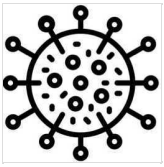

Jul 17, 2021

Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR

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1 Works for me Share dx.doi.org/10.17504/protocols.io.bv5bn82n

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DISCLAIMER

ABSTRACT

This process instruction describes the steps for quantitative analysis of nucleic acid from SARS-CoV-2 with a triplex Reverse Transcriptase droplet digital Polymerase Chain Reaction (RT-ddPCR) assay targeting the N Gene, S Gene and 2 mutation assays (one for HV69-70 and one for E484K/N501Y) in extracted and purified RNA samples from solid wastewater samples for population level SARS-CoV-2 community surveillance. RT-ddPCR is a modified version of conventional RT-PCR workflows which involves separating the reaction mixture into many partitions (~20,000) before thermal cycling which allows for direct absolute quantification of the target RNA molecules.

Future protocols will be published that are complementary to this one and describe assays targeting additional SARS-CoV-2 mutations.

This protocol uses RNA extracted using this protocol: High Throughput RNA Extraction and PCR Inhibitor Removal of Settled Solids for Wastewater Surveillance of SARS-CoV-2 RNA. That RNA is generated from samples subjected to pre-analytical steps outlined in: High Throughput pre-analytical processing of wastewater settled solids for SARS-CoV-2 RNA analyses. It is recommended that these assays be run along assays for PMMoV and BCoV as controls as described in the companion protocol High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR

The readout of this assay is a concentration of each target in the extracted RNA samples (copies/µL).

Scope
This process instruction applies to quantitative analysis of nucleic acid from SARS-CoV-2 RNA from solid wastewater samples with ddPCR using a Bio-Rad AutoDG Droplet Digital PCR system consisting of the AutoDG Automated Droplet Generator and the QX200 droplet reader.

DOI

dx.doi.org/10.17504/protocols.io.bv5bn82n

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

PROTOCOL CITATION

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https://dx.doi.org/10.17504/protocols.io.bv5bn82n

KEYWORDS

null, SARS-CoV-2, wastewater, alpha, beta, gamma, variant, mutation, COVID-10

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MATERIALS TEXT

Equipment

- Bio-Rad QX200 AutoDG
- Bio-Rad QX200 Droplet Reader
- Bio-Rad PX1 PCR Plate Sealer (Bio-Rad, Catalog #:1814000)
- Agilent Bravo
- Eppendorf Master Cycler ProThermal Cycler
- Bio-Rad C1000 Touch Thermal Cycler
- Rainin Single-Channel Pipettes L1000XLS+, L200XLS+, L20XLS+, L10XLS+, , L2XLS+
- Rainin Multi-Channel Pipettes L12-1000XLS+, L12-200XLS+, L12-20XLS+, L12-10XLS+, L8-1000XLS+, L8-200XLS+, L8-20XLS+, L8-10XLS+
- Axygen Plate Centrifuge
- Benchtop vortex
- 96-well plate cold block for AutoDG droplet generator
- 96-well plate cold block for ddPCR plates
- Zebra GX430t label printer

Reagents

- ddPCR™ One-Step RT supermix for Probes
- Reverse Transcriptase
- 300mM DTT
- Automated Droplet Generation Oil for Probes
- Ambion Nuclease Free Water, 50mL
- ddPCR Droplet Reader Oil

Consumables

- Bio-Rad ddPCR™ 96-Well Plates
- Eppendorf twin.tec PCR Plate
- DG32™ Automated Droplet Generator Cartridges
- PCR PX1 Plate Sealer, foil, pierceable
- Pipet Tips for the AutoDG™
- Rainin low retention, pre-sterilized, filter tips 1000 µL, 200 µL, 20 µL
- Axygen Automation Tips, 20 µl, filtered, sterile tips (Axygen 20 µL tips)
- Reagent Reservoirs

●Automation Reservoirs
●Eppendorf Tubes, 1.5mL, 2mL and 5mL.
●2.5” x 0.5” Cryogenic Labels for the Zebra GX430t label printer

Samples and Test Materials

●Purified RNA from wastewater samples as described in High Throughput RNA Extraction and PCR Inhibitor Removal of Settled Solids for Wastewater Surveillance of SARS-CoV-2 RNA
●SARS-CoV-2 gRNA positive control (ATCC® VR-1986D™) diluted and aliquoted at a concentration of 50 copies per µL.
●Synthetic gene block containing the E484K/N501Y mutations purchased from IDT diluted and aliquoted at a concentration of 50 copies per µL.
●Heat inactivated SARS-CoV-2 B.1.1.7 (ATCC VR-**3326HK**™) diluted and aliquoted at a concentration of 50 copies per µL. Note: no RNA extraction is done.

Primer and Probe Sequences

See companion protocol for N and S gene primers and probes: High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR. Additional protocols to be published will describe primer and probe sequences targeting other SARS-CoV-2 mutations.

A	B	C
Target	Primer/Probe	Sequence
HV69-70 del	Forward	ACTCAGGACTTGTTCTTACCT
	Reverse	TGGTAGGACAGGGTTATCAAAC
	Probe	ATGCTATCTCTGGGACCAAT (5’ FAM or HEX/ZEN/3’ IBFQ)
E484K/N501Y	Forward	CTGAAATCTATCAGGCCGGT
	Reverse	GTTGGTAACCAACACCATAAG
	Probe	CACACCTTGTAATGGTGTTAAAGGTT (5’ FAM or HEX/ZEN/3’ IBFQ)

Primer and probes for the mutation assays. They are to be multiplexed with the assays for N and S as described in our companion protocol.

Preparation

1 Retrieve the ddPCR One-Step RT Supermix for probes from the **-20 °C** freezer and thaw the components **On ice** .

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

2 Retrieve ddPCR positive control aliquots (50 copies per uL gRNA) from the **-80 °C** freezer and thaw **On ice**

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

3 **For re-running frozen plates only:**

Thaw the RNA storage plate **On ice** . Before analysis of thawed frozen samples, ensure that the plate is adequately sealed and briefly vortex and centrifuge the plate to ensure the samples are well mixed.

4 Keep extracted RNA samples **On ice** or in a cold block from the freezer at all times.

ddPCR master mix preparation 45m

5 Prepare Master Mix

This is a triplex assay for the N, S, and a mutation gene. Protocols targeting the HV69-70 deletion and E484K/N501 mutation are in this document, but it is possible to swap out one or two of the assays for others that also target low copy number targets using the same procedure. However, we do not recommend multiplexing with a high copy number target like PMMoV because a high copy number target assay would need to use diluted RNA as template.

5.1 Prepare a working stock of fresh nuclease free water by pouring from a 50 mL Ambion Nuclease Free water into a 5mL eppendorf tube.

5.2 Store Master Mix components **On ice** as much as possible during the preparation process.

5.3 Briefly vortex One-Step RT supermix for Probes (Yellow Tube) and Reverse Transcriptase (Orange Tube) to mix contents then briefly spin with the benchtop centrifuge.

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

5.4 In a 2mL eppendorf tube, prepare the master mix according to the table for SARS-CoV-2 quantification with HV69-70 or E484K/N501Y. Store prepared master mix on ice.

Additional protocols will be published for other mutations associated with SARS-CoV-2 variants.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

A	B	C
	20x Concentration	Final Concentration/Rxn
Primers (each)	18 µM	900 nM
N Probe (FAM)	5 µM	250 nM
S Probe (HEX)	5 µM	250 nM
E484K/N501Y Probe (HEX)	2.5 µM	125 nM
E484K/N501Y Probe (FAM)	2.5 µM	125 nM

The 20x SARS-CoV-2 and E484K/N501Y Primer/Probe Mix contains primers and probes in the following concentrations (suspended in molecular grade water).

A	B	C
	20x Concentration	Final Concentration/Rxn
Primers (each)	18 µM	900 nM
N Probe (FAM)	5 µM	250 nM
S Probe (HEX)	5 µM	250 nM
HV69-70 Probe (HEX)	2.5 µM	125 nM
HV69-70 Probe (FAM)	2.5 µM	125 nM

The 20x SARS-CoV-2 and HV69-70 Primer/Probe Mix contains primers and probes in the following concentrations (suspended in molecular grade water).

A	B	C
Reagents	**Volume per Well**	**Volume Per Plate**
ddPCR™ One-Step RT supermix for Probes (Yellow Tube)	5.5 µL	580.8 µL
20x SARS-CoV-2 Primer/Probe Mix	3.3 µL	348.48 µL
Reverse Transcriptase (Orange Tube)	2.2 µL	232.32 µL
300mM DTT (Gray Tube)	1.1 µL	116.16 µL
Nuclease Free Water	4.4 µL	464.64 µL
Total Volume	16.5 µL	1742.4 µL

SARS-CoV-2 ddPCR Master Mix. Volume per plate assumes 96 well plate (with 10% excess).

Transfer Master Mix and Samples to ddPCR plate

6 For each assay, remove a cold block from the freezer and place a new Bio-Rad ddPCR 96-well Plate in it.

Note that these steps may be completed with a liquid handling robot. We use the BRAVO system.

7 Using a new reagent reservoir, manually pipette **16.5 µl** of the appropriate Master Mix to each well of each ddPCR Plate.

8 Transfer samples to the following wells on the plate either manually or using a liquid handling robot such as the Agilent Bravo system:

- Transfer **5.5 µl** of samples from columns 1-10 of the RNA plate into columns 1-10 of the ddPCR plate.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

- Transfer **5.5 µl** of the extraction controls from column 12 of the RNA plate into column 11 of the ddPCR plate.
- Transfer **5.5 µl** of NTC (water) into A-G of column 12.
- Add **5.5 µl** of ddPCR positive control to well H12 of the ddPCR plate (add manually even if using a liquid handler)

Use SARS-CoV-2 genomic RNA (ATCC® VR-1986D™) mixed 1:1 with appropriate mutation standard as ddPCR positive control (see materials section of the protocol). The extraction positive controls are generated during RNA extraction per this companion protocol: https://www.protocols.io/view/high-throughput-rna-extraction-and-pcr-inhibitor-r-btyrnpv6. In that protocol, the extraction positive control consists of SARS-CoV-2 genomic RNA that does not contain the mutations HV69-70 and E484K/N501Y or other mutation targets.

The plate layout should be:

	1	2	3	4	5	6	7	8	9	10	11	12
A	Sample 1										Extraction Negative control	NTC
B	Sample 2											
C	Sample 3											
D	Sample 4											
E	Sample 5											
F	Sample 6											
G	Sample 7											
H	Sample 8										Extraction Positive control	ddPCR Positive control

Note: The set up of this plate is slightly different from the set up of the 96 well extraction plate used in our companion protocol. Column 11 of this plate uses column 12 of the extraction plate. The lower right hand corner was purposely chosen for the placement of the positive controls.

9 Bring the BioRad ddPCR plate with Master Mix and samples to the Bio-Rad PX1 PCR Plate Sealer and place it in the metal carrier inside the plate sealer.

Do not store the metal carrier inside the plate sealer, as it will become warm and warm the samples during sealing.

10 Align a PCR Plate Heat Seal Foil on top of the ddPCR plate with the red line facing up.

11 Seal the plate by pressing the green “seal” button.

12 Briefly vortex the plate using a bench top plate vortexer.

Briefly spin down the plate in the bench top Axygen Plate Centrifuge.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

13

14 Store the ddPCR plate on a white cold block or **On ice** until droplet generation.

ddPCR droplet generation 45m

15 Generate Droplets:

15.1 On the AutoDG droplet generator: select “configure plate” and then press the blue arrow in the upper left corner to highlight all columns.

15.2 Load 3 DG32 cartridge plates and 2 tip boxes with the lid removed onto the AutoDG deck. The icons on the AutoDG display will change from yellow to green if the plates and tips are oriented correctly.

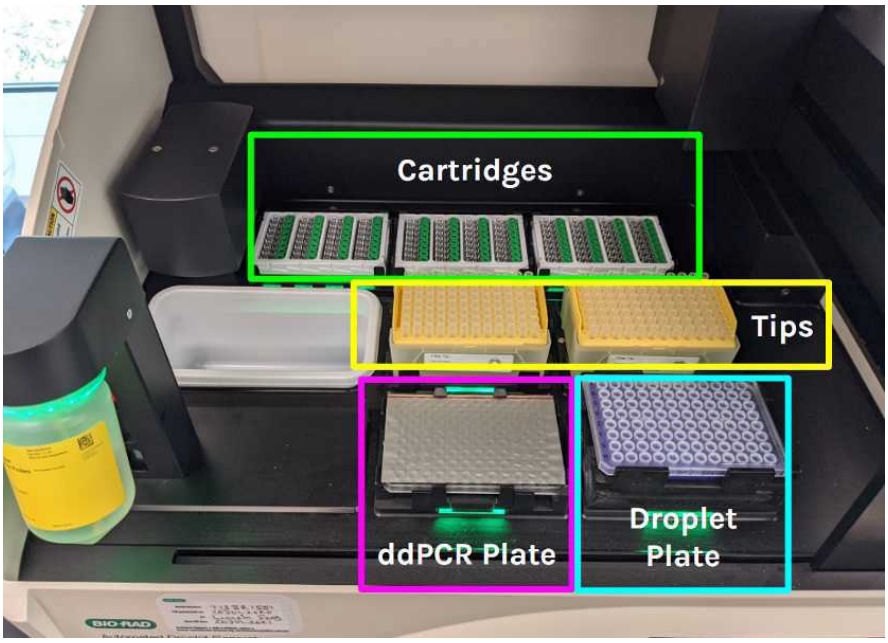
15.3 Place the sealed, vortexed, and centrifuged ddPCR plate in the Sample Plate position on the AutoDG deck.

15.4 Remove the AutoDG cooling block from the freezer and place it in the Droplet Plate slot on the AutoDG deck.

15.5 For the Droplet Plate: label a new Bio-Rad ddPCR™ 96-Well Plate and place it in the Auto DG cooling block.

15.6 Click “Generate Droplets” to begin droplet generation. Droplet generation will take approximately 45m **00:45:00** .

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n



15.7 When droplet generation is done, open the lid and remove the droplet plate from the Auto DG cold block and place it in the metal carrier inside the plate sealer.

The droplets are highly unstable at this stage. The droplets should not be left on the generator longer than they need to be. Once thermocycled the droplets are more stable.

15.8 Align a PCR Plate Heat Seal Foil on top of the droplet plate with the red line facing up.

15.9 Seal the plate by pressing the green “seal” button.

NOTE: Do NOT vortex plate at this point!

Thermocycling 45m

16 Place the plate in the thermocycler. Verify and run the thermocycler program according to the table for SARS-CoV-2 ddPCR.

The annealing temperature for the multiplex assay for N, S, and E484K/N501Y is higher (61°C) than that for N, S, and HV69-70 (59°C). A higher annealing temperature was necessary to ensure high specificity for the E484K/N501Y assay, and we found that the N and S assays performed with the same amplitude/efficiency of reaction at 61°C as at 59°C.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

For additional mutation assays, see those protocols for thermocycling conditions and take note of differences in recommended annealing temperature.

A	B	C	D
Steps	Cycle #s	Temp (°C)	Time
1	1	50	60 min
2	1	95	5 min
3	40	95	30 secs
	40	59	1 min
4	1	98	10 min
5	-	4	hold

Cycling conditions for the N/ S / HV69-70 multiplexed assay

A	B	C	D
Steps	Cycle #s	Temp (°C)	Time
1	1	50	60 min
2	1	95	5 min
3	40	95	30 secs
	40	61	1 min
4	1	98	10 min
5	-	4	hold

Cycling conditions for the N/ S / E484K/N501Y multiplexed assay

16.1 After the PCR program is done, store the ddPCR plate at **4 °C** (in the thermal cycler, on ice or in the fridge) until droplet reading.

Read Droplets

17 Before running the plate on the QX200 droplet reader, open the drawer on the left side of the instrument and check that there is adequate droplet reader oil and that the waste bottle is not overly full.

18 Load template from previous plate of the same assay - ensure that all parameters (supermix, dye, absorbance etc.) are correct for the assay used and that sample names are assigned to the appropriate wells. Save as with a new plate name.

Double check that the correct supermix label is used for the read and that the channels are labeled appropriately. These parameters can NOT be changed after you run the plate.

19 Name the file with a name clearly describing which plate you are reading.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

20 Remove the plate from the thermal cycler and secure it in the droplet reader by placing it in the stage, placing the metal brace on top of it and pressing down the black plastic tabs on either side of the brace.

21 Click “Run” in Quantasoft to commence droplet reading.
Select “Ok” when the next dialog box comes up. Do not change the settings from “Columns” and “FAM/HEX”.

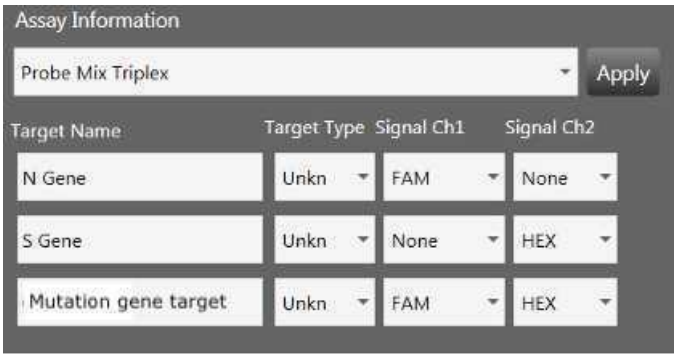
Post-Processing Analysis

22 SARS-CoV-2 triplex assay analysis

22.1 Open QuantaSoft™ Analysis Pro Software

22.2 Open the .qlp file associated with the run

22.3 1.1.1.On the Plate Editor tab, select all the wells then in the dropdown menu for Assay Information select Probe Mix Triplex and complete as follows and click “Apply”:

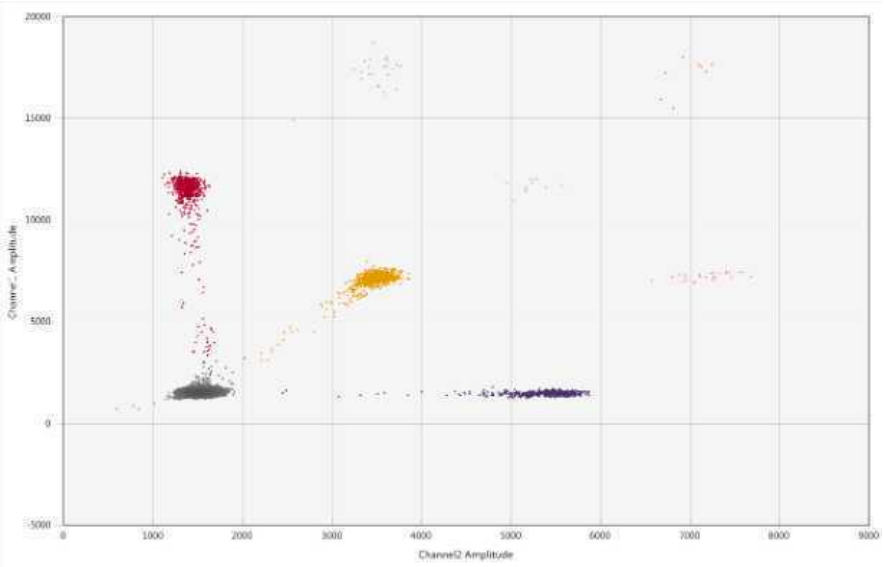


22.4 At this point, if sample names were not added at the start of the run, fill in the sample name by selecting the wells, typing in sample ID and click “Apply”.

22.5 If wells need to be deselected: Go to the Plate Editor tab, select wells to exclude and click “Clear Selected Well”.

22.6 On the 2D Amplitude tab, make sure that all wells are highlighted and select the Threshold Cluster Mode Graph Tool
and draw 2D thresholds around each cluster as follows

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n



22.7 Go through every well to ensure that:
- The droplets are designated properly per well
- There are >10,000 droplets per well

22.8 If any well does not have the appropriate droplets, go to the Plate Editor tab, select the affected well and click “Clear Selected Well”.

22.9 On the 2D Amplitude tab, select Merged Wells on the left side of the page

22.10 Click on the Table Menu Button on the right side of the Well Data table and select Export to CSV to export data.

22.11 Save the QuantaSoft Analysis

Dimensional Analysis and Quality Control

23 For dimensional analysis to express the results of each assay in terms of gene copies/dry weight solids:

23.1 Begin with the concentration provided by the QuantaSoft software and reported in the CSV, as expressed in gc/uL of reaction.

This concentration is expressed in terms of the total volume of the merged wells, rather than the volume of the template added. Determination of gc/g relies on the ratio of of template in the reaction and mass in the volume eluted from extraction, so when beginning with this value expressed in terms of gc/uL the number of wells merged is not considered in the dimensional

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n

analysis.

23.2 To determine the concentration in cp/g dry weight:

$$\left(X\frac{copies}{\mu L}\ rxn\right)*\left(\frac{B\ \mu L\ rxn}{A\ \mu L\ template\ in\ rxn}\right)*\left(\frac{C\ \mu L\ eluent\ from\ extract}{Z\ g\ wet\ mass\ solids\ in\ extract}\right)*(percent\ solids\ of\ sample)$$

When using the assays described in the three protocols that make up this process as written:

- B = 20 µL total reaction volume
- A = 5 µL volume template in reaction
- C = 50 µL elution volume from each well in extraction kit
- Z = 0.0225 g mass of solids added to each extraction well
- Percent solids varies for each sample on each day (typical range 0.05 - 0.3, i.e. 5-30%)

Links to two companion protocols to this protocol can be found in the description of this protocol.

Citation: Bridgette Hughes, Bradley J. White, Marlene K. Wolfe, Krista Wigginton, Alexandria B Boehm (07/17/2021). Quantification of SARS-CoV-2 variant mutations (HV69-70 and E484K/N501Y) in settled solids using digital RT-PCR. https://dx.doi.org/10.17504/protocols.io.bv5bn82n