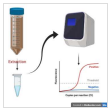

Version 2

Mar 04, 2021

# One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNA V.2

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1 Works for me dx.doi.org/10.17504/protocols.io.bsgvnbw6

Coronavirus Method Development Community | Wastewater-based epidemiology working group
1 more workspace

Hannah Greenwald

SUBMIT TO PLOS ONE

ABSTRACT

The following protocol describes the reverse transcription quantitative polymerase chain reaction (RT-qPCR) methods that we have used at the University of California, Berkeley in the Nelson Lab and COVID-WEB (covid-web.org) for downstream analysis of extracted wastewater samples for the purpose of SARS-CoV-2 surveillance. We have been successfully using this protocol paired with the Sewage, Salt, Silica, and SARS-CoV-2 (4S) method. We are including multiple assays that we have used throughout this project, although not all of them may be in active use. This method may need to be adapted based on your lab's application, available equipment, and safety requirements. 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.

Version 1 included the following assays: N1, PMMoV, BCoV, and SOC.
Version 2 was amended to include the following assays: crAssphage, Bacteroides HF183 rRNA, and human 18S rRNA.

Oscar Whitney, Basem Al-Shayeb, Alex Crits-Cristoph, Mira Chaplin, Vinson Fan, Hannah Greenwald, Adrian Hinkle, Rose Kantor, Lauren Kennedy, Anna Maurer, Robert Tjian, Kara L. Nelson, UC Berkeley Wastewater-based epidemiology consortium. V.4 - Direct wastewater RNA capture and purification via the "Sewage, Salt, Silica and SARS-CoV-2 (4S)" method.
http://dx.doi.org/10.17504/protocols.io.bpdfmi3n

THIS PROTOCOL ACCOMPANIES THE FOLLOWING PUBLICATION

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WHAT'S NEW

Addition of assays for crAssphage, HF183 Bacteroides rRNA, and human 18S rRNA

KEYWORDS

RT-qPCR, SARS-CoV-2, WBE, wastewater surveillance, qPCR, QuantStudio3, COVID-19



IMAGE ATTRIBUTION

Generated via Biorender by Hannah Greenwald

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47349

GUIDELINES

This protocol is intended for research purposes to quantify SARS-CoV-2 and additional targets in RNA extracted from wastewater. It is not intended for diagnostic purposes.

MATERIALS TEXT

**Reagents:**

☒TaqMan™ Fast Virus 1-Step Master Mix **Thermo Fisher Catalog #4444434** Step 3.2

☒1.5 mL LoBind tubes **Eppendorf Catalog #022431021** Step 3.2

☒Microplates Genomic 96-Well Eppendorf Twin.tec PCR Clear Extra-thin polypropylene wells Semi **Eppendorf Catalog #E951020303** Step 5.1

☒MicroAmp™ Optical Adhesive Film **Thermo Fisher Catalog #4311971** Step 6

☒RNase AWAY™ Spray Bottle, RNase in spray bottle; 475mL **Thermo Fisher Catalog #7002** Step 1

☒PCR Water (nuclease free) **Contributed by users Catalog #PCPW**

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 (with adapters for individual tubes, 8-strip tubes, and 96-well plates), a Class II biosafety cabinet, refrigerator/freezers, and vortex.

SAFETY WARNINGS

Please coordinate with your local biosafety committee to adapt wastewater procedures in accordance with biosafety standards for your institution and interim guidance issued by the CDC (https://www.cdc.gov/coronavirus/2019-ncov/lab/lab-biosafety-guidelines.html#environmental).

DISCLAIMER:

> This protocol includes widely accepted methodologies for qPCR and assays adapted from previous publications. The methods have been compiled to protocols.io for those that are new to qPCR and seeking to incorporate wastewater SARS-CoV-2 surveillance into their lab. 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. This protocol is intended for research purposes to quantify SARS-CoV-2 and additional targets in RNA extracted from wastewater. It is not intended for diagnostic purposes.

BEFORE STARTING

This protocol is for molecular analysis of RNA extract, downstream of concentration/extraction procedures on the original wastewater samples. RNA must be extracted from samples prior to beginning this protocol.

Experimental Protocol

1 **Clean and set up:** Clean the work surface and equipment (including recently calibrated pipettes, vortex, tube spinner, and personal gloves) with 10% bleach followed by 70% ethanol followed by ☒RNase AWAY™ Spray Bottle, RNase in spray bottle; 475mL **Thermo Fisher Catalog #7002** .

> We worked with University of California, Berkeley EH&S to adapt our protocol for safety considerations. We conduct all of our experimental steps in a class II biosafety cabinet in a BSL2 lab.

2 **Gather supplies:** Gather materials needed for the assay that you are completing (see Assay Details section), including samples, probe, forward primer, reverse primer, master mix, water, and standard. Place on ice.

Unless otherwise stated, primers are ordered as LabReady DNA oligos from IDT and arrive at a concentration of [M]**100 Micromolar (µM)** . Probes are ordered as LabReady PrimeTime 5' FAM/ZEN/3' IBFQ (Purification:HPLC Purification from IDT) and arrive at a concentration of [M]**100 Micromolar (µM)**

> Unless otherwise stated, the forward primer ( **15.81 µl** ), reverse primer ( **15.81 µl** ), and probe ( **4 µl** ) are mixed together with PCR water ( **200 µl** ) to create a primer/probe mix for more efficient sample processing. The resulting concentrations in the mix are [M]**6.7 Micromolar (µM)** for each primer and [M]**1.7 Micromolar (µM)** probe.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

2.1 ⚠

Keep highly concentrated standards separate from all other components.

2.2 ⚠

From this point forward, all reagents should be kept below 4C-- either in cooling racks or on ice. Ensure components do not freeze again once thawed before they are used

3 **Create the Reaction Mix.**
Assay-specific details are included in section 2.

The reaction mix (sometimes referred to as "Master mix") contains all of the ingredients needed for RT-qPCR, except for the sample/template itself.

3.1 Calculate the total volume of reaction mix needed. Include 15 µL per well in addition to 10-20% excess (to account for losses and pipetting error).

All assays are set up as 20 µL reactions with 5 µL of template.

3.2 ☒TaqMan™ Fast Virus 1-Step Master Mix **Thermo**
Combine **Fisher Catalog #4444434** , the primer/probe mix, and DNase/RNase free water in
☒1.5 mL LoBind tubes
**Eppendorf Catalog #022431021**

When preparing the reaction mix, keep all reagents cold-- either in cooling racks or on ice. Add components starting with the largest volume.

4 **Create the standard dilutions.**

Options for standards for each assay as well as recommended dilution concentrations are described in the following section ("Assay Details").

The standard stock should be resuspended in low EDTA TE buffer with added carrier RNA at a concentration of 0.1-1 mg/L and then aliquotted into single-use Lobind tubes (to avoid freeze-thaw) stored at -80 C. Standard dilutions should be made fresh each day from a newly thawed standard stock.

4.1 ⚠

Aliquot water in the correct volumes into strip tubes.
☒EasyStrip™ Plus Tube Strip with Attached Flat Caps **Thermo**
**Fisher Catalog #AB2000**

Ensure aliquoted water is the correct volume. Pipette back up the water and check for a gap in the tip or water left in the tube.

We find that making dilutions in ~100 uL total volume works well for us. For example, in a 10-fold dilution, 10 uL of the previous standard dilution would be added to 90 uL of water.

4.2

Pipette standard stock solution into the top dilution then proceed with the serial dilution.

Mix thoroughly between each dilution by capping and vortexing, then spin down.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

4.3

Change gloves and clean your work station after handling standards.

5 **Aliquot reaction mix and sample or standard into a 96-well plate**

5.1 Place a 96 well plate on ice or in a cool rack.

The plates we use in our QuantStudio 3 qPCR machine are
Microplates Genomic 96-Well Eppendorf Twin.tec PCR Clear Extra-thin polypropylene wells Semi **Eppendorf Catalog #E951020303**
. We also use cool racks (IsoFreeze, see below) in place of ice.

Check your instrument for plate compatibility by determining if your plates should be unskirted, semi-skirted, or full-skirted in addition to the well volume requirements. We recommend using clear plates over colored plates because it is easier to view all wells and corresponding labels.

IsoFreeze PCR Rack with Lid, Purple to Pink
Cool Rack
**Genesee** **27-400**

5.2

Add 15 µL of reaction mix to each well. Before adding to the plate, ensure the mixture is well mixed but avoid bubbles (pipette up and down).

Using a multichannel pipette can expedite this process. Visually inspect the plate afterwards to ensure every well contains reaction mix as intended.

5.3

Add 5 µL of well-mixed sample RNA, no template control (DNase/RNase free water), or standard dilution to each well. Pipette up and down to mix.

**No template control (NTC):** a qPCR negative control with respect to the target where DNase/RNase free water is input to the reaction instead of sample.

After designing the plate in the qPCR instrument software, print the layout for reference in the lab. This "cheat sheet" can also include volumes of reaction mix components and volumes for standard dilutions.

6 MicroAmp™ Optical Adhesive Film **Thermo**
Seal the plate with **Fisher Catalog #4311971** .

Use a rubber plate sealer to press the adhesive film securely onto the plate. Then, using the edge, score around the wells of the plate, between every column, and between every row to prevent cross-contamination.

7

Gently vortex the plate to mix and spin down in a plate spinner until all liquid is in the bottom of the wells and bubbles have popped or risen to the surface.

Be sure to inspect the plate to verify that all wells have the same volume in the intended wells.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

8 Place the plate in the qPCR machine, load the previously designed plate file with cycling conditions included, and start.

The qPCR machine we use is a cloud-connected QuantStudio3 Real-Time PCR System:

QuantStudio 3 Real-Time PCR System
qPCR machine
**Applied Biosystems** **A28572**

The instrument can be set to Fast cycling mode. Set data collection during the annealing/extension step

We use the following cycling conditions used for all assays listed in this protocol:

| A | B | C | D |
|---|---|---|---|
| **Step** | **Temperature (C)** | **Time (min:sec)** | **Number of Cycles** |
| UNG Incubation | 25 | 2:00 | 1 |
| Rerverse Transcription | 50 | 15:00 | 1 |
| Polymerase Activation | 95 | 2:00 | 1 |
| Denaturation | 95 | 0:03 | 45 |
| Annealing/Extension | 55 | 0:30 | |

9 **Follow-up QA/QC**

Your full SARS-CoV-2 RT-qPCR protocol should include steps listed in the following subsections.

9.1 Ensure MIQE guidelines are met using the following resource:

Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, Mueller R, Nolan T, Pfaffl MW, Shipley GL, Vandesompele J, Wittwer CT (2009). The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments.. Clinical chemistry.
https://doi.org/10.1373/clinchem.2008.112797

9.2 Assess RT-PCR inhibition

Inhibition testing could include:
- Spike and dilute methods
- Internal positive control addition
- Direct dilute methods

For more information, check the following resources:

Centers for Disease Control and Prevention (2020). Coronavirus Disease 2019 (COVID-19). Wastewater Surveillance Testing Methods.
https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/wastewater-surveillance/testing-methods.html

Cao Y, Griffith JF, Dorevitch S, Weisberg SB (2012). Effectiveness of qPCR permutations, internal controls and dilution as means for minimizing the impact of inhibition while measuring Enterococcus in environmental waters.. Journal of applied microbiology.
https://doi.org/10.1111/j.1365-2672.2012.05305.x

9.3 Normalize SARS-CoV-2 signal using a fecal concentration control

The fecal concentration control target could be:
- Pepper Mild Mottle virus
- Human 18S rRNA
- Bacteroides HF183
- crAssphage

For more information, check the following resources:



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

Centers for Disease Control and Prevention (2020). Coronavirus Disease 2019 (COVID-19). Wastewater Surveillance Testing Methods.
https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/wastewater-surveillance/testing-methods.html

Oscar N. Whitney, Lauren C. Kennedy, Vinson Fan, Adrian Hinkle, Rose Kantor, Hannah Greenwald, Alexander Crits-Christoph, Basem Al-Shayeb, Mira Chaplin, Anna C. Maurer, Robert Tjian, Kara L. Nelson (2020). Sewage, Salt, Silica and SARS-CoV-2 (4S): An economical kit-free method for direct capture of SARS-CoV-2 RNA from wastewater.. medRxiv.
http://10.1101/2020.12.01.20242131

9.4 Assess concentration and/or extraction consistency

Process control(s) could include:
- proxy virus (e.g., bovine coronavirus)
- free-RNA (e.g., synthetic oligomer construct)

For more information, check the following resources:

Centers for Disease Control and Prevention (2020). Coronavirus Disease 2019 (COVID-19). Wastewater Surveillance Testing Methods.
https://www.cdc.gov/coronavirus/2019-ncov/cases-updates/wastewater-surveillance/testing-methods.html

Rose S. Kantor, Kara L. Nelson, Hannah D. Greenwald, and Lauren C. Kennedy (2021). Challenges in Measuring the Recovery of SARS-CoV-2 from Wastewater. Environmental Science and Technology.
https://doi.org/10.1021/acs.est.0c08210

Gonzalez R, Curtis K, Bivins A, Bibby K, Weir MH, Yetka K, Thompson H, Keeling D, Mitchell J, Gonzalez D (2020). COVID-19 surveillance in Southeastern Virginia using wastewater-based epidemiology.. Water research.
https://doi.org/10.1016/j.watres.2020.116296

Assay Details

10 **SARS-CoV-2 nucleocapsid N gene (N1)**

The SARS-CoV-2 nucleocapsid N gene (N1) is one of the CDC assays for SARS-CoV-2 and is our primary target for SARS-CoV-2.

Centers for Disease Control and Prevention (2020). Coronavirus Disease 2019 (COVID-19). Research Use Only 2019-Novel Coronavirus (2019-nCoV) Real-time RT-PCR Primers and Probes.
https://www.cdc.gov/coronavirus/2019-ncov/lab/rt-pcr-panel-primer-probes.html

10.1 **N1 Reaction Mix Recipe**

☒2019-nCoV CDC RUO Primers and

The Probes **IDT Catalog #10006713** arrive at a premixed concentration of 15 uM, and the following recipe can be used for the reaction mix:



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 8.5 µL |
| Primer/probe mix | F: 500 nM, R: 500 nM, P: 125 nM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Total | | | 15 µL |

### 10.2 N1 Reaction Mix Recipe -- with optional Vetmax Xeno assay

☒2019-nCoV CDC RUO Primers and Probes **IDT Catalog #10006713** The arrive at a premixed concentration of 15 uM. For this assay, ☒VetMAX™ Xeno™ Internal Positive Control - VIC™ Assay **Thermo Fisher Catalog #A29767** ☒VetMAX™ Xeno™ Internal Positive Control RNA **Thermo Fisher Catalog #A29763** and can be added in order to assess inhibition in samples (through internal positive control addition). The N1 probe uses a FAM reporter while the Xeno assay uses a VIC reporter, allowing them to be duplexed. The following recipe can be used for the reaction mix:

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | - | - | 7.6 µL |
| Primer/probe mix | F: 500 nM, R: 500 nM, P: 125 nM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Xeno Assay | Proprietary | Proprietary | 0.8 µL |
| Xeno RNA | 10000 copies/µL | 50 copies/µL | 0.1 µL |
| Total | | | 15 µL |

Note that the internal positive control assay and RNA are added to the reaction mix. Therefore, the no template controls (NTCs) will become positive for Vetmax Xeno and can serve as a baseline for inhibition testing.

### 10.3 N1 primers/probe/amplicon sequences

☒2019-nCoV CDC RUO Primers and Probes **IDT Catalog #10006713**

The N1 assay has an amplicon length of 72 nucleotides.

| A | B |
|---|---|
| forward | GACCCCAAAATCAGCGAAAT |
| reverse | TCTGGTTACTGCCAGTTGAATCTG |
| probe | FAM/ACCCCGCATTACGTTTGGTGGACC/IABkFQ |
| amplicon | GACCCCAAAATCAGCGAAATGCACCCCGCATTACGTTTGGTGGACCCTCAGATTCAACTGGCAGTAACCAGA |

### 10.4 N1 Standard

There are many options to use as N1 standard. We have used and had success with the following options:

- Twist Bioscience offers a complete genome synthetic RNA standard:
  ☒Twist synthetic SARS-CoV-2 RNA control **Twist Bioscience Catalog #Mt007544.1**
- DNA plasmids ordered and linearized for use as a DNA standard:
  ☒2019-nCoV CDC RUO Plasmid Controls **IDT Catalog #10006625**
- DNA plasmids ordered and *in vitro* transcribed for use as an RNA standard

Our standard curves include the following concentrations: 5, 10, 20, 100, 1000, 10000, and 100000 gene copies/reaction.

## 11 Pepper Mild Mottle virus coat protein gene (PMMoV)

Pepper mild mottle virus can be used as a fecal concentration control to normalize N1 signal. The Pepper Mild Mottle virus coat protein gene (PMMoV) primers and probe sequences that we include here were used by Haramoto et. al, 2013.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

Haramoto E, Kitajima M, Kishida N, Konno Y, Katayama H, Asami M, Akiba M (2013). Occurrence of pepper mild mottle virus in drinking water sources in Japan. Applied and environmental microbiology. https://doi.org/10.1128/AEM.02354-13

### 11.1 PMMoV Reaction Mix Recipe

The PMMoV primer/probe mix is made by adding **20 µl** of forward primer (LabReady 100 uM), **20 µl** of reverse primer (LabReady 100 uM), and **10 µl** of probe (LabReady 100 uM) to **200 µl** of DNAse/RNAse free water or low EDTA TE buffer. The following recipe can be used for the reaction mix:

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 9 µL |
| Primer/probe mix | F: 8 uM, R: 8 uM, P: 4 uM | F: 400 nM, R: 400 nM, P: 200 nM | 1 µL |
| Total | | | 15 µL |

### 11.2 PMMoV primer/probe/amplicon sequences

The PMMoV assay has an amplicon length of 68 nucleotides. Note that the original publication (Haramoto et. al, 2013) had a probe with an MGB quencher as a melt temperature modifier, but the ZEN/IBFQ quencher has been working for us.

| A | B |
|---|---|
| forward | GAGTGGTTTGACCTTAACGTTTGA |
| reverse | TTGTCGGTTGCAATGCAAGT |
| probe | FAM/CCTACCGAAGCAAATG/ZEN/IABkFQ |
| amplicon (Accession AB716964) | GAGTGGTTTGACCTTAACGTTTGAGCGGCCTACCGAAGCAAATGTCGCACTTGCATTGCAACCGACAA |

### 11.3 PMMoV Standard

We have successfully used the following PMMoV standards:
- RNA Ultramer from IDT
- DNA gBlock from IDT

The standard sequence is based on the amplicon plus additional 5 nucleotides from the NCBI sequence (Accession: AB71694) added to each end:

AAUGAGAGUGGUUUGACCUUAACGUUUGAGCGGCCUACCGAAGCAAAUGUCGCACUUGCAUUGCAACC GACAAUUGCA

Our standard curves include the following concentrations: $10^2$, $10^3$, $10^4$, $10^5$,$10^6$, $10^7$, and $10^8$ gene copies/reaction.

## 12 Bovine coronavirus transmembrane protein gene (BCoV)

Bovine coronavirus can be used as a spike-in process control. The bovine coronavirus transmembrane protein gene (BCoV) primers and probe sequences that we include here were used by Decaro, et. al 2008.

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. Journal of virological methods. https://doi.org/10.1016/j.jviromet.2008.05.016

### 12.1 BCoV Reaction Mix Recipe

The BCoV primer/probe mix is made by adding **18 µl** of forward primer (LabReady 100 uM),



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

**18 µl** of reverse primer (LabReady 100 uM), and **5 µl** of probe (LabReady 100 uM) to **200 µl** of DNAse/RNAse free water or low EDTA TE buffer.

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 7.59 µL |
| Primer/probe mix | F: 7.5 uM, R: 7.5 uM, P: 2.1 nM | F: 900 nM, R: 900 nM, P: 250 nM | 2.41 µL |
| Total | | | 15 µL |

### 12.2 BCoV primers/probe/amplicon

The BCoV assay has an amplicon length of 85 nucleotides.

| A | B |
|---|---|
| forward | CTGGAAGTTGGTGGAGTT |
| reverse | ATTATCGGCCTAACATACATC |
| probe | FAM/CCTTCATATCTATACACATCAAGTTGTT/IABkFQ |
| amplicon (Accession AF39154) | CTGGAAGTTGGTGGAGTTTCAACCCAGAAACAAACAACTTGATGTGTATAGATATGAAGGGAAGGATGTATGTTAGGCCGATAAT |

### 12.3 BCoV Standard

We have successfully used the following types of standards for this assay:
- RNA Ultramer from IDT
- DNA gBlock from IDT

The standard curve range will depend on the amount spiked into the sample and the expected recovery. Our standard curves include the following concentrations: $10^3$, $10^4$, $10^5$,$10^6$, $10^7$, and $10^8$ gene copies/reaction.

## 13 Synthetic Oligomer Construct T33-21 free-RNA (SOC)

We designed an assay that targets RNA that is not naturally present in wastewater to serve as a free-RNA recovery control that we could spike into our samples. To this end, we *in vitro* transcribed RNA from a synthetic oligomer construct, T33-21 (12-mer) from Hsia et. al 2016.

Hsia Y, Bale JB, Gonen S, Shi D, Sheffler W, Fong KK, Nattermann U, Xu C, Huang PS, Ravichandran R, Yi S, Davis TN, Gonen T, King NP, Baker D (2016). Corrigendum: Design of a hyperstable 60-subunit protein icosahedron. Nature.
https://doi.org/10.1038/nature20108

### 13.1 SOC reaction mix recipe

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 8.5 µL |
| Primer/probe mix | F: 6.7 uM, R: 6.7 uM, P: 1.7 uM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Total | | | 15 µL |

### 13.2 SOC primers/probe/amplicon sequences

The SOC assay amplicon length is 89 nucleotides.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

| A | B |
|---|---|
| forward | CCACCAAAGTGGGCGATAAA |
| reverse | GGTGCCATTCGCCTCAATAA |
| probe | FAM/TGGCGGTGAGGAAGTTTGGAAAGA/IABkFQ |
| amplicon | CCACCAAAGTGGGCGATAAAGGCAGCACCCGTTTATTTGGCGGTGAGGAAGTTTGGAAAGATAGCCCGATTATTGAGGCGAATGGCACC |

### 13.3 SOC standard and spike-in

SOC RNA standards were generated by *in vitro* transcription of an IDT gBlock corresponding to the amplicon with a T7 promoter added to the 5' end of the sequence. We used

☒ HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**

as per manufacturer's specifications to generate crude RNA and digested with DNase I to remove the gBlock template. We purified this crude RNA with

☒ AMPure XP **Beckman Coulter Catalog #A63881** and quantified the standard by Nanodrop.

The standard curve range will depend on the amount spiked into the sample and the expected recovery. Our standard curves include the following concentrations: $10^4$, $10^5$,$10^6$, $10^7$,$10^8$, and $10^9$ gene copies/reaction.

## 14 CrAssphage CPQ_056 (crAssphage)

CrAssphage has been proposed as a fecal concentration control to normalize N1 signal. The primers and probe described in this protocol were designed by Stachler et. al, 2017.

Stachler E, Kelty C, Sivaganesan M, Li X, Bibby K, Shanks OC (2017). Quantitative CrAssphage PCR Assays for Human Fecal Pollution Measurement.. Environmental science & technology. https://doi.org/10.1021/acs.est.7b02703

### 14.1 CrAssphage Reaction Mix Recipe

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 8.5 µL |
| Primer/probe mix | F: 6.7 uM, R: 6.7 uM, P: 1.7 uM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Total | | | 15 µL |

### 14.2 CrAssphage primers/probe/amplicon

The crAssphage CPQ_056 amplicon is 126 nucleotides long.

| A | B |
|---|---|
| forward 056F1 | CAGAAGTACAAACTCCTAAAAAA CGTAGAG |
| reverse 056R1 | GATGACCAATAAACAAGCCATTA GC |
| probe 056P1 | FAM-AATAACGATTTACGTGATGTAAC |
| amplicon (Accession AF39154) | CAGAAGTACAAACTCCTAAAAAACGTAGAGGTAGAGGTATTAATAACGATTTACGTGATGTAACTCGTAAAAAGTTTGATGAACATACTGATTGTAATAAAGCTAATGGCTTGTTTATTGGTCATC |

### 14.3 CrAssphage Standard

For the crAssphage standard, we ordered a custom geneBlocks DNA (Integrated DNA Technologies).

Our standard curve includes the following concentrations: 1e3, 1e4, 1e5, 1e6, 1e7, 1e8, 1e9 gene copies/reaction.

## 15 *Bacteroides* 16S ribosomal RNA HF183/BacR287 (Bacteroides)



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

Bacteroides has been proposed as a fecal concentration control to normalize N1 signal, although we have not had success with it for SARS-CoV-2 WBE. The primers and probe for this assay were previously presented in Green et. al, 2014.

Green HC, Haugland RA, Varma M, Millen HT, Borchardt MA, Field KG, Walters WA, Knight R, Sivaganesan M, Kelty CA, Shanks OC (2014). Improved HF183 quantitative real-time PCR assay for characterization of human fecal pollution in ambient surface water samples.. Applied and environmental microbiology. https://doi.org/10.1128/AEM.04137-13

### 15.1 Bacteroides Reaction Mix Recipe

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 8.5 µL |
| Primer/probe mix | F: 6.7 uM, R: 6.7 uM, P: 1.7 uM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Total | | | 15 µL |

### 15.2 Bacteroides primers/probe/amplicon

The Bacteroides amplicon is 126 nucleotides long.

| A | B |
|---|---|
| forward | ATCATGAGTTCACATGTCCG |
| reverse | CTTCCTCTCAGAACCCCTATCC |
| probe | FAM/AT CGT TGA C/ZEN/T AGG TGG GCC GTT AC/IBFQ |
| amplicon (Accession MT464394.1) | ATCATGAGTTCACATGTCCGCATGATTAAAGGTATTTTCCGGTAGACGATGGGGATGCGTTCCATTAGATAGTAGGCGGGGTAACGGCCCACCTAGTCAACGATGGATAGGGGTTCTGAGAGGAAG |

### 15.3 Bacteroides Standard

Bacteroides RNA standards were generated by *in vitro* transcription of an IDT gBlock corresponding to the amplicon with a T7 promoter added to the 5' end of the sequence. We used

☒ HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**

as per manufacturer's specifications to generate crude RNA and digested with DNase I to remove the gBlock template. We purified this crude RNA with

☒ AMPure XP **Beckman Coulter Catalog #A63881** and quantified the standard by Nanodrop.

Our standard curve includes the following concentrations: 2e2, 2e3, 2e4, 2e5, 2e6, 2e7, 2e8 gene copies/reaction.

## 16 Human 18S ribosomal subunit RNA (18S rRNA)

The human 18S rRNA assay was designed by our group (first presented in Whitney et. al, 2020) for use as a fecal concentration control to normalize N1 signal. Although we have not had success with it for SARS-CoV-2 normalization, it may be a promising degradation control, indicating when samples may no longer reflect accurate target values.

Whitney ON, Kennedy LC, Fan V, Hinkle A, Kantor R, Greenwald H, Crits-Christoph A, Al-Shayeb B, Chaplin M, Maurer AC, Tjian R, Nelson KL (2020). Sewage, Salt, Silica and SARS-CoV-2 (4S): An economical kit-free method for direct capture of SARS-CoV-2 RNA from wastewater.. medRxiv : the preprint server for health sciences. https://doi.org/pii:2020.12.01.20242131.10.1101/2020.12.01.20242131

### 16.1 18S rRNA Reaction Mix Recipe



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6

| A | B | C | D |
|---|---|---|---|
| **Reagent** | **Stock Concentration** | **Reaction Concentration** | **Volume per reaction** |
| Taqman Master Mix | 4x | 1x | 5 µL |
| Water | NA | NA | 8.5 µL |
| Primer/probe mix | F: 6.7 uM, R: 6.7 uM, P: 1.7 uM | F: 500 nM, R: 500 nM, P: 125 nM | 1.5 µL |
| Total | | | 15 µL |

## 16.2 18S rRNA primers/probe/amplicon

The 18S rRNA amplicon is 138 nucleotides long.

| A | B |
|---|---|
| forward | GGTTCCTTTGGTCGCTCGCT |
| reverse | GGGCTGACCGGGTTGGTTTT |
| probe | FAM/AG AGC TAA T/ZEN/A CAT GCC GAC GGG C/IBFQ/ |
| amplicon (Accession 6G18_2) | GGTTCCTTTGGTCGCTCGCTCCTCTCCTACTTGGATAACTGTGGTAATTCTAGAGCTAATACATGCCGACGGGCGCTGACCCCCTTCGCGGGGGGGGATGCGTGCATTTATCAGATCAAAACCAACCCGGTCAGCCC |

## 16.3 18S rRNA Standard

18S RNA standards were generated by *in vitro* transcription of an IDT gBlock corresponding to the amplicon with a T7 promoter added to the 5' end of the sequence. We used

☒ HiScribe T7 High Yield RNA Synthesis Kit - 50 rxns **New England Biolabs Catalog #E2040S**

as per manufacturer's specifications to generate crude RNA and digested with DNase I to remove the gBlock template. We purified this crude RNA with

☒ AMPure XP **Beckman Coulter Catalog #A63881** and quantified the standard by Nanodrop.

Our standard curve includes the following concentrations: 1e2, 1e3, 1e4, 1e5, 1e6, 1e7, 1e8 gene copies/reaction.



**Citation:** Hannah Greenwald, Lauren C Kennedy, Vinson Fan, Rose Kantor, Kara L Nelson (03/04/2021). One-Step RT-qPCR for SARS-CoV-2 Wastewater Surveillance: N1, PMMoV, BCoV, SOC, CrAssphage, Bacteroides rRNA, 18S rRNAÃÂ . https://dx.doi.org/10.17504/protocols.io.bsgvnbw6