (0.45 µm) and ultrafiltation (30 kDa Centricon-Plus units). The products from each step (centrifugation pellet, membrane filters and ultrafiltration retentate) will be analyzed. Each sample will be processed in duplicate and will result in 4 products: unprocessed sample, pellet, filter, ultrafiltration retentate.

Definitions/Acronyms

BSC	Biological Safety Cabinet
BSL	Biosafety level
CDC	Centers for Disease Control and Prevention
CoV	Coronavirus
COVID-19	Coronavirus disease 2019
LOD	Limit of Detection
PBS	Phosphate buffered saline
PPE	Personal protective equipment
SARS	Severe Acute Respiratory Syndrome
SARS-CoV-1	the viral etiological agent of the 2003 SARS outbreak
SARS-CoV-2	the viral etiological agent of COVID-19
RT-PCR	Reverse transcriptase polymerase chain reaction
UF	Ultrafiltration
WHO	World Health Organization

Health and Safety Warnings

Precautions should be taken when handling samples that may contain SARS-CoV-2. The CDC has issued Interim Biosafety Guidelines for labs processing COVID-19-related samples (CDC. 2020a). Standard Precautions should be implemented when collecting and handling samples; these precautions include hand hygiene and the use of personal protective equipment (safety glasses, lab coats and gloves). Sample manipulation should be performed in a BSL-2 laboratory using Standard Precautions and a certified Class II Biological Safety Cabinet (BSC). Sample processing procedures likely to generate

aerosols or droplets, such as centrifugation and membrane filtration, should be performed in a BSL-2 laboratory with unidirectional airflow, a certified Class II BSC and BSL-3 precautions to include respiratory protection and designated area for donning and doffing PPE. Samples can be disposed in the manner of other biohazardous laboratory waste.

Cautions/Interferences

To date, there are no recommended methods for concentrating SARS-CoV-2 from wastewater, thus creating a challenge for efficient recovery from sewage. Wastewater contains constituents that can readily inactivate virions, degrade genomic material and inhibit detection. Thus, holding times, sample stabilizers and other quality control metrics are being investigated and will be implemented as data are available.

Personal Qualifications/Responsibilities

All personnel must be current in mandatory laboratory safety training, including Hazardous Waste Management, Chemical Hygiene and respiratory protection courses. Personnel must also be aware of the biosafety guidelines for handling specimens likely containing SARS-CoV-2 and be certified to wear respiratory protection. The technique of first-time analysts must be reviewed by an experienced analyst prior to completing this SOP alone. During this review, the new analyst must demonstrate proficiency in completing this procedure alone which must be approved and documented by an experienced analyst/supervisor. Personnel must wear the required personal protective equipment (eye protection, lab coat, gloves and respiratory protection) while performing this protocol.

Equipment and Supplies

Nitrile gloves
Lab coat
Safety glasses
Wypall (Kimberly Clark 34770, or equivalent)
Label tape
Permanent marker
Conical tube, 250 ml, sterile (Corning 430776 or equivalent)
Centrifuge (up to 3000 x g), cups and conical adapters
Stopper, silicone, #8 (Millipore XX2004718 or equivalent)
Disposable Microfunnel, GN-6, 0.45 µm (PALL 4800)
Side arm Erlenmeyer flask, 1L, sterile
Vacuum source
Centricon Plus-70 Centrifugal Filter Units, 30 kDa (EMD Millipore UFC703008)
Microcentrifuge tube, 1.5 ml, low retention, RNase-, DNase-, DNA-, pyrogen-free
RNeasy PowerWater kit PowerBead Tube (Qiagen 14700-50-NF)
Freezer, -20°C
Refrigerator, 4°C

Reagents and Standards

Bleach, 10%
Ethanol, 70%
1X PBS

10X PBS, RNase-free, pH 7.4 (Invitrogen, AM9624)
OC43 process control, 10^7 virus particles/ml

Procedure

1. Don PPE.

2. Disinfect the surfaces of the BSC with 10% bleach and turn on UV light for at least 15 min.

3. For each sample, obtain 2-250 ml conical tubes and label each bottle with the sampleID, replicate number, date and analyst's last name.

4. Obtain the samples in plastic bags for secondary containment from temporary storage at 2-8°C.

5. One at a time, transfer the sample to the BSC, remove the sample bottle from the plastic bag and wipe the outside of the bottle with 70% ethanol.

6. Invert the sample bottle 20 times to mix.

7. Using a sterile graduated cylinder, measure 225 ml and transfer to the appropriately labeled 250 ml conical tube.

8. Add 25 ml of 10X PBS and 1 ml of OC43 process control to each sample and invert sample bottles 10 times.

9. Remove 0.2 ml of each prepared sample to a labeled 1.5 ml tube for nucleic acid extraction. Tubes should be labeled with date, analyst's last name, SampleID and "before processing."

10. Balance the 250 ml conical tubes in the centrifuge cups with conical bottom cushions and safety caps using a dual-pan balance.

11. Centrifuge the samples at 3000 x g for 15 minutes at 10˚C to pellet the larger particulate matter.

12. Pour the supernatant from each of the samples into a new, labeled sterile 250 ml conical tube without disrupting the pellet. If possible, remove all the supernatant leaving a dry pellet.

13. For each sample, assemble a sterile 1L side-arm flask, stopper and disposable Pall MicroFunnel.

14. Filter half of the volume of each 250 ml sample through a disposable Pall MicroFunnel. Remove the filter cup and, using sterile forceps, roll the membrane filter and transfer the filter to a PowerWater DNA bead tube. Label each bead tube with date, analyst's last name, SampleID and "filter." Apply a second Pall MicroFunnel to the flask and filter the remaining volume of the sample. Remove the filter cup and, using sterile forceps, roll the membrane filter and transfer the filter to the same PowerWater DNA bead tube (there should be 2 filters in a bead tube for each 250 ml subsample and 250 ml of filtrate in the flask).

15. Transfer 60-70 ml of 0.45 µm filtered sample to an appropriately labeled Centricon® Plus-70 centrifugal ultrafilter.

16. Centrifuge Centricon unit at 1500 x g for 15 minutes. Discard the filtrate appropriately.

17. Repeat centrifugation until the entire 250 ml sample has been ultrafiltered and retentate volume is 0.2 ml or less (at least 4 cycles of centrifugation to get through 250 ml; a 5th cycle of centrifugation should reduce the volume to the desired 0.2 ml).

18. Remove Centricon Plus-70 unit from centrifuge and separate the sample filter cup from the filtrate collection cup.

19. Carefully invert unit into the retentate cup.

20. Spin at 1,000 × g for 2 minutes.

21. Remove the retentate cup containing the concentrated sample from the sample filter cup.

22. Measure the retentate volume and transfer to a RNase, DNase, DNA-free 1.5 ml tube labeled with date, analysts' last name, SampleID, "UF" and retentate volume (ul).

23. Store the sample products (4 sample tubes per sample replicate: 1) before processing, 2) pellet, 3) filter, and 4) UF retentate) at -20°C.

24. Doff PPE appropriately.

Data and Records Management

A research notebook will be kept by each individual researcher generating or analyzing data. Each laboratory notebook will be used to record or reference research goals, hypotheses, assumptions, experimental data, interim summaries and conclusion. Experimental procedures, apparatus, and data will be recorded with sufficient detail such that another trained person could reconstruct the experiment.

Quality Assurance/Quality Control

The quality control procedures required to demonstrate successful performance of sample concentration includes the use of positive and negative controls. Table 1 describes how controls will be assessed.

References

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WHO. 2020. Surface sampling of coronavirus disease (COVID-19): A practical "how-to" protocol for health care and public health professionals. https://apps.who.int/iris/bitstream/handle/10665/331058/WHO-2019-nCoV-Environment_protocol-2020.1-eng.pdf. Accessed 04/06/2020

Table 1. Quality Control Samples for Concentrating Municipal Wastewater

QC Sample	Description	Frequency	Acceptance Criteria	Corrective Action
Field blank	sterile, buffered solution	Monthly	RT-ddPCR results below LOD of respective RT-ddPCR/ddPCR assay	RT-ddPCR/ddPCR result outside of the acceptance criteria indicates contamination. Corresponding samples may need to be discarded. Alternatively, results of unknown samples could be interpreted in the context of the results of the negative control sample.
Positive Process Control	Predetermined quantity of OC43 spiked into each sample replicate	Each sample replicate	1-200% recovery efficiency	Recovery efficiency outside of the acceptance criteria indicates inefficiency with sample processing and analysis, potentially leading to an underestimation of virus quantification in samples.
Endogenous process control	Viruses of human fecal origin endogenous to municipal wastewater (crAssphage, pepper mild mottle virus)	Each sample replicate	5-200% recovery efficiency	Recovery efficiency outside of the acceptance criteria indicates inefficiency with sample processing and analysis, potentially leading to an underestimation of virus quantification in samples.

Nucleic Acid Extraction of Microbes in Municipal Wastewater Samples

Nichole Brinkman, USEPA/ORD

07/20/2020

Scope and Applicability

The purpose of this protocol is to outline the steps necessary to extract nucleic acids from unprocessed and concentrated municipal wastewater samples for microbial analysis by RT-PCR/PCR. The steps described here are adapted from the manufacturer's protocols (Qiagen A and B).

Summary of Method

Nucleic acids are indiscriminately extracted from virions and prokaryotic/eukaryotic/archaeal cells present in samples using commercially available kits. The Qiagen AllPrep PowerViral DNA/RNA Kit is used to extract nucleic acids from the unprocessed and ultrafiltered sample products and RNeasy PowerWater kit is used with the pellet and membrane filter sample products. The kits utilize the same buffers and silica-based strategy for chemical lysis, precipitation and purification of nucleic acids, but the pellet and membrane filter sample products require mechanical pressure through bead beating to facilitate release of nucleic acids using the RNeasy PowerWater kit bead tubes. Briefly, a small volume of Phi6 bacteriophage stock is added to all sample products to assess extraction efficiency, along with a lysis solution (PM1). The pellet and membrane filter sample products are bead beaded and then an inhibitor removal solution (IRS) is added to pellet organic material and any molecules that could interfere with PCR. A combination of solutions containing chaotropic agents and binding salts (PM3 and PM4) is added to facilitate RNA and DNA to adhere to a silica spin column. The sample lysate is added onto the silica-based spin column to bind nucleic acids followed by washes with alcohol-based solutions to purify RNA/DNA bound to the spin column. Nucleic acids are eluted from the spin column with RNase-free water.

Definitions/Acronyms

B-ME	β-mercaptoethanol
BSC	Biological Safety Cabinet
BSL	Biosafety level
DNA	deoxyribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
PPE	Personal protective equipment
QA	Quality Assurance
QC	Quality Control
RNA	Ribonucleic acid

Health and Safety Warnings

Standard laboratory PPE (i.e., laboratory coat, safety glasses/goggles, and gloves) is required. Nucleic acid extractions must take place in a biological safety cabinet (BSC). Special care must be taken in handling β-mercaptoethanol (CAS# 60-24-2), a volatile liquid that is toxic to the nervous system and can be absorbed through the skin and mucous membranes. β-mercaptoethanol storage container should be opened in a biosafety cabinet that vents to the outside, and all pipetting operations should be carried out in the biosafety cabinet. All solutions containing β-mercaptoethanol, isopropanol, ethanol, detergents, and the proprietary chaotrope should be disposed of appropriately.

Cautions/Interferences

To reduce the risk of potential contamination and/or degradation of nucleic acids during the processing of samples, the analyst should wear appropriate PPE and never handle samples or reagents with bare hands. Prior to and after extractions, samples should only be opened in a disinfected BSC. Where applicable, equipment (i.e., micropipettes) should be wiped with 10% bleach and should be used to disinfect bench top surfaces before and after use to degrade residual amplicons left on lab surfaces.

Personal Qualifications/Responsibilities

All personnel must be current in mandatory laboratory safety training. Personnel must also be aware of the biosafety guidelines for handling specimens likely containing SARS-CoV-2. The technique of first-time analysts must be reviewed by an experienced analyst prior to completing this SOP alone. During this review, the new analyst must demonstrate proficiency in completing this procedure alone which must be approved and documented by an experienced analyst/supervisor. Personnel must wear the required personal protective equipment (eye protection, lab coat and gloves) while performing this protocol.

Equipment and Supplies

Nitrile gloves
Lab coat
Safety glasses
Wypall (Kimberly Clark 34770, or equivalent)
Label tape
Permanent marker
Heat block, 55˚C
Thermometer
Cold block, 2-8˚C
Vacuum source
QIAvac 24 Plus vacuum manifold (Qiagen, 1941), VacValves (Qiagen 19407), VacConnectors (Qiagen 19408)
Microcentrifuge
Microcentrifuge tube, 1.5 ml, low retention, RNase-, DNase-, DNA-, pyrogen-free
AllPrep PowerViral Kit (Qiagen 28000-50)
RNeasy PowerWater Kit (Qiagen 14700-50-NF)
Freezer, -80°C
Freezer, -20°C
Refrigerator, 4°C
Biosafety cabinet
Vortex Genie
Vortex Genie adaptor for 5 ml tubes (Qiagen 13000-V1-5)

Reagents and Standards

Bleach, 10%
Ethanol, 70%
1X PBS
DEPC-treated water
β-mercaptoethanol
Phi6 Extraction control (10^6 PFU/ml)

Procedure

1. Don PPE.

2. Disinfect the surfaces of the BSC with 10% bleach and turn on UV light for at least 15 min.

3. Turn on heat block and warm solution PM1 at 55°C to dissolve any precipitates. Prepare a cold block filled with DI water added to the wells and set aside to cool.

4. Prepare Phi6 extraction calibrator sample by adding 10 ul of the Phi6 stock and 190 ul of DEPC-treated water to each of a 1.5 ml low retention, RNase-, DNase-, DNA-, pyrogen-free microfuge tube and PowerWater DNA bead tube.

5. Prepare the negative extraction controls by adding 200 uL DEPC-treated water to an appropriately labeled a 1.5 ml low retention, RNase-, DNase-, DNA-, pyrogen-free microfuge tube and PowerWater DNA bead tube.

6. Make a solution of PM1 and β-ME (10 uL β-ME/1000 uL PM1) for all samples. Note that each pellet and Filter sample fractions require 1mL of PM1-β-ME, while the subsamples removed before processing and UF fractions require 600 ul of PM1-β-ME.

7. Prepare the pellet sample fractions by resuspending each pellet in 1 mL of PM1-β-ME. Transfer the entire volume to a 5 mL PowerWater bead tube. Ensure all biomass is scraped off the sites; this may require forcefully pipetting up and down. Add 10 ul of the Phi6 stock to each sample tube.

8. Prepare the filter sample fractions by adding 1mL of PM1-β-ME to each 5mL PowerWater bead tubes that have filters. Add 10 ul of the Phi6 stock to each sample tube.

9. Secure the PowerWater bead tubes horizontally to a MO BIO Vortex Adapter. The tube caps should be oriented pointing toward the center of the Vortex Adapter.

10. Vortex at maximum speed for 5 minutes.

11. Centrifuge bead tubes at 4000 x *g* for 1 minute at room temperature.

12. Transfer all the supernatant using a 1mL pipette tip into a clean labeled 2 mL collection tube (provided in the kit) by placing the tip down into the beads and drawing as much volume as possible.

13. Centrifuge the tubes at 13,000 x *g* for 1 minute.

14. Avoiding the pellet, transfer the supernatant to the clean 2mL collection tube pre-loaded with 200uL of IRS. Continue to step 18 for processing all the sample products.

15. Prepare the 0.2 ml subsample removed prior to processing and the ultrafilter retentate by adding dd 10 ul of the Phi6 stock and 600 uL of PM1-β-ME directly into the sample tubes.

16. Vortex each sample for 30 sec. Incubate samples at room temperature for 5 min.

17. Add 150 ml of IRS to each sample.

18. Vortex all sample product tubes loaded with IRS briefly to mix. Incubate at 4°C for 5 minutes

19. Centrifuge the tubes at 13,000 x *g* for 1 minute.

20. Avoiding the pellet, transfer the supernatant to the clean 2.2mL lysate tube pre-loaded with 650 µl of PM3 +650 ul PM4. Mix gently by pipetting.

21. Set up the QIAvac vacuum manifold by adding new vac-connectors and MB RNA Spin Filter to the valves.

22. Load 650 µl of supernatant onto the appropriately labeled MB RNA Spin Filter. Turn on the vacuum and allow the lysate to flow through. Repeat until all the lysate has passed through the spin column.

23. Shake to mix Solution PM5. Add 650 µL of Solution PM5 to the Spin Filter and vacuum though the manifold.

24. Add 650 µL of Solution PM4 and vacuum though the manifold.

25. Turn off the vacuum and place spin filters into the spin filter collection tube and centrifuge at 13,000 x *g* for 2 minutes to remove any residual wash solution.

26. Place the Spin column into the appropriately labeled clean 2 mL Collection Tube.

27. Add 125 µL of RNase-Free Water to the center of the filter membrane. Allow the water to sit on the membrane for at least 5 minutes.

28. Centrifuge at 13,000 x *g* for 1 minute.

29. Remove spin columns and close tubes containing extracted nucleic acids.

30. Store extracts at -80C.

Data and Records Management

A research notebook will be kept by each individual researcher generating or analyzing data. Each laboratory notebook will be used to record or reference research goals, hypotheses, assumptions, experimental data, interim summaries and conclusion. Experimental procedures, apparatus, and data will be recorded with sufficient detail such that another trained person could reconstruct the experiment.

Quality Assurance/Quality Control

The quality control procedures required to demonstrate successful performance of sample concentration includes the use of positive and negative controls. Table 1 describes how controls will be assessed.

References

Qiagen AllPrep PowerViral DNA/RNA Kit Handbook https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/multianalyte-and-virus/allprep-powerviral-dna-rna-kit/#orderinginformation

Qiagen RNeasy PowerWater Kit Handbook https://www.qiagen.com/us/products/discovery-and-translational-research/dna-rna-purification/rna-purification/microbial-rna/rneasy-powerwater-kit/?clear=true#resources

Table 1. Quality Control Samples for Concentrating Municipal Wastewater

QC Sample	Description	Frequency	Acceptance Criteria	Corrective Action
Negative Extraction Control	sterile, buffered solution	Each batch of samples (up to 24 samples)	RT-ddPCR results below LOD of respective RT-ddPCR/ddPCR assay	RT-ddPCR/ddPCR result outside of the acceptance criteria indicates contamination. Corresponding samples may need to be discarded. Alternatively, results of unknown samples could be interpreted in the context of the results of the negative control sample.
Phi6 extraction calibrator sample	Predetermined quantity of Phi6 bacteriophage	1 per batch of samples (up to 24 samples)	RT-ddPCR results within 2 standard deviations of the mean of the expected quantity	RT-ddPCR result outside of the acceptance criteria indicates inefficiency with sample processing and analysis.
Spiked Extraction Control	Predetermined quantity of Phi6 bacteriophage	Every sample	5-200% recovery efficiency	Recovery efficiency outside of the acceptance criteria indicates inefficiency with sample processing and analysis, potentially leading to an underestimation of virus quantification in samples.

U.S Environmental Protection Agency
Office of Research and Development
National Exposure Research Laboratory
Systems Exposure Division
Research Triangle Park, North Carolina, Headquarters
Athens, Georgia
Cincinnati, Ohio
Las Vegas, Nevada

STANDARD OPERATING PROCEDURE

Title: Droplet Digital PCR (ddPCR) Analysis of Genomic Targets

Number: D-SED-EFAB-014-SOP-01	Effective Date: August 16, 2016

SOP was Developed ☒ In-house ☐ Extramural

Alternative Identification: EFAB-014-01

SOP Steward

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NERL-SOP.2(11/2005)

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SOP Title: Droplet Digital PCR (ddPCR) Analysis of Genomic Targets

1.0 Purpose

This standard operating procedure (SOP) describes the amplification and quantitation of genomic targets using droplet digital PCR (ddPCR).

2.0 Scope and Applicability

ddPCR can be used to quantify RNA and DNA targets of prokaryotes, eukaryotes, archaea and viruses in a variety of sample matrices, including, but not limited to, water, air and rubber surfaces. The use of this procedure is contingent upon sample preparation that releases RNA and/or DNA from cells or viral capsids.

3.0 Summary of Method

The use of quantitative PCR (qPCR) to determine the concentrations of targeted microbes in environmental samples has become a standard tool used in microbiology because it is sensitive and specific for the intended microbial targets. The quantitative nature of qPCR relies on the linear relationship that exists between $\log_{10}$ target sequences in a sample and the number of thermal cycles needed to reach a defined threshold (often referred to as the cycle threshold) where fluorescence is detectable above background but is still increasing exponentially due to PCR amplification. Fluorescence can be produced either by the binding of intercalating dyes or by the cleavage of target sequence specific probes that both occur stoichiometrically with the generation of amplified target sequence fragments. Based on this relationship, standard curves of $\log_{10}$ target sequences vs. cycle threshold measurements can be generated from well characterized source material (standards) using a linear regression model and target sequences in unknown samples can be quantified by interpolation of their cycle threshold measurements on the standard curves. In this way, qPCR provides quantitation of unknown samples relative to known standards.

Despite the utility of qPCR in many environmental applications, limitations inherent in the linear regression approach can lead to inconclusive results such as when target concentrations of unknown samples fall outside of the dynamic range of the standard curve and when PCR inhibitors are present in the sample. In the former scenario, quantities of target sequences in unknown samples become inaccurate or are unable to be determined. While in the latter scenario, PCR inhibition directly affects amplification efficiency resulting in an underestimation of the true target sequence concentration in the sample.

Digital PCR (dPCR) utilizes the components of qPCR, but eliminates the need for a standard curve. Therefore, the disadvantages intrinsic to quantity estimates inferred from a curve are also eliminated. However, quantitation is still feasible and offers precision in quantity determinations beyond the capability of qPCR. In dPCR, samples are portioned into thousands of nanoliter sized PCR reactions such that each reaction contains zero or one or more DNA templates. After endpoint PCR amplification, each reaction is screened to determine whether amplification occurred. The number of positive and negative reactions is scored and used to

determine the number of DNA molecules (λ) present in the reaction using the Poisson distribution from which the following equation is derived:

$$\lambda = -\ln\left(1 - \frac{x}{n}\right)$$

Where x is the number of positive PCR reactions and n is the total number of reactions.

Droplet digital PCR (ddPCR) uses an oil-based approach to partition the nanoliter reactions. This SOP describes the steps to perform ddPCR using BioRad's QX200 ddPCR system.

4.0 Definitions

ADG – automated droplet generator
BSC – biological safety cabinet
BSL – biosafety level
ddPCR – droplet digital PCR
DNA – deoxyribonucleic acid
PCR – polymerase chain reaction
RNA – ribonucleic acid
RT-ddPCR – reverse transcriptase-ddPCR
PPE – personal protective equipment

5.0 Health and Safety Warnings

Standard laboratory PPE for Biosafety Level (BSL) 1 microorganisms is required. This PPE consists of lab coat, safety glasses and gloves.

HASP# 2015-046 describes the health and safety considerations for conducting PCR. Use of the ddPCR reader in the post-PCR lab is covered under HASP# 2016-017.

6.0 Cautions/Interferences

PCR is a sensitive tool for detecting genomic targets in samples. To reduce the risk of potential contamination during the processing of samples, the analyst should wear appropriate PPE and never handle samples or reagents with bare hands. Prior to PCR, samples should only be opened in a BSC that has been sanitized with 10% bleach and UV prior to use. Where applicable, equipment (i.e., micropipettes) should be wiped with 10% bleach. After samples have been amplified by PCR, a BSC is not required for sample manipulation. However, 10% bleach should be used to disinfect bench top surfaces before and after use to degrade residual amplicons left on lab surfaces. Also, a unidirectional work-flow will be implemented to prevent cross-contamination of amplicons between labs. Negative PCR controls will be used to monitor potential contamination events during these operational procedures. Samples and master mixes containing enzymes should be kept on ice or in a cold block to maintain integrity/activity during PCR set up.

Care should be taken when generating droplets in the manual mode. Air bubbles can cover the bottom of the well and result in 2500-7000 fewer droplets and poor data quality. They are difficult to see. To avoid creating air bubbles, use the pipetting technique described in detail in section 9 "Manual Droplet Generation," step 3. These techniques, ensures samples wet the bottoms of the wells so they are wicked into the microchannels (necessary for proper droplet generation).

PCR is an enzymatic reaction that can be disrupted if interfering substances are co-extracted and purified during sample processing. For best results, high quality DNA should be used in these procedures.

7.0 Personnel Qualifications/Responsibilities

The techniques of first-time analysts must be reviewed by an experienced analyst prior to completing this SOP alone. During this review, the new analyst must demonstrate his/her ability to complete this procedure alone which must be approved and documented by an experienced analyst/supervisor in the analyst's laboratory notebook.

8.0 Equipment and Supplies

General supplies needed for all steps
Lab coat
Safety goggles
Laboratory gloves (nitrile or latex)
Micropipettes and tips
Low retention polypropylene microfuge tubes
10 mM Tris-HCl, pH 8.5

ddPCR or RT-ddPCR
Forward primer
Reverse primer
Dual labeled probe (non-fluorescent quencher must be used with BioRad platform)
ddPCR Droplet Reader Oil (BioRad cat# 1863004)
Automated Droplet Generation Oil for Probes (BioRad cat# 1864110)
Droplet Generation Oil for Probes (BioRad cat# 1863005)
QX200 Droplet Generation Oil for EvaGreen (BioRad cat# 1864006)
AutoDG Oil for EvaGreen (BioRad cat# 1864112)
DG8 Cartridges and Gaskets (BioRad cat# 1864007)
DG32 Automated Droplet Generator Cartridges (BioRad cat# 1864108)
Pipet Tips for the Auto DG (BioRad cat# 1864120)
Pipet Tip Waste bins for the AutoDG System (BioRad cat# 1864125)
ddPCR Supermix for Probes (no dUTP) (BioRad cat# 1863024)
One-Step RT-ddPCR Advanced Kit for Probes (BioRad cat# 1864021)

QX200 ddPCR EvaGreen Supermix (BioRad cat#186-4033)
Eppendorf 96-well twin.tec PCR plates (Eppendorf cat# 951020346)
Pierceable Foil Heat Seal (BioRad cat# 1814040)
Microseal-A Film (BioRad cat# MSA5001)
Rainin Tips LTS 20UL Filter RT-L10FLR (Rainin cat #17007957)
Rainin Pipet-Lite Multi Pipette L8-50XLS= (Rainin cat #17013804)

The QX200 AutoDG Droplet Digital PCR System consists of several pieces of instrumentation to perform ddPCR analysis. PCR reactions are sequestered into thousands of droplets using either the Droplet Generator or Automated Droplet Generator. PCR plates are sealed using the PX1 PCR Plate Sealer. PCR is performed in a C1000 Touch Thermal Cycler with 96-Deep Well Reaction Module. PCR amplification is assessed with the QX200 Droplet Reader and QuantaSoft Software.

9.0 Procedure

Preparation of Positive (RT-)ddPCR Controls for the FAM and VIC/HEX Channels

1. Identify material to be used as a positive control. This can be a genomic extract of the organism of interest or a synthetic construct, like a minigene containing the targeted genomic sequence.

2. Determine the concentration of the DNA stock to be used a positive control.

3. Dilute the positive control stock to 3.2×10^4 genomic copies/ul in a volume sufficient to prepare the desired number of aliquots. This quantity equates to approximately 10 copies of the ddPCR target per droplet, assuming an average of 16,000 droplets per reaction.

4. Prepare 25 µl aliquots of the positive control and store at -80°C.

Preparation of Exogenous Internal Amplification Controls

1. Identify material to be used as an exogenous internal amplification control. This can be a genomic extract of a specific organism or a synthetic construct, like a minigene containing the targeted genomic sequence.

2. Determine the concentration of the DNA stock to be used an exogenous internal amplification control.

3. Dilute the exogenous internal amplification control stock to 1.3×10^4 genomic copies/ul in a volume sufficient to prepare the desired number of aliquots. This quantity equates to approximately 1.6 copies of the ddPCR target per droplet, assuming an average of 16,000 droplets per reaction.

4. Prepare 200 µl aliquots of the exogenous internal amplification control and store at -80°C.

Preparation of (RT-)ddPCR Reactions

1. Bleach PCR hood in clean room. Turn on UV for at least 15 min.

2. Wipe the barrels of all micropipettors to be used with 10% bleach.

3. In PCR hood, prepare the ddPCR or RT-ddPCR mixes, examples are shown in Section 14 Tables 1-2 below. Move to DNA lab.

4. Bleach Biosafety cabinet in DNA lab. Turn on UV for at least 15 min. Wipe the barrels of all micropipettors to be used with 10% bleach.

5. Obtain samples, positive controls and internal amplification control aliquots from the -80°C freezer.

6. Prepare dilutions of samples as indicated in Table 3 with 10 mM Tris-HCl, pH 8.5.

7. Obtain an Eppendorf 96-well twin.tec PCR plate (these are the ONLY plates that fit in the ADG and reader).

8. Dispense 18 µl of the ddPCR/RT-ddPCR master mix into the 5 NTC wells as outlined in Table 3.

9. To the ddPCR/RT-ddPCR negative control (NTC) wells, load 7 µl of 10 mM Tris-HCl, pH 8.5 to the corresponding wells of the plate as indicated in Table 3.

10. To the remaining ddPCR/RT-ddPCR master mix, add 2 µl of the exogenous internal amplification control (1.3 x 10^4 genomic copies/ul) for the remaining reactions: 2 x (100-5) = 190µl.

11. Dispense 20 µl of the ddPCR/RT-ddPCR master mix with the exogenous internal amplification control to the remaining reactions in the 96 well plate as indicated in Table 3.

12. Add 5 µl of the appropriate positive control sample to the positive control wells as indicated in Table 3.

13. Add 5 µl of the appropriate sample to the wells as indicated in Table 3.

14. If using the ADG to make droplets, seal the plate with the appropriate foil seals and PX1 PCR Plate Sealer. If using the manual Droplet Generator to make droplets, seal the plate with Microseal-A.

15. Mix plate thoroughly by vortexing briefly, followed by centrifugation at 500 x g for 15 sec.

16. Allow the reactions to equilibrate to room temperature for about 3 minutes before loading into the droplet generator.

Manual Droplet Generation

- The QX200 droplet generator prepares droplets for up to eight samples at time. Droplet generation takes ~ 2 minutes for each set of 8 samples (~30 minutes for a 96-well plate).

- All 8 sample wells in the DG8 droplet generator cartridge must contain sample (or 1X buffer control), and all 8 oil wells must contain droplet generator oil.

- Do not load sample or oil into the DG8 cartridge unless it is inserted in the holder.

1. Insert the DG8 cartridge into the holder with the notch in the cartridge at the upper left of holder.
   a. Open the cartridge holder by pressing the latches in the middle.
   b. Slide the DG8 cartridge into the right half of the holder, then drop it down.
   c. Press the halves of the holder together to snap it closed.

2. Dispense the 80 µl of droplet generator oil for each reaction in the reagent reservoir

3. Transfer 20 µl of each prepared sample to the sample wells (middle row) of the DG8 cartridge **with the considerations listed below**:

   a. Use only 20 µl aerosol-barrier (filtered) Rainin pipet tips; do not use 200 µl tips.

   b. Gently slide the pipet tip down the side of the well at a ~15° angle until it passes over the ridge near the bottom. Holding the angle, ground the pipet tip against the bottom edge of the sample well while slowly dispensing a small portion of the sample; do not pipet directly onto the side (wall) of the well.
   c. After dispensing about half the sample, slowly draw the tip up the wall while dispensing the rest of the sample; do not push the pipet plunger past the first stop.

4. Using a multichannel pipet, fill each oil well (bottom row) with 70 µl of DG oil from the reagent reservoir.

5. Hook the gasket over the cartridge holder using the holes on both sides. The gasket must be securely hooked on both ends of the holder; otherwise, pressure sufficient for droplet generation will not be achieved.

6. Open the QX200 droplet generator by pressing the button on the green top and place the cartridge holder into the instrument. When the holder is in the correct position, both the power (left light) and holder (middle light) indicator lights are green.

7. Press the button on the top again to close the door. This initiates droplet generation: a manifold positions itself over the outlet wells, drawing oil and sample through the microfluidic channels, where droplets are created. Droplets flow to the droplet well, where they accumulate. The droplet indicator light (at right) flashes green after 10 sec to indicate droplet generation is in progress.

8. When droplet generation is complete, all three indicator lights are solid green. Open the door by pressing the button and remove the holder (with DG8 cartridge still in place) from the unit. Remove the disposable gasket from the holder and discard it. The top wells of the cartridge contain droplets, and the middle and lower wells are nearly empty with a small amount of residual oil.

9. Pipet 40 µl of the contents of the top wells (the droplets) into a single column of a 96-well PCR plate, **using the following pipetting techniques to avoid shearing or coalescing the droplets**:

   a. Use an 8-channel manual L-50 pipet with 200 µl tips (not wide or narrow bore).

   b. Place the cartridge holder on a flat surface that has been sterilized with 10% bleach and position the pipet tips in each of the 8 top wells at a ~30-45° angle, vertical into the junction where the side wall meets the bottom of the well; do not allow the tips to be flat against the bottom of the wells.

   c. Slowly draw 40 µl of droplets into the pipet tip (should take ~5 sec and ~5 µl air is expected); do not aspirate >40 µl as this causes air to percolate through the droplets. Pipet slowly. Apply a stable resistive force to the plunger to draw and aspirate droplets smoothly into and out of pipet tips.

   d. To dispense droplets into the 96-well plate, position the pipet tip along the side of the well – near, but not at, the bottom of the well – and slowly dispense droplets (~5 sec).

   e. To prevent evaporation and contamination with particulates, cover the plate as you work. To cover the plate, set the tip box lid or a piece of foil over the plate.

10. Once droplets are removed, press the latches on the DG8 cartridge holder to open it. Remove the empty DG8 cartridge and discard it.

Automatic Droplet Generation and ddPCR

1. When powering on the Automated Droplet Generator (ADG), you will see a startup screen while the instrument powers on and performs a self-check. Please note that the door will automatically close.

2. If the instrument deck is empty, the indicator lights on the deck of the ADG should be off, indicating that no consumables are present. The corresponding areas of the touch screen will be gray. If consumables were present in the instrument when power was lost or disconnected, an error message will appear prompting you to check and reset the consumables if necessary. The cartridges, gaskets, tips and plates are all single-use consumables and should be discarded in the biohazard waste after use. If the consumables in the instrument are of questionable use after power loss, please remove and discard.

3. Bring the ADG out of idle mode by touching the screen.

4. Check the indicator lights on the deck of the ADG instrument and the consumable icons on the touch screen.

5. To create a plate of droplets with the ADG:

    a. Touch the Configure Sample Plate button at the bottom center of the screen.

    b. Touch or swipe across the screen to select the columns in which your samples are located on the sample plate. Touching a selected column deselects it. You can touch any orientation of columns.

    c. The plate name and plate notes are optional; touching the files will bring up a keyboard on the screen. Click OK when done.

6. Based on the number of columns selected in the previous step, the consumable icons on the screen will begin to blink yellow to indicate where new consumables need to be loaded into the instrument.

    a. If the blinking yellow icon displays Load on the screen, remove the previously used consumable (if applicable) and load a new consumable into the designated are of the instrument.

    b. If the icon remains gray on the screen, that consumable is not needed to complete the currently configured run.

7. Open the door on the ADG instrument by lifting up on the handle at the front to the instrument. The electronic braking system will assist you in this task and prevent the lid from closing accidentally. Note: the ADG door will close the preserve HEPA-filtered

enclosure if left open for longer than 20 minutes while in idle mode. There is an audible click when the door brake releases, and the door closes slowly. Please observe the pinch points of the instrument and keep hands clear.

   a. To avoid contamination, load the consumables from the back to the front of the instrument. Nothing should be placed on the instrument deck outside to the dedicated consumable holders.

8. Load the DG32 ADG Cartridges along the back row of the instrument:

   a. Remove the plastic wrapping from the DG32 cartridges and place, with the green gaskets to the right, into the three plate holders. The holders are keyed for proper orientation of each DG32 cartridge to prevent incorrect loading.

   b. The lights on the DG32 plate holders will change from yellow to green when the DG32 cartridges are inserted correctly. If a light remains yellow, try repositioning the plate in a different orientation.

   c. As the lights turn green on the deck, the corresponding icons on the touch screen will go from blinking yellow to solid green and Ready will be displayed.

9. To load the ADG Pipet tips along the center row of the instrument:

   a. Remove the plastic wrapping and box lids from the tip boxes and place into the plate holders in the middle of the deck. Only full tip boxes should be loaded.

   b. Remove the tip waste bin containing any tips from a previous run and replace with a clean waste bin.

   c. The lights on the tip box holders will changed from yellow to green when the tip boxes are inserted correctly.

   d. As the lights turn green on the deck, the corresponding areas of the touch screen will go from blinking yellow to solid green, and “Ready” will be displayed.

   e. Please not that only ADG Pipet Tips should be used; other tips can damage the instrument.

10. To load the 96 well PCR plate containing your prepared ddPCR reactions into the front row of the instrument:

    a. The sample plated can be sealed with a PX1 Plate Sealer and heat-sealing foil in advance of loading into the ADG instrument. Each well should contain 25 µl of the

prepared ddPCR load the 96 well PCR plate containing your prepared ddPCR reactions into the front row of the instrument.

b. Place the plate into the front left plate holder, labeled on the screen as Sample Plate. The holder is keyed for proper orientation and contains plate clips to support sealed plates.

c. The light on the Sample Plate holder will change from yellow to green when the plate is inserted correctly. If the light remains yellow, try repositioning the plate in a different orientation.

d. As the light turns green on the deck, the corresponding icon on the touch screen will go from blinking yellow to solid green, and Ready will be displayed.

e. Note that a sample plate is required for every run, regardless of the number of columns selected.

11. To load the Droplet Plate assembly:

a. The cooling block should be placed in a -20C freezer for at least 2 hours before configuring a run on the ADG instrument and inserting into the instrument. The block goes from a pink color at room temperature to a dark purple color when properly cooled.

b. Remove the cooling block from the freezer and place into the front right plate holder, labeled on the screen as Droplet plate. The holder is keyed for proper orientation of the cooling block. The block should be a dark purple color, indicating that it is at the proper temperature. If the block is pink, it has warmed up and should not be used.

c. The light on the instrument will change from yellow to green when the block is interested correctly. If the light remains yellow, try repositioning the block in a different orientation.

d. As the light turns green on the deck, the corresponding area of the screen will go from blinking yellow to solid green, and Ready will be displayed.

e. Place a clean 96-well PCR plate for droplet collection into the cooling block accessory. The cooling block is also keyed for proper orientation of the plate.

f. A clean droplet plate is required for every run, regardless of the number of columns selected. Once generated, the droplets will be dispensed into the sample plate orientation as the ddPCR reactions were taken from the sample plate.

12. To load the ADG Oil into the instrument:

a. Remove the cap from the bottle of ADG Oil and twist the bottle into the tower of the oil delivery system at the front left corner of the instrument. Turn the bottle until it does not move; the label on the bottle should face out.

b. Select the type of ADG oil that was loaded into the instrument by touching the droplet for either Probes or EvaGreen oil, depending on the type of assay being used. The droplet you select will turn blue; touch OK to set the oil type.

c. The oil level icon on the screen will turn blue and display the current oil level of the bottle. They system will display the oil type at the bottom left of the screen as well.

d. If a bottle of ADG oil was previously loaded, you may be prompted to confirm the type of oil currently loaded into the instrument.

e. If the last plates were run on the ADG with a different oil type than the one being currently loaded and selected, the instrument will perform a small volume purge of the oil through the delivery system and into the oil waste reservoir. The total run time will be a few minutes longer when this occurs, but droplet generation will not be impacted.

13. Once all of the consumables are loaded and the corresponding lights are green on the deck and touch screen, a blue Start button will appear at the bottom right of the screen. Touching Start will bring up a confirmation window indicating the type of Droplet Generation Oil loaded, the number and orientation of columns selected for droplet generation and any plate name and details entered during configuration of the sample plate.

14. Once you have confirmed the plate setup, touch the Start Run button to being droplet generation. The door will automatically close at the beginning of the run and must remain closed during the run. Opening the door before the Droplets Ready message appears may cause the instrument to terminate the run and samples to be lost.

15. After a brief initialization the ADG will display a countdown timer on the screen with time remaining until the plate of droplets is ready. No additional action is needed until the plate is ready. Thin initialization can take 1-5 min, depending on whether the oil type has been changed or not.

16. Once the plate of droplets is ready, the screen will display a finalizing window followed by a blue Droplets ready message with a timer showing time elapsed since complete. The droplet plate at the front right corner of the instrument will be illuminated blue. The corresponding icon on the touch screen will also pulse blue… the door on the instrument will unlock and the droplet plate can be removed. Please wait to remove the oil bottle as noted on the screen; the unused oil is being returned to the bottle.

17. Remove the droplet plate containing ddPCR droplets and seal within 30 min of droplet generation completing as described below in "Plate sealing and ddPCR/RT-ddPCR.".

18. Remove any consumables from the ADG that have been completely used and discard. DG32 cartridges, tips and plates are single-use consumables. The ADG will remember the status of the consumables as long as it remains powered on; if a consumable has been only partially used, leave it the ADG for the next run.

<u>Plate sealing and ddPCR/RT-ddPCR</u>

19. Seal the PCR plate with foil (BioRad cat # 181-4040) immediately after transferring droplets to avoid evaporation. Note: the foil seals have to be compatible with the PX1 PCR plate sealer. With the recommended product, the red stripe should be facing up.

20. Set the plate sealer temperature to 180°C and time to 5 sec (remove support block while sealer is heating).

21. Touch the arrow to open the PX1 tray door. Position the support block on the tray with the 96-well side facing up. Place the 96-well plate onto the support block and ensure that all plate wells are aligned with the support block.

22. Secure the plate on the support block and touch the seal button. The tray will close and heat sealing will initiate.

23. When heat sealing is complete, the PX1 door opens automatically. Remove the plate from the block for thermal cycling. Remove the support block from the PX1.

24. Check that all the wells in the plate are sealed; the depressions of the wells should be visible on the foil. Once sealed, the pate is ready for thermal cycling.

    a. Begin thermal cycling within 30 minutes of sealing the plate, or store the plate at 4°C for up to 4 hours prior to thermal cycling.

25 Run reactions through the PCR/RT-ddPCR cycle in the BioRad C1000 Touch instrument with the appropriate conditions.

    a. For ddPCR, the following conditions should be used: 95°C for 5 min*, followed by 50 cycles of 95°C for 30 seconds*, and 55°C for 1 min*, then 98°C for 10 minutes* and a 4°C hold**. (*ramp time should be ~2°C/sec, **ramp time should be ~1°C/sec).
    b. For RT-ddPCR, the following conditions should be used: 50°C for 1 hr, 95°C for 10 min, followed by 50 cycles of 95°C for 30 seconds, and 55°C for 1 min*, then 98°C for 10 minutes and a 4°C hold (* adjust ramp rate to 2°C/sec).

c. Use a heated lid set to 105°C and set the sample volume to 40 µl.

d. Note: After PCR, the plate should be read within 24 hours. If not run immediately, the plate should be stored at 4°C until reading can occur.

Droplet Reader

1. Power on the QX200 droplet reader using the switch at the back. Allow it to warm up for 30 min, then switch on the PC and launch QuantaSoft software.

2. Check the indicator lights on the front of the droplet reader. The first two lights at left should be solid green, indicting power is on, there is sufficient oil in the designated oil reservoir and there is <700 ml in the waste bottle. If the lights are flashing amber, the run cannot be started; clean out the waste bottle by placing a hazardous waste label on it and storing it in the satellite waste secondary container or replace the oil (see manual for instructions).

3. Place the 96-well PCR plate into the plate holder:

   a. Place the 96-well PCR plate containing the amplified droplets into the base of the plate holder. Well A1 of the PCR plate must be in the top left position and place the top on the PCR plate.

   b. Move the release tabs of the top of the plate holder in the “up” position and place the top on the PCR plate. Firmly press both release tables down to secure the PCR plate in the holder.

4. Press the button on the green lid to open the droplet reader. Load the plate holder in to the droplet reader and press the button on the lid again to close the cover. Confirm the first three indicator lights are green.

5. In QuantaSoft software, click SETUP in the left navigation bar to define your experiment.

6. Click TEMPLATE>NEW.

7. Select the wells by double clicking.

   a. In the “Name” field of the Sample well editor, enter the **SampleID.**

   b. In the “Experiment” field of the Sample well editor, select **ABS**.

   c. In the “Supermix” field of the Sample well editor, select **ddPCR Supermix for Probes (no dUTP)** or **One-Step RT-ddPCR Kit for Probes**.

   d. Define Assay 1 (channel 1, the FAM channel) in the Target 1 box:

i. In the "Name" field of the Target 1 well editor, enter the assay target
ii. In the "Type" field, select **Ch1Unknown, Ch1 Positive or NTC.**

e. Define Assay 2 (channel 2, the VIC/HEX channel) in the Target 2 box:
   i. In the "Name" field of the Target 2 well editor, enter the exogenous internal amplification control assay target
   ii. In the "Type" field, select **Ch2Unknown, Ch2 Positive or NTC.**

f. When done, click APPLY or OK to save the information.

8. Save the file using the following format: "YYYY-MM-DD_TCR_PCRX", where YYYY-MM-DD is the date of the ddPCR run and PCRX is the consecutive number of PCR plates run for this sample set.

9. Click RUN. Up to 1 minute later, a green circle appears next to the abort button and flashes periodically to indicate the run is in progress. Active and analyzed wells are also highlighted in green in the plate map.

10. When droplet reading is complete, all four indicator lights are solid green. Open the door and remove the plate holder from the unit. Remove the 96-wel PCR plate from the holder and discard it.

11. Save data files to L:\Lab\Lablan\MCEARD_digitalPCR\dPCR\Brinkman.

12. Use D-SED-EFAB-015-SOP-01 "Quantitative Analysis of ddPCR data" to assess positive and negative droplet populations based on droplet fluorescence and to calculate copies of genomic targets and internal amplification control in each sample.

10.0 Data and Records Management

The data output from the QuantaSoft software is a directory of files that includes files containing fluorescence data of every droplet detected in the 96-well plate and QuantaSoft analysis of the droplet data. The entire directory will be copied to the LabLan for backup and access for further analysis, as described in D-SED-EFAB-015-SOP-01.

11.0 Quality Control and Quality Assurance

The quality control procedures required to demonstrate successful performance of (RT-)ddPCR include the use of positive and negative controls. To ensure quality of data collection during PCR, a negative control consisting of the buffer used to dilute samples will be used. This should result in baseline fluorescence amplitude levels for every droplet detected. A positive control for (RT-)ddPCR consists of DNA containing the targeted amplicon, either from extracts of organisms or synthesized DNA constructs. This control will contain 10^5 copies of the target per reaction, resulting in an average of 10 copies per droplet and amplification in 100% of droplets.

If the (RT-)ddPCR negative controls result in droplets with fluorescence amplitude levels indicating amplification or the PCR positive controls result in the less than 100% positive

droplets, the amplicons will be discarded and the (RT-)ddPCR will be repeated.

The results of the quality control procedures will be documented in the analysts' notebook. All QC failures will be reported to the project lead. A discussion of the overall QC procedures and results will be described in the manuscript.

12.0 References

This SOP was adapted from BioRad documents for the QX200 Droplet Digital PCR System, which can be found at http://www.bio-rad.com/en-us/product/qx200-droplet-digital-pcr-system?tab=Documents

Instruction Manual, QX200 Droplet Generator, Rev C (BioRad # 10031907)

Instruction Manual, QX200 Automated Droplet Generator (BioRad # 1864101)

Instruction Manual QX200 Droplet Reader and QuantaSoft Software, Rev D ((BioRad # 10031906)

Instruction Manual, PX1 PCR Plate Sealer, Rev A (BioRad # 10023997)

Instruction Manual, C1000 Touch Thermal Cycler, Rev B (BioRad # 10021377)

QX200 ddPCR EvaGreen Supermix Product Insert, Rev C (BioRad # 10028376)

ddPCR Supermix for Probes (No dUTP) Product Insert, Rev D (BioRad # 10026868)

One-Step RT-ddPCR Advanced Kit for Probes Product Insert, Rev A (BioRad # 10049226)

13.0 Document History

Date of Revision	Revision #	Description of Changes
8/16/2016	0	First version

14.0 Tables

Table 1. ddPCR Master Mix

Component	Volume/ Reaction (µl)	Volume/Mix (µl) 100 Reactions	Final Concentration	Lot#, Expiration Date
Nuclease-free water	2.45	245	To 20 µl	
2X Supermix for probes (no dUTP)	12.5	1250	1X	
Forward primer (FAM, 50 µM)	0.45	45	900 nM	
Reverse primer (FAM, 50 µM)	0.45	45	900 nM	
Probe (FAM, 10 µM)	0.625	62.5	250 nM	
IAC Forward primer (VIC/HEX, 50 µM)	0.45	45	900 nM	
IAC Reverse primer (VIC/HEX, 50 µM)	0.45	45	900 nM	
IAC Probe (VIC/HEX, 10 µM)	0.625	62.5	250 nM	
Total Volume	**18**	**1800**	-	

IAC = Internal amplification control

Table 2. RT-ddPCR Master Mix

Component	Volume/ Reaction (µl)	Volume/Mix (µl) 100 Reactions	Final Concentration	Lot#, Expiration Date
Nuclease-free water	5.7	570	To 20 µl	
4X One-Step Advanced RT-ddPCR Supermix	6.25	625	1X	
Reverse transcriptase	2	200	20 U/µl	
DTT (300 mM)	1	100	15 mM	
Forward primer (FAM, 50 µM)	0.45	45	900 nM	
Reverse primer (FAM, 50 µM)	0.45	45	900 nM	
Probe (FAM, 10 µM)	0.625	62.5	250 nM	
IAC Forward primer (VIC/HEX, 50 µM)	0.45	45	900 nM	
IAC Reverse primer (VIC/HEX, 50 µM)	0.45	45	900 nM	
IAC Probe (VIC/HEX, 10 µM)	0.625	62.5	250 nM	
Total Volume	**18**	**1800**	-	

IAC = Internal amplification control

Table 3. Sample Layout for (RT-)ddPCR in a 96-well Plate

<table>
<tr><th></th><th>1</th><th>2</th><th>3</th><th>4</th><th>5</th><th>6</th><th>7</th><th>8</th><th>9</th><th>10</th><th>11</th><th>12</th></tr>
<tr><td>A</td><td colspan="3">Sample A, undiluted</td><td colspan="3">Sample C, 1:4</td><td colspan="3">Sample F, 1:2</td><td colspan="3">Sample I, undiluted</td></tr>
<tr><td>B</td><td colspan="3">Sample A, 1:2</td><td colspan="3">Sample D, undiluted</td><td colspan="3">Sample F, 1:4</td><td colspan="3">Sample I, 1:2</td></tr>
<tr><td>C</td><td colspan="3">Sample A, 1:4</td><td colspan="3">Sample D, 1:2</td><td colspan="3">Sample G, undiluted</td><td colspan="3">Sample I, 1:4</td></tr>
<tr><td>D</td><td colspan="3">Sample B, undiluted</td><td colspan="3">Sample D, 1:4</td><td colspan="3">Sample G, 1:2</td><td rowspan="5">Positive (RT-) ddPCR control, FAM target</td><td rowspan="5">Positive (RT-) ddPCR control, VIC/HEX target</td><td rowspan="5">Negative (RT-)ddPCR control NTC</td></tr>
<tr><td>E</td><td colspan="3">Sample B 1:2</td><td colspan="3">Sample E, undiluted</td><td colspan="3">Sample G, 1:4</td></tr>
<tr><td>F</td><td colspan="3">Sample B, 1:4</td><td colspan="3">Sample E, 1:2</td><td colspan="3">Sample H, undiluted</td></tr>
<tr><td>G</td><td colspan="3">Sample C, undiluted</td><td colspan="3">Sample E, 1:4</td><td colspan="3">Sample H, 1:2</td></tr>
<tr><td>H</td><td colspan="3">Sample C, 1:2</td><td colspan="3">Sample F, undiluted</td><td colspan="3">Sample H, 1:4</td></tr>
</table>

Quantifying SARS-CoV-2 in Wastewater Samples

Sample Concentration:

1. Let the wastewater sample thaw overnight from -20°C storage.
2. Spike the sample with the Process Control (Hs_RPP30 or OC43).
3. Filter the sample through 0.45 µm nongridded sterile cellulose membrane filter (Sartorius™).
4. Prerinse a Corning™ Spin-XTM concentrator (100 kDa molecular weight cut-off) with deionized water as described in the manufacturer's manual.
5. Fill the prerinsed concentrator with 20 mL of the filtrate. Ensure screw closure is fully seated. Insert assembled concentrator into a swing bucket centrifuge that can accommodate 50 mL conical bottom tubes. Centrifuge at 3,000 g for 30 min.
6. Repeat the steps 4-5 until the pocket of the concentrator (for concentrated sample) reached approximately 1 mL. Record the volume of filtrate used. Transfer the concentrated sample to a 2-ml centrifuge tube.

RNA Extraction:

1. Extract RNA from concentrated samples with the RNeasy Mini Kit (Qiagen) with 40 µL elution volume following the manufacturer's protocol.

Reverse Transcription Quantitative PCR (RT-qPCR):

1. Use two primer pairs (N1 and N2) and probes from 2019-nCoV CDC EUA Kit.
2. Follow CDC guidelines for reagent and controls preparation. (https://www.fda.gov/media/134922/download)
3. Use 20 µL as the reaction volume, which includes 10 µL iTaq universal probes one-step reaction mix, 0.5 µL iScript advanced reverse transcriptase, 1.5 µL of primer and probe mix, 3 µL of water, and 5 µL of template.
4. Use nuclease-free water as negative template control (NTC).
5. Use 2019-nCoV_N_Positive Control as the positive control.
6. Follow the following protocol for amplification: 50°C for 10 min, 95°C for 1 min followed by 40 cycles of 95°C for 10 sec, and 60°C for 30 sec.

Supplies needed:

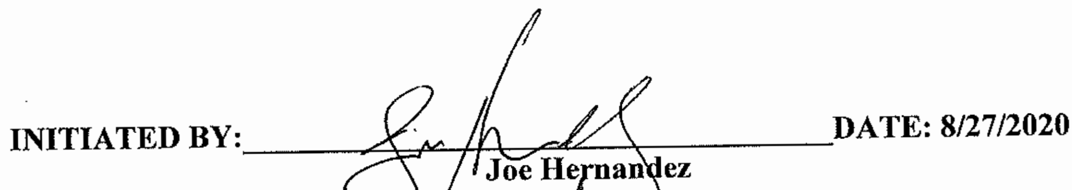
<table>
<tr><th>Item</th><th>Description</th><th>Catalog No.</th><th>Company</th><th>Storage</th></tr>
<tr><td>Membrane</td><td>Sartorius™ nongridded sterile cellulose membrane filters: pore size 0.45 µm, pack of 100</td><td>14-555-577</td><td rowspan="2">Fisher Scientific</td><td rowspan="2">Room temperature in the dark</td></tr>
<tr><td>Concentrator</td><td>Corning™ Spin-X™ concentrators, with 100 kDa molecular weight cut-off, capacity 20 mL, pack of 12</td><td>07-201-354</td></tr>
<tr><td>RNA Extraction Kit</td><td>RNeasy mini kit, 50 rxns</td><td>74104</td><td>Qiagen</td><td></td></tr>
<tr><td>Primers and Probe</td><td>2019-nCoV CDC EUA Kit, 500 rxns, include primers and probes for N1, N2, and RP</td><td>100006606</td><td rowspan="3">Integrated DNA Technologies IDT</td><td rowspan="3">Store dried primers and probes at 2-8°C until re-hydrated for use. Store liquid primers and probes at -20°C</td></tr>
<tr><td>Positive Control</td><td>2019-nCoV_N_Positive Control for N1 and N2</td><td>10006625</td></tr>
<tr><td>Process Control</td><td>Hs_RPP30 Positive Control for RP</td><td>10006626</td></tr>
<tr><td>Reaction Mix</td><td>iTaq® Universal Probes One-Step Reaction Mix, 100 x 20 µL rxns, 1 mL</td><td>1725140</td><td>Biorad</td><td>Store at -20°C</td></tr>
</table>

WATER QUALITY LABORATORY

CITY OF SCOTTSDALE

STANDARD OPERATING PROCEDURE

2020-WQ-PCR-SARS-CoV-2 by RT-qPCR



INITIATED BY: ______________________ **DATE: 8/27/2020**
Joe Hernandez

QA REVIEWED BY: ______________________ **DATE: 08/28/2020**
Lillian Reeves

APPROVED BY: ______________________ **DATE: 8/28/2020**
Laura McCasland

THIS SOP IS SCHEDULED TO BE REVIEWED ANNUALLY

A. SCOPE AND APPLICATION

To determine the presence and quantity of SARS-CoV-2 and HCoV-OC43 enveloped viruses in raw wastewater using the Reverse Transcription Quantitative Polymerase Chain Reaction (RT-qPCR). The PCR platform of use is the Rotor-Gene Q and associated software.

B. HOLDING TIME/SAMPLE PRESERVATION

1. Raw wastewater should be processed as soon as possible but may be held between 2-5 °C for up to 8 hours
2. Sample extracts waiting to be analyzed by RT-qPCR should be stored at -80°C

C. EQUIPMENT

1. Rotor-Gene Q system
2. Quick spin mini centrifuge
3. Dry block heater with micro tube adapter
4. -80°C Ultra low freezer
5. Micropipettes (P-10, P-20, P-100, P-200, P-1000) & Molecular Biology Grade (MBG) filtered tips
6. Pipet-Aid and individually wrapped sterile serological pipets, 1mL, 5mL, 25mL
7. Micro tubes (MBG), 0.5mL, 2mL
8. Vortex mixer
9. Timer
10. 50 mL conical centrifuge tubes
11. Refrigerated centrifuge with swinging bucket rotor, 50mL conical and flat tube adapters (Beckman Coulter, Allegra X-14R or equivalent)
12. Centrifugal concentrator, 50kd molecular cutoff (Centriprep Ultracel YM-50, Millipore item no. 4310)
13. Magnetic Particle Separator (DynaMag™ - 2 Magnet, ThermoFisher part no. 12321D or equivalent)
14. Sterile sample vessel, 120mL (IDEXX item no. 98-09222-00)
15. Refrigerator designated for wastewater samples (2-6 °C)

D. CHEMICALS AND REAGENTS

1. 10% Bleach solution
2. 70% Isopropyl alcohol wipes
3. Molecular Grade Water (MGW)
4. IDEXX Water DNA/RNA Magnetic Bead Kit (IDEXX item no. 98-0014719-00)
5. Water SARS-CoV-2 RT-PCR Test (IDEXX item no. 98-0014718-00)
6. AccuPlex™ SARS-CoV-2 Verification Panel – Full Genome (Seracare item no. 0505-0168)
7. Quantitative Synthetic SARS-CoV-2 RNA (ATCC item no. VR-3276SD)
8. Quantitative Genomic RNA from betacoronavirus 1 OC43 (ATCC item no. VR-1558DQ)

E. PROCEDURE

Concentration

1. Mix sample and transfer 35mL to four 50mL conical tubes.

2. Centrifuge tubes with brake off at 4,300 RCF at 4°C for 30min.
3. Carefully transfer one tube at a time to a rack. Using a 25mL serological pipet, slowly draw 27mL of supernatant from just below meniscus being careful to not disturb pellet. Transfer sample to sterile sample vessel and repeat process with remaining three tubes. Pool all four 27mL volumes into one sample vessel.
4. Place 7 centrifugal filter devices in rack. Turn the twist-lock cap on each sample container counterclockwise, then slide the filtrate collector/cap assembly out and set it aside on a clean surface.
5. Using a 25mL serological pipet, transfer 15mL of the pooled sample to each of the seven sample containers (105mL total volume).
6. Carefully insert the capped filtrate collector into the sample container, gently pushing down so the collector displaces the solution. Turn the twist-lock cap clockwise to seal the sample container. Finally, ensure the air-seal cap is snug on the twist-lock cap.
7. Transfer filter devices to centrifuge, counterbalance as necessary, and centrifuge with brake off at 1,500 RCF and 4°C for 10min.
8. Remove each device from the centrifuge and take off the air-seal cap. With the vent groove oriented upward, decant the filtrate. Replace the cap and spin the device again at 1,500 RCF and 4°C for 5min.
9. Repeat step 8 until liquid level is equal to or less than reference filter device containing 1mL of water.
10. Loosen the twist-lock cap (turn counterclockwise) and remove and discard the filtrate collector. Withdraw the sample from device container using a 1mL serological pipette and transfer concentrate to a new filter device.
11. Using the same serological pipette, repeat step 10 for the remaining 6 filter devices pooling concentrates into a single sample container.
12. Repeat steps 7 and 8 above, spinning once for 10-minutes and then 5-minute intervals until sample concentrate reaches concentration limit (filtrate no longer pours out of inner tube).
13. Draw final concentrate using a new 1mL serological pipette and transfer to a 2mL micro tube.
14. Using a P-1000 micropipette set to 1mL, slowly draw concentrate from microtube and reverse dial plunger until liquid reaches pipette tip and make note of volume.
15. Return sample to micro tube and immediately proceed with extraction, or store concentrate overnight at -25 to -10 °C

RNA Extraction

1. Preheat heat block to 58°C
2. **IDEXX Water DNA/RNA Magnetic Bead Protocol**
   Calculate the amount of Working Solution required. Prepare an additional 10% to allow for pipetting loss.
   Lysis Working Solution calculation:

Reagent	**Volume per Sample**
Binding Buffer (BB)	500 µL
Proteinase K (PK)	50 µL
Magnetic Beads (MB)	20 µL

3. Prepare Working Solution by mixing reagents in the order listed above. Mix beads to ensure homogenous solution prior to pipetting. The Working Solution can be stored at room temperature for up to 1 hour prior to use. Longer storage time may result in diminished lysis efficiency.
4. Mix Working Solution thoroughly by inverting before use to ensure that beads are in a homogenous solution.
5. Add 570µL Working Solution to a microcentrifuge tube.
6. Prior to addition, mix the wastewater concentrate by pipetting up and down several times.
7. Add 200µL of wastewater concentrate to the sample tube and mix well.
8. Incubate 10 minutes at 58°C in a dry heat block. Periodically vortex tube to keep magnetic beads suspended and break apart any aggregates that may form.
9. Flash spin tubes to collect all liquid into the bottom of the tube.
10. Separate the magnetic beads by placing the sample tube on the magnetic separator. Wait 1–2 minutes until all the beads have been attracted to the magnets. Remove supernatant with a pipette, taking care not to disturb the magnetic beads.
11. Remove the tube from the magnetic separator. Add 500µL Wash 1 and mix well by pipetting up and down to completely resuspend the beads.
12. Repeat Step 10 to remove Wash 1.
13. Remove the tube from the magnetic separator. Add 500µL Wash 2 and mix well by pipetting up and down to completely resuspend the beads.
14. Repeat Step 10 to remove Wash 2.
15. Repeat Step 13 to resuspend the beads in Wash 2 again.
16. Repeat Step 10 to remove Wash 2.
17. Dry the beads in the open tube for 5–10 minutes at room temperature.
18. Add 100µL Elution Buffer to the sample tube and mix well by pipetting up and down to completely resuspend the beads.
19. Incubate 10 minutes at room temperature. Periodically mix tube to keep magnetic beads suspended.
20. Separate the magnetic beads by placing the tube on the magnetic separator. Wait 1–2 minutes until all the beads have been attracted to the magnets.
21. Transfer the supernatant containing purified nucleic acid to a new tube.
22. Store the purified nucleic acid at 2–8°C for use within 6 hours, or at –25 to –15°C for up to 1 month, or at –80°C for long-term storage.

RT-PCR Reagent Prep, Template and Control Addition

(It is essential that QA/QC measures be followed; refer to SOP DM# 14591264)

IDEXX PCR Test Procedure

Preparation of the PCR Mix:

1. Mix the thawed RNA MMx by inversion or gentle vortex.
2. The RNA MMx is a viscous solution; always pipette it slowly.
3. To prepare the PCR Mix add 10µL SARS-CoV-2 Mix and 10µL RNA MMx for each reaction.
4. Include reagents for all control reactions.

5. When preparing the PCR Mix, first pipette SARS-CoV-2 Mix into the tube and then add the RNA MMx.
6. Pipette up and down a few times to rinse the MMx pipette tip.
7. Gently vortex the solution to ensure the components are mixed well.

Adapted IDEXX PCR Test Procedure for OC43 Assay

OC43 Betacoronavirus 1 RT-PCR

Date: 8/28/2020

Experiment Name/Label on PCR Plate:

Analyst ________

Rxn Info	In uL	
# of Rxns	1	Note: Include 1 extra Rxn for every 10 to account for pipeting loss
Sample Volume	5	
Final Rxn Vol	25	

	Rxn Conc	Units	Stock	Units
RNA Mix (IDEXX)	10	U	1	U
OC43 Forward Primer (IDT)	400	nM	5	pmol/uL
OC43 Reverse Primer (IDT)	400	nM	5	pmol/uL
OC43 Probe (IDT)	100	nM	2	pmol/uL

Lot#	MASTER MIX	Amt (uL)	Added (check)
	MGW	4.75	
	RNA Mix	10	
	OC43 Forward Primer	2	
	OC43 Reverse Primer	2	
	OC43 Probe	1.25	
	Total MaterMix Volume =	20	
	Add 20 uL MasterMix to each PCR tube/well		

Cycling Program			
Step	Temp	Time	Cycles
RT	50°C	15min	1
Denaturation	95°C	1min	1
Amplification	95°C	15sec	45
	60°C	30sec	

Settings for Reporter and Quencher		
Target	Reporter	Quencher
OC43	FAM	BHQ (none)

a) Populate number of reactions in HumCoV-OC43 RT-PCR Excel template sheet (DM# 18391177) and place paper copy on Pre-PCR workstation hood for reference and recording reagent lots.
b) Thaw OC43 primers and probe.
c) Begin by adding calculated MWG to Master Mix (MM) tube; retrieve IDEXX RNA Mix from PCR reagent freezer and transfer calculated volume to MM tube, gently pipette up and down until viscosity dissipates. Promptly return RNA Mix to freezer.
d) Add each of the primers and probe to MM by first pipet mixing in aliquot tube and then again when transferring calculated volume into MM tube.
e) Gently vortex the final MM followed by a quick spin to ensure the components are mixed well.

Load the PCR tubes within 20 minutes or store MM at 2 to 8°C for up to 4 hours. The MM can be stored at –25 to –15°C for up to 2 weeks. Protect from light.

1. Slowly pipette 20 µL of the PCR Mix into the required PCR tubes.
2. For each wastewater sample, add 5 µL of purified RNA to the appropriate tube. The final reaction volume is 25 µL.
3. For each control reaction, add 5 µL to the appropriate tube. The final reaction volume is 25 µL. Include the OC43 Positive Control (300copies/5µL) and No

Template Control (5µL MGW), for each test run. Include 5µL of the provided IDEXX kit PCR Positive Control.

4. Snap tube caps into place and number caps accordingly using a fine tip sharpie.
5. Remove locking ring and carousel from thermocycler and secure carousel onto carrier. Load tube strips into corresponding numbered slot in carousel and replace carousel and locking ring in thermocycler.

Instrument/Software Set Up

1. Locate toggle switch on back of Rotor-Gene Q and power instrument on. The "Standby" light on the instrument face will come on.
2. Double click on the Rotor-Gene Q Series icon located on the computer desktop. In the Quick Start Wizard screen. Select new run.
3. In the Rotor Selection screen, select the blue-72 place rotor and check box indicating that the locking ring has been attached. Select next.
4. Set up thermocycling conditions at follows:

Step	Temperature	Time	Cycles
Reverse Transcription	50°C	15min	1
Denaturation	95°C	1min	1
Amplification	95°C	15sec	45
	60°C	30sec	

5. Set fluorescence acquisition to FAM reporter following 60°C amplification step.
6. Confirm profile page will display – name and save run by either clicking on Save as or the Start Run icon.
7. After run is started, the Edit Samples screen will display. Select individual or multiple wells by holding the shift key and enter sample IDs under the Name column.
8. Label each sample type by selecting the corresponding cell and under the Type column, clicking the down arrow, and selecting corresponding sample type (i.e. unknown, NTC, positive control, negative control, standard)
9. Select OK when done and the Temp and Profile Progress screens will display. The run time countdown for the assay is displayed in the Profile Progress screen.

Data analysis

All test controls must be examined prior to interpretation of results. If the controls are not valid, the results cannot be interpreted.

To obtain appropriate Ct values, analysis for SARS CoV-2 target is performed by manually setting the threshold. The threshold is adjusted to the inflection point for the exponential phase of the curve and above background signal. This is done while viewing all amplification curves for a given run on a logarithmic scale. It is important to follow the same procedure run to run when setting the manual threshold.

The following control results must be obtained for each PCR run in for the run to be deemed valid. If the plate controls are not valid, the results cannot be interpreted, are not valid, and the plate must be repeated.

Control/Sample	SARS-CoV-2 FAM Ct Value	SARS-CoV-2 FAM Result
Positive Control	<40	Positive
PCR Negative Control	No Signal	Negative
Extraction Positive Control	<40	Positive
Extraction Negative Control	No Signal	Negative
Wastewater Sample	<40	Positive

Control/Sample	OC43 FAM Ct Value	OC43 FAM Result
Positive Control	<40	Positive
No Template Control (NTC)	No Signal	Negative
Wastewater Sample	<40	Positive

F. QUALITY CONTROL

1. Refer to SOP, Quality Control Procedures for the Molecular Biology Laboratory (DM# 14591264) before applying this test procedure
2. An extraction control containing SARS-CoV-2 RNA is extracted and tested with each set of samples. The extraction positive control is used to demonstrate successful recovery of RNA during the extraction process and should test positive for the SARS-CoV-2 target. To perform the extraction positive control, use 200µL of the "Member 1" dilution provided in the AccuPlex™ SARS-CoV-2 Verification Panel – Full Genome, for the sample during extraction.
3. Quantitative SARS-CoV-2 and OC43 RNA is diluted to a final concentration of 60,000copies/µL in MGW and five ten-fold serial dilutions prepared. A six-point standard curve is included with each corresponding assay by adding 5µL of each dilution to corresponding assay. Use 5µL of the 10^{-3} OC43 dilution as a PCR positive control for corresponding assay.
4. A PCR positive control and PCR negative control (NTC) is included in each OC43 test run. An extraction positive control, PCR positive control and PCR negative control (NTC) is included in each SARS-CoV-2 test run.

G. WASTE DISPOSAL

1. Unused wastewater should be carefully poured down laboratory sink with copious amounts of cold water and the surrounding sink area thoroughly disinfected using a 10% bleach solution.
2. Unused sample concentrates should be discarded in biohazardous waste container.
3. RT-PCR product tubes are discarded in Post-PCR section biohazardous waste container.

H. HEALTH AND SAFETY

1. Lab coat, gloves, and safety glasses must always be worn when handling wastewater and or hazardous chemicals.
2. Take care to not aerosolize raw wastewater or wastewater concentrate.
3. Insulated cold temperature gloves must be worn when handling items from the -80°C freezer.

I. REFERENCES

1. Rotor-Gene Q User Manual

2. City of Scottsdale Standard Operating Protocol, DM# 14591264 Molecular Biology Quality Control
3. EPA Quality Assurance/Quality Control Guidance for Laboratories Performing PCR Analyses on Environmental Samples, 2004

Updated Workflow for WRF Consideration
Cresten Mansfeldt
University of Colorado – Boulder
July 20th, 2020

Undergraduate Reception and Processing Team (x 2)

In total, 2 undergraduate students will be employed to process the samples at the facility likely housed in ECSL 1B11. Their routine and exact responsibilities, potential exposure routes, and identification of risk and risk mitigation strategies is presented below.

Workflow

Students will, in order, perform the following tasks at the laboratory at the start of the reception shift (12 PM):

1. Complete medical self-assessment;
2. Login to the Engineering Center Research Activities Logs;
3. Access PPE;
4. Enter the ECSL 1B11 labs;
5. Clean all tables, hoods, and ovens; mop floors;
6. Prep the reception log for the sample arrival;
7. Exit the laboratory and receive the coolers on arrival from the field sites; record the reception and mark the weight of the as received coolers;
8. Weigh and record the weight of the recovered coolers;
9. When all stations have submitted samples, open the coolers, remove the sample vessels from all lines, and immediately spike in a 1/100 by volume 10% Tween-20 surfactant solution. The final concentration of Tween-20 will be 0.1%. This enables better concentration and also the inactivation of the virus. Let sit for 10 minutes;
10. Spike an internal control. The internal control used for this sampling campaign is an inactivated Bovine Coronavirus utilized in immunization of cows (INFORCE-3, Zoetis, https://www.zoetisus.com/products/beef/inforce-3.aspx). The does added is 100 µL/100 mL.
11. Disinfect all components by spraying 70% ethanol; after allowing the ethanol to evaporate for 10 minutes, wipe the interior and exterior of the cooler and the exterior of the cold packs with a dilute bleach solution;
12. Soak the tubing lines in a 70% ethanol solution for 10 minutes;
13. Soak the tubing lines in a soap solution for 30 minutes;
14. Rinse all components under tap water;
15. Flip the coolers to dry overnight; and
16. Return the ice packs to the fridge to freeze for the next day's sampling.

Students will, in order, perform the following tasks after Tween-20 spike:

17. The graduate researcher will remove the volume required for the concentration step (likely 50-mL of media);
18. Measure the post-Tween-20 pH;
19. Setup the vacuum system for the total suspended solids (TSS) measurement;
20. Remove blank and dessicated filters, measure the weight;
21. Filter the sample through the TSS apparatus;
22. Place samples in the oven for drying overnight;

23. Place new filters into the dessicator;
24. Measure and record the weight of the previous day's filters;
25. Withdraw 50 mL for freezing as a backup; place and log backup in freezer;
26. Pellet 2 mL for a secondary backup; place and log backup in freezer;
27. Pore any excess down the drain;
28. Thoroughly wash the sample bottles with soap and water; hang sample bottles to dry;
29. Remove sample lines from soap bath; rinse; hang sample lines to dry;
30. Wash all benchtops, hoods, and surfaces;
31. Logout of the lab; and
32. Exit the lab.

Potential Exposure Routes to Human Fecal Matter

In a preliminary analysis of the major potential exposure routes to human fecal matter, the routine detaching of the internal collection tubes from the sample container to the cooler opens the most potential for exposure to small amounts of fecal matter. For more rare and catastrophic events, drop and spill is highlighted.

Mitigation for the routine risk of exposure includes the use of proper PPE, the use of 70% ethanol, and the immediate spike of the Tween-20 surfactant on the arrival in the lab. No sample properties or assays are allowed on the pre-surfactant treated raw wastewater. Mitigation of potential crash events include the usage of triple barriers (both in the design of the sampler and the transportation method) and the maintenance of on onsite spill cleanup kit (mentioned above).

Required training includes on-site sampling, but also EHS specific training

These students will work in solo alternating shifts. However, they will not be allowed to work in the lab alone. Either the graduate student researcher, postdoctoral researcher, or principal investigator must be onsite and within the lab, following social distancing guidelines.

Graduate Researcher/Postdoctoral Researcher (x 2)

Centrifuge the Sample to Remove Debris

1. Pipette transfer equally-weighted volumes (50-mL) of the Tween-20 treated sewage to 50-mL conical tubes; weight to ensure the balance of the swing-bucket centrifuge rotor;
2. On the Tween-20 treated sewage, centrifuge 50 mL of the media at 10,000×g for 10 minutes at 4°C (or longer at slower speeds; Sorvall 8-R). Continue with the supernatant, discarding the pellet. NOTE: On a rotating schedule, extract from the pellet to ascertain virus lost.

Viral Particle Concentration Using an InnovaPrep CP Select:

1. Set up Concentrating Pipette (CP) as instructed in Section 4 of the CP Select User Guide.
2. Insert a Concentrating Pipette Tip (CPT) and select a menu protocol as instructed in Section 5.2 of the CP Select User Guide for the chosen CPT type.
3. Lower the CPT into the sample.
4. Press "Start Run" on the user screen.
5. When the entire sample has been processed the CP will stop.
6. Place a clean final sample container under the CPT. The menu screen will prompt you to press "Elute".

7. Press “Elute”. The sample will dispense from the pipette tip into the sample container.
8. The sample is ready for subsequent sample preparation and analysis steps.

EXCESS MEDIA DISPOSAL. Any excess sample will be inoculated with a high-concentration of common dish soap, let stand for >20 minutes in a fume hood, and disposed down the drain. This process will return the material to an EPA regulated and appropriate disposal route. This procedure will be required to receive approval from EHS.

RNA Extraction using TRIzol™

The guidelines below are adapted from the manufacturer’s manual

Procedural guidelines

• Perform all steps at room temperature (20–25°C) unless otherwise noted.
• Use cold TRIzol™ Reagent
• Wear disposable gloves while handling reagents and RNA samples to prevent RNase contamination from the surface of the skin; change gloves frequently, particularly as the protocol progresses from crude extracts to more purified materials.
• Use RNaseZap™ RNase Decontamination Solution (Cat. no. AM9780) to remove RNase contamination from work surfaces and non-disposable items such as centrifuges used during purification.
• Ensure that all materials that come into contact with TRIzol™ Reagent are compatible with phenol, guanidine isothiocyanate, and chloroform.

Resuspend RNA

1. Add 1 mL of TRIzol™ to the resultant of the CP Select filter;
2. Pipet the suspension up and down several times to homogenize.
    a. Note: More rigorous homogenization is not anticipated because of the purification of the viral particles within the InnovaPrep CP Select step; use bead beating as a backup in case extraction efficiencies are low.
3. Incubate for 5 minutes to allow complete dissociation of the nucleoproteins complex.
4. Add 0.2 mL of chloroform per 1 mL of TRIzol™ Reagent used for lysis, securely cap the tube, then thoroughly mix by shaking.
5. Incubate for 2–3 minutes.
6. Centrifuge the sample for 15 minutes at 12,000 × g at 4°C. The mixture separates into a lower red phenol-chloroform, an interphase, and a colorless upper aqueous phase.
7. Transfer the aqueous phase containing the RNA to a new tube by angling the tube at 45° and pipetting the solution out.

Isolate RNA

1. Precipitation the RNA
    a. Add 0.5 mL of isopropanol to the aqueous phase, per 1 mL of TRIzol™ Reagent used for lysis.
    b. Incubate for 10 minutes at 4°C.
    c. Centrifuge for 10 minutes at 12,000 × g at 4°C. Total RNA precipitate forms a white gel-like pellet at the bottom of the tube.
    d. Discard the supernatant with a pipette.

2. Wash the RNA

a. Resuspend the pellet in 1 mL of 75% ethanol per 1 mL of TRIzol™ Reagent used for lysis
b. Vortex the sample briefly then centrifuge for 5 minutes at 7500 × g at 4°C.
c. Vacuum or air dry the RNA pellet for 10 minutes.
    i. IMPORTANT! Do not dry the pellet by vacuum centrifuge. Do not let the RNA pellet dry, to ensure total solubilization of the RNA. Partially dissolved RNA samples have an A230/280 ratio <1.6.

3. Solubilize the RNA
    a. Resuspend the pellet in 50 µL of RNase-free water, pipette up and down.
    b. Incubate in a water bath or heat block set at 55–60°C for 10 minutes.

RNA Quality/Quantity

1. Run a quick Qubit analysis. Follow manufacture guidelines.

RT-qPCR on a QuantStudio 3
https://www.thermofisher.com/document-connect/document-connect.html?url=https%3A%2F%2Fassets.thermofisher.com%2FTFS-Assets%2FLSG%2Fmanuals%2FTaqPath_1Step_Multiplex_MasterMix_QR.pdf&title=UXVpY2sgUmVmZXJlbmNlOiBUYXFQYXRoIDEtU3RlcCBNdWx0aXBsZXggTWFzdGVyIE1peA==

Make sure to use appropriate tubes and tips for this RT-qPCR.

Primers and Probes Used:

From Gertjen Medema's KWR SOP wastewater SARS-CoV-2 RNA assays

N2	Nucleocapsid (N)	2019-nCoV_N2-F	200 nM	5'-TTACAAACATTGGCCGCAAA-3'	3
		2019-nCoV_N2-R	200 nM	5'-GCGCGACATTCCGAAGAA-3'	3
		2019-nCoV_N2-P	200 nM	5'-FAM-ACAATTTGCCCCCAGCGCTTCAG- ZEN/Iowa Black-3'	3
E	Envelope (E)	E_Sarbeco_F	400 nM	5'-ACAGGTACGTTAATAGTTAATAGCGT-3'	4
		E_Sarbeco_R	400 nM	5'-ATATTGCAGCAGTACGCACACA-3'	4
		E_Sarbeco_P1	200 nM	5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-ZEN/Iowa Black-3'	4

(3) 2019-Novel coronavirus (2019-nCoV) real-time rRT-PCR panel primers and probes. US Centers for Disease Control and Prevention. (accessed Apr 27, 2020)
(4) Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DGJC, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MPG, Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. *Euro Surveill* **2020,** Jan;25(3):2000045. doi: 10.2807/1560-7917.

Need to change the FAM/Iowa Black

CONTROLS

F+ RNA bacteriophage (Wolf et al. 2008)

F+ Fc TCTATGTATGGATCGCACTCG + 60 1778–1799
F+ Rc GTAGGCAAGTCCATCAAAGTC − 60 1869–1889
F+ Pbc TGCTGTCCGATTTCACGTCTATCTTCA + 67 1805–1831 Amplicon size 112 bp

Bovine Coronavirus (Decaro et al)

BCoV-Fc CTGGAAGTTGGTGGAGTT + 29026–29043
BCoV-Rc ATTATCGGCCTAACATACATC − 29090–29110
BCoV-Pbc Texas RED ? d-CCTTCATATCTATACACATCAAGTTGTT-BHQ1e − 29058–29085
Amplicon size 85 bp

"Quantification of N1, N2 and N3 specific assays can be performed using four 10-fold dilutions (ranging from 1.0E+02 to 1.0E+05 copies per reaction) of the 2019-nCoV_N_Positive Control plasmid DNA from IDT (Leuven, Belgium) with specified concentrations as calibration suspension. Evaluate the standard curve parameters: PCR efficiency should be 80% and 105%, correlation coefficient R2 should be 0.9."

Quantification of E and F+ are run against dilutions of a gel-purified RT-PCR products run WITHOUT probe.

Quantification of BCoV is run against dilutions of a reference controlled purified RNA extraction.

Prepare the reaction mix (from the TaqPath 1-Step Multiplex Master Mix Quick Reference)

1. Thaw all reagents on ice.
2. Calculate the total volume required for each reaction component according to the following table.

Component	Fast System (20-µL Rxn)	Standard System (50-µL Rxn)	Notes
TaqPath™ 1-Step Multiplex Master Mix (4X)	5 µL	12.5 µL	
Up to four user-defined assays (primers and probe)[1]	1 µL/assay	2.5 µL/assay	Use primer concentrations of 150–900 nM and a probe concentration of 100–250 nM.
RNA Sample	Variable	Variable	Use as much sample as needed, up to the maximum allowed by the reaction volume.
RT-qPCR Grade Water (Nuclease Free)	Variable	Variable	Fill to the total reaction volume.
Total Volume per Rxn	20 µL	50 µL	

Note: When working with the above reaction table, remember to include some dead volume that gets stuck on the tubes if premixing all of the primers and probes

Primers and Probes Used in This Study

3. Working on ice, add the components directly to each well of an optical reaction plate.
4. Cover the reaction plate with an optical adhesive cover and invert the plate 3–5 times, making sure that the contents of the wells are moving back and forth between the seal and the bottom of the wells to ensure proper mixing.
    a. IMPORTANT! The TaqPath™ 1-Step Multiplex Master Mix is a 4X formulation and is more viscous than most master mixes. Ensure that all of the components are thoroughly mixed in all the wells before proceeding. It has been observed that inverting the plate gives more uniform mixing across the reaction plate than vortexing.
5. Centrifuge the plate at 150 × g (1000 rpm) for 1 minute to collect the contents at the bottom of the wells and eliminate air bubbles.

Run the RT-real-time PCR plate

Run the plate on an Applied Biosystems™ real-time PCR instrument. See the appropriate instrument user guide for detailed instructions to program the thermal cycling conditions or to run the plate. We will be using a QuantStudio3

1. In the real-time PCR system software, open the plate document or experiment that corresponds to the reaction plate.

Step	Stage	Cycles	Temperature	Time for Fast	Time Standard
UNG Incubation	1	1	25C	2 min	2 min
Reverse transcription	2	1	53C	10 min	10 min
Polymerase activation	3	1	95C	2 min	2 min
Amplification	4	40	95C	3 sec	15 sec
			60C	30 sec	1 min

2. Verify the appropriate reaction volume is selected for your experiment.
3. Select the passive reference dye.
    a. None TaqPath™ 1-Step Multiplex Master Mix (No ROX™) – we are using the passive channel as one of the controls.

1. *Ye, Y., Ellenberg, R.M., Graham, K.E. and Wigginton, K.R., 2016. Survivability, partitioning, and recovery of enveloped viruses in untreated municipal wastewater. Environmental science & technology, 50(10), pp.5077-5085.*

Facilities.

A dedicated lab space in the Engineering Center, ECSL 1B11, may be made available to setup a temporary lab unit

Kjellerup's Lab Sars-Cov-2 Analysis in WRF Samples

Kjellerup Lab's Internal QA/QC Officer: Devrim Kaya, Ph.D.

A) Virus concentration (1hr-2 hr)

1) Centrifuge:

1. Prepare duplicate samples of each wastewater sample for procedure below
2. Add BRSV (our lab matrix spike): for WRF experiments: I added 34 and 10 uL of BRSV in triplicate samples at the sample prep step (to 30 mL of samples that is step 2 below), and RNA extraction step (to 0.25 mL that is used for RNA extraction step, Section B, step 1, below), respectively.
3. Take 30 mL of raw wastewater samples into a sterile 50 mL falcon tube (mark outside/upper part or the falcon tube where the pellet would have more force and accumulate).
4. Centrifuge at 3400 g with swinging-bucket centrifuges (use adapters for falcon tubes) for 15 min at room temperature to remove sediment and large particles
5. Carefully remove supernatant without disturbing the pellet (pellet should be visible).
6. Pour supernatant carefully from the side of the tube opposite to the pellet into a new 50 mL falcon tube.
7. Keep the falcon tubes with pellets at 4°C if can't process immediately, resuspend pellet with RNAse free water (tot. vol. 1 mL) and vortex the sample for 15 sec, this is enough to fully dissolve the pellet. Vortex also horizontally on the side that you expect to have the pellet. Be consisted with the choice of medium and write down final sample volume (pellet+medium).
8. Transfer resuspended pellet into 2 ml RNase-free tubes and store the samples up to 72 hrs at 4°C if can't process immediately, for long term store at -80°C/-20°C.
9. For concentration of supernatant (step 5) continue with Ultrafiltration Method (UF) methods described below

2) Ultrafiltration method (1 hr-2 hr based on number of samples): The supernatant coming from centrifuge part (aqueous phase containing virus particles) can be further concentrated by using ultrafiltration method through **Amicon-15 centrifugal filters** with a molecular cutoff of 100kDa (Merck Millipore) following manufacturer's protocol.

Protocol for Amicon® Ultra-15 Centrifugal Filter Devices

Amicon® Ultra 100K device — 100,000 MWCO (MilliporeSigma, cat no: UFC910024, 100,000 Da)

Sample Concentration

1. Add up to 15 mL of sample (total of 2 loads)
2. Place capped filter device into centrifuge rotor; counterbalance with a similar device.
3. **When using a swinging-bucket rotor**, spin the device at 3400 × g (maximum 4000 x g) for approximately 15–60 minutes (each load about 15 min).
4. Take concentrated sample into RNase free Tubes (2ml) and add RNAse free water into the bottom of the filter device and withdraw the sample using a side-to-side sweeping motion to ensure total recovery, total sample volume should be 1 mL.
5. Store samples at -20C/-80C for long-term storage, keep them at 4C if you will process (RNA/DNA extraction) them in 24-48C

Note: 1) To recover the concentrated solute, insert a pipettor into the bottom of the filter device and withdraw the sample using a side-to-side sweeping motion to ensure total recovery. The ultrafiltrate can be stored in the centrifuge tube. 2) For optimal recovery, remove concentrated sample immediately after centrifugation.

B) RNA extraction: Zymo Quick RNA mini prep (1.5-2 hr. based on number of the samples)

Clean and decontaminate all work surfaces, pipette, centrifuges and other equipment using RNAZap or 10% freshly prepared bleach prior to use.

- Use RNase-free Tips, Tubes, and H_2O.
- Clean and decontaminate all work surfaces, pipettes, centrifuges, and other equipment prior to use.
- Decontamination agents should be used such as 5% bleach, 70% ethanol, and DNAzap or RNase AWAY to minimize the risk of nucleic acid contamination.

Kit: Zymo Quick RNA mini prep (R1055 or 1054 : 200-50 extraction)

All centrifugation steps should be performed between 10,000-16,000 x g (did at 12,000 x g).
Follow manufacturer procedure: briefly:

1. Take 0.25 mL of concentrated sample in to clean RNAse free 2 mL Eppendorf tube
2. Add 600 uL RNA Lysis Buffer to the sample
3. Add 1 volume ethanol (95-100%) to the sample in **RNA Lysis Buffer** (1:1). Mix well.
4. Transfer the mixture to a **Zymo-Spin™ IIICG Column**[1] (green) in a **Collection Tube** and centrifuge for 30 seconds. Discard the flow-through.
5. Add 400 µl **RNA Prep Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
6. Add 700 µl **RNA Wash Buffer** to the column and centrifuge for 30 seconds. Discard the flow-through.
7. Add 400 µl **RNA Wash Buffer** and centrifuge the column for 2 minutes to ensure complete removal of the wash buffer. Transfer the column carefully into an RNase-free tube (not provided).
8. Add 100 µl **DNase/RNase-Free Water** directly to the column matrix and let it sit at RT for 1-2 min and centrifuge for 30 seconds.
9. The eluted RNA can be used immediately or stored at -70°C.

C) Measure RNA Concentration

1. Clean the Nanodrop with RNaseZap in advance.
2. Pipette 2 µL Nuclease-free H_2O to start the Nanodrop
3. Select the RNA-40 in the Sample type option, and pipette 2 µL RNase-free/DEPC-treated H_2O (the water resuspends the RNA pellet) as a blank control.
4. Pipette 2 µL RNA sample and start the measurement.

D) Reverse-transcription: First Strand cDNA Synthesis (Standard Protocol) (NEB #M0368) (1-1.5hr)

1) In the reagent set-up room clean hood, place RT-PCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.
2) Mix buffer, enzyme, and primer/probes by inversion 5 times.
3) Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
4) Label one 1.5 mL microcentrifuge tube for each primer/probe set.
5) Determine the number of reactions (N) to set up per assay. It is necessary to make excess reaction mix for the no template control (NTC) and standard reactions and for pipetting error.

Use the following guide to determine N:

- If number of samples (n) including controls equals 1 through 14, then N = n +2
- If number of samples (n) including controls is 15 or greater, then N = n + 10% of n

For each primer/probe set, calculate the amount of each reagent to be added

Prepare Reaction Mixture: M1

Reagent	Volume (x1), uL	Vol. of Reagent Added per Rxn
Random Hexamer (50 uM Invitrogen Cat. no: N8080127	1.5	N*1.5 µL =
dNTP mix (10mM) Invitrogen Cat. no: 18427013	1.25	N*1.25 µL =
H_2O (RNase-free)	2.25	N*2.25 µL =
total	**5**	**N* 5 uL=**

- After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.
- Centrifuge for 10 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
- Dispense 5 uL of M1 into each well and add 6 uL RNA (see table below)

Dispense M1	5 µL
Total RNA	6 µL (adjust if concentration is low)
total	**11** µL

- Mix well, denature mix (sample RNA/primer) 70C denature for 5mins and then cool down at 4C forever (till next step) or immediately on ice for 2 mins, centrifuge briefly.

Prepare Reaction Mixture: M2

Reagent	**Volume (x1)**, µL	**Volume (N Rxns)**, µL
5X ProtoScript II Buffer Comes with Protoscript II NEB cat no: M0368X	5	5 *N=
0.1M DTT Comes with Protoscript II NEB cat no: M0368X	2.5	2.5 *N=
SUPERase (RNAse inhibitor) (20U/ul) Invitrogen Cat no: AM2696	0.5	0.5 *N=
ProtoScript II RT (200U/ul) M-MLV NEB cat no: M0368X	1.25	1.25 *N=
H_2O (RNase-free)	4.75	4.75 *N=
total	**14**	**14 *N=**

- After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.
- Centrifuge for 10 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.
- Dispense M2 into each well containing M1+RNA, so now the total rxn volume is 25 uL.
- Incubate the samples at 42C* for 1hr.
- Inactivate the enzyme at 70°C for 20 minutes

E) Real-time PCR (RT-qPCR) (1-2 hrs)

Preparing the reaction mix as shown in table:

- Thaw the TaqMan® Fast Advanced Master Mix (Applied Biosystems cat no: 4444557) on ice, then mix thoroughly but gently.
- Thaw samples on ice, then vortex and briefly centrifuge to resuspend.
- Thaw TaqMan® Assays on ice, then vortex and briefly centrifuge to resuspend.
  - If number of samples (n) including controls equals 1 through 14, then N = n + 2
  - If number of samples (n) including controls is 15 or greater, then N = n + 10% of n

Prepare Reaction Mixture (M3) for each Assay: genes N1, N2, OC43 or BRSV (surrogate): check Tables 2-4 for details

Reagents	**Volume (x1), µL**	**Volume for N Rxns, µL**
2x TaqMan Master Mix	10	N*10=
Primer/probe *	X	
H_2O (RNase-free)	W=17-10-X:	N*W=
Total	**17**	**N*17 =**

*see Table 1 for determination of primer/probes and volumes for N1, N2, BRSV, or OC43. Check Table 2-4 for each target of interest (e.g., N1, N2, OC43 or BRSV)

- Vortex M3 briefly to mix
- Centrifuge briefly to bring the reaction mix to the bottom of the tube and eliminate air bubbles.
- Set up reaction strip tubes or plates in a 96-well cooler rack.
- Dispense 17 µL of each master mix into the appropriate wells (of an optical reaction plate)

Nucleic Acid Template Addition

1) Gently vortex nucleic acid sample tubes for approximately 5 seconds.
2) Centrifuge for 5 seconds to collect contents at the bottom of the tube.
3) After centrifugation, place extracted nucleic acid sample tubes in the cold rack.

4) Add cDNA template, 3 µL, or Nuclease-Free Water for NTC, to each well of the specific assay that is being tested. Carefully pipette 3 µL of the first sample into all the wells labeled for that sample. Keep other sample wells covered during addition to prevent cross contamination and to ensure sample tracking. Change tips after each addition

5) Change gloves often and when necessary to avoid contamination.

6) Repeat steps #4 and #5 for the remaining samples

Master mix: M3	17 µL
cDNA	3 µL for each well
Total	20 µL

- Seal the reaction plate with optical adhesive film, then centrifuge briefly to bring the PCR reaction mix to the bottom of the well and eliminate air bubbles.
- Apply a compression pad to the plate, if required by your real-time PCR system.

Settings:

- Set up a plate document or experiment file on the thermocycler using the following conditions for BRSV and see Table 4 for N1 and N2 and Table 5 for OC43:
  - Denature: 95°C 2min;
  - Amplify: (95°C for 10 seconds; 60°C for 30 seconds) x 45 Cycles
  - Fluorescence data (FAM, HEX, etc.) should be collected during the 60°C incubation step.
- Select the appropriate block, if this option applies to your instrument.
- Select the appropriate experiment type, if this option applies to your instrument.
- Select TaqMan® or probe mode to detect the target sequence
- Enter the sample volume, if this option applies to your instrument.
  - 96- and 48-well plates (both Standard and Fast): 20.0 µL

Table 1: Primer/probe info for targets such as N1, N2, BRSV (Kjellerup lab surrogate), and OC43

Genes	Description	Oligonucleotide Sequence (5'>3')	product length, bp	Stock conc., uM	Final Conc., nM /20 uL rxn	Vol, uL/ 20 uL rxn
N1	FWD	GAC CCC AAA ATC AGC GAA AT	72 bp	6.7	500	1.5
	REV	TCT GGT TAC TGC CAG TTG AAT CTG		6.7	500	
	Probe	[FAM]-ACC CCG CAT TAC GTT TGG TGG ACC-[BHQ1] or [ZEN/IOWA Black]		1.7	125	
N2	FWD	TTA CAA ACA TTG GCC GCA AA	67 bp	6.7	500	1.5
	REV	GCG CGA CAT TCC GAA GAA		6.7	500	
	Probe	[FAM]-ACA ATT TGC CCC CAG CGC TTC AG-[BHQ1]		1.7	125	
BRSV RT-qPCR Assay1	BRSV RT A#1_FWD	GCAATGCTGCAGGACTAGGTATAAT	124	10	500	1
	BRSV RT A#1_Rev	ACACTGTAATTGATGACCCCATTCT		10	500	1
	BRSV Probe1	[HEX]-ACCAAGACT-[ZEN]-TGTATGATGCTGCCAAAGCA-[3IABkFQ]		10	250	0.5
OC43 RT-qPCR ref; Dare, R.K. et al. J Infect. Diseases, 2007, 196: 1321-8	FWD	CGATGAGGCTATTCCGACTAGGT	62	10	500	1
	Rev	CCTTCCTGAGCCTTCAATATAGTAACC		10	750	1.5
	Probe	6FAM- TCCGCCTGGCACGGTACTCCCT -BHQ		10	50	0.1

Table 2: Preparation of serial dilutions and RT-qPCR reaction for cov-2 N1 and N2

To make dilutions for the RT-qPCR standard curve, use the following table: postive controls are used as standard

Dilution #	Source Tube for Dilution	Source Conc (gc/uL)	Vol. of the Source (uL)	Diluent Vol. (uL) (PCR water)	Final Vol of Dilution (uL)	Dilution Conc. (gc/uL)	Dilution Conc. (gc/3 uL)
stock: N linearized plasmid	stock: plasmid	2.00E+05	-	-	-	2.00E+05	6.00E+05
1	linearized stock	2.00E+05	10	40	50	4.00E+04	1.20E+05
2	1	4.00E+04	10	90	100	4.00E+03	1.20E+04
3	2	4.00E+03	10	90	100	4.00E+02	1.20E+03
4	3	4.00E+02	10	90	100	4.00E+01	1.20E+02
5	4	4.00E+01	10	90	100	4.00E+00	1.20E+01

qPCR

2 min at 95°C, and then 45 cycles including denaturation at 95°C for 3 s and annealing and elongation at 55°C for 30 s, followed by fluorescence measurement.

Master Mix Preparation		N1 or N2
Reagents	**Final conc. /20 µl rxn**	**Vol/rxn (µl)**
Master Mix (2X)	1X	**10**
Fwd Primer, 6.7 uM	500	1.5
Rev Primer, 6.7 uM	500	
Probe, 1.7 uM	125	
Water		5.5
DNA		**3**
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

Standard concentrations that are used to make calibration curve are shown in red.

Table 3: Preparation of serial dilutions and RT-qPCR reaction for BRSV

To make dilutions for the RT-qPCR standard curve, use the following table: gblock is used as standard

Dilution #	Source Tube for Dilution	Source Conc (gc/uL)	Vol. of the Source (uL)	Diluent Vol. (uL) (PCR water)	Final Vol of Dilution (uL)	Dilution Conc. (gc/uL)	Dilution Conc. (gc/3 uL)
stock: BRSV :1 ng/uL		7.36E+09	-	-	-	7.36E+09	2.21E+10
1	stock: 1ng/uL	7.36E+09	20	80	100	1.47E+09	4.42E+09
2	1	1.47E+09	10	90	100	1.47E+08	4.42E+08
3	2	1.47E+08	10	90	100	1.47E+07	4.42E+07
4	3	1.47E+07	10	90	100	1.47E+06	4.42E+06
5	4	1.47E+06	10	90	100	1.47E+05	4.42E+05
6	5	1.47E+05	10	90	100	1.47E+04	4.42E+04
7	6	1.47E+04	10	90	100	1.47E+03	4.42E+03
8	7	1.47E+03	10	90	100	1.47E+02	4.42E+02
9	8	1.47E+02	10	90	100	1.47E+01	4.42E+01

qPCR

Master Mix Preparation		BRSV
Reagents	**Final conc. /20 µl rxn**	**Vol/rxn (µl)**
Master Mix (2X)	1X	**10**
Water		4.5
Fwd Primer, 10 uM	500	1
Rev Primer, 10 uM	500	1
Probe1: hex/zen, 10 uM	250	0.5
DNA		**3**
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

Standard concentrations that are used to make calibration curve are shown in red.

Table 4: Preparation of serial dilutions and RT-qPCR reaction for OC43

To make dilutions for the RT-qPCR standard curve, use the following table: gblock is used as standard

Dilution #	Source Tube for Dilution	Source Conc (gc/uL)	Vol. of the Source (uL)	Diluent Vol. (uL) (PCR water)	Final Vol of Dilution (uL)	Dilution Conc. (gc/uL)	Dilution Conc. (gc/3 uL)
stock: OC43 :1 ng/uL	stock	1.47E+10	-	-	-	1.47E+10	4.42E+10
1	stock	1.47E+10	20	80	100	2.94E+09	8.83E+09
2	1	2.94E+09	10	90	100	2.94E+08	8.83E+08
3	2	2.94E+08	10	90	100	2.94E+07	8.83E+07
4	3	2.94E+07	10	90	100	2.94E+06	8.83E+06
5	4	2.94E+06	10	90	100	2.94E+05	8.83E+05
6	5	2.94E+05	10	90	100	2.94E+04	8.83E+04
7	6	2.94E+04	10	90	100	2.94E+03	8.83E+03
8	7	2.94E+03	10	90	100	2.94E+02	8.83E+02
9	8	2.94E+02	10	90	100	2.94E+01	8.83E+01

qPCR: 95 °C for 2 min, followed by 40 cycles of 95 °C for 10 sec, and 60 °C for 60 sec (capture fluorescence).

Master Mix Preparation		OC43
Reagents	**Final conc. /20 µl rxn**	**Vol/rxn (µl)**
Master Mix (2X)	1X	**10**
10 uM Fwd Primer	500	1
10 uM Rev Primer	750	1.5
10 uM Probe	50	0.1
Water		4.4
DNA		**3**
rxn mix volume=		20
add 17 uL of Master mix+3 uL of template in well		

Standard concentrations that are used to make calibration curve are shown in red.

SARS-CoV-2 Control Plasmid Digest with ScaI-HF (from NEB)

Reaction Conditions

Component	uL per RXN
DNA	10 uL (< 25% of RXN volume)
10X CutSmart Buffer	5 uL (1X)
ScaI-HF	1 uL of 20 Units
PCR-Grade H_2O	34 uL
Total	50 uL

Protocol:
- 2019-nCoV_N_Positive Control (#10006625)
- ScaI-HF from New England Bio #R3122S
- Prepare enzyme reaction as detailed above.
- Incubate reaction at 37°C for 30 min (and no more than one hour)
- Inactivate enzyme at 80°C for 20 min.
- Plasmid concentration (200,000 copies/uL) diluted to 40,000 copies/uL in final solution.

Notes:
- Our SARS-CoV-2 Control Plasmid (4,012 bp) comes at a concentration of 200,000 copies/uL
- To adjust the digest protocol, we will use 5 units of enzyme at one hour (no more than one hour, as this is a HF enzyme and could result in erroneous cutting)
- Dilute enzyme in 1X CutSmart Buffer to 5 units/uL
- Enzyme should not exceed 10% of the reaction volume (5 uL)
- The DNA sample should be no more than 25% of the reaction volume, according to NEB.

Method is described in the following study:

R. Gonzalez, K. Curtis, A. Bivins, K. Bibby, M. H. Weir, K. Yetka, H. Thompson, D. Keeling, J. Mitchell and D. Gonzalez, COVID-19 surveillance in Southeastern Virginia using wastewater-based epidemiology, *Water Research*, 2020, **186**.

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SARS-CoV-2 Wastewater Testing SOP

1. Safe Handling of Sample

1.1 Any samples with the potential to contain any trace of SARS-CoV or SARS-CoV-2 (2019-nCoV) should be performed in BSL-2 facilities with standard BSL-2 work practices according to the CDC Biosafety in Microbiological and Biomedical Laboratories (BMBL), 5th Edition (https://www.cdc.gov/labs/BMBL.html).

1.1.1 If possible all procedure in which infectious aerosols or splashes may be created should be performed in a Class II BSC or other physical containment equipment.

1.1.1.1 Use safety cups whenever possible to avoid exposure to aerosols.

1.1.2 Personal Protective Equipment (PPE): lab coat, gloves, surgical mask, eye protection & face shield.

1.1.3 Any procedure or process that cannot be conducted in a BSC should be performed while wearing PPE with respiratory protection (ie. N95 or higher) and behind plexiglass screen if possible.

1.1.4 Work surfaces should be decontaminated at every step of work with appropriate disinfectants.

1.1.5 All disposable waste should be autoclaved.

2. Sample Concentration and RNA Extraction

2.1 Centrifuge for 10min at 1500rpm.

2.2 Filter the 500mL wastewater supernatant through a 0.45µm pore size, 47mm diameter negatively charge HA membrane (HAWP04700; Millipore) via the filtration apparatus.

2.3 Re-suspend in 1mL wastewater supernatant.

2.4 Extract the RNA by using the Qiagen QIAamp® Viral RNA MiniKit (Cat# 52906) as per manufacturer's instruction.

3. RT-qPCR analysis

3.1 Follow the protocol"CDC 2019-Novel Coronavirus Real-Time RT-PCR Diagnostic Panel (CDC-006-00019, Rev 5; Effective 7/13/2020) for the detection of SARS-CoV-2 RNA.

3.2 Use 2019-nCoV_N1 primer/probe mix, 2019-nCoV_N2 primer/probe mix, and Quantabio qScript XLT One-Step RT-qPCR tough Mix as described in the CDC protocol.

3.3 All RT-qPCR reactions were performed in triplicate.

3.4 For each qPCR run, a series of three positive and negative controls were included.

4. RT-qPCR inhibition and quality control

4.1 A known concentration of *Oncorhynchus keta (O. keta)* was processed to obtain the Ct value as reference point (as according to EPA Method 1611.1 protocol; April 2015).

4.2 If the Ct value of the sample increases compared to the reference point then the sample is considered to have PCR inhibitors.

4.3 All RNA samples were stored at -80C and subjected to RT-qPCR analysis within the same day after RNA extraction.

4.4 A reagent blank and an extraction blank were tested to ensure no carryover contamination occurred during RNA extraction.

Water sample Filtration Instruction for COVID19 Testing

Purpose:

1. To outline the standard protocol for filtering 2019_nCoV virus from water samples for downstream analysis.
2. To incorporate matrix-spike-in samples for calculating virus recovery.

Scope:

This protocol only applies to quantitative COVID-19 testing of water samples. This protocol does not include BSL2 work guidance.

Responsibility:

Laboratory personnel who are authorized to work in BSL2 lab.

Material Required:

Equipment

Note: a set of equipment designated for the BSL2 lab should not be taken out without thorough decontamination:

- Biosafety cabinet (BSL2 hood)
- Funnels with HA membrane options:
  - Self-prepared funnels: replace PC membrane with HA membrane. Sterile, Mixed cellulose ester (MCE/HA), 0.45μm pore size, 47mm diameter membrane filters (Sigma-Aldrich cat # N9145-100EA) OR
  - Preloaded funnels: 100ml or 300ml MicroFunnel unit with 0.45 μm GN-6 Metricel membrane, white, gridded (PALL, cat # 4801 or 4815)
- Vacuum source with hose
- Vacuum filter manifold to hold Microfunnels (Pall Corp)
- 250ml clear blue-cap PC bottles with graduation
- Sterile forceps
- Flame source
- Bead tubes: 2ml PowerBead tube with 0.1mm glass beads supplied with Qiagen kits or purchased separately (Cat# 13118-50)
- Sterile 50ml centrifuge tubes
- PCR-grade 5ml screw cap or snap cap tubes
- Pipettes and pipette tips

Reagents

- Freshly prepared Lysis Buffer (see Section 1 below)
- 1:10 dilution of heat-inactivated SARS-CoV-2 viral cultural fluid (ZeptoMetrix, cat # 0810587CFHI-0.5ML). No longer than 2 days storage at 4C fridge once taken out from the -80C freezer.
- Sterile 1x PBS
- 2N HCl

- 2N NaOH (freshly made by dissolving the pellet in PCR-grade H2O before use)
- pH Test strips (0.0~6.0)
- 10% bleach solution
- 70% ethanol
- PCR-grade H2O
- 100% absolute ethanol for flame sterilization
- PM1 Solution (Qiagen cat #26000-50-1)
- Beta-mercaptoethanol (Molecular grade, Sigma-Aldrich, cat # 63689-25ML-F)

Quality Control:

- Make sure to clean the BSL2 hood working surface each time before starting a filtration batch.
- Always wear clean gloves to take out unused funnels.
- Make sure the lid of the filtration funnel is always closed unless pouring in a water sample. Only open one lid at a time.
- NPEC shall be prepared with PCR-grade water.
- Wipe manifold holder with 10% bleach after filtration and before used for a different sample (the matrix spike for the same sample is not considered as a different sample).
- Always ensure enough 100% ethanol for complete forceps flaming.
- Decontaminate pipettes and markers in the BSL2 hood regularly with 10% bleach and 70% ethanol.

Procedure:

<u>Section 1: Filtration Preparation (outside BLS2 lab)</u>

1. Turn on the water bath to warm up to 55C. Incubate buffer PM1 bottle at 55C for 10min to dissolve any precipitation.
2. Label the PowerBeads bead tubes (Qiagen) for samples and NPEC. Label a snap-cap 1.5ml tube for positive control “VS POS” sample.
3. Outside the BSL2 lab, prepare the lysis buffer. Fresh lysis buffer needs to be prepared before each filtration batch. Calculate the total number of samples (n), and prepare PM1 with beta-mercaptoethanol (2-Me) lysis buffer:
    a. Lysis buffer volume per filter sample: 700ul.
    b. Lysis buffer volume for VS POS sample: 600ul.
    c. Total volume of PM1: (n+1) * 700ul. (warm and clear, in a 5ml tube or 50ml tube depends on total volume)
    d. Add (n+1) *7 ul of 2-Me to PM1.
    e. Mix by inverting the tube several times.

<u>Section 2: Filtration methods selection and pH adjustment</u> (inside BSL2 hood wearing PPE).

1. Depending on the turbidity of the water sample (may need to open the lid to look into the bottle), one needs to use his or her best judgment on how much water sample to filter and which method to choose from. One will gain enough experience with time, but here is a general reference table to follow.

Table 1. Processing method: CF vs DF

	High turbidity	Medium turbidity	Low turbidity
Heavy granular solid	CF, 20~50ml	CF, 50ml	DF, 50ml
Fecal particles	CF, 20~50ml	CF, 100ml	DF, 100ml
Low level of biomass	DF, 100ml	DF, 100~200ml	DF, 200~400ml

CF: Centrifuge 5min at 5,000rpm and then filter.
DF: Direct filtration

2. Pour out water samples to adjust pH:
    a. For samples to be processed with CF, invert the water bottle several times and pour into 2 or 4 pre-labeled 50ml conical tubes (see Section 3 below)
    b. For samples to be processed with DF of 50ml or 100ml, invert the water bottle several times and pour 200ml into a 250ml bottle.
    c. For samples to be filtered for more than 100ml, adjust the pH in the original 500ml water bottle. No need to pour out.
3. Samples must be titrated to pH3.5 by 2N HCl. ***Make sure the pH test is fairly accurate and not go down to 3.0.*** Record the volume of water and HCl used for future reference.
4. After pH adjustment, label the bottle or tube with “pH3.5”. Incubate for a minimum **10min** (write down the time on the filtration bench sheet will help tracking the time) and shake vigorously before pouring that water sample into a funnel.
5. After pouring each sample into the funnel, make sure to close the lid to prevent cross contamination.
6. Filter the sample at ~60 psi for less than 45min.
7. **DO NOT** use PCR-water or PBS to rinse the bottle because of the pH difference.
8. After filtration, fold the membrane and store it in a bead tube containing 700ul Lysis Buffer. Invert the tube several times to ensure complete membrane coating.

Section 3: Centrifugation and Filtration (aerosol tight centrifuge and BSL2 hood)

For turbid samples, centrifugation can significantly reduce filtration time.

1. Pour 50ml for turbid samples into a conical tube.
    a. For CF 50ml, pour one tube for an un-spiked sample, another tube for matrix-spike sample.
    b. For CF 100ml, pour two tubes for an un-spiked sample, another two tubes for matrix-spike sample.
2. Centrifuge the water sample at >=5,000prm X 5min.
3. Slowly pour out the supernatant to a clean 50ml conical tube or 250ml bottle. Leave about 3ml~4ml liquid with the pellet at the bottom of the tube.
    a. For CF 50ml samples, pour out the supernatant into another 50ml tube labeled as “S” for supernatant.
    b. For CF 100ml samples (combining two 50ml tubes), pour out all the supernatant (~200ml from 4 tubes) to a 250ml bottle.

4. Swirl the pellet at the bottom of the 50ml conical tube in residual liquid, and pour them into a 5ml tube. For CF 100ml samples, combine pellets from two tubes into one 5ml tube.
5. Centrifuge the 5ml tube at maximum speed 7,830rpm x 10min to completely precipitate the pellet.
6. Use a P1000 pipette to take out liquid from the 5ml tube and add it back to the sample tube or bottle.
7. Adjust the pH of the supernatant, label the bottle or tube with “pH3.5”. Incubate for 10min, and filter through an HA membrane. *Note that addition of the matrix spike is done after pH adjustment to the supernatant, prior to incubation, so the pellet would not contain a spike*.
8. While waiting for filtration to finish, add 700ul Lysis buffer to the pellet. Cut the tip of a P1000 tip with clean scissors. Resuspend the pellet with the cut-tip by pelleting up and down several times and transfer the suspension to a pre-labeled bead tube. ***Be careful that if the tip is stuck, it could create vacuum force and splash the sample during the dispensing step.***
9. After all the supernatant has been filtered through the HA membrane, fold the membrane and put it in the same bead tube containing resuspended pellets. Invert the tube several times to ensure complete membrane coating.

Section 4: Matrix-spike-in samples (inside BSL2 hood wearing PPE)

1. After sample pH adjustment, take out one stock of 1:10 dilution of SARS-CoV-2 heat inactivated culture fluid aliquot from -80C freezer. Make a 1:1000 dilution with 1xPBS by adding 2ul of 1:10 viral fluid to 198ul PBS.
2. Add 2ul into the same filtration volume as the non-spiked sample.
    a. For CF 50ml, you can add the 2ul 1:1000 viral dilution to the supernatant tube “S”, and label the tube clearly with “+VS”.
    b. For CF 100ml, after pouring out 100ml water for un-spiked sample filtration, add 2ul 1:1000 viral dilution to the remaining 100ml water in the 250ml bottle. And label the bottle clearly with “+VS”.
    c. For CF >100ml, after pouring out water for un-spiked sample filtration, pour out the same amount of water into a 250ml blue-cap bottle. Add 2ul 1:1000 viral dilution to the bottle, and label the bottle clearly with “+VS”.
3. Mix well by shaking the bottle vigorously. Note that addition of the matrix spike is done after pH adjust, prior to incubation. Incubate for a minimum **10min** before filtration (writing down the time on the filtration bench sheet will help tracking the time). Note that for CF 50ml, the un-spiked sample and matrix-spike sample will have the same incubation time frame.
4. After pouring each sample into the funnel, make sure to close the lid to prevent cross contamination.
5. Filter the sample in the same way as the un-spiked sample.

Section 5: Sample Storage

1. During filtration, store the resuspended pellet in the 4C fridge in the BSL2 hood while waiting for filtration to finish.
2. After filtration, and inversion of the tube several times, store the sample tube at -20°C for less than a week.

Section 6: Cleaning and Decontamination after Filtration

1. Spray the manifold with 10% bleach with the vacuum on.
2. Pour 1:10 of bleach to the wastewater bottle and let it sit for a minimum 20min before dumping it into the sink.
3. Pour 1:10 of bleach to the remaining of the client water sample (if not to be saved) and let it sit for a minimum 20min before dumping it into the sink.
3. Clean the BSL2 hood bench with 10% bleach and 70% ethanol.
4. Empty waste tip beaker into biohazard container under the BSL2 hood.
5. Take out the non-biohazard trash if necessary and replace the trash bag.

University of Wisconsin – Madison
Adelaide Roguet, Sandra McLellan and Shuchen Feng

Filtration method

- Add 25 mL in 50 mL tube.
- Add 5 uL of BCoV (150,000 cp/ul) + add MgCl2 to a final concentration of 25 mM
- Invert for 30 min at 4C
- Filter each sample through a 0.8 um pore size HAWP MF-Millipore Membrane Filter
- Transfer the filter in Garnet PowerBead 2-mL tube (containing the solution PM1-β-ME included in the AllPrep® PowerViral® DNA/RNA Kit)
- Put the samples on ice for 5 min (to avoid the sample to get too hot during the bead beating)
- Bead beat for 2:30 min. Put the samples on ice for 5 min and repeat the bead beating step. Put the samples 5 min on ice.
- Centrifuge at full speed for 1 min
- Transfer the supernatant in a clean 2-mL tube
- Extract nucleic acids using AllPrep® PowerViral® DNA/RNA Kit according to manufacturer's instructions.
- Elution in 60 uL

One-Step

RT-ddPCR using the One-Step RT-ddPCR Advanced Kit for Probes (BioRad 1864021) according to manufacturer's instructions**.** We use 5.5 uL of RNA per 20 uL RT-PCR reaction.

Assays

- SARS-CoV-2 assay: 2019-nCoV CDC ddPCR Triplex Probe Assay (BioRad: 12008202). Use CDC's N1 & N2 sequences.
- BCoV probe: CCTTCATATCTATACACATCAAGTTGTT (ordered from IDT)
- HepG probe: CCTTCATATCTATACACATCAAGTTGTT (ordered from IDT)

PS: BCoV (spiked before the concentration step or before freezing the samples) is quantified to estimate the recovery rate of the whole procedure. HepG (spiked before the RT step) is quantified to estimate the PCR inhibition during the RT-PCR step.

Concentration, Extraction and Amplification Protocols

Version 3.0
Date: 11-10-2020
Prepared by Tyler Radniecki
School of Chemical, Biological and Environmental Engineering
Oregon State University, Corvallis, OR 97331

Concentration of Virus from Wastewater

PPE Requirements

All personnel involved with sewage sampling should adhere to their current PPE requirements enforced by the wastewater utility. Additionally, a face shield, mask and gloves should be worn at all times (even if not required by the local wastewater utility) when collecting wastewater samples.

Thawing (optional) – up to 24 sample bottles at a time

1. Remove bottles from the freezer (if used to preserve the sample) and place in 2 buckets. If they are the 500 mL bottles there should be about 12 bottles in each bucket. Place a bucket full of sample bottles in each sink and turn on the water to a temperature of about 30 °C. Let the water run into the bucket and out of the hole in the side of the bucket continuously. Alternately, if insufficient hot water is available, replace the hot water in the bucket every 10 minutes. Inspect bottles frequently and gently shake. When the ice has melted in a bottle, immediately store it on ice or in a 4 °C refrigerator.

Concentration – three samples at a time

Steps 2 through 12 are typically done with multiple samples at a time depending on manifold size.

2. Add the sample IDs to the Excel sample data sheet. Note which samples are QA/QC samples.

3. If the sample bottle volume is greater than 100 mL, retain 100 mL for analysis, and fill 2 replicate bottles with 200 mL aliquots (or as much as possible) using a graduate cylinder. Gently mix the original sample bottle prior to being poured into a graduated cylinder. Store the labeled sample replicates at -20 °C with the word “SPARE” on the bottle and cap. Use a different graduated cylinder for each sample.

4. Label each stir plate, filter and 2 mL beaded microcentrifuge tube with the sample ID.

5. Add 1 mL of RNA/DNA Shield (Zymo, Cat #R1100-250) to the beaded microcentrifuge tube.

6. Insert HA filters (47 mm diameter, hydrophilic, mixed cellulose ester 0.45 um pore size, Whatman Cat #10401612) into vacuum filters seated on a vacuum manifold with the vacuum turned on.

7. Slowly pour aliquots of 30 - 60 mL from each sample onto the middle of their respective filter. As much volume as possible, up to 60 mL, is preferred, but clogging of the filter usually determines the volume that can be filtered in a reasonable amount of time.

8. After the liquid volume has been filtered, shut the vacuum off. Remove each HA filter with a tweezer, fold in half with the solids on the inside, roll the filter up and place into a 2 mL beaded microcentrifuge tube containing 1 mL of DNA/RNA Shield reagent (to inactivate the virus and prevent RNA degradation).

Nucleic Acid Extraction and Quantification

PPE Requirements

All personnel involved with nucleic acid extraction and quantification should adhere to BSL2+ guidelines. This includes wearing a lab coat, long pants, closed toe shoes, nitrile gloves, facemask and faceshield. All work is to be hperformed in BSL2 certified lab spaces and as much of the work as is possible is to be carried out inside a biosafety cabinet.

Nucleic Acid Extraction

Thaw frozen DNA/RNA Shield-stabilized samples at 4 ºC. Once thawed homogenized samples with 0.7 mm garnet beads using a Qiagen TissueLyser (Qiagen Inc, Germantown, MD) for 2 min at 30/sec. Pellet beads and debris by centrifugation at 12,000 rcf for 1 min. Transfer the lysate from each tube into a 96-well plate. Extract RNA from 200-400 µL of the lysate using the KingFisher MagMAX Viral/Pathogen kit (ThermoFisher Scientific, Waltham, MA). Elute purified RNA into 50 µL volumes. Quantify extraction recovery with a commercial standard (Exact Diagnostics, Fort Worth, TX). Include extraction blanks with every run. Keep extracted RNA at -80 ºC until quantification can be done.

Nucleic Acid Quantification via Reverse Transcriptase Droplet Digital PCR (RT-ddPCR)

Thaw frozen RNA at 4 ºC and keep on ice. Quantify the two viral RNA targets (N1/N2) and an internal control (RNAse P) using a commercial triplex assay (2019-nCoV CDC ddPCR Triplex Probe Assay, Bio-Rad catalog no. 12008202) and the One-Step RT-ddPCR Advanced Kit for Probes. Run the quantification assay on a QX-200 ddPCR system (Bio-Rad, Hercules, CA). Generate droplets using an automated droplet generator. Include commercially prepared RNA standards and negative controls on each plate (Exact Diagnostics, Fort Worth, TX). Run all samples and controls in duplicate. Follow the Bio-Rad assay protocol exactly.

Confirm the reliability of RT-ddPCR results by running a 6-point standard curve in duplicate using the conditions, primers and probes described above. The concentration of the standard curve should range from 10^0 to 10^6 RNA copies/uL. To account to reverse transcriptase efficiency, the standard curve should be made from small RNA transcripts (704 -1363 nt) of the SARS-CoV-2 nucleocapsid (N) genes. These transcripts were kindly provided by the Grubaugh lab at Yale University (grubaughlab.com/).

Additionally, include a no-template control on each plate. The no-template control contains RNA-free water instead of extracted RNA samples.

Data Analysis

Call droplet clusters manually for each target using the QuantaSoft Analysis Pro software (Bio-Rad, Hercules, CA). Conduct all other analyses in R (version 4.0.2) with Rstudio Desktop (version 1.3.1056). Graphics can be created with ggplot2 (Wickham 2016) and tmaps (Tennekes 2018) packages.

Quality Control

Do not accept wells with less than 6,000 droplets. Accept sample data only if the corresponding extraction blank and field blanks, as well as the PCR negative and no-template controls, are all negative for the N1/N2 targets. Reactions are regarded as positive if three or more droplets per well amplified in either target.

Data Management Procedures

Store the data generated from these sampling on Clean Water Services computers and Oregon State University's server. The data on the OSU server is backed up nightly. Digitize the result from the laboratory analysis of collected samples and metadata generated/gathered for each sample into spreadsheets and store on Clean Water Services computers and OSU servers. Include the quality control data for each sample. For cases when any problem is encountered in the analysis, report the problem along with the corrective actions taken and changes to the standard operation procedures. Perform a quality assurance review on the data to ensure that all sample values were entered without error. This review consists of multiple manual checks to make sure that all laboratory data has been entered into the spreadsheet correctly.

The Ohio State University

Virus filtration and concentration from wastewater

Received wastewater samples were processed for viral filtration and concentration using the EPA Method 1615 (USEPA, 2014) with some modifications. 200 mL of each sample was passed through positively charged ViroCap filters (Scientific Methods, Inc., Granger, IN, USA) at a rate of 0.5 L/min using a peristaltic pump. Once filtration was completed, remaining water in the ViroCap was discarded. For elution, 150 mL 1.5% beef extract (BE) (Cat. No. 211520, Becton, Dickinson and Company, USA) containing 0.05 M glycine (pH 9) (Cat. No. G8898, Sigma-Aldrich, USA) was added to the ViroCap filters, which were soaked for 30 min and then the eluent was circulated with the peristaltic pump in the ViroCap filter housing for an additional 5 min at room temperature. With the collected eluents, secondary concentration was performed with organic flocculation. For the flocculation, the pH of the eluent was adjusted to 3.5±0.1 by adding small increments of 1.2 M hydrochloric acid (HCl) while slowly mixing at room temperature followed by a 30-min stirring period. After 15 min of centrifugation at 2,500 g at 4°C, the flocculated virus in the pellet was resuspended in 30 mL of 0.15 M sodium phosphate (pH 9) (Cat. No. 255793, Sigma-Aldrich, USA). The precipitate was shaken at room temperature at 160 rpm for 10 min on an orbital shaker to ensure complete dissolution. The virus-containing supernatant was collected after centrifuging at 2,500 g for 10 min at 4°C. After adjusting the pH to 7.0-7.5 with 1.2 M HCl, the supernatant was filtered through a 0.22 µm sterile filter (Cat. No. SLGPM33RS, Millipore, USA). The filtered supernatant was transferred into the Vivaspin 20 unit (30,000 MWCO, Sartorius Stedim, Cat. No. VS2022, Germany) for tertiary concentration. The filtrate was centrifuged at 2,500 g at 4°C until the final sample volume is less than 400 µL. The sample was then washed with 1 mL of sterile 0.15 M sodium phosphate (pH 7-7.5), followed by centrifuging at 4,000 g at 4°C to give a final volume of 200 µL. The concentrated virus filtrate was stored under -80℃ until further analysis.

RNA extraction and reverse transcription-qPCR

RNA/DNA was directly extracted from the viral concentrate using AllPrep PowerViral DNA/RNA Kit (Cat. No. 28000-50; QIAGEN, Germantown, MD, USA) following manufacturer's instruction. Briefly, 5µL of the DNA/RNA eluent extracted from the above step was applied to RT-qPCR following the manufacture instruction of the Prototype SARS-CoV-2 RT-qPCR detection kit for wastewater (Promega). For N1 and N2 gene quantification, RT-qPCR standard curves were generated by adding 5µL of the serial diluted (1:10) quantitative control dsDNA (2×10^3- 2×10^0 copies/µL) into the wells that contained the mastermix. Three technical RT-qPCR replicates were performed for the standard curves. The PCR cycling condition for the RT-qPCR included an initial reverse transcription at 45 °C for 15 min, reverse transcriptase inactivation/ GoTaq® activation at 95 °C for 2 min, followed by 40 cycles of denaturation at 95 °C for 3 s and annealing/extension at 62 °C for 30 s. Data were collected from FAM (N1, N2

gene), HEX (Internal amplification Control), Cy5 (PMMoV) fluorescence channels at end of each 62°C Annealing/Extension step.

Reference

Fout, Shay, N. Brinkman, J. Cashdollar, S. Griffin, B. McMinn, E. Rhodes, E. Varughese, M. Karim, A. Grimm, S. Spencer, and M. Borchardt. Method 1615 Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. U.S. Environmental Protection Agency, Washington, DC, 2014.

Quality Assurance Plan for SARS-CoV-2 Wastewater Samples

In the

Biosafety Level 2 Laboratory

Of

Dr. Jennifer Weidhaas

Associate Professor

Civil and Environmental Engineering, Floyd and Jeri Meldrum Civil Engineering Building

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1 Purpose

This SOP applies to all research staff, hosted visitors and guests, volunteers, building staff, and service staff who enter the laboratory (MCE 2424). This manual will be reviewed annually by the Principal Investigator, Laboratory Supervisor and graduate students for changes or corrections to ensure that it is accurate. Review of this Manual is mandatory for all employees and staff working in the BSL-2 laboratory of Dr. Jennifer Weidhaas (MCE 2424) on SARS-CoV-2 containing wastewater samples. This Manual must be updated at least annually, and whenever there are changes in laboratory procedures that may change a worker's exposure. A copy of this Manual must remain in the lab, and must be accessible to lab personnel.

2 Laboratory personnel, organization and responsibility

The individuals involved in this project are listed in the table below as well as their responsibilities.

Personnel	Role	Responsibility
Dr. Jennifer Weidhaas	PI, QA manager	Routine assessment of QC data and internal audits, RT- qPCR
Rubayat Jamal	Graduate research assistant	Sample receipt, inactivation and RNA extraction
Danielle Zebelean	Graduate research assistant	Sample receipt and filtering, RNA extraction
Katrina Brown	Graduate research assistant	Sample receipt and filtering
Rachel Lee	Research assistant	Sample receipt and filtering, RNA extraction
Michael Jarzin	Under graduate research assistant	Sample receipt and filtering

Dr. Weidhaas is responsible for all training of graduate and undergraduate research assistants and documents the annual performance evaluations and trainings.

3 Facilities

The lab MCE 2424 is a BSL-2 certified laboratory equipped with the biological safety cabinet for PCR prep, isolated areas for preparation of molecular samples (DNA and RNA extraction) which is separate from nucleic acid amplification (PCR and qPCR) areas. The labs are located adjacent to the PI offices as well as the separate graduate student offices. The labs are equipped with safety stations (eye wash, safety showers, fire blankets, fire aid kits), deionized water unit, biosafety level 2 hoods and low temperature freezers, biotek plate readers equipped with fluorescence filters, qPCR thermocyclers, PCR

thermocyclers, spectrophotometers, ultra-refrigerators (-20, -80 deg C), pH probes, Qubit fluorometer and vortexers.

4 Field sampling procedures

SARS-Cov-2 wastewater samples are received by Rubayat Jamal or Dr. Weidhaas at MCE 2424 or by shipment to the University receiving warehouse. Chain of custody forms are kept in a centralized location and the sample handling tubes and filters are labeled per the COC names. All samples are transported on wet ice the University of Utah within 2 to 8 hours of sample collection.

5 Laboratory test handling procedures and record keeping

Wastewater handling

1. All wastewater samples should be collected by personnel at the wastewater facility rather than University of Utah personnel.
2. Don safety gloves. Collect the wastewater samples from the personnel at the wastewater treatment facility water quality testing laboratory. Bleach the exterior of the bottle with 1:10 freshly prepared bleach solution. Do not open the bottle until back in a BSC at the University of Utah. Transport the bottle in a secondary container labeled with the biosafety hazard label and a list of potential biosafety hazards (i.e., Biosafety hazards: Wastewater and feces potentially containing coronavirus). Dispose of gloves as biohazardous waste.
3. Upon receipt in the lab, inactivate the coronavirus by placing the bottle in a 65 ^{o}C incubator for 1 hour. Note that the coronavirus is likely inactivated, however, the sample will be handled by assuming the virus is still viable and other pathogens are also present.
4. All further procedures will occur in a BSC or a centrifuge with a biosafety cap
    a. After heat treating the sample. Place the bottle in the BSC. Add acid until the pH=3
    b. Centrifuge the sample at 5000xg for 10 minutes. Retain the supernatant. Autoclave all glassware prior to use.
    c. Filter the sample through a 0.45 uM mixed cellulose water membrane filter. Autoclave the filtrate and then dispose of as hazardous waste. Record the volume of water filtered (typical volumes will vary from 80 to 200 mL depending in the influent characteristics)
    d. Place the filter in a falcon tube and store at -80 ^{o}C until further processing
    e. Extract the RNA via a commercially available extraction kit or the Griffiths manual extraction method (See below). Handle the filter and all extraction steps in a BSC until the final elution of the RNA in tris.

Manual RNA extraction method

1. Label all tubes and filters with the chain of custody name and date for each sample
2. Measure sample as per steps below and put into the Lysing Matrix E bead beater tubes (or equivalent)
    a. 10-200 ml of filtered sampled (0.45 uM filter), frozen at -80 ^{o}C, shattered (place the entire filter into the bead beater tube)

3. Add 0.5 ml CTAB mixture (10% wt/vol CTAB with 0.7 M NaCl with equal volume of 0.24 M potassium phosphate, pH=8.0) and 0.5 ml of phenol: chloroform:isoamyl alcohol (25:24:1)
4. Vortex 10 minutes at setting 8 in horizontal position or use a bead beater system to homogenize
5. Spin at 4 ^{o}C, 16,000 x g for 5 minutes
6. Transfer the aqueous phase to a new, sterile 1.5 ml tube (take top layer, avoiding white cell debris layer)
7. Remember the aqueous phase volume, then add the same vol of chloroform: isoamyl alcohol (24:1) to get rid of phenol
8. Spin at 4 ^{o}C, 16,000 x g for 5 min
9. Extract aqueous layer (take top layer, avoiding white cell debris layer) to a new 1.5 ml tube
10. Remember vol, and add 2 vol of 30% wt/vol PEG 6000-1.6 M NaCl. Incubate for 2 h at room temperature for nucleic acid precipitation. Place in drawer to prevent light degradation of DNA
11. Spin at 4 ^{o}C, 17,000 x g for 15 min, remove supernatant by pipette or by dumping out the solution
12. Add 100 ul of ice cold 70% v/v ethanol
13. Spin 4 ^{o}C, 17,000 x g for 15 min
14. Remove ethanol with pipette and evaporate remaining ethanol in PCR hood under air
15. Add 25 µl RNase/DNase free Tris to dissolve the RNA
16. Incubate at 37 ^{o}C for 10 minutes with a final concentration of 100 ug/ml RNase A to obtain DNA or 100 ug/ml DNase I to obtain RNA
    a. Add 2.5 ul of RNase A (stock concentration of 2 mg/ml)
17. Quantify by nanodrop or take3 plate
18. Store extracted nucleic acids at -20 ^{o}C until use.

10% wt/vol CTAB with 0.7 M NaCl (15 mls total)

- 0.614 g NaCl
- 1.5 g CTAB
- Fill to 15 ml PCR grade water in the hood
- Dissolve by heating and mixing

Phosphate buffer (15 ml total)

- 3.53 ml of 0.96 M K_2HPO_4 (make with 2.51 g K_2HPO_4 in 15 ml PCR water)
- 0.225 ml of 0.96 M KH_2PO_4 (make with 1.95 g KH_2PO_4 in 15 ml PCR water)
- 11.25 ml of PCR grade water
- Check pH with strips by taking 25 ul and dropping onto the pH paper strip

30% wt/vol PEG-6000 and 1.6 M NaCl (50 mls)

- 4.675 g NaCl
- 15 g PEG-6000
- Fill to 50 mls with PCR grade water in the hood
- Dissolve by heating and mixing

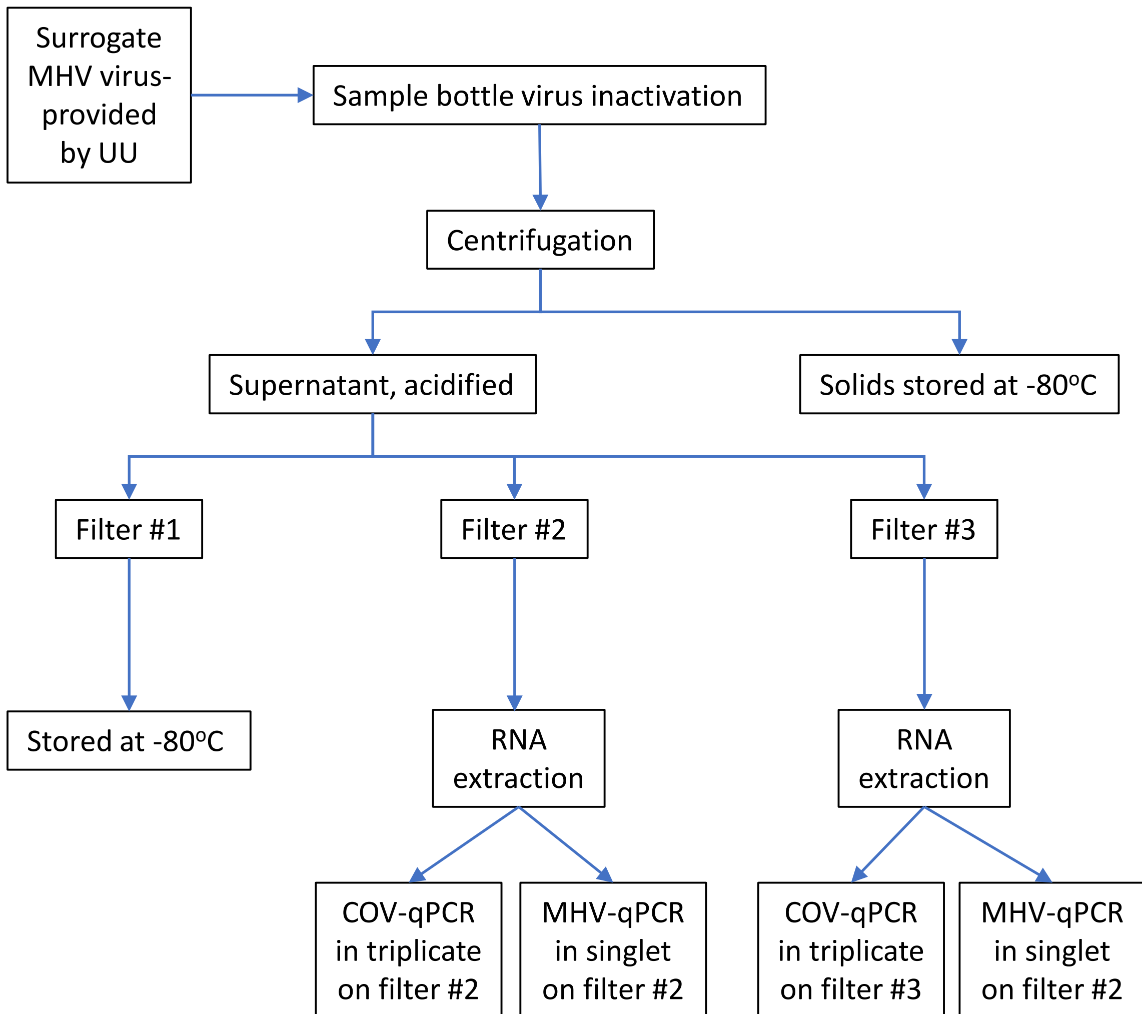
RT-qPCR protocol

1. Dilute the RNA samples to 25-45 ng/ul concentrations using PCR grade 10 mM tris
2. Prepare RT-qPCR mastermix
    a. Per each 20 ul reaction add
        i. 1X TaqPath Mastermix (Thermofisher #A15299)
        ii. 1.5 ul of N1 primer and probe mix (IDT-DNA, 2019-nCoV RUO Kit, # 10006605)
        iii. 1.5 ul of N2 primer and probe mix (IDT-DNA, 2019-nCoV RUO Kit, # 10006605)
        iv. PCR grade water to obtain 15 ul without sample RNA
        v. 5 ul of RNA diluted to appropriate concentrations (25 to 45 ng/ul)
    b. For each RT-qPCR run include a negative control (PCR grade water used to prepare the mastermix) and a positive control (IDT-DNA 2019-nCoV_N_Positive Control #10006625)
3. Using a QuantStudio 3 thermocycler amplify the RNA per the following program:
    a. an initial step of 25 ◦C for 2 minutes; 50 ◦C for 15 minutes; 95 ◦C for 2 minutes; and 45 cycles of denaturation at 95 ◦C for 3 seconds and annealing at 55 ◦C for 30 seconds. At BYU the thermocycler conditions were: an initial step of 50 ◦C for 10 minutes; 95 ◦C for 3 minutes; and 45 cycles of denaturation at 95 ◦C for 3 seconds and annealing at 55 ◦C for 30 seconds.

RT-qPCR data analysis:

1. Ensure there is no amplification in the negative control and the positive control Cts are as expected compared to the standard curve and the dilution used. If the positive control fails, re-run the analysis. If the negative control amplifies, use new primers, mastermix and PCR grade water and re-run the analysis.
2. Average all qPCR replicates on filter #2 and filter #3
3. If any triplicate qPCR has 1 of 3 or 2 of 3 ND,
    a. but the MHV-qPCR is positive flag the sample as present but below detection limits
    b. If the MHV-qPCR is negative, rerun the qPCR for the ND sample and include a replicate matrix spike sample
    c. If after re-analysis the qPCR still has any ND, flag the sample as present but below detection limits
4. Using the standard curve, estimate the gene copies per mL of wastewater based on the volume of water filtered, and the PCR dilution factor use to reach the target RNA concentrations
5. Keep a copy of the result in excel, on the Utah wastewater database and on the computer associated with the QS3.

Scenario 1: data correlates with public health cases

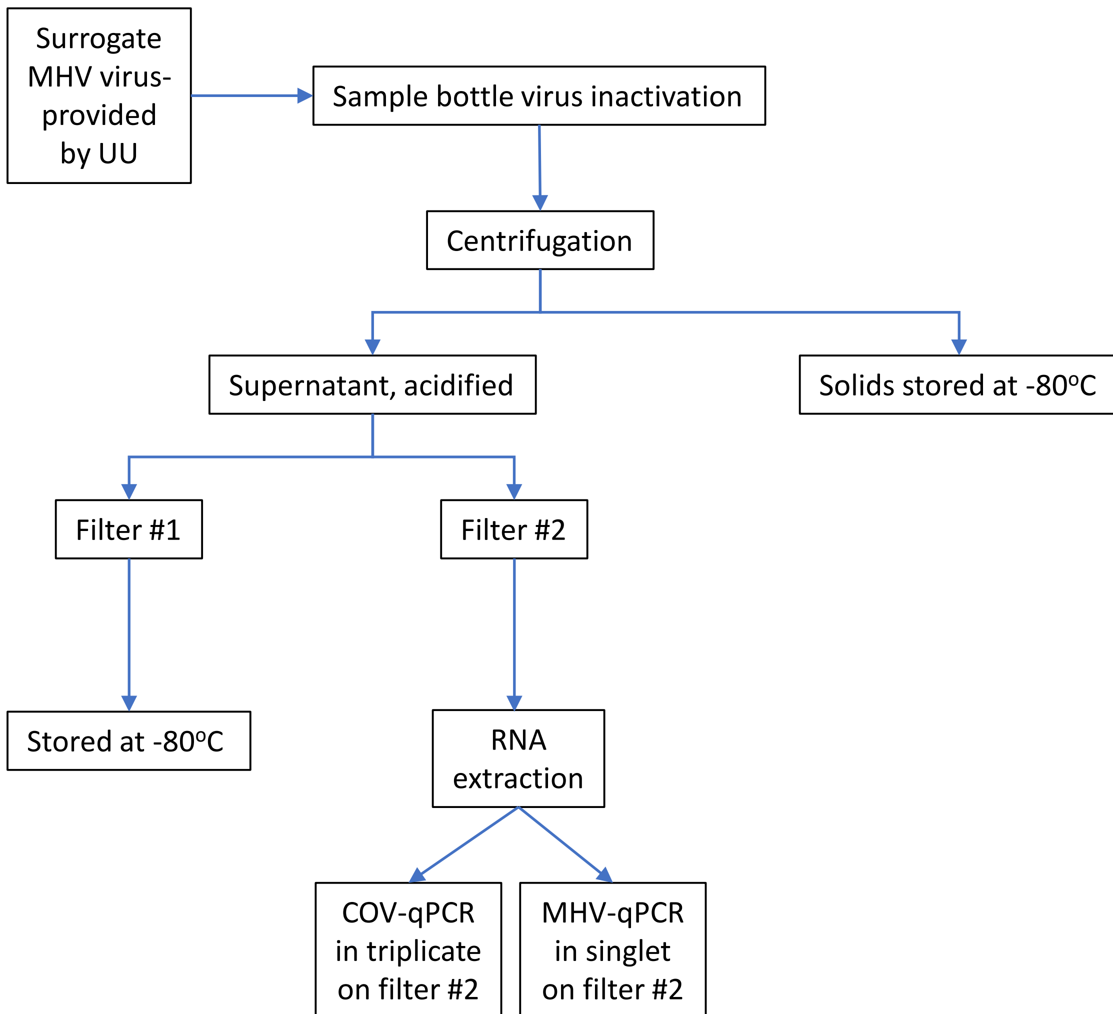


RT-qPCR data analysis:

1. Average all qPCR replicates on filter #2 and filter #3
2. If any triplicate qPCR has 1 of 3 or 2 of 3 ND,
    a. but the MHV-qPCR is positive flag the sample as present but below detection limits
    b. If the MHV-qPCR is negative, rerun the qPCR for the ND sample and include a replicate matrix spike sample
    c. If after re-analysis the qPCR still has any ND, flag the sample as present but below detection limits

SOP 3S.2H

Scenario 2: data used just for trend analysis or hot spot detection



RT-qPCR data analysis:

1. Average all qPCR replicates on filter #2
2. If any triplicate qPCR has 1 of 3 or 2 of 3 ND,
    a. but the MHV-qPCR is positive flag the sample as present but below detection limits
    b. If the MHV-qPCR is negative, rerun the qPCR for the ND sample and include a replicate matrix spike sample
    c. If after re-analysis the qPCR still has any ND, flag the sample as present but below detection limits

Standard Operating Procedure for Sampling, Concentration and RNA Extraction of SARS-CoV-2 in Sewage

Prepared by:
Tiong Gim Aw, PhD

Last Updated July 10th, 2020

Equipment

1. **Biosafety Level 2 laboratory with BSL2 cabinet**
2. Magnetic stir plates for PEG precipitation
3. Refrigerated centrifuge (capable of achieving centrifugal speeds up to 4,700 x g, for example Beckman Coulter Avanti J-E Series)
4. Microcentrifuge for RNA extraction
5. Vortex
6. -20^0 C or -80^0 C freezer
7. Pipette (1000 µl, 200 µl, 100 µl)
8. Pipet-aid (Drummond or equivalent)

Supplies

Field Sampling

1. Sterile 1-L or 500ml sample bottle
2. Markers for labeling
3. Ice packs
4. Cooler
5. PPE (Gloves, eye protector, mask)

Virus concentration

1. Polyethylene Glycol (PEG) 8000 (Promega, Cat No. V3011 or Fisher Scientific, Cat No. BP233-100)
2. Sodium Chloride (NaCl) (Fisher Scientific, Cat No. BP358-1 or equivalent)

3. Serological pipet, 50ml, 10ml
4. Bacteriophage phi6 as process control

<u>Viral RNA extraction</u>

1. QIAamp Viral RNA Mini Kit (QIAGEN Cat No. 52906)
2. Ethanol (96-100%) (Fisher Scientific, Cat No. BP2818100 or equivalent)
3. 1.5 ml DNA low binding tube (Eppendorf, Cat No. 022431021 or equivalent)
4. Bacteriophage phi6 as inhibition control

Procedure

1. Sample Collection

1.1. On the day of sampling, put ice packs in the cooler.

1.2. **Wear PPE** (gloves, mask, eye protector)

1.3. Label the bottle(s) (sampling location, date and time).

1.4. Copy Sample ID/description, date and time onto the Chain of Custody Form provided.

1.5. Remove the bottle top when ready to sample and protect from contamination.

1.6. Pour from the 24-hour composite sampler of raw sewage (from influent to primary treatment, post grit chamber) from the refrigerated wastewater sampler slowly into the sample container.

- No need to have a full bottle, approx. 500ml is sufficient.
- If manual composting is employed, the individual sample portions must be thoroughly mixed before pouring the individual aliquots into the composite container.
- If wastewater sampler is not available, collect one grab raw sewage sample in the morning using a bucket from a sample site post grit chamber.

1.7. Tightly close the bottle and place in the cooler with the ice packs. Fill with newspaper or other filler so that the bottles don’t move around.

1.8. Sign and add the Chain of Custody form to the cooler (Putting the form in a ziplock bag will preserve it during shipping).

1.9. Tape up the ice chest.

1.10. Ship the sample to the following address on the day of sampling **(Use FedEx Priority Overnight)**:

School of Public Health and Tropical Medicine
Tulane University
GEHS, Tidewater Building, Suite 2100
1440 Canal Street
New Orleans LA 70112

Note: Samples once received in the laboratory can be stored at -80° C if not processed within 72 hours.

2. Virus concentration

Adapted from:

Borchardt M.A., Spencer S.K., Hubbard L.E, Firnstahl A.D., Stokdyk J.P, Kolpin D.W. (2017) Avian Influenza Virus RNA in Groundwater Wells Supplying Poultry Farms Affected by the 2015 Influenza Outbreak. Environmental Science & Technology Letters 4(7): 268-272

Note:

- **All the steps should be conducted in the Biosafety Level 2 cabinet except centrifugation and sample incubation.**
- **Negative Control: For every concentration process, include 1 negative control using 100ml of ultrapure water.**
- **Process control (to determine virus recovery) should be done for each new sampling site and sample type by spiking phi6 phages in the sample. After initial determination of the recovery, it is recommended that process control to be included at least once a month.**

2.1. Place a stir bar in the 250-ml sterile centrifuge bottle.

2.2. Using pipet-aid, add 100 ml of sewage (or 100ml of ultrapure water as negative control) to the centrifuge bottle.

2.2.1. If process control is done, spike 100 µl of bacteriophage phi6 (titer: 10^6 PFU/ml) into 101 ml of sewage, mix well and reserve 1 ml of sample for RNA extraction before step 2.3.

2.3. Add 1.17 g NaCl (0.2 M NaCl) and mix.

2.4. Add 8 g PEG (8% PEG 8000 w/v) and mix.

2.5. Transfer container to a cold room or refrigerator (4 ^{o}C) and place on magnetic stirrer.

2.6. Stir at a speed sufficient to develop a vortex, but minimizes foaming (which inactivates viruses). Mix slowly for a minimum of 2 hours.

2.7. Remove from the magnetic stirrer and hold sample overnight at 4 ^{o}C (16 hrs)

2.8. Centrifuge the precipitated sample at **4,700 x g for 45 minutes at 4 °C**. (Remove stir bar before centrifuging)

2.9. Remove **supernatan**t by pipeting and reserve some liquid in the bottle. Pipet at least 10 times to re-suspend the pellet.

2.10. Let the sample set for at least 30 min (in the refrigerator) to allow the residual liquid to drain to the bottom of the tube.

2.11. Mix with pipette. If sample is too thick, sterile 1X PBS may be added (2 to 3ml).

2.12. Final concentrate volume should be 5 – 6 ml. Record the volume.

2.13. Aliquot the concentrate before storing them at -80 °C

3. Viral RNA extraction

Note:

- **All the steps should be conducted in the Biosafety Level 2 cabinet except centrifugation.**
- **Refer to the QIAGEN QIAamp Viral RNA Mini Handbook for detailed protocol: https://www.qiagen.com/us/resources/resourcedetail?id=c80685c0-4103-49ea-aa72-8989420e3018&lang=en**
- **Negative control: For each RNA extraction, include 2 negative controls using AVE buffer.**
- **PCR inhibition control (only when process control is not done): Prior to extraction, spike bacteriophage phi6 in the Buffer AVL. All samples will be quantified for matrix inhibition.**

3.1 Prepare Buffer AVL + Carrier RNA in AVE (1 µg/µl) by adding 8 µl of Carrier RNA per sample to 800 µl of Buffer AVL per sample (e.g., for 10 samples: add 80 µl of Carrier RNA (8 µl x 10 samples) to 8 ml of Buffer AVL (800 µl x 10 samples)

Add 10 µl of bacteriophage phi6 (titer: 10^2 PFU/ml) into Buffer AVL+ Carrier RNA per sample.

3.2 Pipet 800 µl prepared Buffer AVL containing carrier RNA and phi6 into a 2.0 ml microcentrifuge tube.

3.3 Add 200 µl of concentrate (AVE buffer for negative controls) to the Buffer AVL–carrier RNA in the microcentrifuge tube. Mix by pulse-vortexing for 15 s.

Note: To ensure efficient lysis, it is essential that the sample is mixed thoroughly with Buffer AVL to yield a homogeneous solution.

3.4 Incubate at room temperature (15–25°C) for 10 min.

3.5 Briefly centrifuge (for 10 seconds) the tube to remove drops from the inside of the lid.

3.6 Add 800 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge (for 10 seconds) the tube to remove drops from inside the lid.

3.7 Carefully apply 630 µl of the solution from step 5 to the QIAamp Mini column (in a 2 ml collection tube) without wetting the rim. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column into a clean 2 ml collection tube, and discard the tube containing the filtrate.

3.8 Carefully open the QIAamp Mini column, and repeat step 3.6 until all of the lysate has been loaded onto the spin column.

3.9 Carefully open the QIAamp Mini column, and add 500 µl Buffer AW1. Close the cap, and centrifuge at 6000 x g (8000 rpm) for 1 min. Place the QIAamp Mini column in a clean 2 ml collection tube (provided), and discard the tube containing the filtrate.

3.10 Carefully open the QIAamp Mini column, and add 500 µl Buffer AW2. Close the cap and centrifuge at full speed (20,000 x g; 14,000 rpm) for 3 min.

3.11 Place the QIAamp Mini column in a new 2 ml collection tube and discard the old collection tube with the filtrate. Centrifuge at full speed for 1 min.

3.12 Performing a double elution: Place the QIAamp Mini column in a clean 1.5 ml Lo Bind microcentrifuge tube. Discard the old collection tube containing the filtrate.

3.12.1 Carefully open the QIAamp Mini column and add 40 µl Buffer AVE

3.12.2 Close the cap, and incubate at room temperature for 1 min.

3.12.3 Centrifuge at 6000 x g (8000 rpm) for 1 min.

3.12.4 Repeat the step 3.12.1 to 3.12.3 by adding another 40 µl Buffer AVE. Final RNA volume is approx. 80 µl.

3.12.5 Aliquot and store viral RNA at –80°C.

Note: If you are analyzing the viral RNA the same day store RNA at 4°C for up to 5 hours until analysis.

Standard Operating Procedure (SOP) for detection and quantification of SARS-CoV-2 RNA signatures by Droplet Digital PCR (ddPCR) for BIO RAD QX200

Prepared by:

Matthew Flood, M.S. (Michigan State University)

1. Purpose and Scope

This Standard Operating Procedure (SOP) is for digital droplet PCR (ddPCR) assay detection and quantification of SARS-CoV-2 RNA. This SOP is intended to be used for BIO RAD QX200™ Droplet Digital PCR system. The purpose of this ddPCR assay is to identify and quantify RNA signatures in water, wastewater, and other environmental samples.

2. Recommended Quality Control Procedures:

1.1. Users must wear gloves at all times and avoid any direct hand contact with ddPCR reagents to avoid any possible contamination

1.2. Users must use the separate designated spaces for reagent preparation and sample addition. **DO NOT** move any supplies from one space to another (e.g., pipet tips, microtube racks, microcentrifuge tubes, pipettes, etc.)

1.3. Routinely wipe down the hoods and lab benches with 70% alcohol and UV – disinfect for 15 minutes prior to and after use.

1.4. Store reagents in appropriate conditions as per the manufacturer's instructions.

3. Equipment

3.1. BIO-RAD QX200™ droplet generator

3.2. BIO-RAD QX200™ droplet reader

3.3. BIO-RAD plate sealer

3.4. Plate centrifuge ("salad spinner" with 96 well plate attachments)

3.5. Vortex (i.e. Vortex Genie II)

3.6. Biological safety cabinets (biohood)

3.7. BIO-RAD C1000 96-Deep Well Thermocycler (cat# 1851197)

4. Reagents and Supplies

4.1. Primers and Probe for ddPCR – specific to target SARS-CoV-2 and Phi6 bacteriophage

- Based on qPCR primers and probes from scientific literature
- **Table 1. SARS CoV-2 primer and probe set particulars**

Target	Primer/Probe name	Primer/Probe Sequence	Reference
SARS CoV-2	2019-nCoV_N1-F 2019-nCoV_N1-R 2019-nCoV_N1-P	5'-GACCCCAAAATCAGCGAAAT-3' 5'-TCTGGTTACTGCCAGTTGAATCTG-3' 5'-FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1-3'	CDC, 2020
	2019-nCoV_N2-F 2019-nCoV_N2-R 2019-nCoV_N2-P	5'-TTACAAACATTGGCCGCAAA-3' 5'-GCGCGACATTCCGAAGAA-3' 5'-HEX-ACAATTTGCCCCCAGCGCTTCAG-BHQ1-3'	CDC, 2020
	E_Sarbeco_Forward E_Sarbeco_Reverse E_Sarbeco_Probe	5'-ACAGGTACGTTAATAGTTAATAGCGT-3' 5'-ATATTGCAGCAGTACGCACACA-3' 5'-FAM-ACACTAGCCATCCTTACTGCGCTTCG-BHQ1-3'	Corman et al., 2020
Phi6	Φ6Tfor Φ6Trev Φ6Tprobe	5'-TGGCGGCGGTCAAGAGC-3' 5'-GGATGATTCTCCAGAAGCTGCTG-3' 5'- FAM-CGGTCGTCGCAGGTCTGACACTCGC-BHQ1-3'	Gendron et al. 2010

4.2. Qiagen QIAamp Viral RNA Mini Kit (cat# 52906)
4.3. One-step RT-ddPCR Advanced Kit for Probes (Bio-Rad, cat# 1864021 or 1864022)
4.4. Droplet generator Oil (Bio-Rad, cat# 1863005)
4.5. ddPCR 96-Well Plates (cat# 12001925)
4.6. Foil Plate seals (Bio-Rad cat# 1814040)
4.7. Droplet reader oil (Bio-Rad, cat# 1863004)
4.8. Droplet reader waste bottle
- When a reader oil bottle is emptied, it becomes the waste bottle

4.9. DG8 Gaskets (Bio-Rad, cat# 1863009)

4.10. DG8 Cartridges (Bio-Rad, cat# 1864008)

5. Sample Preparation for ddPCR

IMPORTANT! All Sample Preparation is to be done in PCR hoods (Except Droplet Generation).

5.1. Thermocycling program for SARS-CoV-2 primer and probe sets N1 and N2 duplex reaction and E gene simplex reaction

- 25°C for 3 min
- 50°C for 60 min
- 95°C for 10 min
- 40 Cycles* of:
  - ❖ 95°C for 30 sec*
  - ❖ 55°C for 1 min*
- 98°C for 10 min
- Hold at 4°C for ∞

Note: Up to 60 cycles can be run if a large amount of inhibition is present. If this is the first optimization test, run gradient PCR (55°C to 65°C range) to find optimum annealing temperature, as this typically is slightly different from the optimum qPCR temperature. Settings: NO hot start, 40 uL reaction, *Slow ramp speed of 2°C/second

5.2. Thermocycling program for Phi6 gene simplex reaction

- 25°C for 3 min
- 50°C for 60 min
- 95°C for 10 min
- 40 Cycles* of:
  - ❖ 95°C for 30 sec*
  - ❖ 60°C for 1 min*
- 98°C for 10 min
- Hold at 4°C for ∞

Note: Up to 60 cycles can be run if a large amount of inhibition is present. If this is the first optimization test, run gradient PCR (55°C to 65°C range) to find optimum annealing temperature, as this typically is slightly different from the optimum qPCR temperature. Settings: NO hot start, 40 uL reaction, *Slow ramp speed of 2°C/second

5.3. Make sure to wear a lab coat and gloves at all times.

5.4. Disinfect the PCR Preparation stations using 10% Bleach and at least 10 min of UV light.

5.5. **Thaw the One-step RT-ddPCR Advanced Kit for Probes (Supermix, Reverse transcriptase (RT), and 300 mM DTT), 10 µM working stock forward and reverse primers and 10 µM working stock target probes at 4°C for at least 30 minutes.

5.6. Samples should be thawed at 4°C until ready to use and then brought to room temperature at least 3 mins before loading into the DG8™ cartridge for droplet formation.

5.7. Vortex the Supermix, RT, and DTT thoroughly for 15 to 30 seconds to ensure homogeneity, as a concentration gradient may form during storage at -20°C. Centrifuge briefly to collect contents at the bottom of the tube before dispensing.

5.8. Prepare a Master Mix for n +2 reactions (n = total samples + controls) in a 1.5 ml microcentrifuge tube at the PCR prep station according to the following table.

Components of the PCR mix for one reaction in the ddPCR assay:

Table 2. N1 N2 Duplex assay Reaction Mixture

Ingredients	Volume/Reaction***
1-Step RT-ddPCR Supermix (20x)	5.5 µl
Reverse Transcriptase	2.2 µl
300mM DTT	1.1 µl
N1 Forward Primer (20µM stock)	1 µl
N2 Forward Primer (20µM stock)	1 µl
N1 Reverse Primer (20 µM stock)	1 µl
N2 Reverse Primer (20 µM stock)	1 µl
N1 Probe (20 µM stock)	0.3 µl
N2 Probe (20 µM stock)	0.3 µl
PCR-grade Water	3.1 µl
Total Volume (w/ 5.5 µl of Sample)	22 µl

Volumes may differ from qPCR volumes in literature

Table 2. Simplex assay Reaction Mixture for the E gene and Phi6

Ingredients	Volume/Reaction***
1-Step RT-ddPCR Supermix (20x)	5.5 µl
Reverse Transcriptase	2.2 µl
300mM DTT	1.1 µl
N1 Forward Primer (20µM stock)	2 µl
N1 Reverse Primer (20 µM stock)	2 µl
N1 Probe (20 µM stock)	0.6 µl
PCR-grade Water	3.1 µl
Total Volume (w/ 5.5 µl of Sample)	22 µl

*****Volumes may differ from qPCR volumes in literature***

5.9. Aliquot 16.5 ul reaction mixtures in vials or in a 96-well PCR plate. **Note:** The advantage of using a PCR plate is that you can use an 8-channel pipet to load samples in the DG8 cartridge.

5.10. Insert the DG8 cartridge into the holder with the notch in the cartridge at the upper left of the holder.
- The cartridge holder is opened by pressing the latches in the middle
- Slide the DG8 cartridge into the right half of the holder, allow it to drop down and then press the two halves of the holder until they snap closed.

5.11. Transfer 20 µl of each prepared sample to the sample wells (Middle row) of the DG8 cartridge.
- **CAUTION!! Air bubbles can cover the bottom of the well and result in 2,500 to 7,000 less droplets and poor quality data.**
- Use only 200 µl aerosol-barrier (filtered) Rainin pipet tips
- Gently slide the pipet tip down the side of the well at a ~15° angle until it passes over the ridge near the bottom. Holding the pipet at this angle, ground the pipet tip against the bottom edge of the sample well while **VERY SLOWLY** dispensing a small portion of the sample; do not pipet directly onto the side (wall) of the well.
- After dispensing about half of the sample, **SLOWLY draw the tip up the wall** while dispensing the remaining sample volume; **DO NOT push the pipet plunger past the 1st stop**.

5.12. Dispense 70 µl of the droplet generation (DG) oil into each oil well.

5.13. Hook the red gasket over the cartridge holder using the holes on both sides. Make sure that the gasket is completely flush against the wells. The gasket

must be securely hooked on both ends of the holder; otherwise, pressure sufficient for droplet generation will not be achieved.

5.14. Open the QX200 droplet generator by pressing the button on the green top of the machine and place the cartridge holder into the instrument. When the holder is in the correct position, both the power (left light) and holder (middle light) indicator lights will be green.

5.15. Press the button on the top of the machine again to close the lid and begin the droplet generation process. The droplet indicator light (at right) flashes green after 10 sec to indicate droplet generation is in progress.

5.16. When droplet generation is complete, all three indicator lights will be solid green. Open the lid of the machine by pressing the button, and remove the holder (with the DG8 cartridge still in place) from the unit. Remove the gasket from the holder and discard it. The top wells now contain the droplets, while the middle and lower wells are nearly empty with a small amount of residual oil in them.

5.17. Make sure to keep the DG8 cartridge in the holder.

5.18. Using an 8-chanel manual L-50 pipet with 200 µl tips, pipet 40 µl of the contents of the top wells (the droplets) into a single column of a 96 well digital PCR plate.

- Place the cartridge holder on a flat surface and position the pipet tips in each of the top 8 wells at a ~ 30 - 45° angle, vertical into the junction where the side wall meets the bottom of the well. **DO NOT position the pipet tip in a vertical orientation or against any flat surface of the well; do not allow the tips to be flat against the bottoms of the wells.**

- Slowly draw up 40 µl of droplets into the pipet tip **(should take ~ 5 sec and ~5** µl **of air is expected)**; do not aspirate >40 µl, as this causes air to percolate through the droplets

- Pipet slowly. Apply a stable resistive force to the plunger to draw and aspirate droplets smoothly into and out of the pipet tips.

- **To dispense droplets into the 96-well plate,** position the pipet tip along the side of the well – near, but not at, the bottom of the well – and slowly dispense the droplets (~5 sec).

- **Make sure to keep the plate covered while pipetting in order to prevent evaporation and contamination with particulates.**

5.19. Seal the PCR plate with foil (keeping the **red line at the top of plate**) immediately after transferring droplets to avoid evaporation.

- Make sure the plate sealer is set to 180°C and time to 5 sec
- Open the plate sealer by touching the arrow and then position the support block with the 96-well side facing up.
- Place the plate with the foil seal on top of it into the support block and then close the plate sealer.
- Touch the seal button in order to start sealing the plate. The door will open automatically after sealing is complete.
- Make sure to remove the support block along with the 96-well plate after sealing to avoid overheating the support block.
- Check to make sure the wells are sealed. Once this is confirmed the plate is ready for thermal cycling.

5.20. Once droplets are removed, press the latches on the DG8 cartridge holder to open it. Remove the empty DG8 cartridge and discard it.

5.21. Begin thermal cycling with the protocol provided above within 30 min of sealing the plate, or you may store the plate at 4°C for up to 4 hr prior to thermal cycling. Settings: NO hot start, 40 uL reaction, ramp speed 2°C per second

6. *ddPCR droplet reader use protocol*

6.1. Power up the QX200 droplet reader using the switch at the back, if it is not already on. Allow it to warm up for 30 min, then launch the QuantaSoft software on the laptop.

6.2. Make sure to check the indicator lights on the droplet reader. The first two lights at the left should be solid green, indicating that the power is on and there is sufficient oil in the designated oil reservoir, and there is <700 ml in the waste bottle. If the lights are flashing amber, a run cannot be started. You must clean out the waste bottle or replace the oil.

6.3. Place your 96-well plate in the plate holder. To do so you must first undo the two black latches on either side of the holder and remove the metal top portion. Then you may place your 96-well plate in the base of the holder and re-latch the top of the plate holder over your plate.

6.4. Press the button on the green lid to open the droplet reader. Load the plate holder with your 96-well plate insider into the droplet reader and press the button on the lid again to close the cover. **Make sure to confirm the three indicator lights are green before proceeding.**

6.5. In the QuantaSoft software, click the **Setup** button in the upper left of the screen in order to define your experiment and then click **Run** to start the ddPCR program.

7. *ddPCR reaction programing*

7.1. Once the QuantaSoft software is up and running click Setup to enter information about your samples, assays, and experiments.

- To open saved details (settings and data) from another experiment click **Plate > Load** and then select the file.
- To open a saved template for a plate map (settings only, no data) click **Template > Load** and select the file
- To create a new template, click **Template > New**

7.2. Use the well editor to define the settings for the plate.

- Hold **Ctrl** to select multiple wells.
- In the Sample panel, enter the sample **Name** and select the **Experiment** rare event detection"RED" from the drop-down menu.
- Select the **RT-Supermix** from the drop-down menu
- Define Assay 1 (channel 1, the FAM channel) and Assay 2 (channel 2, the VIC or HEX channel). Then assign each assay a **Name (e.g. N1, N2, E, or Phi6)** and sample **Type** (Unknown for experimental samples, Positive, Negative, or NTC)

7.3. After all wells are set and you have chosen "RED" from the experiment drop-down menu you then click the **Run** button in the left navigation bar to start the run.

7.4. In the Run Options window, select the detection chemistry:

- If a probe supermix is selected in the well editor, the probe dye sets appear. Select FAM/HEX or FAM/VIC.
- Up to 1 minute late, a green circle appears next to the abort button and flashes to indicate the run is in progress. Active and analyzed wells are also highlighted in green in the plate map.
- As each well is analyzed, the data appear across the top navigation area. Once the run is complete, all data are reanalyzed for the final data file.

8. ddPCR QA/QC data Requirements and Data Analysis/Interpretation

8.1. QA/QC Requirements

8.1.1. Samples must have >10,000 accepted droplets in order to be considered for further analysis. If a sample has <10,000 accepted droplets it should not undergo further analysis

8.1.2. If a sample has < 3 positive droplets after the threshold is set then this sample is considered negative and is to be reported as having 0 positive droplets

8.1.3. If a sample has < 3 negative droplets after the threshold is set then the sample is disregarded and should be rerun in order to determine whether of not it was a false positive

8.1.4. The Threshold for positive and negative droplets is set at 1 Standard Deviation above the negative droplets (As determined by calculating the Standard deviation from each run's NTC raw amplitude data.

8.2. Calculating Amplitude Threshold for each run:

8.2.1. After the run is completed, choose 1 of your NTC samples with >10,000 accepted droplets to be used for threshold calculation (If more than one of your NTC are above 10,000 droplets choose the one with the greatest accepted droplet count)

8.2.2. Go to the **Setup** Tab in the QuantaSoft program and click the **Options** button to the right of the right of the setup menu

8.2.3. Then choose the **Export Amplitude and Cluster Data** option and export the raw amplitude data to a folder of your choice.

8.2.4. Now open the raw amplitude data for your NTC well in Microsoft Excel

8.2.5. In an empty cell input the following formula (=STDEV()) and proceed to highlight all of the raw amplitude scores and then hit enter. This value will be your Standard Deviation and will be added to the highest droplet amplitude value in the NTC well you selected. This new amplitude value should then be applied to the rest of the samples in the run as a common Threshold value.

8.2.6. After this threshold has been set for all of the samples in your run you should then proceed to export the CSV file for the run so that it can be opened in Excel as well.

8.2.7. Once you have opened your .csv file for your run in Excel you should create a new sheet in that workbook where you will copy and paste all of the sample that had >10,000 accepted droplets before looking at which samples came back positive/negative and the concentrations of DNA in those wells.

8.2.8. Next you must calculate the Cell equivalents for each of your positive samples based on the following instructions:

8.2.9. Concentration (copies/ul) x 20ul reaction volume = Total copies in the 20 ul that was loaded into the droplet generator

8.2.10. Then take that copy # and divide it by the 5ul of Template that was added to the reaction mixture to get the concentration (copies/ul) that was originally added to the reaction mixture.

8.2.11. Then multiply that concentration by the final volume of eluted DNA from the DNA extraction procedure (Usually 100ul) to get the total copy # in that was extracted

8.2.12. Finally divide that number by the total volume or mass that was filtered/used for DNA extraction to get the concentration of copies in your initial sample volume.

8.2.13. To determine your Cell Equivalent for the copies of your target DNA you must divide the total copy # that you calculated for your sample volume by the # of copies of that target DNA in a single cell of your target organism

References:

BIO RAD QX200 Droplet Digital PCR System Instruction Manual

Corman VM, Landt O, Kaiser M, Molenkamp R, Meijer A, Chu DKW, Bleicker T, Brünink S, Schneider J, Schmidt ML, Mulders DGJC, Haagmans BL, van der Veer B, van den Brink S, Wijsman L, Goderski G, Romette JL, Ellis J, Zambon M, Peiris M, Goossens H, Reusken C, Koopmans MPG, Drosten C. Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Euro Surveill 2020 Jan;25(3):2000045. doi: 10.2807/1560-7917.

2019-Novel coronavirus (2019-nCoV) real-time rRT-PCR panel primers and probes. US Centers for Disease Control and Prevention.

Standard Operating Procedures
Title: Standard Laboratory Practices for the Dennehy Research Laboratory
SOP#: Dennehy 1
Purpose: To provide safe handling, operations and procedures for all personnel working in the laboratory tasked with detecting SARS-CoV-2 in NYC wastewater.

I. Research Protocol

A. Sample Preparation and Virion Concentration

The New York City Department of Environmental Protection provides, on a weekly basis, 1-liter 24-hr composite samples from the wet-well of all 14 wastewater treatment plants located in NYC. On arrival at our laboratory, 500 mL from each location is processed, and the remainder is frozen at -80℃. Detailed information on sample transport and safe handling and receiving is below in section III.F.

B. **Pretreatment:** Prior to processing, samples are rendered non-infectious by pasteurization at 60℃ for 60 min. Following this, the samples are centrifuged at 5,000 x g to pellet solids, then the supernatants are passed through a 0.22 filter using a disposable vacuum filter system (Corning 430769).

C. **Quality Assurance:** For quality control assurance, a matrix spike of a known titre of rotavirus RNA, a non-enveloped dsRNA virus to determine RNA recovery during RNA isolation from wastewater. In addition, samples may be spiked with a known titre of phi6, an enveloped dsRNA bacteriophage to test the effect of various processing methods on the recovery of infectious virions. Both spike-ins allow the determination of recovery rates of both viable and non-viable enveloped viruses. Inactivated and filtered samples are divided into six 40-mL parts to determine the effect of various processing methods (e.g., different concentration protocols, different means of storage). While establishing protocols, efforts are made to perform the experiment at least in triplicate within a sample if possible while testing the outcomes across multiple distinct samples. For qPCR experiments, all assays are performed in triplicate and include standards and template and enzyme free controls according to best practices (MIQE Guidelines). 500 mls of each wastewater sample is maintained at -80℃ as a recovery sample.

D. **Viral Concentration Protocols**

To determine the best method of virion concentration, virions in the filtrate are concentrated using one of four methods: PEG precipitation, $AlCl_3$ flocculation, skim milk flocculation, or ultracentrifugation.

1. **PEG Precipitation Protocol**
    a) 4 g PEG 8000 (100 g/L) and 0.9 g NaCl (22.5 g/L), are added to 40 mL filtrate.
    b) The filtrate is allowed to rest overnight at 4℃.
    c) After resting, the sample is centrifuged in a 50 mL Nalgene Polycarbonate Oak Ridge tube (Thermo Fisher Scientific 31380050) at 12,000 x g at 4℃ for 10 min.
    d) The supernatant is removed, pellet is resuspended in 1.5 mL TRIzol (Invitrogen 15596018) and transferred to a 2 mL centrifuge tube for RNA isolation.

2. **$AlCl_3$ Flocculation Protocol**
    a) 400uL of 0.3M $AlCl_3$ is added to 40 mL filtrate, then the pH is adjusted to 6.0 with 1M NaOH.
    b) The resulting mixture is incubated with agitation 100 rpm for 15 min at RT, then centrifuged at 3000 x g for 20 min at RT.
    c) The supernatant is removed, and the pellet is resuspended in 10 mL of 3% beef extract (Gibco Bacto DF0115173) at pH 7.4, incubated again with agitation (150 rpm for 10 min at RT), then centrifuged at 3000 x g for 20 min at RT.
    d) The pellet is resuspended in 1.5 mL TRIzol and transferred to a 2 mL centrifuge tube for RNA isolation.

3. **Skim Milk Flocculation Protocol**
    a) 1350 µl skimmed milk mixture (25g/L) is added to 40 mL filtrate.
    b) The pH is adjusted to between pH 3 to 4 with HCl 1N and NaOH.
    c) The sample is then incubated 2 hours at room temperature on a rocker set to ~ 400.
    d) To concentrate the virions, the sample is centrifuged 12,000 x g at 4℃ for 30 min.
    e) After removing the supernatant, the pellet is resuspended in 1.5 mL TRIzol and transferred to a 2 mL centrifuge tube for RNA isolation.

4. **Ultracentrifugation Protocol:**
    a) Filtrate is placed in a 5 mL thick walled polycarbonate centrifuge tube (Beckman Coulter 362305) and centrifuged at 200.000 x g at 4℃ for 60 min.
    b) The supernatant is removed and the pellet is resuspended in 1.5 mL TRIzol and transferred to a 2 mL centrifuge tube for RNA isolation.

5. **RNA Extraction Protocol:** To test the efficiency of the RNA extraction,

some samples are spiked with rotavirus RNA of known concentration.

a) Samples are processed in a dedicated RNA biosafety cabinet to minimize exposure to RNAses. All surfaces, such as bench tops, pipettes, and tip boxes, are cleaned with RNAse Zap and cross-contamination minimization strategies are employed (e.g. barrier tips).
b) To extract RNA suspended in TRIzol, cold (4℃) chloroform is added in a 1:5 ratio, allowed to rest at RT for 5 min, then the sample is centrifuged at 12,000 x g at 4℃ for 15 min.
c) The top aqueous phase is transferred to a fresh tube, and cold (-20℃) isopropanol is added in a 1:1 ratio.
d) The resulting mixture is allowed to rest at RT for 15 min, and then centrifuged at 12,000 x g at 4℃ for 15 min to pellet the RNA.
e) The supernatant is removed and the pellet was washed twice by adding 1 mL 75% ethanol, centrifuged at 12,000 x g at 4℃ for 3 minutes, then the ethanol is removed.
f) After the second wash, the sample is air-dried for 10 min, then resuspended in 30μl RNA Protect. Samples are stored at -20℃.

6. **Quantification:**
    a) The concentration and purity of RNA assessed using a QuBit 3 fluorometer (Thermo Fisher Scientific).
    b) A working solution is prepared and samples (1-20 ul) are diluted in up to 200 ul of working solution.
    c) Two standards (0 ng/ul and 10 ng/ul concentration) are used to generate a calibration curve on the Qubit before reading each sample. This process is repeated each time the Qubit is used for quality assurance.
    d) Before reading the samples, they are allowed to reach room temperature for 2 mins.
    e) The assay is highly selective for RNA over double-stranded DNA (dsDNA) and is accurate for initial sample concentrations from 250 pg/μL to 100 ng/μL.

7. **Quantitative PCR: Primers and probes**
    a) Samples are tested for the presence of SARS-CoV-2 RNA using the CDC 2019-nCoV Real-Time RT-PCR Diagnostic Panel (IDT Inc.) and according to the instructions provided by the CDC.
    b) Each RT qPCR assay is performed with two no-template controls, and a ten-fold serial dilution of standards (nCoVPC positive control) across five orders of magnitude. Each sample is assayed in triplicate with N1 or N2 primers/probes.
    c) Reverse transcription is performed using the Thermofisher

TaqPath 1-Step RT-qPCR Master Mix and the ABI StepOne instrument.

d) ROX reference dye is used to normalize the fluorescent reporter signal.

e) Thermal cycling is performed using the conditions established for the TaqPath 1-Step RT-qPCR amplification.

f) Reaction efficiency is assessed using plots of standard Cts plotted against standard concentration. Each ten-fold standard dilution should be dilution should be ~3.3 cycles apart.

g) A sample is considered negative if all 2019-nCoV marker (N1, N2) cycle threshold growth curves DO NOT cross the threshold line within 40.00 cycles (< 40.00 Ct). A sample is considered positive for 2019-nCoV if all 2019-nCoV marker (N1, N2) cycle threshold growth curves cross the threshold line within 40.00 cycles (< 40.00 Ct).

II. Authority for Microbiology Lab and Prep Room Regulations

The Lab will follow the guidelines posted by the U.S. Department of Health and Human Services, Centers for Disease Control and Prevention, and National Institutes of Health. These guidelines describe acceptable biosafety practices in biomedical and microbiological laboratories and can be found at: **http://www.cdc.gov/OD/ohs/biosfty/bmbl5/bmbl5toc.htm.** D343 is a research laboratory and classified as BSL2. All work conducted in the BSL2 lab must adhere to BSL2 guidelines and regulations. All biohazardous waste generated in D343 is classified as BSL2 biohazardous waste.

The CDC currently recommends that routine viral testing of specimens, such as the proposed activities, can be handled in a BSL-2 laboratory using Standard Precautions (https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html). While the ability for COVID-19 to be transmitted via wastewater has not been documented, we will assume samples contain infectious viral particles as well as potentially hazardous bacteria. To date, no study has shown that SARS-CoV-2 in human feces is infectious. Moreover, current thinking is that given the dilute nature of wastewater and the presence of inactivating substances (e.g. detergents, solvents) will render any wastewater-borne SARS-CoV-2 non-infectious. Despite being a significant concern, no studies have reported being able to isolate viable virus from wastewater. Current CDC recommendations do not stipulate that personnel take any extra precautions beyond standard practices when handling wastewater (https://www.cdc.gov/coronavirus/2019-ncov/php/water.html and https://www.who.int/publications-detail/water-sanitation-hygiene-and-waste-management-for-covid-19).

III. Regulations

A. Access, Training and Responsibilities

1. Access is limited to individuals involved directly in growth media prep,

clean up, lab prep, and research. One technician will work at a time in D343 with duties being rotated among three trained individuals.

2. Beth Lamanna, Director of Public Safety, will be alerted as to who will be working each day 24hrs in advance.
3. The individuals listed below will work on this project; before engaging in this work, they must be trained in the safe manipulation of recombinant DNA molecules and the organisms that contain them and/or infectious agents and such training must be documented:

Dr. John Dennehy (PI, QC, C14 Certificate of Fitness)
Dr. Monica Trujillo (Co-PI, QCC, C14 Certificate of Fitness)
Dr. Sherin Kannoly (postdoctoral fellow, C14 Certificate of Fitness)
Kaung Myat San (QC MA Biology 2018, technician, C14 Certificate of Fitness)
Anna Gao (QC BA Biology 2020, technician, C14 Certificate of Fitness)
Nanami Kubota (QC BA Biology 2020, technician, C14 Certificate of Fitness)

4. Access for other individuals is with permission of lab staff:
   - **Construction/Maintenance:** Any construction/maintenance work requires advanced 24hr notice to the lab staff.
   - **Cleaning:** Only as requested by Lab staff through the work order system.
   - **Emergency:** Entry permitted in conjunction with notification of Lab staff by text and email (cell phones numbers on are on the doors)
5. The lab doors will be closed when a BSL2 agent is in use.
6. All staff and students are required to read, understand, and follow these regulations before working in D343.
7. All staff and students working in D343 will receive training from their instructor concerning use of the equipment.
8. John Dennehy will train permanent and part-time college laboratory technical staff on aseptic techniques appropriate for handling pathogenic agents. This will include the potential hazards associated with the work involved, the necessary precautions to prevent exposures, and the exposure evaluation procedures. Records of training are maintained in the Dennehy Laboratory.
9. Personnel will receive annual updates or additional training as necessary for procedural or policy changes.
10. Personnel are advised of special hazards and are required to read and follow instructions on practices and procedures.
11. Any staff or students found in violation of the regulations may have their access to room D343 and other laboratory spaces terminated.
12. The Chair of the Department of Biology is responsible for seeing that the consequences of student or staff actions are rectified, including correction of damages and violations.

B. Apparel

1. Gloves and N95 masks will be provided to all personnel for use in transit to the laboratory.
2. Once at the lab, these items will be removed and a different set will be provided for use in the laboratory. New PPE will be provided for all employees after they are finished working for their travel home.
3. Requisite PPE, including coat, gloves, safety goggles, and N95 masks, will be provided to all personnel, who will be required to wear them at all times in the laboratory.
4. All PPE is single use and will be autoclaved prior to disposal.
   a) The use of N95 masks requires that workers be enrolled in a respirator program that is run by the Environmental Health and Safety Office. We will coordinate with Parmanand Panday or William Graffeo to ensure this is completed.
   b) Personnel will be medically cleared by a physician whose written certification is sent to the EHS Office by June 1st or as soon as possible.
5. Personnel entering room D343 will be required to wear closed-toe shoes and have long hair tied back.
6. Personnel working in D343 at BSL2 must wear lab coats at all times, lab coats are assigned to an individual. This protective clothing is removed and left in the laboratory before leaving for non-laboratory areas (e.g., restroom, cafeteria, library, or administrative offices). All protective clothing is either autoclaved or laundered at least once a semester.
7. Gloves are worn when handling microorganisms or hazardous chemicals. Gloves are disposed of when contaminated, and removed when work with infectious materials is completed or when the integrity of the glove is compromised. Gloves are placed in a biohazard bag and autoclaved prior to disposal. Disposable gloves are not washed, reused, or used for touching “clean” surfaces (keyboards, telephones, etc.), and they should not be worn outside the lab. Hands are washed with soap and water following removal of gloves.
8. In a BSL2 lab, safety goggles or safety glasses are worn for normal lab procedures involving liquid cultures that do not generate a splash hazard (e.g., proper pipetting, spread plates, etc.). Safety goggles and visors or safety goggles and masks are worn when performing procedures that may create a splash hazard.
9. When working in a biosafety cabinet, only lab coats and gloves are needed for personal protection.

C. Standard Microbiological Practices

1. Eating, drinking, smoking, handling contact lenses, and applying

cosmetics are not permitted in the lab. Food for human consumption is never stored in the lab.

2. A red biohazard sign must be posted on the entrance to the laboratory when etiologic agents are in use. Information to be posted includes the agent(s) in use, biohazard symbol, biosafety level 2, the investigator's name and telephone number, any personal protective equipment that must be worn in the laboratory, and any procedures required for exiting the laboratory.
3. Persons wash their hands upon entering the lab, after they finish working in the lab, after removing gloves, and before leaving the laboratory.
4. Work surfaces are decontaminated prior to beginning any work in these rooms, on completion of work or at the end of the day with 10% bleach solution. Any small spill or splash of viable material should be decontaminated with an equal volume of 20% bleach solution (Using an equal volume of 20% bleach makes the final concentration 10%).
    a) All procedures are performed carefully to minimize the creation of splashes or aerosols. Any procedure that would potentially create aerosols will be performed within the biosafety cabinet. Centrifuges in the BSL2 laboratory must have sealed rotors.
        (1) Centrifugation - All centrifugation in the BSL2 lab use Thermo Scientific™ Sorvall™ Legend™ Micro 21 centrifuges that have a click seal bio lid.
        (2) Homogenization - This can be performed using a special adaptor on the vortex that allows tubes to be shaken at high speeds that contain cells or tissues and shearing beads. This action is performed in the BSC to avoid potential spills and/or aerosols. Alternatively, a bead beater is used. The unit in the lab is the FastPrep-24 which has a lid that maintains a tight seal during homogenization preventing aerosols. Following both procedures, the organisms are killed and the cellular contents are released. A kit is used to isolate the DNA, RNA or proteins.
        (3) Vortexing - When using the vortex to resuspend cells or break-up pellets, this procedure is carried out in the BSC to prevent spills or aerosols.
5. Mouth pipetting is prohibited; mechanical pipetting devices are used.
6. A limited number of needles and syringes are used for reconstituting reagents. After use, these materials are placed in a puncture-proof red sharps container placed near the area of work. Do not recap needles.

D. Special Practices

1. Access to the laboratory is limited or restricted by the BSL2 laboratory director when work with infectious agents is in progress. Persons who are

at increased risk of acquiring infection – e.g., those who are immunocompromised or immunosuppressed – or for whom infection may have serious consequences, should consult with their physician to determine the appropriate level of participation in the lab.

2. If two individuals are working the same day, a one-hour gap between their hours will be imposed to allow any potential airborne virus to settle (we will minimize occasions where multiple people work per day).

E. Transfer and receipt of materials

1. On Fridays, Dr. Dennehy will take delivery of wastewater samples and bring them to QC, likely in the afternoon.
2. On Fridays, 14 liters of wastewater (in 500 mL bottles) will be picked up from Newtown Creek Wastewater Treatment Plant on Fridays. Samples are prepared by DEP personnel in sealed 500 mL bottles that have been double-bagged to prevent leakage.
3. Collection personnel will wear a respirator, face shield, lab coat and gloves when receiving the samples from the DEP.
4. The samples will be placed in a large cooler and transported to the Dennehy Lab at QC. The inside and outside of the cooler will be wiped down with bleach or hydrogen peroxide solution following use.
5. Upon arrival, bottles will be wiped down with a bleach solution in a Class 2/A2 biosafety cabinet. SARS-CoV-2, if present in sample, will be rendered non-infectious by heating bottles to 60°C for 60 min. The temperature treatment is intended to inactivate viruses and pathogens, but not disrupt viral genomes.
6. Cultures, tissues, or potentially infectious wastes are placed in a container that prevents leakage during collection, handling, processing, storage, and transport.
7. For each sample site, 500 ml is processed as above and a second 500 ml maintained in the -80°C as a backup.

F. Disposal of Materials and Decontamination

1. All cultures, swabs, tips, plates and waste containers are decontaminated by autoclaving prior to disposal by incineration. Liquid bacterial cultures are inactivated by adding an equal volume of 20% bleach prior to autoclaving (Using 20% bleach will ensure that a final concentration of 10% has been achieved). Red-bag materials to be decontaminated outside of the immediate laboratory should be placed in a durable, leak-proof container for transport from the laboratory. **All red-bag waste generated in the Dennehy lab is BSL2 biohazardous waste.**
2. Laboratory equipment and work surfaces should be decontaminated with 5% Hydrogen Peroxide or 10% bleach on a routine basis and after work with infectious materials is finished.

3. The biosafety cabinet should be wiped down with a 5% Hydrogen Peroxide or 10% bleach solution before and after use.
4. Following any procedures, the cabinet will be sterilized using its UV lamp.

G. Spills

1. Overt spills, splashes, or other contamination by infectious materials should be decontaminated with an equal volume of 20% bleach (Using an equal volume of 20% bleach will ensure a final concentration of 10%). An overt spill is defined as the following:
   a) Spills greater than 5 mL (BSL-2, toxic chemicals) outside primary containment
   b) Spills that may contain broken glass or other sharps
   c) Spills that result in an exposure
   d) Spills that present an inhalation hazard
   e) Spills that cannot be easily cleaned
   f) Spills that endanger people or the environment
2. In the event of an overt spill, EHS, Dr. Dennehy, and Fire/Safety Director should be informed and a lab incident form should be filed (cell phones on doors). Appropriate PPE should be used when cleaning. All cleanup material should be disposed of as biohazardous waste.
3. For spills outside the biosafety cabinet, the spill should be decontaminated by covering with paper towels and soaking in an equal volume of freshly made 20% bleach solution or disinfectant for at least 30 minutes. The area at least 2 feet around the spill should also be cleaned with 10% bleach or disinfectant (Including furniture/walls). Spills that contain broken glass or sharps should also be treated with bleach but the material should be picked up using a dustpan and brush.
4. For spills inside the Biosafety Cabinet, the spill should be covered in paper towels and the paper towels soaked in an equal volume of freshly made 20% bleach solution or 70% alcohol for at least 30 minutes. The work surfaces and equipment in the BSC should be wiped down with disinfectant while the spill is soaking. All cleanup material should be disposed of in the biohazardous waste container.
5. For spills inside equipment like the centrifuges or incubators, turn off the equipment and leave the door closed for at least 30 minutes to reduce aerosol exposure before cleaning up the spill. Treat the spill as in step 3 and 4.
6. For spills inside equipment like the centrifuges or incubators, which involve glass or a breakage, turn off the equipment and leave the door closed for at least 30 minutes to reduce aerosol exposure before cleaning up the spill. Treat the spill as in step 3 and 4 with the addition that glass should be removed post decontamination, with a forceps and transferred

to the glass biohazardous waste container using a designated dust-pan and broom.

7. A sign should be taped on the equipment, indicating a spill occurred until the decontamination period has passed.
8. In the event of a chemical spill, clean it up immediately after consulting the SDS and contacting EHS and the Fire/Safety Director.
9. Spills and accidents that result in overt exposures to infectious materials are immediately reported to Dr. Dennehy, and to EH&S. Medical evaluation, surveillance, and treatment are provided as appropriate and written records are maintained.
10. Broken glassware that is not contaminated with biohazardous materials should be swept up with the D343 designated broom and dustpan and discarded in the glass disposal box.
11. Broken glassware that contains live cultures or other biohazardous material should be saturated with an equal volume of 20% bleach solution. After 15 minutes, the debris should be “swept” up into an autoclave bin using a plastic beaker and/or paper towels. After being autoclaved, the glassware can go into the glass disposal box and the paper towels can go into the regular trash.

H. Hygiene and Housekeeping

1. Bench tops are impervious to water and are resistant to moderate heat and the organic solvents, acids, alkalis, and bleach used to decontaminate the work surfaces.
2. Laboratory furniture is capable of supporting anticipated loading and uses. Spaces between benches, cabinets, and equipment are accessible for cleaning. Chairs used in laboratory work should be covered with a nonporous material that can be easily decontaminated.
3. Access for other personnel (facilities/housekeeping) will be with the authorization of the Dr. Dennehy, in consultation with EHS.

WRF 5089 Study -- IDEXX Water SARS-CoV-2 RT-PCR Procedure

The following procedure was used in August of 2020 by IDEXX Laboratories for WRF Study 5089. Protocols for using the IDEXX Water DNA/RNA Magnetic Bead Kit and the IDEXX Water SARS-CoV-2 RT-PCR Test have been updated and improved since that date. Please refer to the IDEXX website for the most up to date procedures and test kits: https://www.idexx.com/en/water/water-products-services/water-sars-cov-2-rt-pcr-test/

IDEXX has validated both the procedures below and updated procedures to provide consistent results in wastewater. The concentration protocol below is provided as an example. The IDEXX Water extraction and PCR test kits can also be used with other concentration or sample preparation protocols.

Contact IDEXX Technical Support for more information (1 800 321 0207).

Sample Handling

- All work will be performed in a BSL-2 laboratory in accordance with recommended procedures for handling and processing of wastewater samples associated with SARS-CoV-2 according to CDC guidelines in: *Interim Laboratory Biosafety Guidelines for Handling and Processing Specimens Associated with Coronavirus Disease 2019 (COVID-19)* (https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html).
- All manipulations of wastewater samples in open containers will be performed in a Class II biosafety cabinet.
- All centrifugation steps will be performed using sealed rotors or buckets with sealed safety cups. Rotors and buckets will be loaded and unloaded only in a biosafety cabinet.
- Upon receipt, the exterior surfaces of sample containers will be decontaminated with 70% isopropanol and exposed to short-wave (UV-C) light using controlled contact and exposure times as detailed below.
- Liquid waste will be decontaminated by dilution with bleach to a final concentration of 10% and incubated at ambient temperature for ≥ 60 minutes before disposal.

Wastewater Concentration Protocol

Procedural Notes

- Concentration is performed according to the instructions in the Water SARS-CoV-2 RT-PCR Kit product insert (available here: https://www.idexx.com/files/product-insert-water-sars-cov-2-rt-pcr-test.pdf), using the example procedure detailed below.
- This procedure concentrates SARS-CoV-2 from 105 mL of wastewater.

- Samples will be stored at 2–8°C upon receipt.
- Samples are maintained at cold temperatures near 4°C throughput the procedure using refrigerated centrifuge equipment and by minimizing handling time outside of the centrifuge.
- Pasteurization is optional. In the WRF 5089 study, samples will be processed with and without pasteurization.
- Pasteurization will be performed on the same day the sample is received at the laboratory, if possible.

Pasteurization:

1. Decontaminate exterior surface of container with 70% isopropanol, using a wet contact time of ≥ 5 minutes. Expose container surface to short-wave (UV-C) light using controlled exposure time and distance between container and light source.
2. Incubate sample in a 60 ±1°C water bath for 1.5 hours, mixing once during incubation.
3. Cool sample to 2–8°C before proceeding with concentration. Sample may be stored overnight at 2–8°C.

Concentration:

1. Mix sample well, then add 35 ±1 mL to each of three (3) empty 50 mL centrifuge tubes.
2. Centrifuge all three tubes at 4,700 RCF for 30 minutes at 4 ±1°C in a swinging bucket rotor. Use little or no braking force to prevent the bacterial pellet from being disturbed.
3. Add 3.5 ±0.1 g PEG 8000 and 0.788 ±0.01 g NaCl into three (3) empty 50 mL centrifuge tubes.
4. Promptly and gently, remove centrifuge buckets from centrifuge. Carefully decant supernatant, smoothly and in one consistent motion, from each tube into a 50 mL tube containing PEG and NaCl to prevent the pellet from being disturbed.
5. Mix the tubes containing PEG and NaCl at ambient temperature until completely dissolved.
6. Open tubes carefully and decant liquid into a new 50 mL centrifuge tube. This step prevents leaks during centrifugation due to powder interference with the cap seal.
7. Mark a location near the bottom of the tube and orient to the outside of the rotor during centrifugation. This will aid the resuspension of viral pellets that are not clearly visible
8. Centrifuge at 12,000 RCF for 120 minutes at 4 ±1°C. Use little or no braking force to prevent the viral pellet from being disturbed.
9. Promptly and gently, remove rotor from centrifuge. Carefully decant and discard most of the supernatant from each tube. Decant smoothly in one consistent motion to prevent the pellet from being disturbed. It is not necessary to remove all liquid as the remainder will be removed in the next step.
10. Centrifuge at 12,000 RCF for 5 minutes at 4 ±1°C. Use little or no braking force to prevent the viral pellet from being disturbed.

11. Promptly and gently, remove rotor from centrifuge. Use a pipette to carefully remove and discard the remaining supernatant from each tube. Do not contact or disturb pellet with pipet tip.
12. Use a pipette to transfer 0.4 mL nuclease-free water to one of the tubes containing a viral pellet.
13. Resuspend the viral pellet by repeatedly pipetting to rinse the inside surface of the tube around the expected location of the pellet. Rinse a wide area surrounding the expected pellet location to ensure all precipitated virus is recovered. Some of the resuspension liquid will adhere to the sides of the tube. This liquid will be recovered in the next step.
14. Flash spin the tube at 1,000 to 3,000 RCF to collect all the liquid at the bottom of the tube.
15. Pipette up and down several times to homogenize the concentrate, then transfer the entire volume to the second tube containing a viral pellet. Repeat steps 13 and 14 to resuspend the pellet and collect all the liquid.
16. Pipette up and down several times to homogenize the concentrate, then transfer the entire volume to the third tube containing a viral pellet. Repeat steps 13 and 14 to resuspend the pellet and collect all the liquid.
17. Transfer the recovered concentrate to a RNase free microtube. The volume should be approximately 0.4 mL
18. Proceed with extraction immediately, or store concentrate overnight at –25 to –15°C.

Extraction of RNA from Concentrates*:*

Procedural Notes:

- Extraction of nucleic acids from the wastewater concentrate is performed according to the instructions in the Water DNA/RNA Magnetic Bead Kit product insert (available here: https://www.idexx.com/files/product-insert-water-dna-rna-magnetic-bead-kit.pdf)
- A Positive Control and Negative Control will be included with each set of samples as described below in the "*Quality Control Procedures*" section below.

Preparation of Lysis/Binding Working Solution and Sample Lysis

1. Calculate the amount of Working Solution required. Prepare an additional 10% to allow for pipetting loss:

Reagent	Volume per Sample
Binding Buffer (BB)	500 µL
Proteinase K (PK)	50 µL
Magnetic Beads (MB)	20 µL

2. Prepare Working Solution by mixing reagents in the order listed above. Mix beads to ensure homogenous solution prior to pipetting. The Working Solution can be stored at 18 to 26°C for up to 1 hour prior to use. Longer storage time may result in diminished lysis efficiency.
3. Mix Working Solution thoroughly with inversion before use to ensure that beads are in a homogenous solution.
4. Add 570 ±10 µL Working Solution to a microcentrifuge tube.
5. Prior to addition, mix the wastewater concentrate by pipetting up and down several times.
6. Add 200 ±5 µL of wastewater concentrate to the sample tube and mix well.
7. Incubate 10 minutes at 58 ±2°C in a dry heat block. Periodically vortex tube to keep magnetic beads suspended and break apart any aggregates that may form.
8. Flash spin tubes to collect all liquid into the bottom of the tube.

Wash Magnetic Beads

1. Separate the magnetic beads by placing the sample tube on the magnetic separator. Wait 1–2 minutes until all the beads have been attracted to the magnets. Remove supernatant with a pipette, taking care not to disturb the magnetic beads.
2. Remove the tube from the magnetic separator. Add 500 ±20 µL Wash 1 and mix well by pipetting up and down to completely resuspend the beads.
3. Repeat Step 1 to remove Wash 1.
4. Remove the tube from the magnetic separator. Add 500 ±20 µL Wash 2 and mix well by pipetting up and down to completely resuspend the beads.
5. Repeat Step 1 to remove Wash 2.
6. Repeat Step 4 to resuspend the beads in Wash 2 again.
7. Repeat Step 1 to remove Wash 2.
8. Dry the beads in the open tube for 5–10 minutes at 18–26°C.

Elute Nucleic Acids

1. Add 100 µL Elution Buffer to the sample tube and mix well by pipetting up and down to completely resuspend the beads.
2. Incubate 10 minutes at 18–26°C. Periodically mix tube to keep magnetic beads suspended.
3. Separate the magnetic beads by placing the tube on the magnetic separator. Wait 1–2 minutes until all the beads have been attracted to the magnets.
4. Transfer the supernatant containing purified nucleic acid to a new tube.
5. Store the purified nucleic acid at 2–8°C for use within 6 hours, or at –25 to –15°C for up to 1 month, or at –80°C for long-term storage.

Extraction control samples:

The following control samples will be processed in parallel with each set of wastewater samples to be tested.

- Extraction Positive Control (AccuPlex SARS-CoV-2 Verification Panel): An extraction control containing SARS-CoV-2 RNA will be extracted and tested along with each set of samples that are processed. The extraction positive control is used to demonstrate successful recovery of RNA during the extraction process and should test positive for the SARS CoV-2 target. The AccuPlex Verification Panel contains a recombinant virus engineered to harbor SARS-CoV-2 RNA sequences; this provides a full extraction control that requires lysis and recovery of single-stranded RNA from an encapsulated viral particle to produce a positive result. To perform the extraction positive control, 200 µL of the "Member 1" dilution provided in the Verification Panel is used as the sample during extraction. Results are interpreted according to the table in the "PCR Results Interpretation" section.

- Extraction Negative Control (PCR Grade Water): A "no template" (negative) control containing no nucleic acids will be extracted and tested with each set of wastewater samples to verify the absence of nucleic acid contamination in the extraction reagents and materials. The Extraction Negative Control should give a negative result for the SARS CoV-2 target. To perform the extraction negative control, 200 µL of PCR Grade Water will be used as the sample during extraction.

RT-PCR Quantification:

Reaction Setup

1. Preparation of the PCR Mix.
    a. Mix the thawed RNA Master Mix (MMx) by inversion or gentle vortex.
    b. The RNA MMx is a viscous solution; always pipette it slowly.
    c. To prepare the PCR Mix add 10 µL SARS-CoV-2 Mix and 10 µL RNA MMx for each reaction. Include reagents for all control reactions.
    d. When preparing the PCR Mix, first pipette SARS-CoV-2 Mix into the tube and then add the RNA MMx.
    e. Pipette up and down a few times to rinse the MMx pipette tip.
    f. Gently vortex the solution to ensure the components are mixed well.
    g. Load the PCR plate within 20 minutes or store at 2 to 8°C for up to 4 hours. The PCR Mix can be stored at –25 to –15°C for up to 2 weeks. Protect from light.
2. Slowly pipette 20 µL of the PCR Mix into the required wells of the multi-well plate.
3. For each wastewater sample, add 5 µL of purified RNA to the appropriate well. The final reaction volume is 25 µL.
4. For each control reaction, add 5 µL to the appropriate well. The final reaction volume is 25 µL.

a. Include the PCR Positive Control (5 µL PC), PCR Negative Control (5 µL PCR Grade Water), Extraction Positive Control (5 µL), and Extraction Negative Control (5 µL) for each test run.
b. Include 5 µL of each dilution of reference material that will be used to produce a standard curve for quantification (see below).

5. Cover the plate and briefly spin the plate, if necessary, to settle contents and remove air bubbles.
6. Perform real-time PCR with settings and cycling program below. Ensure the instrument is set to record fluorescence following the 60°C amplification step.

Settings for Reporter and Quencher

Target	Reporter	Quencher
SARS-CoV-2	FAM™	BHQ® (none)
Passive Reference	ROX™	N/A

Cycling Program (used for all instruments)

Step	Temperature	Time	Cycles
Reverse transcription (RT)	50°C	15 min.	1
Denaturation	95°C	1 min.	1
Amplification**	95°C 60°C	15 sec. 30 sec.	45

PCR control reactions:

The following control reactions will be performed in parallel with each set of wastewater samples that are extracted and tested in RT-PCR.

- PCR Positive Control (PC): A positive template control is needed to confirm the PCR plate is valid. The positive control will be included on each PCR run and should test positive for the SARS CoV-2 target. To perform the PCR positive control, 5 ul of a linear DNA containing SARS-CoV-2 N1 target region will be added to a RT-PCR reaction. This material is provided in the Water SARS-CoV-2 RT-PCR Test kit.

- PCR Negative Control (PCR Grade Water): A “no template” (negative) control is needed to confirm the PCR plate is valid. PCR Grade water is used and will be included for each PCR run. The negative control should test negative for the SARS CoV-2 target. To perform the PCR negative control, 5 ul of a PCR Grade Water will be added to a RT-PCR reaction. This material is provided in the Water SARS-CoV-2 RT-PCR Test kit.

- Extraction Positive Control: 5 ul of the Extraction Positive Control sample (see “*Extraction control samples*” above) is added to a RT-PCR reaction.

- Extraction Negative Control: 5 ul of the Extraction Negative Control sample (see “*Extraction control samples*” above) is added to a RT-PCR reaction.

Standard Curve

- A standard curve for quantification of SARS-CoV-2 and OC43 will be prepared using at least three (3) ten-fold dilutions of the respective target reference material in nuclease-free water.
- Each standard curve will be analyzed by RT-PCR at the same time as the unknown samples to be quantified.
- The quality of the standard curve will be assessed by plotting the results as a linear regression of Ct versus the log of the RNA quantity and evaluating the slope and coefficient of correlation (R^2).

Data Analysis and Results Interpretation

- All test controls will be examined prior to interpretation of results. If the controls are not valid, the results cannot be interpreted. See “*Quality Assurance Plan*” section below .

- To obtain the most appropriate Ct values, analysis for SARS CoV-2 target will be performed by manually setting the fluorescence threshold. The threshold will be adjusted to the inflection point for the exponential phase of the curve and above background signal. This will be done while viewing all amplification curves for a given run, on a logarithmic scale. The same procedure will be used for analysis of each PCR run.

Control/Sample	SARS-CoV-2 FAM Ct Value	SARS-CoV-2 FAM Result
Positive Control	<40	Positive
PCR Negative Control	No Signal	Negative
Extraction Positive Control*	<40	Positive
Extraction Negative Control*	No Signal	Negative
Wastewater Sample	<40	Positive

Quality Assurance Plan

Each RT-PCR run requires the following specific validity criteria to be met for the results to be considered valid.

Control reactions:

- Expected results for the (1) PCR positive control, (2) PCR negative control, (3) Extraction positive control, and (4) Extraction negative control shown in the table above must be obtained for each PCR run in order for the run to be deemed valid. If the plate controls are not valid, the results are not valid, and the testing must be repeated.

Standard Curve

- Dilutions used must encompass the range of Ct results to be analyzed and for which quantitative data will be reported.
- The quality of each standard curve must display acceptable slope and coefficient of correlation (R^2).

Unknown samples

- Each sample is considered valid when analyzed on a plate demonstrating the expected control results as described in the table above.
- Positive samples should display a characteristic amplification curve.

University of Wisconsin – Madison
Adelaide Roguet, Sandra McLellan and Shuchen Feng

Centrifugation method
- Add 45 mL in 50 mL tube
- Add 5 uL of BCoV (~150,000 cp/ul)
- Invert for 30 min at 4ºC
- Centrifuge samples at 4700xg for 45 minutes at 4ºC.
- Transfer the supernatant in a new clean 50 mL tube
- Add 0.53 g NaCl (0.2 M NaCl final) + 3.6 g Polyethylene glycol (PEG) (8% PEG 8000 w/v final)
- Invert for 2h minimum at 4ºC
- Place samples overnight at 4ºC refrigerator
- The next day, centrifuge samples at 4700xg for 45 minutes at 4ºC
- Discard the supernatant and resuspend the pellet in 800 uL of 1X TE buffer (pH=8).
- Extract nucleic acids from 140 uL of the resuspended pellet using the QIAamp Viral RNA Mini Kit (Qiagen) according to manufacturer's instructions.
- Elution in 60 uL

One-Step
RT-ddPCR using the One-Step RT-ddPCR Advanced Kit for Probes (BioRad 1864021) according to manufacturer's instructions**.** We use 5.5 uL of RNA per 20 uL RT-PCR reaction.

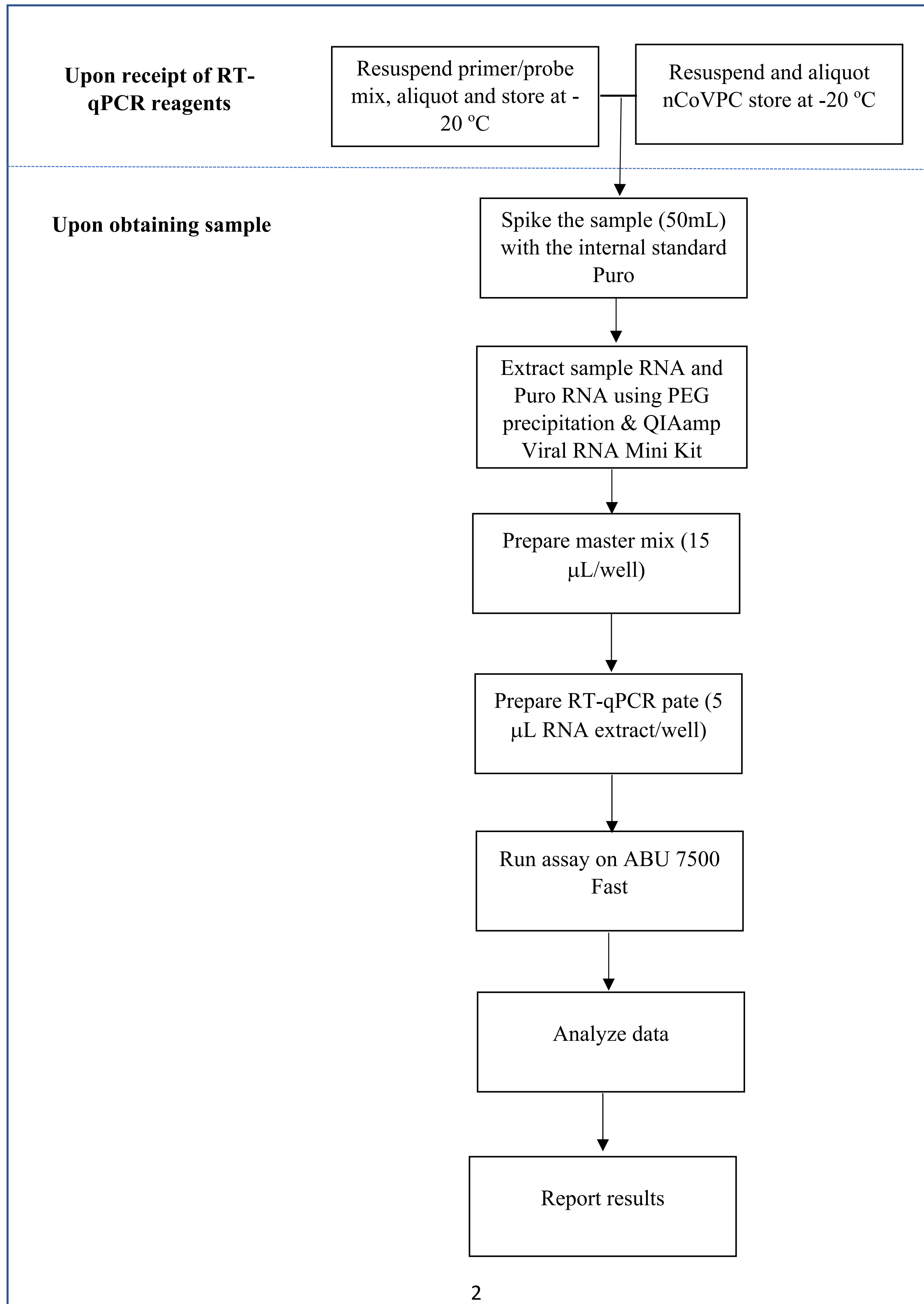
Assays
- SARS-CoV-2 assay: 2019-nCoV CDC ddPCR Triplex Probe Assay (BioRad: 12008202). Use CDC's N1 & N2 sequences.
- BCoV probe: CCTTCATATCTATACACATCAAGTTGTT (ordered from IDT)
- HepG probe: CCTTCATATCTATACACATCAAGTTGTT (ordered from IDT)

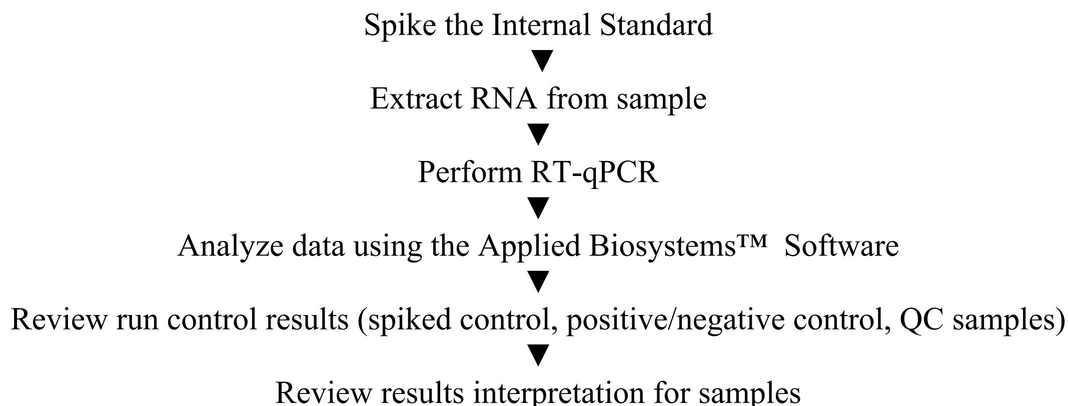
PS: BCoV (spiked before the concentration step or before freezing the samples) is quantified to estimate the recovery rate of the whole procedure. HepG (spiked before the RT step) is quantified to estimate the PCR inhibition during the RT-PCR step.

Standard Operating Procedure for Quantification of SARS-COV-2 in Wastewater

University of Missouri
Marc Johnson and Chung-Ho Lin

Workflow





The workflow begins with fortification of the internal standards (spike), non-infectious retroviral VLPs (which have the same size and properties as SARS-COV-2) that contain an RNA genome with an engineered sequence, followed by the extraction of the nucleic acid from the wastewater samples collected from the facilities. Nucleic acids are isolated, concentrated and precipitated by polyethylene glycol (PEG) and the RNA will be purified from the extracts using the QIAGEN QIAamp Viral RNA Mini Kit. The purified RNA is reverse transcribed into cDNA and amplified using the ThermoFisher TaqPath™ RT-qPCR Master Mix and Applied Biosystems™ 7500 Fast Real-Time PCR Instrument.

In the process, the probes anneal to the specific SARS-CoV-2 target sequences located between unique forward and reverse primers for the following genes:

- N1 (N protein)
- N2 (N protein)
- Puro (internal standard)

During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle by the real-time PCR instrument.

The data are analyzed, then interpreted by the Applied Biosystems™ Software.

Materials Required

Primers and Probes:

Reagent	Part#	Description	Quantity/Tube	Reactions/Tube
2019-nCoV_N1	10006770	2019-nCoV_N1 CDC EUA Kit (FAM) Combined Primer/Probe Mix	22.5 nmole	1000
2019-nCoV_N2	10006770	2019-nCoV_N2 CDC EUA Kit (FAM) Combined Primer/Probe Mix	22.5 nmole	1000

Internal Standard (Puro)	
Forward primer	5'CCCGATCGCCACATAGAGC 3'
Reverse primer	5' CCATTCTAGGGCCAATTTCTGC 3'
Taqman Probe	5' CGGTAAGGTTTGGGTCGCCGAC 3'
	Reporter dye: VIC Quencher: QSY Probe Size: 20,000 pmole

Positive Control

Reagent Label	Part #	Description	Quantity	Notes
nCoVPC 2019-nCoV Noninfectious Positive Control (nCoVPC)	10006625	Control plasmids contain the complete nucleocapsid gene from 2019-nCoV, SARS, and MERS. The 2019-nCoV CDC qPCR Probe Assays target regions within the nCoV nucleocapsid gene which is present in the 2019-nCoV_N Positive Control plasmid	1 tube (200K/µL; 1mL)	nCoV-N control: Severe acute respiratory syndrome coronavirus 2 isolate Wuhan-Hu-1, complete genome (GenBank: NC_045512.2)

RT Enzyme Mastermix

Reagent	Part#	Quantity
Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix, CG	A15299 (Thermo Fisher)	1000 reactions

RNA Extraction

Filters and Extraction Kit	Manufacturer	Catalog No.
MilliporeSigma™ Steriflip™ Sterile Disposable Vacuum Filter Units	MilliporeSigma™	SCGP00525
QIAamp Viral RNA Mini Kit (250x)	QIAGEN	52906

Equipment and Consumables Required

 Vortex mixer
 Microcentrifuge
 Micropipettes (2 or 10 µL, 200 µL and 1000 µL)
 Multichannel micropipettes (5-50 µl)
 Racks for 1.5 mL microcentrifuge tubes
 2 x 96-well -20°C cold blocks
 7500 Fast Real-Time PCR Systems with SDS 1.4 software (Applied Biosystems; catalog #4406985 or #4406984)
 Extraction systems (instruments): QIAGEN EZ1 Advanced XL
 Molecular grade water, nuclease-free
 10% bleach (1:10 dilution of commercial 5.25-6.0% hypochlorite bleach)
 DNA*Zap*TM (Ambion, cat. #AM9890) or equivalent
 RNAse AwayTM (Fisher Scientific; cat. #21-236-21) or equivalent
 Disposable powder-free gloves and surgical gowns
 Aerosol barrier pipette tips
 1.5 mL microcentrifuge tubes (DNase/RNase free)
 0.2 mL PCR reaction plates (Applied Biosystems; catalog #4346906 or #4366932)
 MicroAmp Optical 8-cap Strips (Applied Biosystems; catalog #4323032)

Sample Collection, Handling and Storage

1. Collecting Wastewater: Samples
   3 x 50mL of 24 hrs composite waste water samples are collected

2. Transporting Samples

   Water samples are packaged with ice (2-8ºC), shipped, and transported to MU campus within 24 hrs.

3. Storing Samples

Water samples can be stored at 2-8ºC for up to 72 hours after collection.

• If a delay in extraction is expected, store specimens at -70ºC or lower.

• Extracted nucleic acid should be stored at -70ºC or lower.

RNA Extraction

1. Wastewater samples (approximately 50 ml) are filtered through a 0.22 micron filter.

2. Approximately 36 mL of filtered wastewater is mixed with 12 mL of 50% (W/V) PEG, 1.2 M NaCl, mixed until solution is homogeneous, and incubated for at least 2 hours at 4ºC.

3. Samples are then centrifuged at 12,000xg for 2 hr.

4. Supernatant is removed and RNA is extracted from the pellet using Qiagen Viral RNA extraction kit following the manufactures instructions and eluted in a final volume of 60 microliters.

5. Store the samples at -20 oC if they can't be processed immediately.

Prepare RT-*q*PCR Reactions

1) In the reagent set-up room clean bench, place RT-qPCR buffer, enzyme, and primer/probes on ice or cold-block. Keep cold during preparation and use.

2) Mix buffer, enzyme, and primer/probes by inversion 5 times.

3) Centrifuge reagents and primers/probes for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.

4) Label one 1.5 mL microcentrifuge tube for each primer/probe set.

5) For each primer/probe set, calculate the amount of each reagent to be added for each reaction mixture (N = # of reactions).

Thermofisher TaqPath™ 1-Step RT-qPCR Master Mix

Step #	Reagent	Vol. of Reagent Added per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/Probe Mix	N x 1.5 µL
3	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	N x 5.0 µL
	Total Volume	**N x 15.0 µL**

8) Dispense reagents into each respective labeled 1.5 mL microcentrifuge tube. After addition of the reagents, mix reaction mixtures by pipetting up and down. Do not vortex.

9) Centrifuge for 5 seconds to collect contents at the bottom of the tube, and then place the tube in a cold rack.

10) Set up reaction strip tubes or plates in a 96-well cooler rack.

11) Dispense 15 µL of each master mix into the appropriate wells going across the row as shown below (Figure 1):

	1	2	3	4	5	6	7	8	9	10	11	
A-N1	S1-N1A	S2-N1A	S3-N1A	S4-N1A	S5-N1A	S6-N1A	S7-N1A	S8-N1A	S9-N1A	S10-N1A	S11-N1A	S12-N1
B-N1	S1-N1B	S2-N1B	S3-N1B	S4-N1B	S5-N1B	S6-N1B	S7-N1B	S8-N1B	S9-N1B	S10-N1B	S11-N1B	S12-N1
C-N2	S1-N2A	S2-N2A	S3-N2A	S4-N2A	S5-N2A	S6-N2A	S7-N2A	S8-N2A	S9-N2A	S10-N2A	S11-N2A	S12-N2
D-N2	S1-N2B	S2-N2B	S3-N2B	S4-N2B	S5-N2B	S6-N2B	S7-N2B	S8-N2B	S9-N2B	S10-N2B	S11-N2B	S12-N2
E-Puro	S1-Puro	S2-Puro	S3-Puro	S4-Puro	S5-Puro	S6-Puro	S7-Puro	S8-Puro	S9-Puro	S10-Puro	S11-Puro	S12-Pu
F N1	N1-100K	N1-100K	N1-10k	N1-10k	N1-1k	N1-1k	N1-100	N1-100	N1-10	N1-10	NTC	
G N2	N2-100k	N2-100K	N2-10k	N2-10k	N2-1k	N2-1k	N2-100	N2-100	N2-10	N2-10	NTC	
H	N1 S-13	N1 S-13	N2 S-13	N2 S-13	Puro S-13	O-QC	O-QC	Puro NTC	Puro 5x10^6	Puro 500k	Puro 50k	

Figure 1. Plate layout for the RT-qPCR

12) Prior to moving to the nucleic acid handling area, prepare the No Template Control (NTC) reactions for in the assay preparation area.

13) Pipette 5 µL of nuclease-free water into the NTC sample wells (Figure 1). Securely cap NTC wells before proceeding.

14) Cover the entire reaction plate and move the reaction plate to the specimen nucleic acid handling area.

15) Pipette 5 µL of the RNA extract from each sample into each well.

16) Seal the plate with MicroAmp™ Optical Adhesive Film, vortex the plate for 10 seconds to ensure proper mixing, then centrifuge for 1 minute at 2000 rpm to collect the liquid at the bottom of the reaction plate.

Perform RT-PCR using the Applied Biosystems™ 7500 Fast Real-Time PCR Instrument

Dye calibration for the 7500 Real-Time PCR Instrument series

A maintained instrument will be calibrated for many dyes. In addition to those dyes, the instrument operator must calibrate the instrument for ABY™ dye and JUN™ dye that are used with this kit. For all other assays, refer to the standard calibration process.

Set up and run the 7500 Fast Real-Time PCR Instrument

This procedure is specific for the 7500 Fast Real-Time PCR Instrument using SDS Software v1.5.1. For more information, see the documents listed in “Related documentation” on page 73.

1. Using SDS Software v1.5.1, access the appropriate template file.

    a. Create a new experiment.

    b. In the Template field, browse to, then open the SDT file that you transferred in “Transfer the template (SDT or EDT) file for the 7500 Fast Real-Time PCR Instrument” on page 44.

2. Confirm the run settings in the template and adjust as necessary.

    • Assay: Standard Curve (Absolute Quantitation)
    • Run mode: Standard 7500
    • Passive reference: None
    • Sample volume: 20 µL

3. Confirm that the reporter dye and the detector pairs are correct in the Detector Manager in the Toolsmenu.

Name	Report Dye	Quencher Dye
N1	FAM	None (MGB)
N2	FAM	None (MGB)
Puro	VIC	QSY

4. Confirm that the targets above are assigned to each well in the plate layout.

5. Confirm the labeling of the control wells.

    • The template included positive control and one negative control assigned to wells for reference.
    • Move the control well assignments by copying the existing control wells and pasting them according to their location on the physical plate.

6. For wells with a positive control, confirm that **Task** is set to **Standard**.

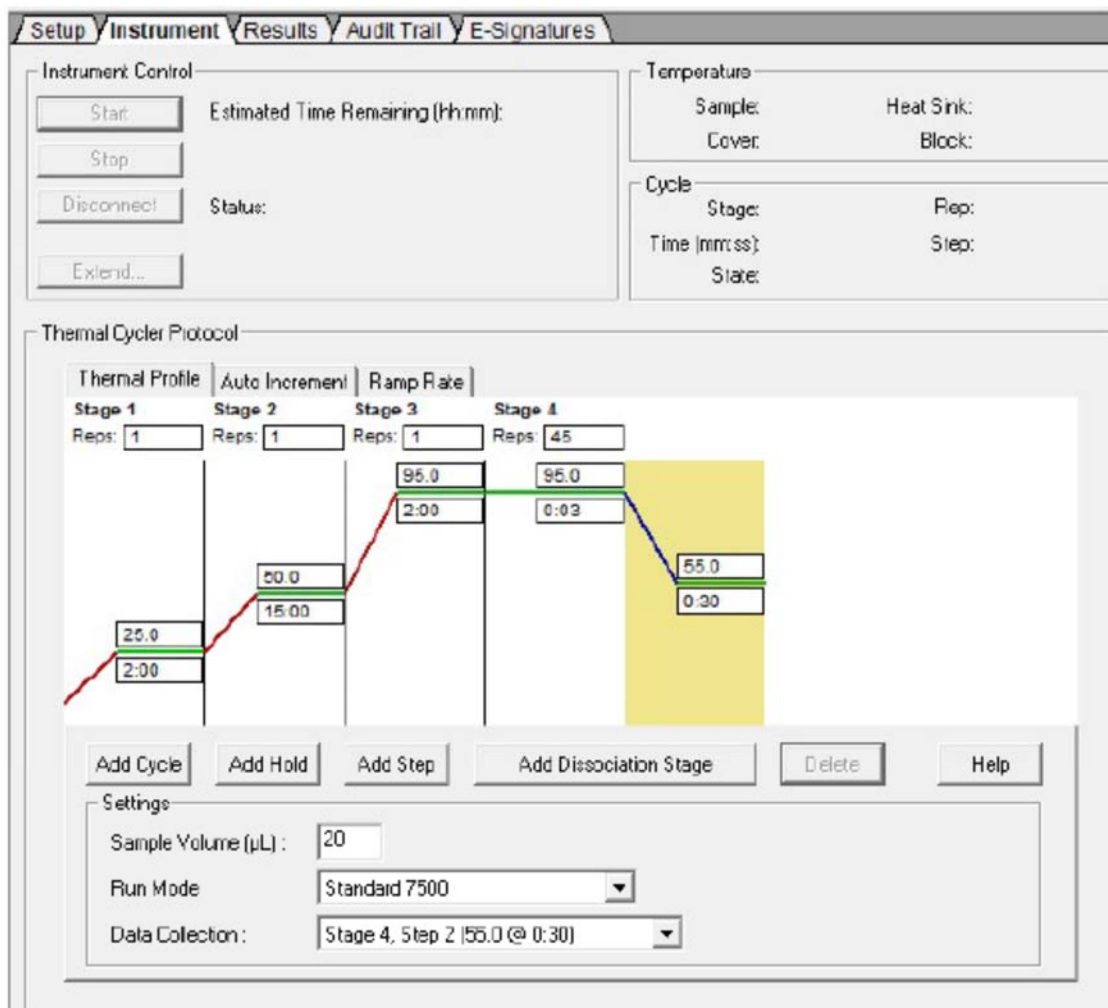
7. For wells with a negative control, confirm that **Task** is set to **NTC**.

8. Edit the plate layout to assign a unique sample name to each well with a patient sample in the physical plate.

For wells with a wastewater sample, confirm that Task is set to Unknown for all detectors.

9. Confirm the thermal protocol.

a. In Stage 1, Set to 2 min at 25°C; 1 Rep.
b. In Stage 2, Set to 15 min at 50°C; 1 Rep.
c. In Stage 3, Set to 2 min at 95°C, 1 Rep.
d. In Stage 4, Step 1 set to 3 sec at 95°C.
e. In Stage 4, Step 2 set to 30 sec at 55.0°C.
f. In Stage 4, Reps should be set to 45.
g. Under Settings (Figure 12), bottom left-hand box, change volume to 20 µL.
h. Under Settings,
Run Mode selection should be Standard 7500.
i. Step 2 of Stage 4 should be highlighted in yellow to indicate data collection (see Figure 12).



10.Click **Save As**, enter a file name, then click **Save**.

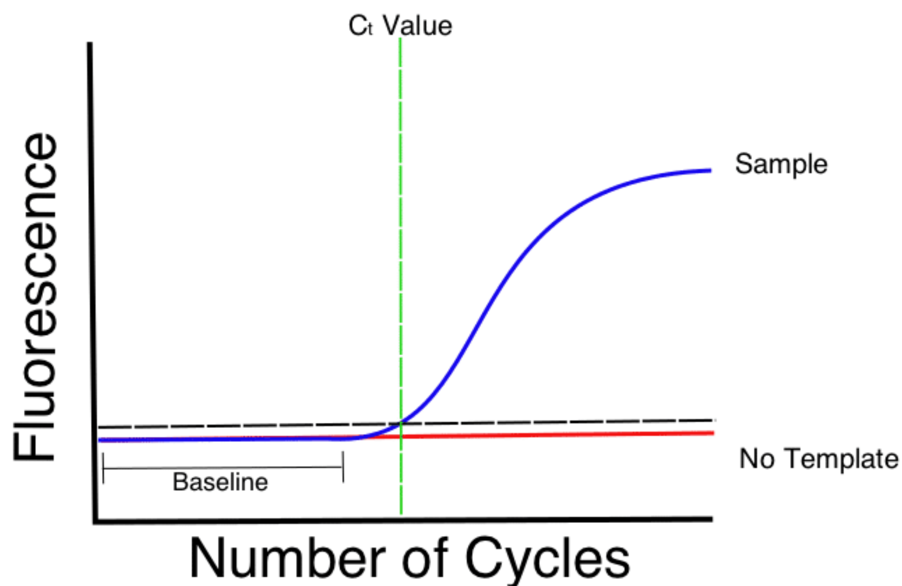
11. Reopen the file to connect the computer to the instrument, load the plate, then start the run on the real-time PCR instrument.

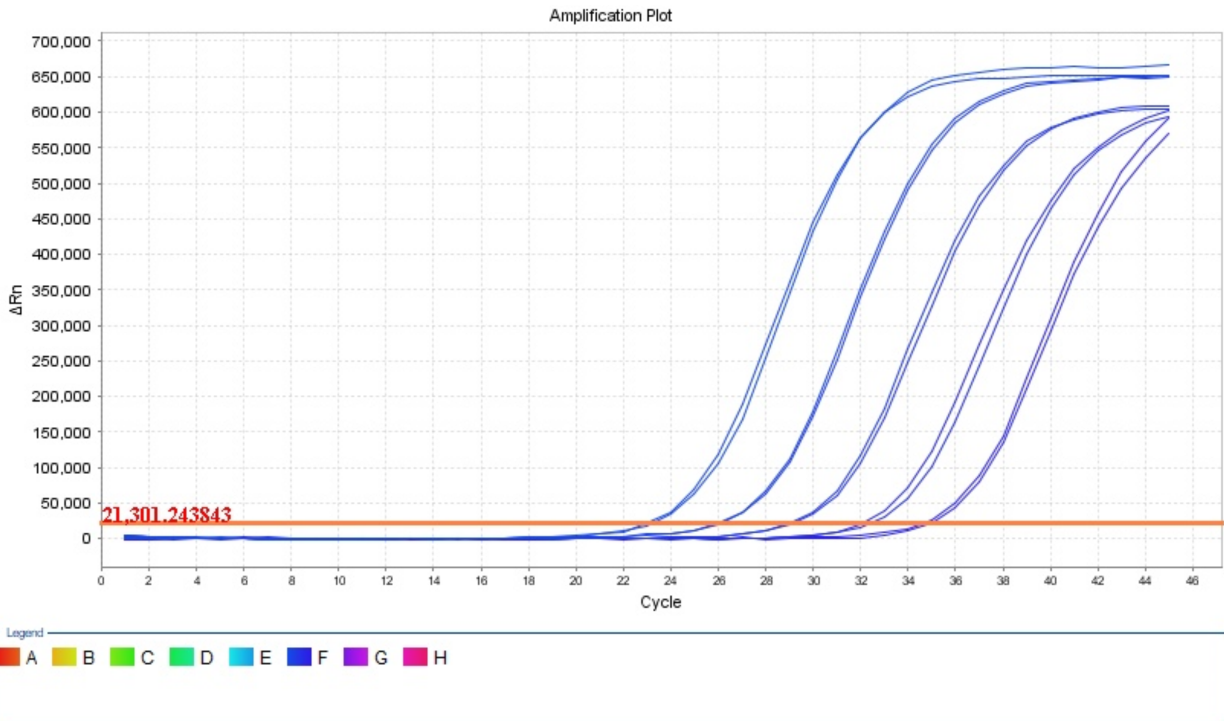
12. After the instrument run is complete, open the SDS file in SDS Software v1.4.1. Analyze, then save the file.

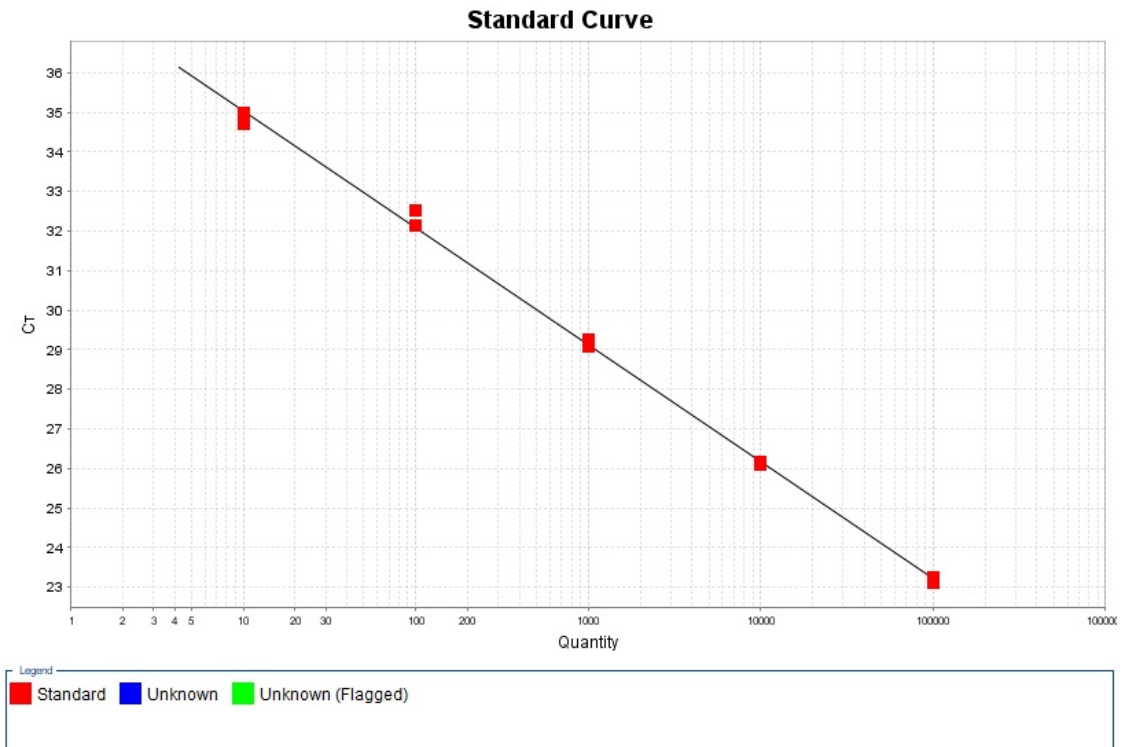
Data Analysis

1) After the run has completed, select the Results tab at the upper left corner of the software.
2) Select the Amplification Plot tab to view the raw data (Figure 17).

The copies numbers of the genetic targets N1 and N2 of SARS-CoV-2 in wastewater samples will be determined by the developed calibration equation between the Ct (cycle threshold) and known copy numbers in the standards. The Ct defined as the number of cycles required for the fluorescent signal to cross the threshold (i.e. exceeds background level). Ct levels are inversely proportional to the amount of target nucleic acid in the sample (i.e. the lower the Ct level the greater the amount of target nucleic acid in the sample). The Ct values generated from the standards and samples from each experiment will be compared.



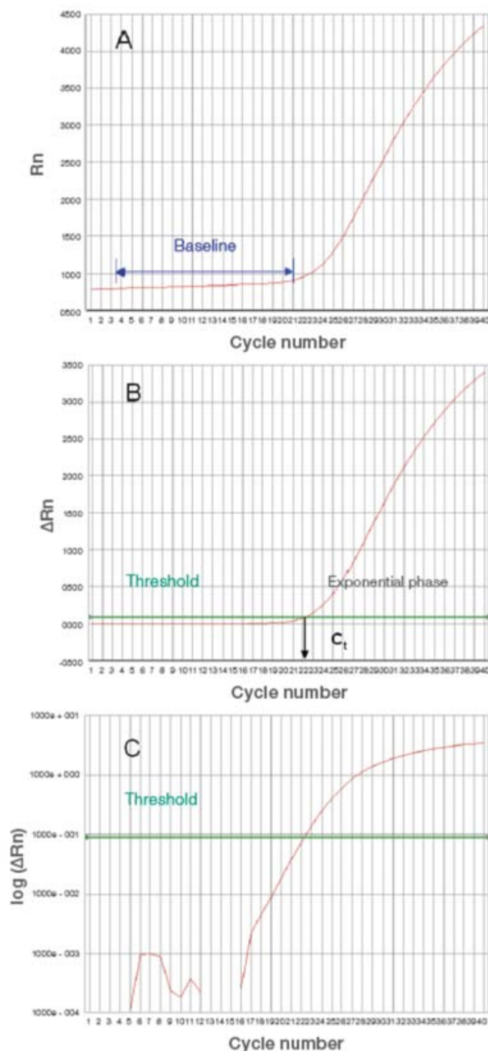

Amplification Plot
ΔRn
700,000
650,000
600,000
550,000
500,000
450,000
400,000
350,000
300,000
250,000
200,000
150,000
100,000
50,000
0
21,301.243843
0 2 4 6 8 10 12 14 16 18 20 22 24 26 28 30 32 34 36 38 40 42 44 46
Cycle
Legend
A B C D E F G H


Standard Curve
CT
36
35
34
33
32
31
30
29
28
27
26
25
24
23
1 2 3 4 5 10 20 30 100 200 1000 10000 100000
Quantity
Legend
Standard
Unknown
Unknown (Flagged)

Measurement Performance Criteria

The samples should provide a written description of facility, location and sampling time at the site. This information is to be recorded on the chain of custody record.

The coy numbers will be determined by the values of the Ct (threshold cycle Figure 2A). The Ct is the intersection between an amplification curve and a threshold line (Figure 2B). The threshold must be set in the linear phase of the amplification plot in Figure 2C. The Ct value increases with a decreasing amount of template. Detection limits, accuracy, and precision will be calculated.



Quality Control Requirements

Quality control requirements must be performed to monitor reagent, extraction and assay performance. Duplicate samples and standards should be included for each RT-qPCR analysis. All positive controls will be tested prior to running diagnostic samples with each new kit lot to ensure all reagents and kit components are working properly. Both negative control (NTC), and the appropriate positive control (nCoVPC) should be included in each amplification and detection run. The internal control was generated using non-infectious retroviral VLPs (which have the same size and properties as SARS-COV-2) that contain an RNA genome with an engineered sequence. The internal control engineered virus with Puro gene will be fortified into the wastewater sample to control for assay quality and extraction efficiency. Five points (with duplicates) calibration equations will be developed for quantification the copy numbers of the SARS-CoV-2 in wastewater samples. The efficiency of RT-qPCR will be calculated from the slope of a standard curve (S) using the recommended value -3.3 +/- 10% with coefficients > 0.95. A low efficiency indicates that primers and/or probes are performing poorly. No single efficiency (as a percentage) has been set as a limit for data to be acceptable, although 90% or greater is an accepted norm. The recovery of the internal standard should be greater than 10%, which conformed to ISO15216-2:2017 standards (>1%).

Since there are no synthetic SARS-COV-2 RNA available at this moment, the spiked internal standard non-infectious retroviral VLPs genome with an engineered RNA sequence was used as the positive controls to assess the PCR inhibitory effects.

Limit of detection (LOD)

The LOD study established the lowest SARS-CoV-2 viral concentration (Genomic Copy Equivalents or GCE) that can be detected by the method in a particular specimen type at least 95% of the time.

Recovery Rates

Each waste water sample was spiked with purified RNA with 4.6 x 10^6 copies of Puro [internal standards (spike), non-infectious retroviral VLPs which have the same size and properties as SARS-COV-2) that contain an RNA genome with an engineered Puro sequence].

The recovery rate of each sample was determined by:

Recovery Rate = Copy number of Puro in each sample/(4.6 x 10^6) x 100%

SARS CoV-2 RT-qPCR SOP
Version 4

NYC DEP Sewage Monitoring

Revision History

Version	Date	Version Notes/Comments
1	2020/05/13	Original revision for preliminary standard curve work
2	2020/06/10	Revised to include full workflow before preliminary testing (in progress)
3	2020/08/10	Revised to perform CUNY protocol, including one step RT qPCR protocol adapted from CDC (versions 1 and 2 were the NYU protocol)
4	2020/08/18	Revised to include change in RNA extraction kit (QIAamp Viral RNA Mini Kit)

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1. SOP Overview

This SOP describes the analysis by RT-qPCR of wastewater influent samples to detect RNA from the coronavirus SARS CoV-2 (which causes COVID-19). Quantification of SARS CoV-2 RNA is accomplished through the **N1/N2 assay** (see Section 1.3 for details). Bovine coronavirus (BCoV) is used as a control to assess recovery from the entire process and is quantified through the **BCoV control assay**.

1.1. Summary

This document describes the steps related to sample processing, virus concentration, RNA extraction, and RT-qPCR:

1. Pasteurization and Solids Separation
2. Supernatant Filtration
3. Virus Concentration (Liquid Samples Only)
4. RNA Extraction
5. RT-qPCR: N1 and N2 Targets
6. RT-qPCR: BCoV Recovery Control

Please see Section 1.6 for safety guidelines.

1.2. Terminology

Controls

Recovery Control - A known amount of a recovery control can be spiked into the sample at desired points throughout the SOP in order to assess recovery (i.e. loss due to SOP steps). For any given spike point along the workflow, recovery results capture loss due to all downstream steps, though comparisons of replicates of the same sample spiked at different points may be used to assess loss due to individual steps.

In this SOP, bovine coronavirus (BCoV) is used as a control. The amount of BCoV recovered at the end of the protocol is assessed using a relative qPCR assay targeting the transmembrane gene of BCoV (Decaro et al. 2008). Possible additional points for spiking BCoV include:

- Before pasteurization and after pasteurization- a comparison of the relative amount of BCoV recovered in samples spiked before and after pasteurization indicates loss due to pasteurization.
    - On their own, samples spiked before pasteurization can be used to assess loss due to the entire process.
    - On their own, samples spiked after pasteurization can be used to assess loss due to all steps following pasteurization.

(qPCR controls)
Negative control - contains all reaction components for PCR except one
No-template control - no template (i.e., extracted nucleic acids from standard or sample) added; nuclease-free water is added instead. Used to check contamination and primer dimer formation.

Positive control - known amount of template added (e.g. 2019-nCoV_N_Positive Control); to check that the primers, probe, and reaction conditions worked and can detect the target. For this SOP, the 2019-nCoV_N_Positive Control serves as both a positive control and the standard.

gBlock gene fragments (synthesized by IDT) - Double-stranded, linear, nucleic acids. In this protocol, a gBlock gene fragment of the BCoV transmembrane gene is used as a standard in qPCR for the BCoV control.

Master mix - premixed solution of most of the required components for RT-qPCR. The purchased master mix for this protocol TaqPath™ 1-Step RT-qPCR Master Mix (4x Concentration) - contains thermostable MMLV reverse transcriptase, dNTPs, UNG, ROX™ dye, and thermostable Fast DNA polymerase, facilitating easy reaction set up—just add user-supplied assay and sample.

Multiplex - when multiple genes (targets), each with their own unique probe, are detected in the same reaction (tube/well). This protocol does not currently use multiplexing.

Plasmid - small, circular DNA molecule; in this protocol, the 2019-nCoV_N_Positive Control (used as a positive control and for standard curves for the N1 and N2 targets) is a plasmid that contains the complete nucleocapsid gene from 2019-nCoV.

Primer - Short strands of nucleic acid (for this protocol, DNA) designed to attach (anneal) to two regions flanking the target region to be amplified. Once annealed, primers allow DNA polymerase to begin replication from either end of the target region (during the elongation or extension step). Both a forward (f) and reverse (r) primer are needed. Because this protocol looks to amplify two target regions (N1 and N2), two sets of f and r primers are required. Another set of primers (f and r) are needed for the BCoV control assay.

Probe - labelled strands of short DNA (oligonucleotides) that bind to a spot on the target region that give off a fluorescent signal during the reaction, allowing for detection and quantification. Probes for the N1 and N2 assay: 5’ FAM / 3’ Black Hole Quencher® (BHQ). Probes for the BCoV control assay: 5’ FAM/ZEN/ 3’ IBFQ (Iowa Black FQ). Information from IDT about probes and quenchers.

quantitative **p**olymerase **c**hain **r**eaction (qPCR) - often used interchangeably with “real-time polymerase chain reaction”. qPCR allows for quantification of the target nucleic acid sequence,

usually through comparison to a standard curve. “Real-time” indicates that measurements are made in real-time, throughout amplification (rather than at the end of PCR).

Reaction mix - premixed solution that contains all of the required components for RT-qPCR except the template. The reaction mix for the N1/N2 assay contains the purchased master mix (TaqPath™ 1-Step RT-qPCR Master Mix), forward and reverse primers, probe,. Each target (N1 and N2) will require its own separate reaction mix, since the primers/probe for N1 are different from the primers/probe for N2 and this protocol does not currently use multiplexing. Similarly, for the BCoV control assay, the BCoV target will require its own separate reaction mix with its own primers/probe.

Reverse **t**ranscription (RT) - the process which generates complementary DNA (cDNA) from RNA. RT is required for this protocol because SARS-CoV-2 is an RNA virus.

Target - region of nucleic acid to be amplified (region that the primers “target”); can be a gene or part of a gene.

For the N1/N2 assay, the two targets include two regions in the SARS-CoV-2 nucleocapsid (N) gene (N1 and N2).

```
N1 Forward Primer       2019-nCoV_N1-F2019-nCoV_N1 GACCCCAAAATCAGCGAAAT
N1 Reverse Primer       2019-nCoV_N1-R2019-nCoV_N1 TCTGGTTACTGCCAGTTGAATCTG
N1 Probe                2019-nCoV_N1-P2019-nCoV_N1 ACCCCGCATTACGTTTGGTGGACC
N2 Forward Primer       2019-nCoV_N2-F2019-nCoV_N2 TTACAAACATTGGCCGCAAA
N2 Reverse Primer       2019-nCoV_N2-R2019-nCoV_N2 GCGCGACATTCCGAAGAA
N2 Probe                2019-nCoV_N2-P2019-nCoV_N2 ACAATTTGCCCCCAGCGCTTCAG
```

>2019-nCoV_N_Positive Control:

```
ATGTCTGATAATGGACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAG
TAACCAGAATGGAGAACGCAGTGGGGCGCGATCAAAACAACGTCGGCCCCAAGGTTTACCCAATAATACTGCGTCTT
GGTTCACCGCTCTCACTCAACATGGCAAGGAAGACCTTAAATTCCCTCGAGGACAAGGCGTTCCAATTAACACCAAT
AGCAGTCCAGATGACCAAATTGGCTACTACCGAAGAGCTACCAGACGAATTCGTGGTGGTGACGGTAAAATGAAAGA
TCTCAGTCCAAGATGGTATTTCTACTACCTAGGAACTGGGCCAGAAGCTGGACTTCCCTATGGTGCTAACAAAGACG
GCATCATATGGGTTGCAACTGAGGGAGCCTTGAATACACCAAAAGATCACATTGGCACCCGCAATCCTGCTAACAAT
GCTGCAATCGTGCTACAACTTCCTCAAGGAACAACATTGCCAAAAGGCTTCTACGCAGAAGGGAGCAGAGGCGGCAG
TCAAGCCTCTTCTCGTTCCTCATCACGTAGTCGCAACAGTTCAAGAAATTCAACTCCAGGCAGCAGTAGGGGAACTT
CTCCTGCTAGAATGGCTGGCAATGGCGGTGATGCTGCTCTTGCTTTGCTGCTGCTTGACAGATTGAACCAGCTTGAG
AGCAAAATGTCTGGTAAAGGCCAACAACAACAAGGCCAAACTGTCACTAAGAAATCTGCTGCTGAGGCTTCTAAGAA
GCCTCGGCAAAAACGTACTGCCACTAAAGCATACAATGTAACACAAGCTTTCGGCAGACGTGGTCCAGAACAAACCC
AAGGAAATTTTGGGGACCAGGAACTAATCAGACAAGGAACTGATTACAAACATTGGCCGCAAATTGCACAATTTGCC
CCCAGCGCTTCAGCGTTCTTCGGAATGTCGCGCATTGGCATGGAAGTCACACCTTCGGGAACGTGGTTGACCTACAC
AGGTGCCATCAAATTGGATGACAAAGATCCAAATTTCAAAGATCAAGTCATTTTGCTGAATAAGCATATTGACGCAT
ACAAAACATTCCCACCAACAGAGCCTAAAAAGGACAAAAAGAAGAAGGCTGATGAAACTCAAGCCTTACCGCAGAGA
CAGAAGAAACAGCAAACTGTGACTCTTCTTCCTGCTGCAGATTTGGATGATTTCTCCAAACAATTGCAACAATCCAT
GAGCAGTGCTGACTCAACTCAGGCCTAA
```

The target for the BCoV control is a region of the transmembrane gene (Decaro et al. 2008, correspondence with Southern Nevada Water Authority, Water Quality R&D)

BCoV Forward Primer **CTGGAAGTTGGTGGAGTT**
BCoV Reverse Primer **ATTATCGGCCTAACATACATC**
BCoV Probe **CCTTCATATCTATACACATCAAGTTGTT**

>BCoV Standard gBlock gene fragment:
GTATCAGGTTGTTTATTAGAA**CTGGAAGTTGGTGGAGTT**TCAACCCAGAA
ACA**AACAACTTGATGTGTATAGATATGAAGG**GAAG**GATGTATGTTAGGCCGATAAT**TGAGGACTACCATA
CCCTTA

Template - Extracted nucleic acids from a standard or unknown sample. For the N1/N2 assay, the standard template is plasmid DNA (2019-nCoV_N_Positive Control). For the BCoV control assay, the standard template is a gBlock gene fragment. For both assays, the sample template is cDNA reverse transcribed from RNA extracted from wastewater samples.

See also IDT's overview of qPCR terminology.

1.3. Workstations

Workstations for each step of the workflow are described below. Required equipment for each workstation can be found in Section 2.1.

It is best to have separate equipment, consumables, and PPE (e.g. designated lab coat) for each workstation to avoid contamination. **When working in each station, clean gloved hands by carefully spraying gloves you are wearing with 70% ethanol and rub gloved hands together until dry.**

1. Pasteurization

Water baths

2. Supernatant Filtration

BSC, fume hood, and other stations with vacuum lines

3. Virus Concentration

Biological safety cabinet (BSC)
Note: Before working in the biosafety cabinet, be sure that it has been decontaminated according to Section 1.5.1; open the sash and wait 5 minutes to allow the cabinet to purge.

4. RNA Extraction

BSC

5. and 6. RT-qPCR

For RT-qPCR, two workstations are maintained, each in a separate LABCONCO purifier filtered PCR enclosure:

1. **REACTION MIX PREP** - reagents only, no samples, no standards, no other controls. Where the master mix is combined with primers and probes to make the reaction mix and the wells are loaded with reaction mix.
2. **TEMPLATE PREP** -
   Where the different template forms (samples (RNA), standards (plasmid DNA), no template controls (nuclease-free water)) are diluted and loaded into the RT-qPCR plate wells.
   Note: because we do not have separate workstations separating controls and standards, thorough decontamination after working with standards/control plasmids is critical to prevent contamination of the samples during plate loading.

1.4. Nucleic Acid ***FULL DECONTAMINATION*** Procedure

Refer to this procedure throughout the SOP, whenever ***FULL DECONTAMINATION*** is called for. This procedure is meant to minimize the risk of nucleic acid contamination.

- Wear appropriate PPE (gloves, lab coat, eye protection).
- Treat all surfaces in the area (***including bench tops, pipettes, tube racks and lids, freezer handles, other touch points***) with a fresh dilution of a **10% bleach solution**.
  - Allow the bleach to contact the work surface for a minimum of **10 minutes** (or 15 minutes for extra decontamination after a spill).
    - Wipe surfaces with the bleach solution.
      - When wiping down pipettes, do not allow the decontamination solution to come in contact with the gaskets or seals.
    - If decontaminating at the end of the day, used tube racks can be soaked in a bleach solution, rinsed, and air-dried overnight.
    - Corrosive cleaning agents are not recommended for centrifuges and rotors. Instead, wipe down with 70% ethanol. Please see page 64 of the Eppendorf Centrifuge 5804 R Manual and page 33 of the Eppendorf Centrifuge 5424 R Manual for detailed cleaning and disinfecting instructions.
  - Wipe the area/equipment down with sterile/de-ionised **water**. *Note: Wiping with water is important because bleach is corrosive and can cause damage over time if not properly removed.*
- Wipe surfaces down with a paper towel dampened with **70% ethanol**.
- For extra decontamination for RT prep:
  - Apply **RNase AWAY** to surfaces. Rub the surface with a kimwipe.
  - Dry the surface with a fresh wipe. The entire surface must be completely dry because residual RNase AWAY can degrade an RNA or DNA sample.
  - Again, when wiping down pipettes, do not allow the decontamination solution to come in contact with the gaskets or seals.
  - Do not use RNase AWAY on aluminium, soft metal or gaskets and seals.
- For those workstations with a UV light, use the **UV light** to UV-sterilize pipettes and tube racks.
  - Close the sash and turn on the UV light for **5 minutes**.
  - Turn off the UV light and turn over pipettes and tube racks.
  - Close the sash and turn on the UV light for **5 additional** minutes to sterilize the other side of the pipettes and racks.
  - Turn off the UV light
- Discard and replace gloves.

(References: EPA 1696, ThermoFisher, LuminexCorp, MilliPoreSigma, van den Hurk, Mark et al. 2018).

1.4.1 Nucleic Acid ***FULL DECONTAMINATION*** Procedure: Biological Safety Cabinet (BSC)

Before working in the BCS (including for cleaning), open the sash and allow the unit to purge for 5 minutes.

Consult User Manual for detailed warnings and cleaning instructions.

In general, the BSC can be cleaned according to the steps outlined in Section 1.5. *Note, however, that bleach is corrosive. DO NOT allow disinfectants with high concentrations of free chlorine to contact the stainless steel components of the biosafety cabinet for a long period of time. Free chlorine will corrode stainless steel after extended contact (Reference).*

- Wear appropriate PPE (gloves, lab coat, eye protection).
- Treat all surfaces in the area (***including bench tops, pipettes, tube racks and lids, freezer handles, other touch points***) with a fresh dilution of a **10% bleach solution**.
    - Allow the bleach to contact the work surface for a minimum of **10 minutes**.
        - Wipe surfaces with the bleach solution.
            - When wiping down pipettes, do not allow the decontamination solution to come in contact with the gaskets or seals.
        - Wipe the area/equipment down with sterile/de-ionised **water**. *Note: Wiping with water is important because bleach is corrosive and can cause damage over time if not properly removed.*
- Wipe surfaces down with a paper towel dampened with **70% ethanol**.
- Use the **UV light** in BSC to UV-sterilize pipettes and tube racks.
    - Close the sash and turn on the UV light for **5 minutes**.
    - Turn off the UV light and turn over pipettes and tube racks.
    - Close the sash and turn on the UV light for **5 additional** minutes to sterilize the other side of the pipettes and racks.
    - Turn off the UV light
- Discard and replace gloves.

1.4.2 Nucleic Acid Full Decontamination of Oak Ridge Tubes:

- After each sample use, rinse each Oak Ridge 50mL tube with diH20 before adding ~2mL of 10% bleach. Rinse the entire tube with bleach solution by inverting it 5 times. Leave at room temp to incubate for 10 minutes.
- Submerge lids in 10% bleach for 10 minutes
- Discard bleach and rinse tubes and lids at least 5 times with diH20.
- Dry tubes and lids at 55°C for 24 hours

1.5. Safety Guidelines

Please refer to the safety data sheet (SDS), provided in the SDSs subfolder, for each reagent as well as equipment manuals for safe handling before use.

Resource about chemical use in the biosafety cabinet:
https://www.ehs.washington.edu/system/files/resources/ChemicalUseinBSC-final.pdf

All lab personnel should wear appropriate PPE at all times (including gloves, lab coats, and goggles). Gloves should be changed frequently for safety and to prevent contamination of the reagents, samples, and assays.

Safety warnings and precautions found here. All raw samples should be handled in the biosafety cabinet. (Additional safety guidelines will be established with DEP)

- Most recent CDC biosafety requirements: https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html#environmental
- Disinfecting with Bleach
- Disinfection after handling unpasteurized samples
    - EPA-approved disinfectants: https://www.epa.gov/pesticide-registration/list-n-disinfectants-use-against-sars-cov-2-covid-19

2. Materials

2.1. Equipment

Steps Used	Item	QTY	Comment
	–20°C freezer		Sample/reagent storage Keep reagents and samples in separate boxes and areas in the freezer
	–80°C freezer		Sample storage
	4°C refrigerator		Reagent storage. Keep reagents in a decontaminated area separate from any samples
all	Ice machine		
all	Ice bucket/pans	several	
all	P10, P100 or P200, and P1000 pipettes (P20 and P300 pipettes can also be used)	several	Nice to have separate for different workstations (otherwise clean and decontaminate thoroughly between uses). Adjustable rather than fixed volume pipettes are required.
all	Receptacle for used tips		Enough for each workstation
all	Vortex Mixer	several	Nice to have separate for different workstations (otherwise clean and decontaminate thoroughly between uses)
all	Small cooler/insulated box/ice bucket for ice	3	Have enough to keep samples, reagents, and standards in separate containers
4,5,6	1.5 mL/2 mL microcentrifuge tube rack	several	
1, 2, 3	Fume hood	1	

2, 5, 6	Centrifuge (up to 12,000 x g) and rotor for 50 mL tubes	1	
1	Analytical balance	1	Accessories such as lab spatula/spoon and weighing dishes also required
2	Pipette filler or graduated cylinder	1	If using a graduated cylinder, should be one that can be sterilized
1, 2, 3, 4	Biosafety cabinet	1	
3	Vortex Adapter for 6 (5/15 ml) tubes	1	
3, 4, 5, 6	Nanodrop spectrophotometer	1	
4, 5, 6	Laminar flow cabinet	2	For designated **REACTION MIX PREP** and **TEMPLATE PREP** workstations
4, 5, 6	Mini microcentrifuge	2	
5, 6	96-well PCR tube rack with lid (Cat. No. 50-550-279)	several	
4, 5, 6	96-well cold block	1	Store upside down in the freezer to prevent formation of ice in the wells
4, 5, 6	qPCR system (StepOne Plus instrument and software)	1	
5, 6	Adhesive seal applicator	1	
4	MicroAmp™ 96-Well Tray for VeriFlex™ Blocks	1	ThermoFisher 4379983
4	QIAvac 24 Plus	1	Qiagen (19413) manifold for RNA extraction

2.2. Consumables

Steps Used	Item	Supplier	Cat. No.	QTY
all	Filter tips to fit all pipette sizes	For all steps, sterile, nuclease-free (barrier/filter) tips with aerosol filters, appropriate for RNA and PCR applications should be used (and never re-used).		
all	Kimwipes	Fisher Scientific	06-666A	
all	Labeling pen/marker			Separate pens for each workstation
2	50 mL conical tubes	Thermo Fisher Scientific	339652	
4	Syringes			For resuspension of BCoV vaccine
2	Corning® 150 mL Vacuum Filter/Storage Bottle System, 0.22 µm Pore 13.6cm² CA Membrane	Corning	431154 (case of 12) 430767	
3, 4, 5, 6	LoBind tubes 1.5 mL	Thermo Fisher Scientific	13-698-791	
3, 4, 5, 6	LoBind tubes 2 mL	Thermo Fisher Scientific	13-698-792	
4	MicroAmp™ Fast 8-Tube Strip, 0.1 mL	Fisher scientific	4358293	

4	MicroAmp™ Optical 8-Cap Strips	Fisher scientific	4323032	
5, 6	Microamp fast 96-well reaction plate (Store these plates in the REACTION MIX PREP workstation)	Fisher scientific	4346907	
5, 6	MicroAmp™ Optical Adhesive Film	Thermo Fisher Scientific	4311971	
	Freezer boxes	Thermo Fisher Scientific	330821PK	
2	50 mL Nalgene Polycarbonate Oak Ridge tube	Thermo Fisher Scientific	31380050	
4	VacConnectors (500)	Qiagen	19407	
	Pasteur pipettes, glass			

2.3. Solutions/Reagents/Kits

Steps Used	Item	Supplier	Cat. No.	QTY	Comment	Storage Conditions
all	10% bleach solution				Decontamination	
	70% ethanol					
	RNase AWAY	Thermo Fisher Scientific	7002PK			
Varied, 6	Calf-Guard® Bovine Rota Coronavirus vaccine	QC Supply	540463		Recovery control	2°-7°C in the dark (cover with foil)
2	Polyethylene glycol 8000	Thermo Fisher Scientific	BP233-1 (1 kg)			RT
2	sodium chloride	Fisher Scientific	BP358-1			
2, 3, 6	TE Buffer	Fisher Scientific	BP2473100			
3, 4, 5, 6	Nuclease-free DEPC-treated water	Thermo Fisher Scientific	AM9906 (10 x 50 mL)		Maintain separate containers for each workstation	Room temp
4	Ethanol, Absolute (200 Proof), Molecular Biology Grade	Fisher Scientific	BP2818500			

4	QIAamp Viral RNA Mini Kit (50) Or (250)	Qiagen	52904 or 52906			Room Temperature
5	2019-nCoV_N_Positive Control	IDT	10006625 (250µL; 200,000 copies/µL)		Plasmid control used for N1 and N2 target standards	–20°C (in aliquots)
5, 6	TaqPath™ 1-Step RT-qPCR Master Mix (4x)	Thermo Fisher Scientific	A15299 (1x5mL) Or A15300 (1x10mL)		One Step RT qPCR mastermix	-5 to -30°C
5	2019-nCoV RUO Kit	IDT	10006713 (750 µL; 500 reactions)		premixed probes and primers for N1, N2 and RP	–20°C (in aliquots)
6	Custom BCoV qPCR assay (primers/probe)	IDT			Pre-mixed primer and probe for BCoV assay	–20°C (in aliquots)
6	BCoV Standard gBlocks® Gene Fragments	IDT			Standard for BCoV assay	–20°C (in aliquots)

Kit/Reagent Aliquot and Storage Information

Upon receipt, initial, date, and store all reagents in Section 2.3 as indicated in the "Storage Conditions" column. For those reagents for which aliquots are required, prepare and store as follows, after a ***FULL DECONTAMINATION*** (Section 1.5) of the **REACTION MIX PREP**, **TEMPLATE PREP,** or Biological Safety Cabinet workstations.

Gloves should be changed frequently, including after decontamination.

- 2019-nCoV RUO Kit containing 3 sets (N1, N2, and RP targets) of premixed primers and probes (Cat. No. 10006713; 750 µL)

  In the ***previously decontaminated*** workstation designated for **REACTION MIX PREP**:
  - Mix the N1 target primer/probe mix by inversion 5 times.
  - Centrifuge the N1 target primer/probe mix for 5 sec in a **mini microcentrifuge** then place on ice.
  - Aliquot the N1 target primer/probe mix in **1.5 mL LoBind tubes**:
    - Using a **P200 pipette**, divide into **7** separate **107 µL** aliquots and label "N1 primer/probe 107 µL" with the date and initials.
      - These aliquots contain enough primer/probe mix for a plate containing 13 samples, a NTC, and one standard curve (60 reactions).
      - Place these aliquots in a plastic freezer box labeled "N1 primer/probe" and store at –20°C.
  - ***FULL DECONTAMINATION*** of the workstation designated for **REACTION MIX PREP**

  Change gloves and repeat the above process for the N2 target.
  - Mix the N2 target primer/probe mix by inversion 5 times.
  - Centrifuge the N2 target primer/probe mix for 5 sec in a **mini microcentrifuge** then place on ice.
  - Aliquot the N2 target primer/probe mix in **1.5 mL LoBind tubes**:
    - Using a **P200 pipette**, divide into **7** separate **107 µL** aliquots and label "N2 primer/probe 107 µL" with the date and initials.
      - These aliquots contain enough primer/probe mix for a plate containing 13 samples, a NTC, and one standard curve (60 reactions).
      - Place these aliquots in a plastic freezer box labeled "N2 primer/probe" and store at –20°C.
  - The mix for the RP target will not be used in this protocol. RP can be labeled with the date and initials and stored as delivered at –20°C.

  FULL DECONTAMINATION of the workstation designated for **REACTION MIX PREP**.

- Custom BCoV qPCR assay (shipped dry at room temperature) pre-mixed primers and probe
  In the ***previously decontaminated*** workstation designated for **REACTION MIX PREP**:
  - Confirm on the specifications sheet that the delivered assay contains 5.0 nm of Primer 1, 5.0 nm of Primer 2, and 2.5 nm of probe.
    - The mass of the primers/probe delivered is used to determine the volume in which to resuspend the pellet in the next step (1000 µL). If the IDT spec sheet indicates a different mass delivered, adjust the volume of nuclease-free water added to obtain 5 µM of each primer in the final stock, based on the equation provided in the "BCoV Reaction Mix" tab of the accompanying spreadsheet.
  - Using a **P1000 pipette**, add 1000 µL **nuclease free water** and gently vortex the primer/probe mix using a **vortex mixer** on low speed. Ensure that the pellet is completely resuspended.
  - Mix the primer/probe mix by inversion 5 times.
  - Centrifuge the primer/probe mix for 5 sec in a **mini microcentrifuge** then place on ice.
  - Aliquot the primer/probe mix in **1.5 mL LoBind tubes**:
    - Using a **P100 pipette**, divide into **16** separate **60 µL** aliquots and label "**BCoV primer/probe 60 µL**" with the date and initials.
      - These aliquots contain enough primer/probe mix for a plate containing 13 samples, a NTC, and one standard curve (60 reactions).
      - Place these aliquots in a plastic freezer box labeled "BCoV primer/probe" and store at –20°C.
  - ***FULL DECONTAMINATION*** of the workstation designated for **REACTION MIX PREP**

- 2019-nCoV_N_Positive Control (Cat. No. 10006625; 250 µL)
  In the ***previously decontaminated*** workstation designated for **TEMPLATE PREP**:
  - Vortex the positive control on low speed using a **vortex mixer**.
  - Spin down briefly with a **mini microcentrifuge**, and place on ice.
  - Aliquot the positive control in **1.5 mL LoBind tubes**:
    - Using a P10 or P20 pipette, divide into **31** separate **8 µL** aliquots and label "**N_Std-stock 8 µL**" **with the date** and initials.
      - These will be used for making standards for future plates (1 standard curve/aliquot). *Note: when the standards are made, the contents of this tube will be diluted to make the first standard, so be sure to leave space on the tube to re-label as "STD 1" later on.*
      - It is critical to include the date on the tube, as the stock concentration may vary for different shipments (see following section on Nanodrop: Standards Concentration Check).

            - Place these aliquots in a plastic freezer box labeled "2019-nCoV_N_Positive Control Standard-stock" and store at –20°C.
        - Set aside one aliquot for concentration check with the Nanodrop spectrophotometer and log results in accompanying Excel Spreadsheet.
    - Store any remaining positive control in the "2019-nCoV_N_Positive Control-stock" plastic freezer box at –20°C.

- BCoV Standard gBlocks® Gene Fragments (250 ng shipped dry at room temperature)
  In the ***previously decontaminated*** workstation designated for TEMPLATE PREP:
    - Confirm on the specifications sheet that the delivered assay contains close to 250 ng of standard.
    - Using a **P100 pipette**, add **100 µL nuclease free water** and gently vortex the primer/probe mix on low speed using a **vortex mixer**. Ensure that the pellet is completely resuspended.
    - Vortex the positive control on low speed using a **vortex mixer**.
    - Spin down briefly with a **mini microcentrifuge**, and place on ice.
    - Aliquot the positive control in **1.5 mL LoBind tubes**:
        - Using a **P10 or P20 pipette**, divide into **16** separate **6 µL** aliquots and label "**BCoV Std-stock 6 µL**" **with the date** and initials.
            - These will be used for making standards for future plates (1 standard curve/aliquot). *Note: when the standards are made, the contents of this tube will be diluted to make the first standard, so be sure to leave space on the tube to re-label as "STD 1" later on.*
            - It is critical to include the date on the tube, as the stock concentration may vary for different shipments (see following section on Nanodrop: Standards Concentration Check).
            - Place these aliquots in a plastic freezer box labeled "BCoV Standard-stock" and store at –20°C.
        - Set aside one aliquot (any standard remaining after aliquots) for concentration check with the Nanodrop spectrophotometer and log results in the accompanying Excel Spreadsheet.
    - Store any remaining positive control in the "BCoV Standard-stock" plastic freezer box at –20°C.

- Calf-Guard® Bovine Rota Coronavirus vaccine
  In the ***previously decontaminated*** biological safety cabinet:
    - Dissolve the vaccine in **1 mL** of **TE buffer**. Label the container "**BCoV Control Spike**" with the date and indicate that TE buffer has been added.
    - Using a **P100 pipette**, divide the resuspended BCoV Control spike into 10 separate 100 µL aliquots in **1.5 mL LoBind tubes**. Label each tube "BCoV Control Spike" with the **date** and initials.
    - **These are single use aliquots**.
    - Store the spike aliquots at -80°C in the dark, covered with foil or in a freezer box labeled "BCoV Control Spikes."

Nanodrop: Standards Concentration Check

Concentrations of the standard stocks shipped from IDT may vary. This step checks the stock concentration (in ng/μL) of standards using the Nanodrop Spectrophotometer. The “N1/N2 Plasmid Standard Log” and “BCoV Standard Log” tabs of the accompanying Excel Spreadsheet calculate and log the concentration in (copies/μL).

Ensure that the **Nanodrop** is calibrated and ready to collect data. Detailed instructions for the calibration check can be found on page 7-2 of the Nandrop 2000/2000c user manual.

- Using a **P10 pipette**, blank the Nanodrop with 2 μL of **TE buffer**.
  - Note: If you are checking the concentration of a standard diluted in nuclease-free water rather than the stock shipped directly from IDT, blank the Nanodrop with **nuclease-free water** instead.
- Wipe the upper and lower pedestals using a dry **kimwipe**.
- Obtain the aliquot of the standard set aside for the concentration check.
  - Record the date written on the aliquot tube in the “Aliquot Date” column of the “N1/N2 Plasmid Standard Log” tab or “BCoV Standard Log” tab in the Excel spreadsheet.
- Record duplicate measurements of the plasmid standard using 2 μL each time.
  - Wipe the upper and lower pedestals using a dry kimwipe in between and after each measurement.
- Enter the measured concentrations (ng/μL) in the “Measured Concentration” column of the “N1/N2 Plasmid Standard Log” tab or “BCoV Standard Log” tab in the Excel spreadsheet. The spreadsheet will automatically calculate the average concentration in ng/μL as well as the concentration in copies/μL based on the plasmid length.

After storing the aliquots, complete a ***FULL DECONTAMINATION*** of the workstation designated for **TEMPLATE PREP** as well as area around the Nanodrop Spectrophotometer. Instructions for cleaning the Nanodrop 2000 can be found on page 6-2 of the Nandrop 2000/2000c user manual.

Table 2.3.1. Aliquots of Kits/Reagents

Product Name	Reagent	Aliquot name	Number of aliquots	Volume of each aliquot
2019-nCoV RUO Kit (Cat. No. 1000671)	N1 primer/probe mix	N1 primer/probe 107 µL	7	107 µL
	N2 primer/probe mix	N2 primer/probe 107 µL	7	107 µL
	RP primer/probe mix	None- freeze as shipped		
2019-nCoV_N_Positive Control (Cat. No. 10006625)	2019-nCoV_N_Positive Control	N_Stdl-stock 8 µL	31	8 µL
Calf-Guard® Bovine Rota Coronavirus vaccine	BCoV Control Spike	BCoV Control Spike	10	100 µL
Custom qPCR assay (IDT)	BCoV primer/probe mix	BCoV primer/probe 60 µL	16	60 µL
BCoV Standard gBlocks® Gene Fragments (IDT)	BCoV standard stock	BCoV Std-stock 6 µL	16	6 µL

Note: For the N1/N2 assay: It is best to avoid multiple freeze/thaw cycles of the primer/probe mixes and positive control, which is why aliquots are made upon receipt and immediately frozen. In the event that aliquots cannot be made immediately, the 2019-nCoV RUO kit and 2019-nCoV_N_Positive Control should be frozen upon receipt, thawed later (on ice) to make aliquots and frozen again until aliquots are used (for a total of two freeze/thaw cycles).

For the BCoV assay: In the event that the coronavirus vaccine cannot be immediately resuspended and frozen, it can be stored at 2°-7°C in the dark (cover with foil). The custom

qPCR assay primer/probe mix is delivered dry at room temperature. In the event that it cannot be immediately resuspended in nuclease-free water and frozen, the dry mix can be stored frozen (or at 4°C for a short period of time). The BCoV Standard gBlock ships dry; while dry, it should be stable almost indefinitely. After resuspending in nuclease-free water, the BCoV standard should be separated into aliquots and stored at –20°C to avoid more than 2 or 3 freeze/thaw cycles.

3. Procedure

The samples are processed on the day of delivery.

DAY 1

3.1. Pasteurization and Solids removal

3.1.1. Pasteurization

Keep sample containers closed throughout the entire procedure

- In the biosafety cabinet, spray the outside of the sample containers with 70% ethanol to decontaminate. Wipe down the containers and ensure that they are dry before proceeding to the next step, but be careful not to wipe off any sample labels/markings.
- Transfer 40 mL of each sample into a separate, labeled **50 mL conical tube**.
- For one of the samples, do the step above in duplicate. This duplicate to be handled in the same manner as the samples throughout the procedure.
- Prepare a method blank by adding 40mL of DI water into a separate, labeled **50 mL conical tube**.
- Place all 50mL conical tubes in the dedicated 60°C water bath.
- Wait until samples reach an internal temperature 60°C (around 30 minutes), then pasteurize samples for 60 minutes. Mix by inverting one time halfway.
- After pasteurization, remove all tubes from the water bath and wipe away any remaining liquid outside of each conical tube.
- Place all tubes in a room temperature water bath and then on ice until the internal temperature of the samples is 23°C ± 5°C.
- ***FULL DECONTAMINATION*** of biosafety cabinet.

3.1.2. Solids Separation

- ***FULL DECONTAMINATION*** of the centrifuge, and rotor.
- Thaw a tube of Bovine Coronavirus vaccine (BCoV) on ice.
- Dilute BCoV 1:10 in DI water.
- Add 40μL of the **1:10 BCoV Control Spike** to each tube.
- Weigh each tube and each cap. Adjust the mass with addition of dH_2O via pasteur pipette.
- The Ensure that all centrifuge tubes are fully closed.
- Load centrifuge tubes balanced in the FA-45-6-30 rotor.

- Centrifuge the samples at 5000 x g for 10 min at 4 °C with brake setting at 0.

3.1.3 Supernatant Filtration

- Without disturbing the pellet, carefully transfer 40 mL of each sample supernatant to a Corning™ Disposable Vacuum Filter/Storage System (0.22μm filter) using a disposable vacuum filter system (Corning 431154).
- Attach disposable vacuum filter system to laboratory stationary vacuum line, or manifold connected to electric vacuum pump.
- Turn on vacuum line and filter until all liquid has passed through the filter.
- Transfer filtrate to previously bleach sterilized 50mL Nalgene Polycarbonate Oak Ridge tube (Thermo Fisher Scientific 31380050).

3.2. Virus Concentration

- Using an **analytical balance**, weigh out individual dishes/tubes of 0.9 g **NaCl** and 4.0 g **PEG 8000** for each sample.

In the biosafety cabinet:

- Add 0.90 g of solid NaCl to each 40-mL liquid sample and mix through inversion.
- Add 4.0 g of solid PEG to each tube and mix through inversion until PEG/NaCl has dissolved.
- Shake until liquid sample and PEG mix is translucent.
- Hold samples overnight in the refrigerator at 4 °C (for at least 16 hrs).

DAY 2

- Remove tubes from 4 °C refrigerator and transfer to centrifuge.
- Prior to centrifugation, mark tube with black marker where the pellet is expected to precipitate (Note this will help determine pellet location when examining later).
- Centrifuge the tubes at 12,000 x g for 120 minutes at 4 °C with no brake applied (setting for the brake is 0). The pellet may be invisible.
- In the biosafety cabinet, remove the majority of supernatant by decanting.
    - Pour supernatant carefully from the side of the tube opposite to the pellet.
    - Collect the supernatant in a liquids container as biohazardous waste for future autoclave.
- Centrifuge tubes again at 12,000 x g for 5 minutes at 4 °C.
- Remove final remaining liquid with a sterile glass Pasteur pipette.
- ***FULL DECONTAMINATION*** of biosafety cabinet, centrifuge, and rotor.

3.3. RNA Extraction

The QIAamp Viral RNA Mini Kit is used.
The protocol below is modified from the instructions provided by QIAgen for this kit. These changes affect (1) the lysis buffer mixture and (2) the tube transfers.

Perform all steps in the biosafety cabinet.
Extract RNA from liquid samples in the biosafety cabinet according to instructions provided for the QIAamp Viral RNA Mini Kit (Manual also available from Qiagen) Volume changes to lysis buffer mixture and tube transfers are noted below.

1. Lysis Buffer Changes and tube changes to Viral RNA Mini Kit
   - Prepare AVL buffer and carrier RNA lysis buffer mixture (=lysis buffer) and transfer to Oak Ridge tubes.
     - 1.68 mL AVL buffer + 16.8µL carrier RNA for each sample
     - Add 1696.8 µL of lysis buffer mixture to each Oak Ridge Tube.
     - Shake, vortex by hand, and rinse tube walls for at least 1 minute.
     - Centrifuge tubes for 2 minutes at 1,000 x g to bring all drops down.
2. Incubate tube at room temperature for 10 min.
3. Briefly centrifuge the tube to remove drops from the inside of the lid.
4. Add 1696.8 µl ethanol (96–100%) to the sample, and mix by pulse-vortexing for 15 s. After mixing, briefly centrifuge the tube to remove drops from inside the lid
5. Insert a QIAamp Mini column into the VacConnector on the QIAvac 24 Plus vacuum manifold.
6. Make sure that the main vacuum valve (between the vacuum pump and the vacuum manifold) and the screw cap valve (on the end of the QIAvac 24 Plus vacuum manifold) are closed. Switch on the vacuum pump by pressing the power switch. The vacuum is applied only to the connecting system (if used) and not to the vacuum manifold.
7. Carefully apply 630 µl of the lysate from step 4 into the QIAamp Mini column without wetting the rim. Avoid touching the QIAamp Mini column membrane with the pipette tip.
8. Open the main vacuum valve. Be sure to leave the lid of the QIAamp Mini column open while applying vacuum. After all lysates have been drawn through the QIAamp Mini column, close the main vacuum valve and open the screw cap valve to vent the manifold. Close the screw cap valve after the vacuum is released from the manifold.
   - Note: If the lysates from individual samples have not completely passed through the membrane despite the VacValves of all other QIAamp Mini columns being closed, place the QIAamp Mini column into a clean 2 ml collection tube, close the cap, and centrifuge at full speed for 3 min or until it has completely passed through. Continue with step 7.
   - Centrifugation is performed at 6000 x g (8000 rpm) to limit microcentrifuge noise. Centrifugation at full speed will not affect the yield or purity of viral RNA

9. Repeat steps 7 and 8. If the sample volume was greater than 140 µl, repeat these steps until all of the lysate has been drawn through the QIAamp Mini column.
10. Apply 750 µl Buffer AW1 to the QIAamp Mini column without wetting the rim. Avoid touching the QIAamp Mini column membrane with the pipette tip. It is not necessary to increase the volume of Buffer AW1 even if the original sample volume was larger than 140 µl.
11. Open the main vacuum valve. After all Buffer AW1 has been drawn through the QIAamp Mini column, close the main vacuum valve and open the screw cap valve to vent the manifold. Close the screw cap valve after the vacuum is released from the manifold.
12. Apply 750 µl Buffer AW2 to the QIAamp Mini column without wetting the rim. Avoid touching the QIAamp Mini column membrane with the pipette tip. Leave the lid of the column open
13. Open the main vacuum valve. After all Buffer AW2 has been drawn through the QIAamp Mini column, close the main vacuum valve and open the screw cap valve to vent the manifold. Close the screw cap valve after the vacuum is released from the manifold.
14. Close the lid of the QIAamp Mini column. Remove it from the vacuum manifold and discard the VacConnector. Place the QIAamp Mini spin column in a clean 2 ml collection tube, and centrifuge at full speed for 1 min to dry the membrane completely.
15. Place the QIAamp Mini spin column into a clean 1.5 ml microcentrifuge tube. Discard the collection tube containing the filtrate. Carefully open the QIAamp Mini spin column. Add 30 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min. Add another 30 µl Buffer AVE equilibrated to room temperature. Close the cap, and incubate at room temperature for 1 min. Centrifuge at 6000 x g (8000 rpm) for 1 min (2 x 30 µl Buffer AVE).
16. Dispense the RNA into several aliquots of appropriate volumes.
17. Store eluted RNA at -80°C.

3.4. RT-qPCR for N1 and N2 Targets

See Appendix A for general guidelines for PCR.

Maintain a record of where standards will be placed in the 96-well PCR plate using the provided “plate” tab of the qPCR set up spreadsheet. Also enter this information into the PCR instrument software.

Ensure that the computer, software, and PCR instrument are on, connected, and functioning properly prior to starting. **Real-time PCR run information and plate set-up should be entered and saved in the software before starting step 3.5 to ensure that the PCR can begin as**

soon as the plate has been prepared. Ideally, a template can be used to expedite this process (See Appendix B for information and resources for the StepOnePlus instrument and software).

3.4.1. Preparation of Standard Curves

A separate curve will be required for each target/plate (N1 or N2), though the same standard is used for both.

All steps are performed in the workstation designated for **TEMPLATE PREP**.

- ***FULL DECONTAMINATION*** (See Section 1.5.)
- Gloves should be changed frequently, including after decontamination.

Serial Dilution of Standards

Note: The following instructions are for making enough standards for 1 curve with 6 standards from approximately 3.08×10^5 cp/5 µL – 3.08 cp/5 µL.

- Remove an aliquot of the **N_Std-stock 8 µL** from the –20°C freezer and thaw on **ice**. This aliquot will be diluted and used for the first standard. Strike through the label which reads "original stock" and label tube with "STD 1" with a **lab pen or marker**.
    - Record the date indicated on this aliquot. Enter this date in the appropriate cell of the "N1/N2 Standard Curve" tab of the accompanying spreadsheet. This date identifies the stock standard concentration based on the Nanodrop concentration check logged in the "N1/N2 Plasmid Standard Log" tab.
        - After the date is entered, the spreadsheet will automatically update the standard curve information for the corresponding "N1/N2 Standard Curve" and "N1/N2 Plate" tabs.
- Obtain a decontaminated **microcentrifuge tube rack** and five (5) **1.5 mL LoBind tubes** and label the tubes "STD 2" - "STD 6."
- Place the tube rack on ice.
- Pipette 18 uL of nuclease-free water (using a **P100 or P200 pipette**) into each of the tubes, starting with STD 6, STD 5, STD 4, STD 3, STD 2 (*note: the empty tubes are filled first to avoid contamination of all the tubes with the positive control plasmid already in the STD 1 tube*). Close each tube immediately after adding water.
- Pipette 18 µL of nuclease-free water into the STD1 tube containing 8 µL of positive control plasmid. Vortex using a **vortex mixer**, spin down briefly with a **mini microcentrifuge**, and place on ice.
- Using a **P10 pipette**, transfer 2 µL of STD 1 to the STD 2 tube containing 18 µL of water, to make a 1 in 10 dilution. Vortex, spin down briefly, and place on ice.
- Using a P10 pipette and new tip, transfer 2 µL of STD 2 to the STD 3 tube containing 18 µL of water, to make a 1 in 10 dilution. Vortex, spin down briefly, and place in the tube rack. Perform a serial dilution by repeating this procedure according to Table 3.4.1 to make all 6 standards. Be sure to use a new tip for each transfer and immediately vortex and spin down each new standard after it is made (Figure 3.4.1).

- Close the tubes and keep the standards on ice as they are made.

Table 3.4.1. Dilution Series for N1/N2 Standard Curve

Standard	Vol of nuclease-free water (µL)	Stock to use in serial dilution	Vol of stock (µL)
STD 1	**18**	stock as shipped, frozen in aliquots upon receipt	will already be in stored aliquot tube 8
STD 2	**18**	**STD 1**	**2**
STD 3	**18**	**STD 2**	**2**
STD 4	**18**	**STD 3**	**2**
STD 5	**18**	**STD 4**	**2**
STD 6	**18**	**STD 5**	**2**

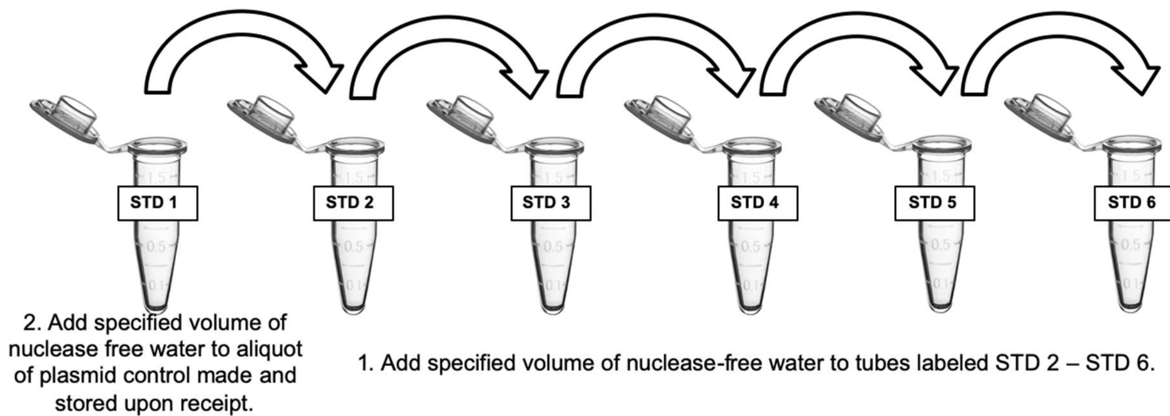

Figure 3.4.1. Summary of Standard Curve Serial Dilution Procedure

- Cover or close the cooler containing the standards on ice and place on a nearby ***decontaminated*** lab bench so that the workstation can be decontaminated (*Note: we are removing the standards so that we can use the UV light for decontamination in the cabinet and avoid any potential damage to the standards)*. Alternatively, the standards can be stored at 4°C while the cabinet is cleaned and the reaction mix is prepared.
- ***FULL DECONTAMINATION*** of workstation designated for **TEMPLATE PREP**.
    - *Note: because we do not have separate workstations separating controls and standards, thorough decontamination after working with standards/control plasmids is critical to prevent contamination of the samples during plate loading. If additional personnel are available, this cleaning can be carried out while the reaction mix is prepared, prior to loading the plate with samples and standards.*

- Remove and discard gloves.
- Remove frozen sample RNA from the -80°C freezer and allow to thaw on ice in the workstation designated for **TEMPLATE PREP**.

3.4.2. Preparation of RT-qPCR Reaction Mix

All steps completed in the workstation designated for **REACTION MIX PREP**.

- ***FULL DECONTAMINATION*** of workstation designated for **REACTION MIX PREP**. Be sure to decontaminate tube racks and the **lid of a 96-well PCR tube rack (part of Cat. No. 50-550-279)** for this step.
- Gloves should be changed frequently, including after decontamination.

Reaction Mix Prep

The number of samples can be changed in this spreadsheet to update required volumes for reaction mix components. For a full plate, with 6 standards, one duplicate and one NTC, set the number of samples = 24 (96 reactions). This sheet will also indicate whether to use a 1.5 mL or 2 mL tube for the reaction mix. For 12 samples, use a 1.5 mL tube.

- If running an N1 plate, remove an **N1 primer/probe 107 µL** aliquot from the –20°C freezer and thaw on ice. Alternatively, if running an N2 plate, instead remove an **N2 primer/probe 107 µL** aliquot from the –20°C freezer and thaw on **ice**.
- Obtain the TaqPath™ 1-Step RT-qPCR Master Mix (4x), from the refrigerator. Keep on ice.
- Mix the TaqPath™ 1-Step RT-qPCR Master Mix (4x), N1 or N2 primer/probe by inversion 5 times and return to ice.
- Centrifuge the primer/probe for 5 sec in a **mini microcentrifuge** then place on ice.
- Obtain a **1.5 mL or 2.0 mL tube**. Label the tube “N1 Reaction Mix” or “N2 Reaction Mix” depending on the target being run. Place the labeled tube in a previously decontaminated tube rack.
- For a stock of reaction mixture master mix, use the table below. N = number of samples (note: if N < 15, add N + 2 amount of volume for each step).

Table 3.4.2

Step #	Reagent	Vol. of Reagent Added Per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/ Probe Mix	N x 1.5 µL
3	TaqPathTM 1-Step RT-qPCR Master Mix (4x)	N x 5 µL
	Total Volume	N x 15 µL

- Mix each reaction mix by pipetting up and down three times, and briefly vortex on the **lowest speed**.
- Spin each reaction mix down briefly (5 sec) in a mini microcentrifuge and store on ice.
- Change gloves and return the TaqPath™ 1-Step RT-qPCR Master Mix (4x) to the refrigerator as soon as possible.
- Proceed immediately to the next step (Reaction Mix Loading).

Reaction Mix Loading

- Place a new **PCR plate** on a pre-cooled **96-well cold block**.
- Using a **P100 or P200 pipette**, transfer 15 µL of the N1 or N2 Reaction Mix to the designated wells, using a new tip each time. *Tip tip! Model the pipette tip box after the 96 well plate and keep track of loaded wells based on empty spots in the tip box.*
- Keeping the plate on the cold rack, cover with a ***decontaminated*** **lid of a PCR tube rack (part of Cat. No. 50-550-279)** and proceed immediately to Step 3.5.4 (Preparation of Reaction Plate).

3.4.3. Preparation of Reaction Plate

Carefully transfer the covered plate to the ***previously decontaminated*** workstation designated for **TEMPLATE PREP**.

- Use a ***previously decontaminated*** **P10 pipette** to transfer nuclease-free water and standards (volumes indicated below) into the appropriate wells. While adding, pipette up and down three times to mix the template with the reaction mix in the well. Change tips after each addition.
  - No template control
    - Add 5 µL of **nuclease-free water** into wells designated for no template control (NTC).
  - Samples
    - Briefly vortex and spin down each sample and store on ice.
    - Add 5 µL of each sample RNA into the designated wells.
  - Standards
    - Change gloves and retrieve standards from storage (ice or 4°C).
    - Add 5 µL of each standard into designated wells.

  Tip tip! Model the pipette tip box after the 96 well plate and keep track of loaded wells based on empty spots in the tip box.

- Seal the reaction plate with **optical adhesive film** using the **adhesive seal applicator** (video instructions).
- **Centrifuge** the plate briefly (<1500 rpm, 1 min, 4°C, based on recommendation for StepOnePlus calibration plates) to bring the contents to the bottom of the well and

eliminate air bubbles. Proceed immediately to step 3.4.4. (Run of the real-time PCR instrument).

3.4.4. Run of RT-qPCR Instrument

- Retrieve the plate from the centrifuge and load into the real-time PCR instrument, orienting the wells in the correct direction.
- Check that the saved run has all the correct conditions and that the plate set-up is correct and saved.

RT-qPCR run information and plate set-up should be entered and saved in the software before starting step 3.4 to ensure that the RT-qPCR can begin as soon as the plate has been prepared. Ideally, a template can be used to expedite this process. References for the StepOnePlus can be found in Appendix B.

StepOnePlus Run Set-up

To operate the StepOnePlus instrument and software, refer to the instructions on Pages A-1 to A-2 of EPA Method 1696. Please note that the conditions required for the SARS-CoV-2 qPCR SOP are **different** from those in EPA Method 1696. Differences are summarized below, with reference to the steps defined in EPA Method 1696:

- **1.3.4 Ramp Speed: Fast**
- **Table A-1 Targets**
    - Enter the target desired for the plate (either N1 or N2). Be sure that sample, standard and NTC wells have the correct target selected.
        - Target 1: N1
            - Name: N1
            - Reporter: FAM
            - Quencher: Black Hole Quencher (select NFQ-MGB in the software)
        - Target 2: N2
            - Name: N2
            - Reporter: FAM
            - Quencher: Black Hole Quencher (select NFQ-MGB in the software)
- **1.9.2 Reaction Volume Per Well** → 20 µL
- **1.9.3 Thermal Profile** (Be sure to set the number of cycles to 45)

Table 3.4.4.

Cycling Parameters	Temp ˚C	Time	Reps
Stage 1	25	2 min	1
Stage 2	50	15 min	1
Stage 3	95	2 min	1
Denature	95	3 sec	PCR (45 cycles)
Anneal	55	30 sec	
Extension	-	-	-

Standard curve(s) setup

- 6 points
- 3 replicates
- Starting quantity: 307692 copies per reaction
- 1:10 dilution
- Make any necessary changes to the plate set-up and save the run.
- Start the run.

Ensure that the StepOnePlus is set up as shown below, and that ROX signal will be appropriately collected:

At end of day: Ensure a ***FULL DECONTAMINATION*** of all workstations after use.

3.4.5. Data Analysis

General data analysis guidelines

Adapted from the [TaqPathTM 1-Step RT-qPCR Master Mix, CG USER GUIDE]:

1. View the amplification plot, and modify as needed:
   a. Use the automatic settings for baseline and threshold values.
   b. Identify and work with the QAO to determine whether it is appropriate to remove outliers from the analysis.

2. In the well table or results table, view the CT values for each well and for each replicate group.

3. Review the standard curve with the QAO for:
   - Slope

- Amplification efficiency
- R2 values
- Y intercept
- CT values
- Outliers

3.5. RT-qPCR for Bovine Coronavirus Control

3.5.1. Preparation of Standard Curve

All steps completed in the workstation designated for TEMPLATE PREP.

- ***FULL DECONTAMINATION*** (See Section 1.5.)
- Gloves should be changed frequently, including after decontamination.

Serial Dilution of Standards

Note: The following instructions are for making enough standards for 1 curve with 6 standards from approximately RANGE. To calculate volumes needed for a different number of curves, use the "BCoV Standard Curve" tab in the accompanying spreadsheet.

- Remove an aliquot of the **BCoV Std-stock 6 μL** from the –20°C freezer and thaw on **ice**. This aliquot will be diluted and used for the first standard. Strike through the label which reads "original stock" and label tube with "STD 1" with a **lab pen or marker**.
  - Record the date indicated on this aliquot. Enter this date in the appropriate cell of the "BCoV Standard Curve" tab of the accompanying spreadsheet. This date identifies the stock standard concentration based on the Nanodrop concentration check logged in the "BCoV Standard Log" tab.
    - After the date is entered, the spreadsheet will automatically update the standard curve information for the corresponding "BCoV Standard Curve" and "BCoV Plate" tabs.
- Obtain a decontaminated **microcentrifuge tube rack** and five (5) **1.5 mL LoBind tubes** and label the tubes "STD 2" - "STD 6."
- Place the tube rack on ice.
- Pipette 18 uL of nuclease-free water (using a **P100 or P200 pipette**) into each of the tubes, starting with STD 6, STD 5, STD 4, STD 3, STD 2 (*note: the empty tubes are filled first to avoid contamination of all the tubes with the positive control plasmid already in the STD 1 tube*). Close each tube immediately after adding water.
- Pipette 18 μL of nuclease-free water into the STD1 tube containing 6 μL of positive control plasmid. Vortex using a vortex mixer, spin down briefly with a mini microcentrifuge, and place on ice.
- Using a **P10 pipette**, transfer 2 μL of STD 1 to the STD 2 tube containing 18 μL of water, to make a 1 in 10 dilution. Vortex, spin down briefly, and place on ice.

- Using a P10 pipette and new tip, transfer 2 µL of STD 2 to the STD 3 tube containing 18 µL of water, to make a 1 in 10 dilution. Vortex, spin down briefly, and place in the tube rack. Perform a serial dilution by repeating this procedure according to Table 3.5.1 to make all 6 standards. Be sure to use a new tip for each transfer and immediately vortex and spin down each new standard after it is made (Figure 3.5.1).
- Close the tubes and keep the standards on ice as they are made.

Table 3.5.1. Dilution Series for BCoV Standard Curve

Standard	Vol of nuclease-free water (µL)	Stock to use in serial dilution	Vol of stock (µL)
STD 1	**18**	stock as shipped, frozen in aliquots upon receipt	will already be in stored aliquot tube 6
STD 2	**18**	**STD 1**	**2**
STD 3	**18**	**STD 2**	**2**
STD 4	**18**	**STD 3**	**2**
STD 5	**18**	**STD 4**	**2**
STD 6	**18**	**STD 5**	**2**

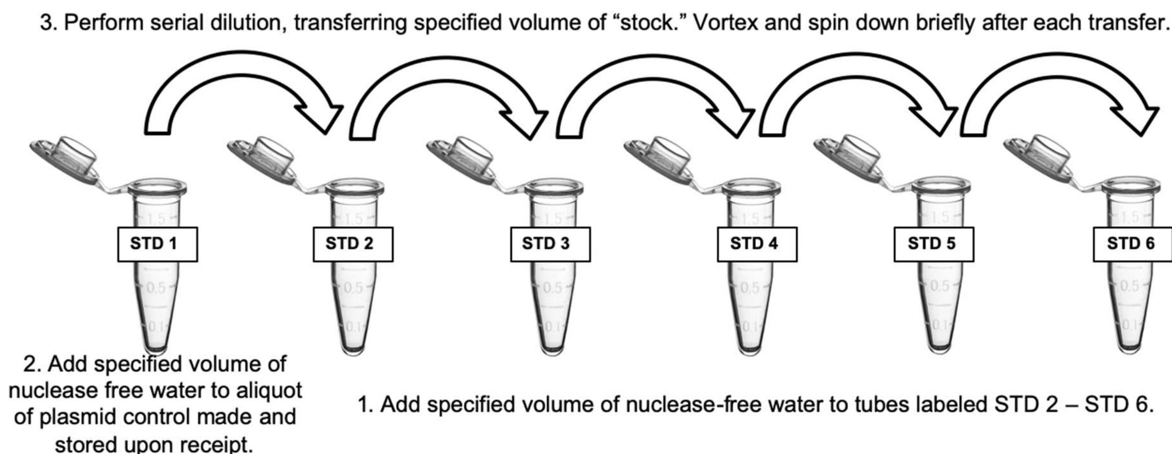

Figure 3.5.1. Summary of Standard Curve Serial Dilution Procedure

- Cover or close the cooler containing the standards on ice and place on a nearby ***decontaminated*** lab bench so that the workstation can be decontaminated (*Note: we are removing the standards so that we can use the UV light for decontamination in the cabinet and avoid any potential damage to the standards)*. Alternatively, the standards can be stored at 4°C while the cabinet is cleaned and the reaction mix is prepared.

- ***FULL DECONTAMINATION*** of workstation designated for **TEMPLATE PREP**.
    - *Note: because we do not have separate workstations separating controls and standards, thorough decontamination after working with standards/control plasmids is critical to prevent contamination of the samples during plate loading. If additional personnel are available, this cleaning can be carried out while the reaction mix is prepared, prior to loading the plate with samples and standards.*
    - Remove and discard gloves.
- Remove frozen sample RNA from the -80°C freezer and allow to thaw on ice in the workstation designated for **TEMPLATE PREP**.

3.5.2. Preparation of RT-qPCR Reaction Mix

All steps completed in the workstation designated for **REACTION MIX PREP**.

- ***FULL DECONTAMINATION*** of workstation designated for **REACTION MIX PREP**. Be sure to decontaminate tube racks and the **lid of a 96-well PCR tube rack (part of Cat. No. 50-550-279)** for this step.
- Gloves should be changed frequently, including after decontamination.

Reaction Mix Prep

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- Remove a **BCoV primer/probe 60 µL** aliquot from the –20°C freezer and thaw on ice.
- Obtain the TaqPath™ 1-Step RT-qPCR Master Mix (4x) from the -20 freezer. Keep on ice.
- Mix the TaqPath™ 1-Step RT-qPCR Master Mix (4x), and BCoV primer/probe by inversion 5 times (CDC) and return to ice.
- Centrifuge the primer/probe for 5 sec in a **mini microcentrifuge** then place on ice (CDC).
- Obtain a **1.5 mL or 2.0 mL tube**. Label the tube “BCoV Reaction Mix” Place the labeled tube in a previously decontaminated tube rack.
- Add the following components to the Reaction Mix tube (volumes specified in Table 3.5.3):
        - **Nuclease-free water**
        - **TaqPath™ 1-Step RT-qPCR Master Mix (4x)**
        - Add the **BCoV primer/probe mix** (volume specified in Table 3.5.3).

Note: Add the nuclease-free water and master mix, before the primer/probe mix to avoid contaminating the master mix and nuclease-free water stocks. Be sure to close each tube/container immediately to avoid contamination.

Table 3.5.2. Reaction Mix For BCoV

Note: This table reflects volumes required for each reaction well .

Step #	Reagent	Vol. of Reagent Added Per Reaction
1	Nuclease-free Water	N x 8.5 µL
2	Combined Primer/ Probe Mix	N x 1.5 µL
3	TaqPathTM 1-Step RT-qPCR Master Mix (4x)	N x 5 µL
	Total Volume	N x 15 µL

- Mix each reaction mix by pipetting up and down, and briefly vortex on the **lowest speed**.
- Spin each reaction mix down briefly (5 sec) in a mini microcentrifuge and store on ice.
- Change gloves and return the TaqPathTM 1-Step RT-qPCR Master Mix (4x) to the freezer as soon as possible.
- Proceed immediately to the next step (Reaction Mix Loading).

Reaction Mix Loading

- Place a new **PCR plate** on a pre-cooled **96-well cold block**.
- Using a **P100 or P200 pipette**, transfer 15 µL of the BoV Reaction Mix to the designated wells, using a new tip each time. *Tip tip! Model the pipette tip box after the 96 well plate and keep track of loaded wells based on empty spots in the tip box.*
- Keeping the plate on the cold rack, cover with a ***decontaminated*** **lid of a PCR tube rack (part of Cat. No. 50-550-279)** and proceed immediately to Step 3.5.4 (Preparation of Reaction Plate).

3.5.3. Preparation of Reaction Plate

Carefully transfer the covered plate to the ***previously decontaminated*** workstation designated for **TEMPLATE PREP**.

- Use a ***previously decontaminated*** **P10 pipette** to transfer nuclease-free water and standards (volumes indicated below) into the appropriate wells. While adding, pipette up and down three times to mix the template with the reaction mix in the well. Change tips after each addition.
    - No template control
        - Add 5 µL of **nuclease-free water** into wells designated for no template control (NTC).
    - Samples
        - Briefly vortex and spin down each sample and store on ice.
        - Add 5 µL of each sample into the designated wells.
    - Standards

        - Change gloves and retrieve standards from storage (ice or 4°C).
        - Add 5 μL of each standard into designated wells.

Tip tip! Model the pipette tip box after the 96 well plate and keep track of loaded wells based on empty spots in the tip box.

- Seal the reaction plate with **optical adhesive film** using the **adhesive seal applicator** (video instructions).
- **Centrifuge** the plate briefly (<1500 rpm, 1 min, 4°C, based on recommendation for StepOnePlus calibration plates) to bring the contents to the bottom of the well and eliminate air bubbles. Proceed immediately to step 3.5.5. (Run of the real-time PCR instrument).

3.5.4. Run of RT-qPCR Instrument

- Retrieve the plate from the centrifuge and load into the real-time PCR instrument, orienting the wells in the correct direction.
- Check that the saved run has all the correct conditions and that the plate set-up is correct and saved.

Real-time PCR run information and plate set-up should be entered and saved in the software before starting step 3.5 to ensure that the PCR can begin as soon as the plate has been prepared. Ideally, a template can be used to expedite this process. References for the StepOnePlus can be found in Appendix B.

StepOnePlus Run Set-up

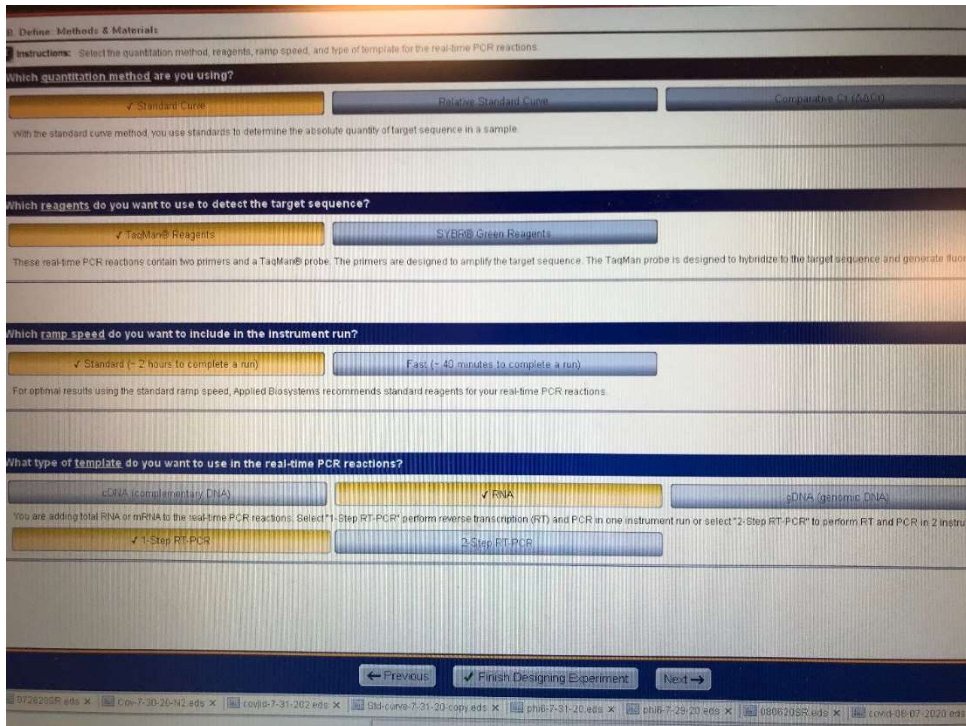
To operate the StepOnePlus instrument and software, refer to the instructions on Pages A-1 to A-2 of EPA Method 1696. Please note that the conditions required for the SARS-CoV-2 qPCR SOP are **different** from those in EPA Method 1696. Differences are summarized below, with reference to the steps defined in EPA Method 1696:

- **1.3.4 Ramp Speed: Fast**
- **Table A-1 Targets**
    - Enter the target desired for the plate.
        - Target: BCoV
            - Name: BCoV
            - Reporter: FAM
            - Quencher: IBFQ
- **1.9.2 Reaction Volume Per Well** → 20 μL
- **1.9.3 Thermal Profile** (Be sure to set the number of cycles to 45)

Cycling Parameters	Temp ˚C	Time	
Stage 1	25	2 min	1
Stage 2	50	15 min	1
Stage 3	95	2 min	1
Pre-denature	95	10 min	
Denature	95	15 sec	PCR (45 cycles)
Anneal	60	30 sec	
Extension	60	1 min	

Standard curve(s) setup

- 6 points
- 3 replicates
- Starting quantity: 4.21 E+5 copies per reaction
- 1:10 dilution
- Make any necessary changes to the plate set-up and save the run.
- Start the run.



At end of day: Ensure a ***FULL DECONTAMINATION*** of all workstations after use.

3.5.5. Data Analysis

General data analysis guidelines

Adapted from the TaqPathTM 1-Step RT-qPCR Master Mix, CG USER GUIDE:

1 View the amplification plot, and modify as needed:
   a. Use the automatic settings for baseline and threshold values.
   b. Identify and work with the QAO to determine whether it is appropriate to remove outliers from the analysis.

2 In the well table or results table, view the CT values for each well and for each replicate group.

3 Review the standard curve with the QAO for:
   - Slope
   - Amplification efficiency
   - R2 values
   - Y-intercept
   - CT values
   - Outliers

To determine recovery, compare copies of BCoV recovered in spiked samples to the copies of BCoV in the spike volume (RNA extracted directly from 4 µL of BCoV spike).

4. References

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(CDC) CDC 2019-Novel Coronavirus (2019-nCoV) Real-Time RT-PCR Diagnostic Panel: For Emergency Use Only: Instructions for Use https://www.fda.gov/media/134922/download

(Decaro et al. 2008) Decaro N, Elia G, Campolo M, et al. Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. J Virol Methods. 2008;151(2):167 171. doi:10.1016/j.jviromet.2008.05.016 https://www.ncbi.nlm.nih.gov/pmc/articles/PMC7112840/

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(Boehm Group, Stanford) Personal correspondence with Boehm group, notes provided by Stanford group

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Appendix A: Guidelines for PCR techniques

General guidelines for PCR techniques, take directly from the CDC protocol:

“Amplification technologies such as PCR are sensitive to accidental introduction of PCR product from previous amplifications reactions. Incorrect results could occur if either the clinical specimen or the real-time reagents used in the amplification step become contaminated by accidental introduction of amplification product (amplicon). Workflow in the laboratory should proceed in a unidirectional manner.

Maintain separate areas for assay setup and handling of nucleic acids.

Always check the expiration date prior to use. Do not use expired reagent. Do not substitute or mix reagent from different kit lots or from other manufacturers.

Change aerosol barrier pipette tips between all manual liquid transfers.

During preparation of samples, compliance with good laboratory techniques is essential to minimize the risk of cross-contamination between samples, and the inadvertent introduction of nucleases into samples during and after the extraction procedure. Proper aseptic technique should always be used when working with nucleic acids.

Maintain separate, dedicated equipment (e.g., pipettes, microcentrifuges) and supplies (e.g., microcentrifuge tubes, pipette tips) for assay setup and handling of extracted nucleic acids.

Wear a clean lab coat and powder-free disposable gloves (not previously worn) when setting up assays.

Change gloves between samples and whenever contamination is suspected.

Keep reagent and reaction tubes capped or covered as much as possible.

Primers, probes (including aliquots), and enzyme master mix must be thawed and maintained on cold block at all times during preparation and use.

Work surfaces, pipettes, and centrifuges should be cleaned and decontaminated with cleaning products such as 10% bleach, “DNAZapTM” or “RNase AWAY®” to minimize risk of nucleic acid contamination. Residual bleach should be removed using 70% ethanol.

RNA should be maintained on cold block or on ice during preparation and use to ensure stability.

Dispose of unused kit reagents and human specimens according to local, state, and federal Regulations.

Protect fluorogenic probes from light.

Primers, probes (including aliquots), and enzyme master mix must be thawed and kept on a cold block at all times during preparation and use.

Do not refreeze probes. Controls and aliquots of controls must be thawed and kept on ice at all times during preparation and use."

Taken directly from the TaqPathTM qPCR Master Mix, CG user guide:

"When preparing samples for PCR or RT-PCR amplification:

Wear clean gloves and a clean lab coat. Do not wear the same gloves and lab coat that you have previously used when handling amplified products or preparing samples. Change gloves if you suspect that they are contaminated.

Maintain separate areas and dedicated equipment and supplies for:

- Sample preparation and reaction setup.
- Amplification and analysis of products.
- Do not bring amplified products into the reaction setup area.
- Open and close all sample tubes carefully. Avoid splashing or spraying samples.
- Keep reactions and components capped as much as possible.
- Use a positive-displacement pipettor or aerosol-resistant barrier pipette tips.
- Clean lab benches and equipment periodically with 10% bleach solution or DNA decontamination solution."

Appendix B: StepOnePlus Resources and Run Set-up

Resources for the StepOnePlus System and Software

StepOnePlus Product Page

Installation, Networking, and Maintenance

Applied Biosystems StepOne and StepOnePlus Real-Time PCR Systems Standard Curve Experiments: Getting Started Guide

SOP for SARS-CoV-2 Detection in Wastewater by PEG Precipitation

Jiang Lab
University of California, Irvine
Version: August 2020

1. Collect 250ml primary influent 24-hour composite sample in sterilized sample bottle
2. Keep sample on ice or 4 °C during storage and transport (<6 hours)
3. Upon arrival in lab, place sample in 60 °C in incubator for 90 mins
4. Set up extraction efficient control samples by seeding sample with various concentrations of heat-inactivated SARS-Related Coronavirus 2
5. Centrifuge at 4,000×g for 30 min to remove large debris
6. Filter supernatant after centrifugation through 0.45 μm pore size low-binding membrane to remove bacterial and eukaryotic cells
7. Mix each 40 mL filtrate with 5 mL of glycine buffer (0.05 M glycine, 3% beef extract, pH 9.6)
8. Add PEG 8000 and NaCl to the mixture from the previous step to a final concentration of 100 g/L and 0.5 M, respectively
9. Incubate the mixture from the previous step overnight at 4°C for precipitation
10. Next day centrifugation the mixture from the previous step for 90 min at 12,000×g
11. Resuspend viral-containing pellet in 140 μL RNase-free water
12. Set up negative control using RNase-free water only
13. Set up RNA extraction efficiency control/positive control using heat-inactivated SARS-Related Coronavirus 2
14. Perform RNA extraction using QIAamp Viral RNA Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocols
15. Quantify RNA using nano-drop
16. Use RNA (8-12 μL) from previous step for cDNA synthesis using QuantiTect Reverse Transcription Kit (Qiagen, Valencia CA, USA) following the manufacturer's protocol
17. Proceed directly with PCR and ddPCR or store at -20 °C until analysis
18. Perform ddPCR using N1, N2 primers and probes reported by CDC
19. Prepare ddPCR reaction following the instruction from Bio-Rad (Hercules CA, USA) for QX200 Droplet Digital PCR System
20. Assemble the ddPCR reaction mixture according to Table 1
21. ddPCR thermocycling conditions is set for: 95 °C for 10 min (DNA polymerase activation), followed by 40 cycles of 94 °C for 30 s (denaturation) and 55 °C for 60 s (annealing), and 98 °C for 10 min followed by an infinite 4 °C hold
22. Analyze the ddPCR data using QuantaSoft Analysis Pro software v.1.0.596 (Bio-Rad, Hercules CA, USA) to calculate the concentration of the target.

Table 1. ddPCR mixture for SARS-CoV-2 detection

Component	**Volume per Reaction (µl)**	
	N1	**N2**
Primers/probe (FAM)	1	1
	500nM/125nM	500nM/125nM
Sample	2 (Variable)	
2x ddPCR Supermix for Probes (No dUTP)	10	
RNase-/DNase-free water	7 (Variable)	
Total volume	20	

Southern Nevada Water Authority

WRF 5089: SOP for Wastewater Surveillance of SARS-CoV-2

Purpose: Provide standard operating procedures (SOPs) performed by SNWA personnel for sample concentration and processing related to wastewater surveillance of SARS-CoV-2.

General reagents/labware

1. Sterile 250-mL Nalgene bottles (with conical bottom)
2. Sterile 50-mL conical tubes
3. Sterile 2-mL microcentrifuge tubes
4. DNA/RNA extraction kit (PureLink™ Viral RNA/DNA Mini Kit, ThermoFisher Scientific)
5. cDNA synthesis kit (Maxima First Strand cDNA Synthesis Kit, ThermoFisher Scientific)
6. Bovine coronavirus (BCoV) (Calf-Guard, Zoetis, 174 Parsippany, NJ, USA) (recovery spike)
7. QuantiFast Pathogen PCR Kit + IC (Qiagen, Carlsbad, CA) (internal positive control)
8. qPCR assays (see table below for additional details)
    a. N1/N2 (CDC, 2020)
    b. E_Sarbeco (WHO, Corman et al., 2020)
    c. *orf1a* (Lu et al., 2020)
    d. BCoV (Decaro et al., 2008)
    e. OC43 (Dare et al. 2007)
9. qPCR standards
    a. N1/N2 (VR-3276SD, ATCC)
    b. All others (gBlock Gene Fragment, Integrated DNA Technologies)

Assay	Target Virus	Primer name	Sequence (5' → 3')	Annealing Temp. (°C)	Ref.
N1	SARS-CoV-2	2019-nCoV_N1-F	GACCCCAAAATCAGCGAAAT	55	CDC (2020)
		2019-nCoV_N1-R	TCTGGTTACTGCCAGTTGAATCTG		
		2019-nCoV_N1-P	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1		
N2	SARS-CoV-2	2019-nCoV_N1-F	TTACAAACATTGGCCGCAAA	55	CDC (2020)
		2019-nCoV_N1-R	GCGCGACATTCCGAAGAA		
		2019-nCoV_N1-P	FAM-ACAATTTGCCCCCAGCGCTTCAG-BHQ1		
E_Sarbeco	SARS-CoV-2	E_Sarbeco_F	ACAGGTACGTTAATAGTTAATAGCGT	58	Corman et al. (2020)
		E_Sarbeco_R	ATATTGCAGCAGTACGCACACA		
		E_Sarbeco_P1	FAM-ACACTAGCCATCCTTACTGCGCTTCG-BBQ		
orf1a	SARS-CoV-2	orf1a_F	AGAAGATTGGTTAGATGATGATAGT	58	Lu et al. (2020)
		orf1a_R	TTCCATCTCTAATTGAGGTTGAACC		
		orf1a_Pb	FAM-TCCTCACTGCCGTCTTGTTG ACCA-BHQ1		
BCoV	Bovine Coronavirus	BCoV-F	CTGGAAGTTGGTGGAGTT	60	Decaro et al. (2008)
		BCoV-R	ATTATCGGCCTAACATACATC		
		BCoV-Pb	FAM-CCTTCATATCTATACACATCAAGTTGTT-BHQ1		
OC43	Human Coronavirus	OC43_F	CGATGAGGCTATTCCGACTAGGT	55	Dare et al. (2007)
		OC43_R	CCTTCCTGAGCCTTCAATATAGTAACC		
		OC43_Pb	FAM- TCCGCCTGGCACGGTACTCCCT-BHQ1		

Cross-reactivity note: OC43 assay will detect BCoV so both spikes should not be used simultaneously

Centricon sample processing consumables

1. Centricon Plus-70 Centrifugal Filter Devices (30kDa) (MilliporeSigma, Burlington, MA)

PEG sample processing consumables

1. Polyethylene glycol (PEG) MW 8000 (FisherScientific, Waltham, MA)
2. Sodium chloride

Instrument requirements

1. Refrigerated freestanding centrifuge (Avanti J-HC, rotor JS-4.2M, Beckman Coulter)
2. Refrigerated tabletop centrifuge (CENTRA CL3R, rotor 243, Thermo Electron Corporation)
3. Microcentrifuge
4. Tube rotator or stir plate
5. Scale
6. Thermal PCR cycler (ABI 2720 Thermal Cycler, Applied Biosystems, Foster City, CA)
7. Real-time PCR detection system (CFX384 Touch TM Real-Time PCR Detection System, Bio-Rad Laboratories, Hercules, CA)

Sample preparation

1. Transfer received sample into a 250-mL sterile Nalgene bottle with conical bottom:
    a. Centricon: 150 mL
    b. PEG: Up to 200 mL
2. Pipette aliquot of bovine coronavirus (BCoV) stock into the sample to target 1000-fold dilution and then close bottles and rock gently for 10 minutes to mix
    a. Centricon: 150-µL spike into 150-mL sample aliquot
    b. PEG: 40-µL spike into 40-mL aliquot or 200-µL spike into 200-mL aliquot
3. Centrifuge samples at 4,000 rpm (~3,200×g) for 30 minutes at 4°C to pellet solids.
    a. Use supernatant for subsequent sample processing with Centricon or PEG
    b. Discard pellet or archive for separate analysis

Sample concentration with Centricon Plus-70 Centrifugal Filter Devices (30 kDa)

1. Split 150-mL supernatant sample from solids pelleting step into 3x50-mL conical tube aliquots
2. Pre-rinse Centricon filter to remove glycine residue:
    a. Fill the sample filter cup with up to 70 mL of DI or Nanopure water
    b. Close the cup with lid provided
    c. Centrifuge at 4,000 rpm (~3,200×g) for 5 min at room temperature
    d. Discard the filtrate and perform a recovery spin as follows:
        i. Turn the concentrate cup upside down and place on top of the sample filter cup
        ii. Centrifuge inverted at 2,250 rpm (~1,000×g) for 1 minute at room temperature
        iii. Remove the device from the centrifuge
        iv. Remove the concentrate cup and discard the recovered water
3. Concentrate sample with pre-rinsed Centricon filter:
    a. Fill the sample filter cup with the contents from one 50-mL conical tube
    b. Close the cup with lid provided
    c. Centrifuge at 4,000 rpm (~3,200×g) for 30 min at 10°C
    d. Remove the device from the centrifuge, record filtrate volume, and discard filtrate
    e. Repeat steps a-d up to 2 more times (3 total centrifugations)

   f. Perform a recovery spin:
      i. Turn the concentrate cup upside down and place on top of the sample filter cup
      ii. Centrifuge inverted at 2,250 rpm (~1,000×g) for 1 minute at 10°C
      iii. Remove the device from the centrifuge
      iv. Remove the concentrate cup and transfer the recovered concentrate into a 2-mL microcentrifuge tube using a pipette
4. Record total filtrate volume and recovered concentrate volume
5. Freeze the concentrate or immediately proceed to nucleic acid extraction
6. Discard the Centricon filter device and sample bottle appropriately

Sample concentration with PEG precipitation

1. Transfer supernatant from solids pelleting step (depends on needs/expected concentration)
   a. Transfer all 200 mL into a sterile 250-mL centrifuge bottle w/ stir bar
   b. Transfer 40 mL into a 50-mL conical tube
2. Add PEG 8000 (final concentration of 9%) to the spiked supernatant
   a. 200 mL volume = 18.0 g
   b. 40 mL volume = 3.6 g
3. Add sodium chloride (final concentration of 1 M) to the spiked supernatant
   a. 200 mL volume = 11.7 g
   b. 40 mL volume = 2.34 g
4. Close the sample container and gently mix the contents to mostly dissolve the PEG and NaCl
5. Mix the sample overnight at 4°C
   a. Place centrifuge bottle on stir plate with moderate mixing
   b. Place conical tube (sealed w/ parafilm) in tube rotator operating at 40 rpm
6. Centrifuge sample at 4,000 rpm (~3,200×g) for 60 min at 4°C
   a. Contents of centrifuge bottle should first be transferred to 250-mL sterile Nalgene bottle with conical bottom (conical tube can be centrifuged directly)
   b. PEG pellet will likely be visible at bottom of sample after centrifugation
7. Carefully discard the supernatant
8. Resuspend PEG pellet in residual supernatant volume
   a. Allow pellet to sit at room temperature for 15 minutes
   b. Gently pipette-mix the pellet and residual supernatant
   c. Transfer the resuspended pellet into a 2-mL microcentrifuge tube
   d. Record volume of resuspended pellet

Nucleic acid extraction

1. Extract nucleic acids from 350 µL of Centricon concentrate or resuspended PEG pellet using the PureLink™ Viral RNA/DNA Mini Kit according to manufacturer's instructions (final volume of nucleic acid extract = 60 µL)
2. Include at least one negative extraction control
3. (Optional) Quantify nucleic acids using the Qubit 3.0 Fluorometer and associated dsDNA HS or RNA HS Assay kits

cDNA synthesis

1. Synthetize complementary DNA (cDNA) from 10 µL of nucleic acid extract using the Maxima First Strand cDNA Synthesis Kit according to manufacturer's instructions (final volume of cDNA = 40 µL)
2. Include at least one negative reverse transcription (RT) control

qPCR assays

1. Spike cDNA sample with a DNA internal positive control (QuantiFast Pathogen PCR Kit+IC)
2. Perform duplex qPCR assays:
    a. N1/N2 = 5 µL of cDNA template
    b. E_Sarbeco/*orf1a*/OC43/BCoV = 1 µL of cDNA template
3. Include a calibration standard curve in each assay
    a. 10-fold serial dilutions of the ATCC standard for N1/N2
    b. 10-fold serial dilutions of gBlock gene fragment standards for E_Sarbeco/*orf1a*/OC43/BCoV
4. Determine concentration of molecular target
    a. Convert Cq values to quantities based on standard curves
    b. Convert quantities to concentrations based on equivalent sample volume
    c. Correct concentrations for BCoV recovery