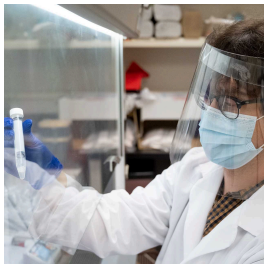
Apr 09, 2024 Version 1

Concentration, extraction and quantification of SARS-CoV-2 in wastewater via droplet digital PCR V.1



DOI
dx.doi.org/10.17504/protocols.io.dm6gpbb31lzp/v1

Jim Huang[1], August Luc[1], Sarah Kane[2], Rose Nash[3], Susan De Long[4], Carol Wilusz[5]

[1]Department of Microbiology, Immunology & Pathology, Colorado State University; [2]GT Molecular;
[3]Burst Diagnostics, Fort Collins, CO;
[4]Department of Civil and Environmental Engineering, Colorado State University; [5]Colorado State University

Carol Wilusz
Colorado State University

OPEN ACCESS

DOI: https://dx.doi.org/10.17504/protocols.io.dm6gpbb31lzp/v1

Protocol Citation: Jim Huang, August Luc, Sarah Kane, Rose Nash, Susan De Long, Carol Wilusz 2024. Concentration, extraction and quantification of SARS-CoV-2 in wastewater via droplet digital PCR. **protocols.io** **https://dx.doi.org/10.17504/protocols.io.dm6gpbb31lzp/v1**Version created by **Carol Wilusz**

Protocol status: Working
We used this protocol from Aug 2020 through July 2023 to test samples from treatment plants and manholes in Colorado.

Created: May 24, 2022

Last Modified: April 09, 2024

Protocol Integer ID: 63162

Keywords: wastewater, SARS-CoV-2, droplet digital PCR, ultrafiltration, quantification via digital droplet reverse transcription pcr, digital droplet reverse transcription pcr, droplet digital pcr, quantification of sar, droplet digital pcr this protocol, wastewater treatment plant, based purification, concentration via ultrafiltration, extraction, purification, pcr

Funders Acknowledgements:
Colorado Department of Public Health & Environment
Grant ID: x
Office of the Vice President for Research, Colorado State University
Grant ID: n/a

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Abstract

This protocol describes the method used at Colorado State University to extract and quantify SARS-CoV-2 in wastewater collected from sewers and at wastewater treatment plants. The method relies on concentration via ultrafiltration, silica column based purification, and quantification via digital droplet reverse transcription PCR. Endogenous and exogenous controls are described.

Materials

Reagents

- Bovilis Coronavirus Intranasal Vaccine (Merck) **Valley Vet Supply Catalog #**43817
- SARS-Related Coronavirus 2 Isolate USA-WA1/2020 Heat Inactivated **BEI Resources Catalog #**NR-52286
- Tween 20 **Research Products International Corp (RPI) Catalog #**P20370-0.5
- Quick Extract DNA Extraction Solution **Lucigen Catalog #**QE0905T
- One-Step RT-ddPCR Advanced Kit for Probes **Bio-Rad Laboratories Catalog #**1864022
- Automated Droplet Generation Oil for Probes **Bio-Rad Laboratories Catalog #**1864110
- Droplet Generation Oil for Probes **Bio-Rad Laboratories Catalog #**1863005
- ddPCR Buffer Control for Probes **Bio-Rad Laboratories Catalog #**1863052
- FluidPrep Elution Fluid Can - Tris **Innovaprep Catalog #**HC08001
- Buffer AVL (w/o carrier RNA 155 ml) **Qiagen Catalog #**19089
- Buffer AW1 (concentrate 242 ml) **Qiagen Catalog #**19081
- Buffer AW2 (concentrate 324 ml) **Qiagen Catalog #**19072

Primers and Probes

	A	B	C	D	E
	Target	**Forward Primer**	**Reverse Primer**	**Probe**	**Citation**
	SARS-CoV-2 CDC-N1	GAC CCC AAA ATC AGC GAA AT	TCT GGT TAC TGC CAG TTG AAT CTG	FAM-ACC CCG CAT /ZEN/ TAC GTT TGG TGG ACC-3IABkFQ	Lu et al (2020)
	BCoV	CTG GAA GTT GGT GGA GTT	ATT ATC GGC CTA ACA TAC ATC	FAM-CCT TCA TAT /ZEN/CTA TAC ACA TCA AGT TGT T-3IABkFQ	Decaro et al (2008)
	PMMoV	GAGTGGTTTGACC TTAACGTTTGA	TTGTCGGTTGCAA TGCAAGT	HEX - CC+T+ACCGA+AG+CA+A A+TG-3IABkFQ	Haramoto et al (2013)
	F+ coliphage	TCT ATG TAT GGA TCG CAC TCG	GTA GGC AAG TCC ATC AAA GTC	FAM-TGC TGT CCG /ZEN/ATT TCA CGT CTA TCT TCA -3IABkFQ	Wolf et al (2008)

Primers and Probes were synthesized by Integrated DNA Technologies. The PMMoV probe was synthesized with Affinity Plus (+) LNA modifications.

Consumables

- Ultrafiltration PS Hollow Fiber Concentrating Pipette Tips - Unirradiated - 200pk **Innovaprep** **Catalog #**CC08004-200
- Stericup Quick Release Millipore 0.22uM PES Filters **Merck MilliporeSigma (Sigma-Aldrich)** **Catalog #**S2GPU01RE
- TempAssure 0.2ml PCR 8-tube strips and optical caps **USA Scientific** **Catalog #**1402-2500
- ddPCR™ 96-Well Plates **Bio-Rad Laboratories** **Catalog #**12001925
- DG8™ Cartridges for QX200™/QX100™ Droplet Generator **Bio-Rad Laboratories** **Catalog #**1864008
- DG8™ Gaskets for QX200™/QX100™ Droplet Generator **Bio-Rad Laboratories** **Catalog #**1863009
- Econospin Mini Spin Column 250ct **Epoch Life Science** **Catalog #**1920-250

Equipment

Clinical Centrifuge (centrifuge protocol only)
Microcentrifuge
BSL2 level Biosafety Cabinet with Vacuum System
Multi-channel pipetter

Equipment	
Concentrating Pipette Select	NAME
Ultrafiltration Device	TYPE
Innovaprep	BRAND
CP Select	SKU
https://www.innovaprep.com/store#!/Concentrating-Pipette-Select-*Contact-for-quote*/p/70397045/category=20718430	LINK

Equipment

QX200 Droplet Digital PCR System	NAME
Digital PCR Device	TYPE
BioRad	BRAND
1864001	SKU
https://www.bio-rad.com/en-us/life-science/digital-pcr/qx200-droplet-digital-pcr-system	LINK

Equipment

C1000 Touch™ Thermal Cycler with 96–Deep Well Reaction Module	NAME
Thermal Cycler	TYPE
BioRad	BRAND
1851197	SKU
https://www.bio-rad.com/en-us/sku/1851197-c1000-touch-thermal-cycler-with-96-ndash-deep-well-reaction-module?ID=1851197	LINK

References:

Decaro N, Elia G, Campolo M, Desario C, Mari V, Radogna A, Colaianni ML, Cirone F, Tempesta M, Buonavoglia C. (2008) Detection of bovine coronavirus using a TaqMan-based real-time RT-PCR assay. *J Virol Methods.* 151(2):167-171

Haramoto E, Kitajima M, Kishida N, et al. (2013) Occurrence of pepper mild mottle virus in drinking water sources in Japan.*Appl Environ Microbiol*. 79(23):7413-7418.

Lu, X., Wang, L., Sakthivel, S. K., Whitaker, B., Murray, J., Kamili, S., Lynch, B., Malapati, L., Burke, S. A., Harcourt, J., Tamin, A., Thornburg, N. J., Villanueva, J. M., & Lindstrom, S. (2020). US CDC Real-Time Reverse Transcription

PCR Panel for Detection of Severe Acute Respiratory Syndrome Coronavirus 2.*Emerging infectious diseases*,*26*(8), 1654–1665.

Wolf S, Hewitt J, Rivera-Aban M, Greening GE. (2008) Detection and characterization of F+ RNA bacteriophages in water and shellfish: application of a multiplex real-time reverse transcription PCR. *J Virol Methods*.149(1):123-8

Protocol materials

- One-Step RT-ddPCR Advanced Kit for Probes **Bio-Rad Laboratories Catalog #**1864022
- ddPCR Buffer Control for Probes **Bio-Rad Laboratories Catalog #**1863052
- Buffer AVL (w/o carrier RNA 155 ml) **Qiagen Catalog #**19089
- SARS-Related Coronavirus 2 Isolate USA-WA1/2020 Heat Inactivated **BEI Resources Catalog #**NR-52286
- Buffer AW2 (concentrate 324 ml) **Qiagen Catalog #**19072
- Stericup Quick Release Millipore 0.22uM PES Filters **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2GPU01RE
- DG8™ Gaskets for QX200™/QX100™ Droplet Generator **Bio-Rad Laboratories Catalog #**1863009
- Econospin Mini Spin Column 250ct **Epoch Life Science Catalog #**1920-250
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- ddPCR™ 96-Well Plates **Bio-Rad Laboratories Catalog #**12001925
- Bovilis Coronavirus Intranasal Vaccine (Merck) **Valley Vet Supply Catalog #**43817
- Stericup Quick Release-GP Sterile Vacuum Filtration System **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2GPU02RE
- Ultrafiltration PS Hollow Fiber Concentrating Pipette Tips - Unirradiated - 200pk **Innovaprep Catalog #**CC08004-200
- FluidPrep Elution Fluid Can - Tris **Innovaprep Catalog #**HC08001
- Quick Extract DNA Extraction Solution **Lucigen Catalog #**QE0905T
- SARS-Related Coronavirus 2 Isolate USA-WA1/2020 Heat Inactivated **BEI Resources Catalog #**NR-52286
- QuickExtract DNA Extraction Solution **Lucigen Catalog #**QE09050

Troubleshooting

Preparation

1 **Sample Description and Treatment**

This protocol is designed for use with untreated influent from wastewater treatment plants. Samples should be provided in 50ml conical tubes with ~40ml of influent in each tube. Optimally, the samples should be flow proportional, collected over a 24 hour time period, and stored at ~4°C for transport to the laboratory for processing. The protocol is also appropriate for grab samples and for flow/time proportional samples collected from sewers.

Safety information

Wastewater contains infectious human pathogens, including SARS-CoV-2, Hepatitis A, Hepatitis B and noroviruses. It should be handled only under BSL2+ conditions with both medical or K95 mask, and face shield.

Note

Up to 80 ml of wastewater can be processed using a single Innovaprep tip to increase sensitivity.

2 **Work Area Set Up** 15m

- Wipe down Biosafety Cabinet (BSC) with 70% ethanol
- Thaw sufficient BCoV spike for the number of samples [(N+2)x 14.5µl] - store on ice
- Perform Start Up Procedure on Innovaprep and insert effluent tube into beaker containing 10% bleach
- Ensure sufficient Elution Fluid, Ultrafiltration Tips etc are available to complete the run

Safety information

If processing 10 or more samples, the BSC will be crowded. Be careful to ensure the air flow is not blocked and that you remove any unnecessary items.

3 30m

Bovine Coronavirus Spike-In

Bovilis Coronavirus Intranasal Vaccine (Merck) **Valley Vet Supply Catalog #**43817

- Lyophilized BCoV (10 dose) is reconstituted in 5 ml PBS, 0.01% Tween 20 and stored in aliquots at -80°C

- A working stock at 1:500 dilution in PBS, 0.01% Tween 20 is prepared and stored in 500 µl aliquots at -80°C
- The concentration of the working stock should be in the range of 3000 genome copies per µl

Safety information

Bovilis is a LIVE viral vaccine for cattle and should be handled with care as it has some ability to infect humans.

Clarification and Concentration of Wastewater

4 **Sample Pre-Treatment**

Addition of BCoV allows virus recovery and inhibition of PCR due to the wastewater matrix to be detected. Tween-20 is added as recommended by Innovaprep. Perform this step on ice.

- Thaw the BCoV aliquot on ice
- Vortex and centrifuge at 16,000g for 5 minutes
- Transfer the supernatant to a fresh tube. This is to ensure the viral particles are evenly distributed.
- Adjust the volume of each wastewater sample to 40 ml
- Add 400 µl of 10% Tween 20
- Add 14.3 µl of BCoV spike (~30-50,000 genome copies)
- Mix well by inverting
- Store remaining BCoV spike on ice until Step 6 is complete

5 **Filtration to remove particulates**

Stericup Quick Release-GP Sterile Vacuum Filtration System **Merck MilliporeSigma (Sigma-Aldrich) Catalog #**S2GPU02RE

Clarification of the sample is necessary to avoid clogging at the ultrafiltration step. This protocol uses filtration but centrifugation can also be employed and is cheaper.

- Pass each sample through a 0.22µM PES filter (diameter >8 cm)

6 **Innovaprep Ultrafiltration**

Ultrafiltration PS Hollow Fiber Concentrating Pipette Tips - Unirradiated - 200pk **Innovaprep Catalog #**CC08004-200

FluidPrep Elution Fluid Can - Tris **Innovaprep Catalog #**HC08001

- Insert a fresh ultrafiltration tip into the device
- Insert the tip into the sample
- Start Run (see note for program details)

- If run stops before all liquid is aspirated, Press Return and Start Run again. This is more likely to happen with turbid samples, or if the tip is faulty.
- Remove the empty sample bottle and replace with a 15 ml conical tube
- Press Elute
- Store the eluate on ice until all samples have been concentrated
- Transfer 140 µl of eluate to a fresh tube containing 560 µl of Qiagen AVL buffer and mix well before proceeding to Step 8
- The remaining eluate can be stored at -80°C

Expected result

The eluate volume is usually in the range of 200-800 µl (50-100 fold concentration)
The volume should be estimated by drawing the eluate up into a 1ml pipette tip and recorded as it is needed to calculate the final viral load.

Note

Innovaprep Program Details

Valve Open	Pulse	Foam Factor	Valve Closed	Flow Start	Flow End	Flow Min Start	Ext Delay	Pump %	Ext Pump Delay
570	2	10	100	3.0	0.2	40	3	100	1

These settings should allow efficient aspiration and elution without interruption using the ultrafiltration tips on wastewater that has been passed through a 0.22uM pre-filter.

Required

Enter eluate volume for Sample 1

Enter eluate volume for Sample 2

Spike Lysis

7 **Spike Lysis**

Quick Extract DNA Extraction Solution **Lucigen Catalog #**QE0905T

- Add 5µl Quick Extract DNA Extraction Solution to 10µl BCoV Spike from Step 5
- Heat in thermocycler at 65°C for 15 min then 98°C for 2 min, then 4°C forever
- Add 85 µl H_2O and store on ice for use in ddPCR
- Can store at -80°C for reference

Note

Altenative Lysis Protocol using GT Molecular Lysis Buffer

Extraction of RNA

8 **QiaAMP Viral RNA Extraction Protocol**

Safety information

Wear gloves, labcoat and goggles or glasses.
Virus is inactivated upon 10 minute incubation with AVL and can be removed from the Biosafety Cabinet at that point.
NEVER mix Qiagen reagents that contain guanidinium with bleach as it results in toxic fumes!

- After 10 min incubation, briefly spin down the AVL/Eluate mix
- Add 560 µl 100% ethanol
- Vortex for 15 sec
- Apply 630 µl to Epoch column in a 2ml collection tube
- Centrifuge 6,000xg for 1 minute.
- Decant the filtrate
- Load the remaining sample onto the column
- Centrifuge 6,000xg for 1 minute
- Add 500µl of AW1 and spin 1 minute at 6,000xg for 1 minute
- Add 500µl of AW2 and spin at max speed for 3 minutes
- Place the column in a fresh 2mL collection tube and spin again for 1 minute at max speed

- Place the column in a fresh, labeled 1.5ml tube and add 60µl of RNase free water to each column
- Incubate for 1 minute
- Spin at 6,000xg for 3 minutes
- Store samples on ice (if using immediately) or at -80°C for long term storage

Expected result

By this stage there is generally very little RNA, so quality control via nanodrop is not informative.
If samples are frozen before processing then more RNA is recovered, presumably because freezing lyses cells and microbes and releases nucleic acids.

Note

Double volume of eluate can be loaded onto a single Epoch column to increase sensitivity. In this case, double volumes of ethanol and AVL must also be used.

Preparation for ddPCR

9 **SARS-CoV-2 Positive Control**

This reagent is used as a positive control for ddPCR detection of SARS-CoV-2. The variant listed below works well with the primer set described here, other variants may or may not be detected.

SARS-Related Coronavirus 2 Isolate USA-WA1/2020 Heat Inactivated **BEI Resources Catalog #**NR-52286

QuickExtract DNA Extraction Solution **Lucigen Catalog #**QE09050

- Add 5µl Quick Extract DNA Extraction Solution to 10µl heat-inactivated virus
- Heat in thermocycler at 65°C for 15 min then 98°C for 2 min, then 4°C forever
- Add 85 µl H_2O
- Dilute to desired concentration, aliquot and store at -80°C

Expected result

The resulting solution will be at around 10^5 copies per µl. We recommend further dilution to between 10 and 100 copies per µl for use as a positive control in RT-ddPCR.

10 **Primer / Probe Mixes**

See Materials for Sequences.
Primer/probe mixes are made as described below, aliquoted, and stored at -20°C in the dark. After thawing on ice, aliquots are stored at 4°C.

100 µM Forward Primer	100 µl
100 µM Reverse Primer	100 µl
100 µM Probe	25 µl
H_2O	1275 µl

One-Step Reverse Transcription Droplet Digital PCR

11 **Reaction Set Up**

- Thaw all required reagents on ice:

> Reverse Transcriptase
> 300mM DTT
> Primer Probe Mixes
> BioRad 1-step RT-ddPCR supermix for probes
> Wastewater RNA
> Positive controls

SARS-CoV-2 /BCoV Reactions

- Create a mastermix sufficient for all reactions adding reagents *in the order shown*
- Include 1-2 safety reactions to ensure there is enough mastermix i.e. if you have 10 reactions make a mastermix for 11-12 reactions

	A	B
	Reagent	**Volume per reaction (µl)**
	Nuclease free H2O	0.9
	SARS-CoV-2 N1 primer/probe mix	1.65
	BCoV primer/probe mix	1.65
	1-step supermix	5.5
	300 mM DTT	1.1
	Reverse Transcriptase	2.2
	Template RNA	*9.0*

	A	B
	Final Volume	**22.0**

Table: Reaction mixture for RT-ddPCR.

- Pipette each template RNA into one well of an 8-tube strip.
- Add 13µl mastermix to each well
- Vortex briefly and spin to the bottom of the tube

F+ / PMMoV Reactions

- Dilute template RNA 1:100 for F+ and PMMoV reactions - add 3 µl RNA to 273 µl H_2O
- Create a mastermix sufficient for all reactions adding reagents *in the order shown*
- Include 1-2 safety reactions to ensure there is enough mastermix i.e. if you have 10 reactions make a mastermix for 11-12 reactions

	A	B
	Reagent	**Volume per reaction (µl)**
	Nuclease free H2O	0.9
	F+ primer/probe mix	1.65
	PMMoV primer/probe mix	1.65
	1-step supermix	5.5
	300 mM DTT	1.1
	Reverse Transcriptase	2.2
	Template RNA (1:100 diluted)	9.0
	Final Volume	**22.0**

Table: Reaction mixture for RT-ddPCR.

- Pipette each diluted template RNA into one well of an 8-tube strip.
- Add 13µl mastermix to each well
- Add ddPCR buffer control for probes to any empty wells in the 8 tube strip
- Mix and spin to the bottom of the tube

It is important to include both positive and negative controls. The negative control should contain H_2O instead of RNA template.

12 **Droplet Generation**

Place a DG8 cartridge in the holder and click to close

- Using a multi-channel pipette, transfer 20 µl of each reaction to the "Sample" row
- Dispense 70 µl AutoDG* (droplet generator) oil for probes in the "Oil" row
- Seal with a clean gasket, insert into droplet generator and press Start
- Using a p200 gently transfer the droplets to a 96-well plate
- Repeat until all samples are in the 96-well plate
- Turn on the plate sealer.
- Place a foil seal on top of the plate. Seal at 180 °C for 5 sec

* We find that Auto-DG oil reproducibly gives a higher droplet count than Manual-DG oil, *even when using a manual droplet generator.*

13 **Thermocycling**

Using a BioRad C1000 Touch deep-well device:

- 50°C for 1 hr (reverse transcription step)
- 95°C for 10 min
- [94°C for 30 sec; 55°C for 1 min] x 44;
- 98°C for 10 min
- 4°C forever

14 **Droplet Reading**

Follow the **manufacturer's instructions** for using the QX200 reader
Allow 2.5 min per sample plus 10-15 minutes for set up

- Open the reader, insert the plate, lock in the metal grid on top and close the device
- Open the Quantasoft program
- For each well, select ABS (for Absolute Quantification), Ch1 FAM (SARS-CoV-2 or F+), Ch2 HEX (BCoV or PMMoV) and 1-step RT-ddPCR kit for probes.
- Click RUN

Data Analysis

15 **Setting Thresholds in Quantasoft Analysis Pro**

Follow the **manufacturer's instructions** for using the software

- On the Droplets tab, select all wells. Verify that the droplet count is over 10,000 in all wells

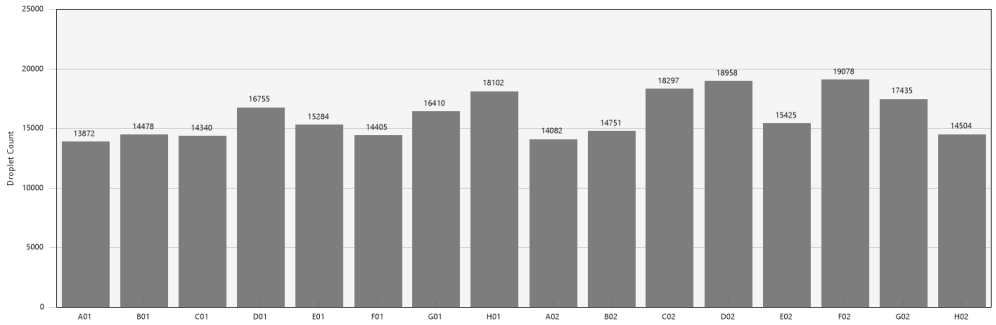

Figure: Droplet counts should be over 10,000. They generally fall within the 12,000-19,000 range.

- In the 2D Amplitude view, select all wells containing the SARS/BCoV probes
- Position the thresholds (pink +) such that they fall between positive and negative droplets

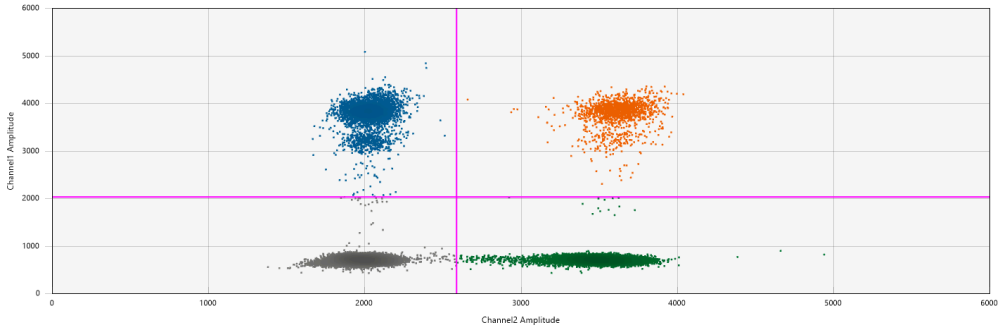

Figure : F+ or SARS-N1 positive droplets are blue, PMMoV or BCoV positive droplets are green, negative droplets are grey. Droplets that are positive for both F+ *and* PMMoV, or BCoV *and* SARS-N1 are orange.

- Check on the 1D Amplitude view that the threshold in both channels lies above all the droplets in the no template control.
- Set the thresholds for the F+/PMMoV wells in the same way (but *not* at the same time because the correct position will be different).

16 Exporting Data from Quantasoft Manager Pro to MS Excel

- On 1D or 2D Amplitude view, select **all** the wells
- On the Well Data panel menu select "Export to Excel"
- While you can select all the data, the values required for downstream calculations or reporting to the NWSS/CDC are:

> Well
> Sample
> Target
> Copies/20 ul

> Accepted *[# droplets]*
> PoissonConfMax
> PoissonConfMin

17 **Calculating SARS-CoV-2 Genome Copies per L**

The following values are required to convert the copies per 20µl [**C**] into copies per L:
> Volume of wastewater sample [**W**=40ml]
> Total volume of eluate from innovaprep [$\mathbf{I_P}$ = ranges from 200-900 µl]
> Volume of eluate used in RNA extraction [$\mathbf{I_E}$ = 140µl]
> Total volume of RNA eluted after extraction [$\mathbf{R_T}$ = 60 µl]
> Volume of RNA used in reaction [$\mathbf{R_U}$ = 8.2 µl]

$$Genomes/L = C(R_T/R_U)(1000/W)(I_P/I_E)$$

Using the protocol described above:
$$Genomes/L = 1.3066C * I_P$$

Expected result

The LOD for this assay is ~3-5 copies per reaction which represents ~3,000-5,000 copies per L
The LOQ for this assay is ~15 copies per reaction which represents ~13,000-15,000 copies per L

The LOD and LOQ are specific to each sample because the eluate from the Innovaprep step is different for each sample.

18 **Calculating Genome Copies per L for Endogenous Controls**

The same calculation is used as above, but should be multiplied by 100 to take into account that the RNA template for the PMMoV and F+ reaction is diluted 100 fold.

Expected result

The endogenous controls reflect the amount of fecal matter in a sample. WWTPs tend to have a characteristic level of F+ and PMMoV which depends on the flow rate, population served etc.
PMMoV falls in the 10^8-10^9 copies per L range.
F+ coliphage is less abundant, in the 10^7-10^8 copies per L range.

$$Genomes/L = C(R_T/R_U)(1000/W)(I_P/I_E) * 100$$

Using the protocol described above:

$$Genomes/L = 130.66C * I_P$$

19 **Determining BCoV Recovery**

Calculate the copies of BCoV that were added at the start of the protocol:

- Use the copies per 20µl in the BCoV positive control reaction to determine how many copies of BCoV were added to each sample at the beginning.

$$BCoV\,copies_{in} = BCoV\,copies_{20\mu l} * BCoV\,vol_{WW} * dilution/BCoV\,vol_{rxn}$$
$$BCoV\,copies_{in} = BCoV\,copies_{20\mu l} * 14.3\mu l * 10/8.2\mu l$$
$$BCoV\,copies_{in} = BCoV\,copies_{20\mu l} * 17.439$$

- Then calculate the number of copies of BCoV that would have been recovered if you had processed the entire 40ml of wastewater and express as a percentage of the copies that were added.

$$BCoV\,recovery = 100 * BCoV\,copies_{20\mu l} * (R_T/R_U)/BCoV\,copies_{in} * I_E/I_P$$

Expected result

BCoV recovery should fall in the 15-25% range
Recovery below 5% could indicate degradation of the RNA, degradation of the positive control spike, or inhibition of the reverse transcription step.

20 **Quality Controls and Troubleshooting**

One possible use of the controls is for normalization. In our experience with Colorado WWTPs normalization to either exogenous (BCoV) or endogenous controls (PMMoV, F+) did not improve correlations with community infection rate or reduce variability.

The controls can also be used to detect failure of reactions due to inhibitors and to distinguish between inhibition and degradation:

Both the reverse transcription step and the PCR step can be inhibited by compounds that carry through the extraction step (either reagents such as phenol or guanidine, or contaminants such as humic acids). Inhibition of the RT step will generally result in fewer positive droplets (because there will be less cDNA produced) and confounds

accurate quantification. Inhibition of the PCR step can result in reduced fluorescence amplitude i.e. poor separation of the positive and negative droplets, because each cycle will produce fewer copies of the cDNA template). This may not affect quantification if the separation remains adequate for threshold setting. Inhibition is not template specific - all targets in a reaction mix will be affected. Low BCoV recovery (<5%) can indicate inhibition of the RT step. Importantly, inhibition can often be abrogated by using less of the RNA containing the inhibitor. Therefore the PMMoV/F+ reaction will generally give good results even when inhibitors are present because it uses a 100 fold diluted RNA template. If BCoV recovery fails QC, it is helpful to rerun the reaction with half or quarter the amount of RNA template (assuming the SARS-N1 levels are high enough to still be detected).

In contrast, degradation of the RNA sample will result in fewer positive droplets for all four targets (i.e. Low BCOV recovery and low PMMoV/F+). This problem can only be remedied by repeating the extraction to make fresh RNA.

RNA quality cannot be easily evaluated prior to the ddPCR assay because even good quality RNA is generally very low abundance (too low for nanodrop), partially degraded and represents a mixture of nucleic acids from a variety of microbes, viruses and human cells (confounds Tapestation/Bioanalyzer). Fluorescent measurements (such as Qubit) may be informative but add time and expense.