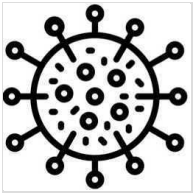

4

Apr 28, 2022

High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR V.4

Aaron Topol (Verily Life Sciences)[1], marlene.wolfe [1], Brad White (Verily Life Sciences)[1], Krista Wigginton[1], Alexandria B B Boehm[1]

[1]Stanford University

dx.doi.org/10.17504/protocols.io.e6nvw5orwvmk/v4

Wastewater-based epidemiology working group

Alexandria B Boehm
Stanford University

Citation: Aaron Topol (Verily Life Sciences), marlene.wolfe , Brad White (Verily Life Sciences), Krista Wigginton, Alexandria B B Boehm High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR https://dx.doi.org/10.17504/protocols.io.e6nvw5orwvmk/v4

v3 updates: changes to the S gene primers and probes for detection of all SARS-CoV-2 variants

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 ORF1a and a duplex assay targeting Bovine Coronavirus Vaccine (BCoV) and Pepper Mild mottle virus (PMMoV) 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.

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.

This protocol describes 2 separate PCR reactions, one containing primer/probe mixtures targeting the three SARS-CoV-2 targets and one containing primer/probe mixtures targeting BCoV and PMMoV. BCoV is spiked into samples before nucleic acid extraction and serves as a process control as well as an indicator of PCR inhibition. PMMoV is an enveloped virus which is abundant in human fecal waste and serves as an endogenous control for data normalization. PMMoV RNA is abundant at such high levels in wastewater samples that the samples must be diluted by a factor of 100 before quantification. The readout of this assay is a concentration of each target in the extracted RNA samples (copies/uL).

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.e6nvw5orwvmk/v4

Aaron Topol (Verily Life Sciences), marlene.wolfe , Brad White (Verily Life Sciences), Krista Wigginton, Alexandria B B Boehm 2022. High Throughput SARS-COV-2, PMMOV, and BCoV quantification in settled solids using digital RT-PCR. **protocols.io** https://dx.doi.org/10.17504/protocols.io.e6nvw5orwvmk/v4
Alexandria B Boehm

Corrected dimensional analysis equation in step 29.2

protocol ,

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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 XXXX
●SARS-CoV-2 gRNA positive control (ATCC® VR-1986D™) diluted and aliquoted at a concentration of 50 copies per µL.
●BCoV and PMMoV gene block positive control diluted and aliquoted at a concentration of 50 copies per µL.

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Target	Primer/Probe	Sequence
N Gene	Forward	CATTACGTTTGGTGGACCCT
	Reverse	CCTTGCCATGTTGAGTGAGA
	Probe	CGCGATCAAAACAACGTCGG (5' FAM/ZEN/3' IBFQ)
S gene (v3)	Forward	CAGACTAAKTCTCVTCGGCG
	Reverse	TGCACCAAGTGACATAGTGT
	Probe	AGCTAGTCAATCCATCATTGCCT (5' HEX/ZEN/3' IBFQ)
ORF1a	Forward	CAGAACTGGAACCACCTTGT
	Reverse	TACAGTTGAATTGGCAGGCA
	Probe	TGCCACAGTACGTCTACAAGC (5' FAM or HEX/ZEN/3' IBFQ)
BCoV	Forward	CTGGAAGTTGGTGGAGTT
	Reverse	ATTATCGGCCTAACATACATC
	Probe	CCTTCATATCTATACACATCAAGTTGTT (5' FAM/ZEN/3' IBFQ)
PMMoV	Forward	GAGTGGTTTGACCTTAACGTTTGA
	Reverse	TTGTCGGTTGCAATGCAAGT
	Probe	CCTACCGAAGCAAATG (5' HEX/ZEN/3' IBFQ)

:

Preparation (both assays)

1 Retrieve all kit components from the One-Step RT-ddPCR advanced kit 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 and 100 copies per µL BCoV and PMMoV gene blocks) from the **-80 °C** freezer and thaw **On ice**

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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.

IMPORTANT NOTE: The PMMoV assay is very sensitive to contamination due to the high copy number of PMMoV RNA molecules in the lab. It is essential to always use freshly prepared water and other reagents when setting up the BCoV/PMMoV assay and the change gloves and wipe down the working space and pipettes frequently with clean water.

ddPCR master mix preparation (SARS-CoV-2 targets) 45m

5 Prepare SARS-CoV-2 Target Master Mix

This is a triplex assay for the N, S, and ORF1a gene. It is possible to swap out one or two of the assays for others that also target low copy number targets. 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), Reverse Transcriptase (Orange Tube), and DTT (Grey Tube) to mix contents then briefly spin with the benchtop centrifuge.

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

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5.4 In a 2mL eppendorf tube, prepare the master mix according to the table for SARS-CoV-2 quantification. Store prepared master mix on ice.

The 20x SARS-CoV-2 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
ORF1a Probe (HEX)	2.5 µM	125 nM
ORF1a Probe (FAM)	2.5 µM	125 nM

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).

ddPCR master mix preparation (BCoV and PMMoV)

6 Prepare Master Mix for the BCoV/PMMoV assay

Store Master Mix components on ice as much as possible during the preparation process.

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

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centrifuge. Thaw DTT (grey tube).

6.2 In a 2mL eppendorf tube, prepare the master mix according to the table for PMMoV/BCoV quantification. Store prepared master mix on ice.

The 20x BCoV/PMMoV 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
BCoV Probe (FAM)	5 µM	250 nM
PMMoV Probe (HEX)	5 µM	250 nM

A	B	C	D	E
Reagents	Volume per Sample	Volume Per Plate		
ddPCR™ One-Step RT supermix for Probes (Yellow Tube)	5.5 µL	580.8 µL		
BCoV/PMMoV Primer/Probe Mix	2.2 µL	232.32 µL		
Reverse Transcriptase (Orange Tube)	2.2 µL	232.32 µL		
300mM DTT (Gray Tube)	1.1 µL	116.16 µL		
Nuclease Free Water	5.5 µL	580.8 µL		
Total Volume	16.5 µL	1742.4 µL		

BCoV and PMMoV ddPCR Master Mix. Volume per plate assumes 96 well plate (with 10% excess).

ddPCR plate set up (BCoV and PMMoV)

7 Dilute the RNA 1:100 for BCoV/PMMoV RT-ddPCR

The RNA must be diluted because of the high expected concentration of these targets.

The dilution of the RNA can be completed using a liquid handling robot. We use the Agilent

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The dilution of the RNA can be completed using a liquid handling robot. We use the Agilent Bravo system.

7.1 Using a new reagent reservoir, manually pipette **198 µL** of nuclease free water into every well of an Eppendorf twin.tec PCR Plate labeled “100x RNA Dilution”.

7.2 Manually pipette **2 µL** of RNA from the stock RNA plate into each corresponding well in the dilution plate.

7.3 Seal the plate and briefly vortex the plate using a bench top plate vortexer.

7.4 Briefly spin down the plate in the Axygen Plate Centrifuge.

8 Store plate **On ice** until ready to add sample to ddPCR plate.

Transfer Master Mix and Samples to ddPCR plate (both assays)

9 For each assay, remove a white 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.

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

11 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.
- Transfer **5.5 µL** of the extraction controls from column 12 of the RNA plate into column 11 of

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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)

For SARS-CoV-2 PCR: Add SARS-CoV-2 genomic RNA
For PMMoV/BCoV PCR: Add PMMoV/BCoV gene block positive control

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.

12 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.

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

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

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

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

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

ddPCR droplet generation (both assays) 45m

18 Generate Droplets:

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

18.2 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 red if the plates and tips are oriented correctly and to green if placed correctly.

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

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

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

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

During Droplet Generation for the SARS-CoV-2 plate, set up the PMMoV/BCoV plate.

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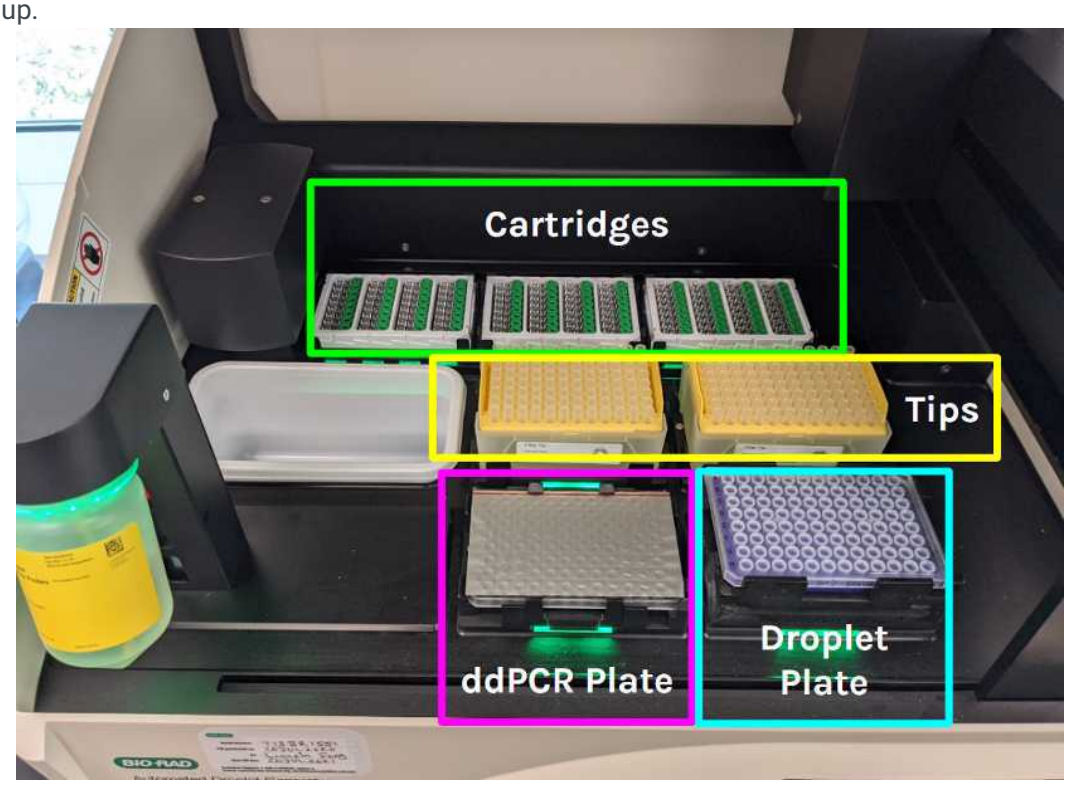
18.7 When droplet generation is done, open the lid and remove the droplet plate from the black cold block and place it in the metal carrier inside the plate sealer.

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



Photograph showing proper setup of samples and consumables in the AutoDG. After droplet generation, samples have been emulsified and deposited into the "Droplet Plate," which is ready for thermocycling.

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

NOTE: Do NOT vortex plate at this point!

Thermocycling (SARS-CoV-2) 45m

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

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Table 3. Thermocycler Program for SARS-CoV-2 ddPCR: “ddpcr covid rt”			
Steps	**Cycle #s**	**Temp (°C)**	**Time**
1	1	50	60 mins
2	1	95	5 mins
3	40	95	30 secs
	40	59	1 min
4	1	98	10 mins
5	-	4	hold

Cycling conditions for the SARS-CoV-2 RNA target assay

19.1 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.

Sample storage

20 Place the plate in the thermocycler. Verify and run the thermocycler program according to the table for BCOV/PMMoV ddPCR.

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Table 4. Thermocycler Program for BCoV and PMMoV ddPCR: "ddpcr bcov pmmov"			
Steps	**Cycle #s**	**Temp (°C)**	**Time**
1	1	50	60 mins
2	1	95	5 mins
3	40	95	30 secs
	40	56	1 min
4	1	98	10 mins
5	-	4	hold

Cycling conditions for the PMMoV / BCoV assay

21 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 (both assays)

22 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.

23 Load template from previous plate of the same assay - ensure that all parameters (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.

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

25 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.

26 Click "Run" in Quantasoft to commence droplet reading.

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Select “Ok” when the next dialog box comes up. Do not change the settings from “Columns” and “FAM/HEX”.

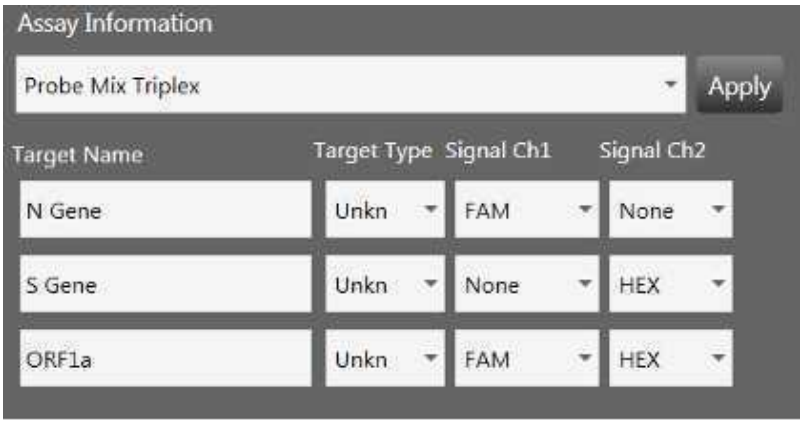
SARS-CoV-2 Post-Processing Analysis

27 SARS-CoV-2 triplex assay analysis

27.1 Open QuantaSoft™ Analysis Pro Software

27.2 Open the .qlp file associated with the run

27.3 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”:



27.4 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”.

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

27.6 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

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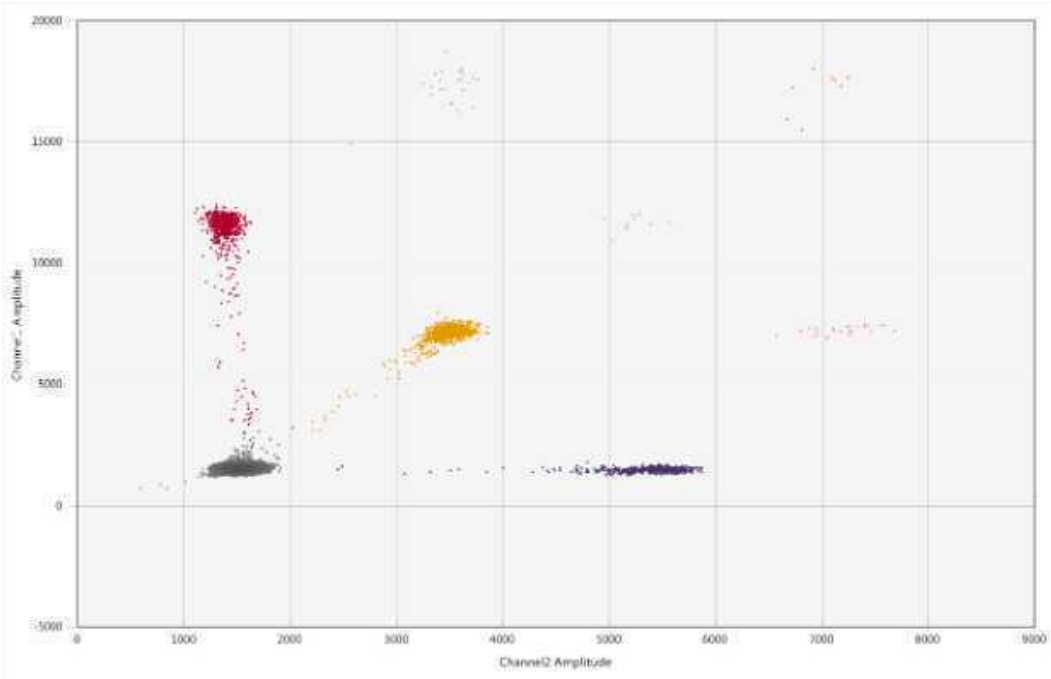

27.7 Go through every well to ensure that:
- The droplets are designated properly per well
- There are >10,000 droplets per well

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

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

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

27.11 Save the QuantaSoft Analysis

BCoV/PMMoV post-processing analysis

28 For BCoV and PMMoV analysis:

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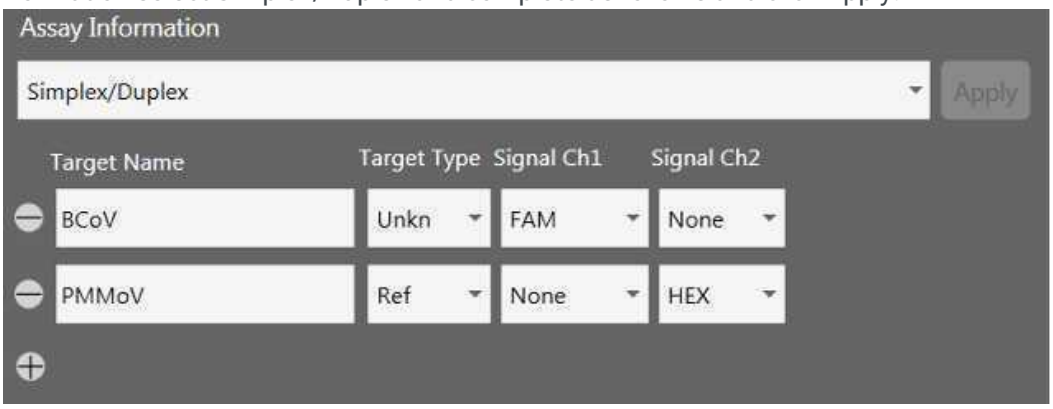
28.1 Open QuantaSoft™ Analysis Pro Software

28.2 Open the .qlp file associated with the run

28.3 On the Plate Editor tab, select all the wells then in the dropdown menu for Assay Information select Simplex/Duplex and complete as follows and click Apply:



28.4 .On the 1D Amplitude tab, make sure that all wells are highlighted and select the Threshold Multiple Wells button to automatically set the threshold between the positive and negative populations
BCoV threshold = 5000
PMMoV threshold = 1700

28.5 Go through every well to ensure that:
- The threshold accurately differentiates positive and negative droplets
- There are >10,000 droplets per well

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

28.7 On the 1D Amplitude tab, select Merged Wells on the left side of the page

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

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https://dx.doi.org/10.17504/protocols.io.e6nvw5orwvmk/v4

28.9 Save the QuantSoft Analysis

Dimensional Analysis and Quality Control

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

29.1 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 analysis.

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

$$\frac{X\ copies}{\mu l\ rxn} * \frac{B\ \mu l\ rxn}{A\ \mu l\ template} * \frac{C\ \mu l\ total\ eluent\ from\ extraction\ kit}{Z\ g\ wet\ mass\ solids\ added\ to\ extraction\ kit} * \frac{1}{\%\ solids}$$

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 = 60 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.

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