



Wastewater Concentration by Adsorption and Direct Extraction for SARS-CoV-2 RNA Detection and Quantification using RT-ddPCR V.2

Version 2 ▾

Jul 01, 2020

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1 Works for me dx.doi.org/10.17504/protocols.io.bhiuj4ew

Coronavirus Method Development Community | 2019-nCoV Wastewater Epidemiology

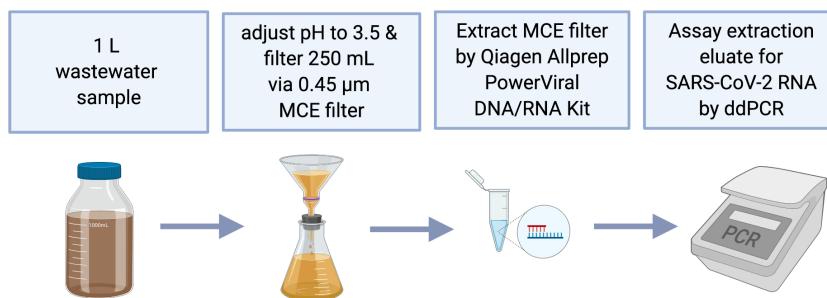


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ABSTRACT

The following protocol describes the GERM Lab workflow for processing wastewater for SARS-CoV-2 via pH adjustment, MCE filtration, extraction, and assay by droplet digital PCR (ddPCR). It is synonymous to method A as described in Ahmed *et al.* 2020 (the referenced manuscript) with modifications to the consumables and PCR format. It is intended for use in a BSL2+ lab with extra precautions for processing environmental specimens for SARS-CoV-2 as described in the CDC Interim Laboratory Biosafety Guidelines.



Graphical abstract of the GERM Lab protocol for concentration SARS-CoV-2 RNA from wastewater and assaying by droplet digital PCR.

EXTERNAL LINK

<https://www.sciencedirect.com/science/article/pii/S004896972033480X>

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

Ahmed W, Bertsch P, Bivins A, Bibby K, Farkas K, Gathercole A, Haramoto E, Gyawali P, Korajkic A, McMinn BR, Mueller J. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. *Science of The Total Environment*. 2020 Jun 5:139960.

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KEYWORDS

wastewater, SARS-CoV-2 RNA, ddPCR

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GUIDELINES

Please coordinate with your local biosafety committee to execute any wastewater handling and concentration procedures in accordance with biosafety standards for your institute and interim guidance issued by the CDC as referenced in the safety warning.

MATERIALS

NAME	CATALOG #	VENDOR
INFORCE 3 Intranasal Bovine Vaccine	INF-00089	Zoetis
Hydrochloric Acid	87003-253	VWR International
2-Mercaptoethanol	0219470580	MP Biomedicals

STEPS MATERIALS

NAME	CATALOG #	VENDOR
INFORCE 3 Intranasal Bovine Vaccine	INF-00089	Zoetis
Hydrochloric Acid	87003-253	VWR International
2-Mercaptoethanol	0219470580	MP Biomedicals
RT-ddPCR Oligonucleotides	Varies	Integrated DNA Technologies
2019-nCoV RUO Kit	10006713	Integrated DNA Technologies
2019-nCoV_N_Positive Control	10006625	Integrated DNA Technologies
One-Step RT-ddPCR Advanced Kit for Probes	186-4021	BioRad Sciences
Droplet Generation Oil for Probes	1863005	BioRad Sciences
ddPCR™ Droplet Reader Oil	1863004	BioRad Sciences

MATERIALS TEXT

In addition to the reagents listed above and the equipment and consumables listed within, this protocol requires access to typical microbiology equipment including pipettes and tips, microcentrifuge, an autoclave, a Class II biosafety cabinet, central vacuum line, and vortex.

EQUIPMENT

NAME	CATALOG #	VENDOR
HAWP MF-Millipore Membrane Filter, 0.45 µm pore size	HAWP04700	Millipore Sigma
GN-6 Metrical 0.45 um 47 mm MCE gridded	63020	Pall
Vacuum Filtration Assembly	Z290408	Sigma Aldrich
pH Test Strips 0 - 14	BDH35309.606	VWR International
PowerBead Tube, Garnet	13123-50	Qiagen
Allprep PowerViral DNA/RNA Kit	28000-50	Qiagen
MP Bio FastPrep 24	116004500	MP Biomedicals
DNA LoBind Tube 1.5 mL	022431021	Eppendorf
DG8™ Cartridges for QX200™/QX100™ Droplet Generator	1864008	BioRad Sciences
DG8 Cartridge Holder	1863051	BioRad Sciences
DG8™ Gaskets for QX200™/QX100™ Droplet Generator	1863009	BioRad Sciences
QX200™ Droplet Generator	1864002	BioRad Sciences
ddPCR™ 96-Well Plates	12001925	BioRad Sciences
PCR Plate Heat Seal, foil, pierceable	1814040	BioRad Sciences
PX1 PCR Plate Sealer	1814000	BioRad Sciences
C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module	1851197	BioRad Sciences
QX200™ Droplet Reader	1864003	BioRad Sciences

SAFETY WARNINGS

Prior to processing wastewater samples for SARS-CoV-2, please review the CDC recommended biosafety guidelines for environmental specimen testing available at <https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html#environmental>. Work with your local biosafety committee to establish applicable biosafety protocols.

DISCLAIMER:

DISCLAIMER – FOR INFORMATIONAL PURPOSES ONLY; USE AT YOUR OWN RISK

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BEFORE STARTING

We are making this protocol available as a courtesy to our colleagues. We make no guarantees or warranties concerning its performance in other laboratories or settings.

Using this procedure at Notre Dame, we have successfully detected and quantified SARS-CoV-2 and pepper mild mottle virus in raw wastewater influent samples via the workflow described herein. Based on results described for murine hepatitis virus by Ahmed *et al.* 2020 (Method A: mean recovery 27%) adsorption direct extraction with pH adjustment will allow for reasonable recovery of SARS-CoV-2 RNA from wastewater. **Please note** that it is possible to perform this protocol without acidification (Ahmed *et al.* 2020 Method B mean recovery 61%), and with MgCl₂ amendment (Ahmed *et al.* 2020 Method C mean recovery 66%). **We have chosen to use acidification so that** we can compare our results in the current project with our historical sampling results.

This procedure relies on vacuum filtration for up to 4 hours to concentrate a 250 mL wastewater sample. It is advisable to use a central vacuum line for this task. However, it is also possible to filter a 100 mL sample in about 30 minutes.

Sample Collection

3h

- 1 Collect a **1 L** sample of primary influent or raw wastewater (grab sample or composite) in a sterile sample collection bottle. Maintain the sample at **-4 °C** during transport to the lab. 2h
- 2  Upon sample receipt at the laboratory spike **1 mL** of resuspended INFORCE 3 into the wastewater sample. 30m

Upon sample receipt at the laboratory spike **1 mL** of resuspended INFORCE 3 into the wastewater sample.

INFORCE 3 contains bovine respiratory syncytial virus (BRSV) which serves as an RNA process control. BRSV is an enveloped single-stranded RNA virus. Spiking 1 mL of INFORCE 3 into 1 liter of wastewater yields approximately 10,000 BRSV gene copies per mL in the wastewater matrix. Mix thoroughly after spiking INFORCE 3 and allow the sample to incubate at **-4 °C** for **00:30:00**. Spiking the process control prior to any freezing and thawing allows for the effect of these processes on recovery to be examined. Other enveloped RNA process controls are also in use by other laboratories including murine hepatitis virus and bovine coronavirus.



INFORCE 3 Intranasal Bovine Vaccine
by Zoetis
Catalog #: INF-00089

- 3 If sample is to be concentrated immediately temporary storage at **-4 °C** is sufficient. If sample is to be processed at a future date (more than 2 days later), mix well, aliquot as suggested below, and store at **-80 °C** until concentration is to be completed. Aliquot **500 mL** of the wastewater sample for concentration as described below and store the remaining **500 mL** at **-80 °C** for any future analyses that may be required. 10m

HA Concentration

10h

- 4 Prior to concentration, sterilize the funnel top, funnel stem/membrane support, aluminum clamp, and filtration flask^{1h} and GN-6 MCE filters by autoclaving. Alternatively, Millipore HAWP MCE filters can also be used in this protocol.



HAWP MF-Millipore Membrane Filter, 0.45
μm pore size
Membrane filter
Millipore HAWP04700 
0.45 um 47 mm



GN-6 Metrcil 0.45 um 47 mm MCE gridded membrane filter

Pall Corporation 63020 [🔗](#)



Vacuum Filtration Assembly

Glassware

Sigma-Aldrich Z290408 [🔗](#)

for 47 mm filters with glass support, NS 40/35 joints, 1 L flask (included)



- 5 Assemble two vacuum filtration assemblies with GN-6 MCE filters for each sample that will be processed (two biological replicates). Handle filters and assemblies using proper aseptic technique. All concentration and processing should be carried out within a Class II Biosafety Cabinet. Attach each filtration flask to a central vacuum line using vacuum tubing. Include a bleach trap to avoid contaminating the vacuum line.

**Note vacuum times to filter the entire volume are up to 8 hours depending on wastewater sample turbidity, so a central vacuum line is strongly recommended.



Filtration columns and bleach traps set up within the BSC.

- 6 Using 2N Hydrochloric acid adjust the pH of each **500 mL** wastewater sample to be concentrated to ~3 to 4. Add Hydrochloric acid in increments of 100 μ L to 10 μ L and measure the pH at each step using pH strips and sterile forceps. After acidification, gently mix the wastewater sample in a sealed vessel prior to beginning filtration. Please note that the protocol can also be performed with two variations: (1) no acidification netural pH or (2) amendment with $MgCl_2$. Please see Ahmed *et al.* 2020 for more details on these variations.



pH Test Strips 0 - 14

pH test strips

VWR International BDH35309.606



Hydrochloric Acid

by VWR International

Catalog #: 87003-253

CAS Number: 7647-01-0

- 7 Slowly add **250 mL** of wastewater sample to a single filtration assembly funnel top in increments of approximately **50 mL**. Allow the suspended settles to settle. Add the remaining **250 mL** of wastewater sample to a second filtration assembly funnel top in the same manner. After wastewater sample has been added to each filtration assembly, slowly turn on the main vacuum line to begin filtration. Monitor for at least the first minute or two to ensure there is no leakage. Continue the vacuum until the entire **250 mL** of each wastewater replicate has passed through the GN-6 MCE filter. Depending on the turbidity of the wastewater, filtration could take up to 8 hours.



Raw influent sample at the beginning of filtration.



Filter with solids caked on after filtration.

- 8 After filtration, use a pair of sterilized forceps and aseptic technique to fold the GN-6 MCE filter and slide it into a 2mL^{15m} PowerBead Tube, Garnet. If the filter is dry, use sterile water to rehydrate the filter prior to folding to prevent breakage.



PowerBead Tube, Garnet

bead beating tube

Qiagen 13123-50 [🔗](#)

PowerBead Tubes, Garnet 0.70 mm

9 Store filters in PowerBead tubes at **8 -80 °C** until extraction.

RNA Extraction

3h

10 RNA Extraction is performed using the Qiagen Allprep PowerViral DNA/RNA Kit with modifications.



Allprep PowerViral DNA/RNA Kit

DNA/RNA extraction kit

Qiagen 28000-50 [🔗](#)

AllPrep PowerViral DNA/RNA Kit (50)

11 30m

Sample Homogenization: Briefly centrifuge each PowerBead tube ~ **5000 x g** then add **650 µl** of Warm Solution PM1 and **6.5 µl** Beta-mercaptoethanol (Beta-ME) to each PowerBead tube. Homogenize the samples with 4 cycles for 20 seconds at 4.0 M/s on an MP Bio FastPrep 24. In between each 20 second homogenization cycle, briefly centrifuge the PowerBead tubes ~ **5000 x g**. After the fourth and final homogenization cycle, centrifuge the PowerBead tubes at **13000 x g 00:01:00** at room temperature. Transfer **450 µl** of the resulting supernatant to a clean 2 mL Collection Tube.



2-Mercaptoethanol

by MP Biomedicals

Catalog #: [0219470580](#)

CAS Number: 60-24-2



MP Bio FastPrep 24
bead beating grinder

MP Bio 116004500



Homogenized filters after bead beating using an MP Bio FastPrep 24.

- 12 Add **150 µl** of Solution IRS and vortex briefly. Incubate at **4 °C** for **00:05:00**.
- 13 Centrifuge the 2 mL Collection Tube at **13000 x g 00:01:00**. Transfer **500 µl** of supernatant to a clean 2.2 mL Collection Tube avoiding the pelleted material.
- 14 Add **600 µl** of Solution PM3 and **600 µl** of Solution PM4 to each 2.2 mL Collection Tube and vortex briefly to mix.
- 15 Load **625 µl** of mixture onto an MB Spin Column and centrifuge at **13000 x g 00:01:00**. Discard the flow through and repeat until all the supernatant has been filtered through the Spin Column.

16 Shake to mix Solution PM5 and add **600 µl** to the MB Spin Column. Centrifuge at **13000 x g 00:01:00**.

17 

Discard the flow through and add **600 µl** of Solution PM4. Centrifuge at **13000 x g 00:01:00**. Discard the flow through and centrifuge at **13000 x g 00:02:00**.

18 Place the MB Spin Column into a clean 2 mL Collection Tube. Add **80 µl** of RNase-free water to the center of the white column membrane and incubate for at least **00:01:00**. Centrifuge at **13000 x g 00:01:00** and discard the MB Spin Column.

19 

Spin the 2 mL Collection Tube at **13000 x g 00:02:00** and transfer **60 µl** of supernatant to a 1.5 mL Eppendorf LoBind Tube.



DNA LoBind Tube 1.5 mL

Microcentrifuge tube

Eppendorf 022431021 

20 The DNA/RNA is now ready for downstream analysis. Store at **-80 °C** until further analysis.

RT-ddPCR Oligos

21 The RT-ddPCR is performed using the One-Step RT-ddPCR Advanced Kit for Probes from BioRad. The concise steps below are taken from the package insert with a few modifications. For full details, please reference the BioRad Droplet Digital PCR Applications Guide and the User Manuals for the referenced equipment.

Please note that the protocol excludes details regarding experimental design such as technical replicates, biological replicates, negative extraction controls, no-template controls etc. Users are referred to the digital MIQE Guidelines for recommendations concerning quality assurance and control.

Huggett JF, Foy CA, Benes V, Emslie K, Garson JA, Haynes R, Hellemans J, Kubista M, Mueller RD, Nolan T, Pfaffl MW. The Digital MIQE Guidelines: Minimum Information for Publication of Quantitative Digital PCR Experiments. Clinical chemistry. 2013 Jun 1;59(6):892-902.

22 Primers, probes, and control materials:

Unless otherwise specified, all oligonucleotide materials are purchased from Integrated DNA Technologies (IDT, Coralville, IA).



RT-ddPCR Oligonucleotides

by Integrated DNA Technologies

Catalog #: Varies

22.1 BRSV primers and probes:

Boxus M, Letellier C, Kerkhofs P. Real Time RT-PCR for the detection and quantitation of bovine respiratory syncytial virus. J Virol Methods. 2005 May;125(2):125-30.

The probe for the BRSV assay specified below is on HEX (Channel 2)

Target region: beta-actin

Amplicon length: 124 bp

FWD Primer	5' GCA ATG CTG CAG GAC TAG GTA TAA T 3'
REV Primer	5' ACA CTG TAA TTG ATG ACC CCA TTC T 3'
Probe	/5HEX/AC CAA GAC T/ZEN/T GTA TGA TGC CAA AGC A/3IABkFQ/

Table 1 - BRSV primers and probes for RT-ddPCR

22.2 SARS-CoV-2 primers, probes, and control plasmid:

The primer and probes set targeting the N1 and N2 genes are available premixed from IDT. The probes for both the N1 and N2 probes are on FAM (Channel 1).



2019-nCoV RUO Kit

by Integrated DNA Technologies

Catalog #: 10006713

The N gene control plasmid is also available from IDT.



2019-nCoV_N_Positive Control

by Integrated DNA Technologies

Catalog #: 10006625

RT-ddPCR

7h

23 The RT-ddPCR is performed using the One-Step RT-ddPCR Advanced Kit for Probes from BioRad.

The kit referenced below is for 200 reactions and contains:

- (1) Supermix: **500 µl** x 2
- (2) Reverse Transcriptase, RT: **200 µl** x 2
- (3) Dithiothreitol, DTT solution: **1 mL [M]300 Milimolar (mM)** x 2



One-Step RT-ddPCR Advanced Kit for
Probes
by BioRad Sciences
Catalog #: 186-4021

All components on the One-Step RT-ddPCR Advanced Kit for Probes are stable for 12 months when stored at **-20 °C**. Repeated freezing and thawing of the supermix is not recommended. DTT should be aliquoted to multiple tubes and stored at **-20 °C** to minimize freezing and thawing.

24

Thaw all components on ice for **00:30:00**. Mix each component thoroughly by vortexing each tube several times for **00:00:30** to ensure homogeneity because a concentration gradient may form during **-20 °C** storage. Centrifuge briefly to collect contents at the bottom of each tube.

25 Prepare samples at the desired concentration before setting up the reaction mix. Store samples at **4 °C** while preparing the reaction mix.

26

Prepare the reaction mix for the number of reactions needed as shown below. The reactions should be setup on ice before droplet generation to prevent a nonspecific reverse transcription reaction from occurring. Assemble all required components except the sample, dispense equal aliquots into each reaction tube or well, and add sample to each tube or well as the final step.

BRSV reagent volumes per reaction:

Reagent	Stock Conc.	Reaction Conc.	Volume per reaction (uL)
Water	n/a	n/a	5.59 μl
Supermix	4x	1x	5.25 μl
RT	n/a	n/a	2.1 μl
DT	[M]300 Millimolar (mM)	[M]15 Millimolar (mM)	1.05 μl
Probe	[M]100 Micromolar (μM)	[M]250 Nanomolar (nM)	0.055 μl
Fwd	[M]10 Micromolar (μM)	[M]900 Nanomolar (nM)	1.98 μl
Rev	[M]10 Micromolar (μM)	[M]900 Nanomolar (nM)	1.98 μl
WW RNA	Variable*	Variable*	4 μl

Total Volume per reaction: **22 μl**

N1 or N2 reagent volumes per reaction:

Reagent	Stock Conc.	Reaction Conc.	Volume per reaction (uL)
Water	n/a	n/a	6.45 μl
Supermix	4x	1x	5.25 μl

RT	n/a	n/a	2.1 μl
DT	[M]300 Millimolar (mM)	[M]15 Millimolar (mM)	1.05 μl
IDT PreMix	Probe @ 250 nM		3.15 μl
	Primers @ 1000 nM		
WW RNA	Variable*	Variable*	4 μl
Total Volume per reaction:			22 μl

*Suggested input quantities of total RNA are 100 fg - 100 ng per reaction

- 27 Mix thoroughly by vortexing the reaction tubes or, if preparing reactions in a 96-well plate, by pipette mixing 5 times prior to transferring the reaction mix to the droplet generation cartridge.
- 28 Once the reaction mixtures are ready, place a DG8 Cartridge into a DG8 Cartridge Holder. Next load **20 μ l** of each reaction mix into the sample well of a DG8 Cartridge for QX200/QX100 Droplet Generator followed by **70 μ l** of Droplet Generation Oil for Probes into the oil wells, according to the QX100 or QX200 Droplet Generator Instruction Manual. After loading the sample and droplet generation oil, seal the DG8 cartridge in the DG8 Cartridge Holder using a DG8 Gasket.



DG8™ Cartridges for QX200™/QX100™
Droplet Generator
Droplet Generation Cartridges

BioRad 1864008 [🔗](#)



DG8 Cartridge Holder
Cartridge Holder
BioRad 1863051 [🔗](#)



Droplet Generation Oil for Probes
by BioRad Sciences
Catalog #: 1863005



DG8™ Gaskets for QX200™/QX100™

Droplet Generator

DG8 Gasket

BioRad 1863009 [🔗](#)

- 29 Load the DG8 Cartridge Holder onto the QX200 Droplet Generator and close the lid to begin droplet generation.



QX200™ Droplet Generator

QX200™ Droplet Generator

BioRad 1864002 [🔗](#)

- 30 Once droplet generation is complete, carefully transfer **40 µl** of the droplet oil emulsion from the DG8 Cartridge to a 96-well ddPCR plate.



ddPCR™ 96-Well Plates

ddPCR™ 96-Well Plates

BioRad 12001925 [🔗](#)

- 31 Once the droplets from all samples have been transferred to the 96-well ddPCR plate, seal the plate with plate heat seal foil using the PX1 PCR Plate Sealer.



PCR Plate Heat Seal, foil, pierceable

Heat Seal foil

BioRad 1814040 [🔗](#)



PX1 PCR Plate Sealer
Plate Sealer
BioRad 1814000 [🔗](#)

32

After sealing the 96-well plate, incubate the plate at $\text{at } 4\text{ °C}$ for $\text{at least } 00:15:00$.

33 Place the sealed 96-well plate on the C1000 Touch Thermal Cycler and perform thermal cycling as specified in Table 2 below.



C1000 Touch™ Thermal Cycler with 96-Deep Well Reaction Module
Thermal Cycler
BioRad 1851197 [🔗](#)

Cycling Step	Temperature (C)	Time	Number of Cycles
Reverse Transcription	$\text{at } 50\text{ °C}$	$\text{at least } 01:00:00$	1
Enzyme Activation	$\text{at } 95\text{ °C}$	$\text{at least } 00:10:00$	1
Denaturation	$\text{at } 95\text{ °C}$	$\text{at least } 00:00:30$	40x
Annealing/Extension	$\text{at } 59\text{ °C}$	$\text{at least } 00:01:00$	above
Enzyme Deactivation	$\text{at } 98\text{ °C}$	$\text{at least } 00:10:00$	1
Hold	$\text{at } 4\text{ °C}$	Infinite	1

34

When thermal cycling is complete, allow the 96-well plate to incubate at $\text{at } 4\text{ °C}$ for $\text{at least } 00:20:00$.

35 Place the 96-well plate in the QX200 Droplet Reader and ensure there is adequate ddPCR Droplet Reader Oil in the reservoir.



QX200™ Droplet Reader
QX200™ Droplet Reader
BioRad 1864003 [\(G\)](#)



ddPCR™ Droplet Reader Oil
by BioRad Sciences
Catalog #: 1863004

- 36 Open QuantaSoft Software to set up a new plate layout according to the experimental design. Refer to the QX100 or QX200 Droplet Reader and QuantaSoft Software Instruction Manual.
- 37 Under Setup, double click on a well in the plate layout to open the Well Editor dialog box.
- 38 Designate the sample name, experiment type (ABS), ddPCR Supermix for Probes (No dUTP) as the supermix type, target name(s), and target type(s): Ch1 for FAM and Ch2 for HEX or VIC.
- 39 Select Apply to load the wells and, when finished, select OK.
- 40 Once the plate layout is complete, select Run to begin the droplet reading process. Select the appropriate dye set used and run options when prompted.
- 41 After data acquisition (~80s per well), select samples in the well selector under Analyze. Examine the automatic thresholding applied to the 1-D or 2-D amplitude data and, if necessary, set thresholds or clusters manually.
- 42 The concentration reported is copies per uL of RNA in the final 1x ddPCR reaction.