



# Quantification by Droplet Digital PCR (ddPCR) V.2

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Version 2

Jan 05, 2021

1

Works for me

This protocol is published without a DOI.

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## PROTOCOL CITATION

Shuchen Feng, Adélaïde Roguet 2021. Quantification by Droplet Digital PCR (ddPCR). **protocols.io**  
<https://protocols.io/view/quantification-by-droplet-digital-pcr-ddpcr-bpg6mjze>  
Version created by [McLellan Lab](#)

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## CREATED

Nov 07, 2020

## LAST MODIFIED

Jan 05, 2021

## PROTOCOL INTEGER ID

44286

## GUIDELINES

This protocol is for quantification of SARS-CoV-2 RNA in wastewater using one-step RT-ddPCR.  
This protocol does not include variable ddPCR settings such as sample wells and assay arrangements.

## MATERIALS TEXT

One-Step RT-ddPCR Advanced Kit for Probes **BioRad**

**Sciences Catalog #186-4021**

MicroAmp<sup>®</sup> Optical Adhesive Film **Thermo**

**Fisher Catalog #4360954**

Integra Assist  
Liquid Handler

Integra

4505



## BEFORE STARTING

1. Cleaning working space by wiping down using 70% ethanol and RNase away.
2. Always make sure the pipettes are calibrated.
3. Prepare an ice bucket. Take out reagents from -20°C freezer, including master mix, reverse transcriptase, DTT,

primers, probes and other reagents may need. Thaw reagents on ice for half an hour.

4. Prepare a cold low temp PCR rack/ice box for reaction set up.

5. Take out RNA samples from -80°C and thaw on ice before setting up reaction.

- 1 When all reagents are thawed on ice, vortex Supermix, Reverse transcriptase and DTT thoroughly for 30 seconds. Vortex to mix primers and probes stocks.

2



Plate Set up: see sub-steps below.



This step requires to keep the mixture cold/on ice.

- 2.1 Prepare the reaction matrix (for one well, beside sample RNA) according to the table below. Prepare Use a low-binding tube of appropriate volume to mix all the components according to the reaction numbers. Always include extra wells when setting up reaction to avoid potential volume shortage caused by pipetting.

Component	Volume per reaction, uL	Final concentration
Supermix	5.5	1x
Reverse transcriptase	2.2	20 U/uL
300 mM DTT	1.1	15 mM
Primer mix (forward + reverse)	1.1	900 nM
Probe	1.1	250 nM
RNase-free water*	5.5	/
Total	16.5	/

\* Note: Water volume can be replaced accordingly by other ingredients, such as another assay (e.g., duplex assay) or another RNA template (e.g., inhibition test).



1. Keep all reagents on ice during the process as well as the matrix.
2. Always prepare a total of 8\* (N columns) wells for droplet generation, or use ddPCR Buffer Control for Probes (#1863052) to fill empty wells on the last cartridge.
3. Make sure sticky reagents are added the correct volumes and not left in the tips, i.e., Supermix, RT.

- 2.2 Place a 96-well PCR plate onto a low temp PCR rack, or on ice. Disperse 16.5 µL of reaction matrix into

each well of a 96-well PCR plate. For runs with multiple columns, calculate (the matrix total volume/8) and evenly distribute the matrix into an 8-well PCR strip. Then use an appropriate multichannel pipette (e.g., 2- 20  $\mu$ L range) to add 16.5  $\mu$ L of matrix into each well, column by column.



1. Keep the PCR plate cold/on ice during the process.
2. Lower the pipetting speed to avoid liquid leftover to the inner side of the tips.

- 2.3 Gently vortex at half speed to mix the RNA sample. Make sure no liquid is attached on the lid. Add 5.5  $\mu$ L of sample RNA into each well containing 16.5  $\mu$ L of reaction matrix, making the total volume of each reaction 22  $\mu$ L.



1. Keep the PCR plate and RNA samples cold/on ice throughout the process.
2. Do not over vortex the RNA samples.
3. Pipetting robot may be used to add RNA samples to the matrix.

- 2.4 Seal the PCR plate. Centrifuge down gently at 1000 rpm for 30s. Take out the plate and vortex on a 96-well plate mixer at 1600 rpm for 30s. Centrifuge again at 1000 rpm for 30s to settle down the plate.

3




Droplet generation handling.




It is NOT required to keep the plate cold/on ice during this process.

- 3.1 Prepare materials/reagents on the working bench top, e.g., cartridges, gaskets, ddPCR 96-well plate, droplet generation oil, foil cover. Label the cartridges with corresponding column numbers (e.g., 1 to 12 for a full plate).
- 3.2 Remove the sealing on the PCR plate. Place the cartridge in the cartridge holder. Align well the PCR plate and the cartridge on the bench.  
Use an appropriate multichannel pipette (e.g., 2- 20  $\mu$ L) for liquid transfer. Adjust the pipette at 20  $\mu$ L and make sure the tips are well positioned. Gently mix the liquid by aspirating up to 2/3 of the tip height and then releasing to a lower level of the tip height, i.e., not to the end of the tip, to avoid creating bubbles. Repeat this mixing step 10 times. In the last movement, slowly aspirate to the full volume of 20  $\mu$ L.
- 3.3 Transfer the 20  $\mu$ L reaction matrix to the middle column of the cartridge. Position the tip end to the ridge in well (where the well wall connects to the bottom) at 15° angle. Avoid creating bubbles when releasing liquid from tips into the cartridge; this can be realized by only pressing the plunger to position 2 and not position 3 before pull the tips out from the cartridge. Make sure the cartridge wells are in the same direction as on the PCR plate.
- 3.4 Fill in 70  $\mu$ L of Droplet Generation Oil into bottom wells of the cartridge and cover the cartridge with a


red gasket. Loop outer holes of red gasket around hooks on left and right sides of cartridge holder. Place the gasket equipped cartridge into the droplet generator.

 You can start preparing the next column of droplet generation while waiting for the previous column to be done.

3.5 When droplet generation is done, take out the cartridge from the droplet generator and remove the red gasket. Using a Rainin multichannel with recommended Rainin tips, in a leaning position, count to 5 to aspirate all the liquid (i.e., 40 µl) from the droplets column, and press against side of wells of the corresponding column in the ddPCR 96-well plate (i.e., not the previous PCR plate), count to 5 to expel the droplets into the wells.

 1. Avoid multiple times of liquid transfer.  
2. Eye ball the ddPCR plate when droplet generation and transferring are done.

4 Turn on the PX1 PCR plate sealer and let heat to 180°C. Correctly place the plate support block, the ddPCR plate, foil cover (i.e., red line up) and the metal holder. Seal the plate at 180°C for 5vs and remove the plate immediately from the sealer.

 Sealed plate should have indentations around wells. Always check the sealing before loading the plate on the PCR thermal cycler.

5 Load the plate on to a PCR thermocycler. Our lab's assay conditions are shown as below. We use Eppendorf Mastercycler Pro and the ramp speed is set to 50% for RT-ddPCR.

Assay	Step 1	Step 2	Step 3 (40 cycles)	Step 4 (40 cycles)	Step 5	Step 6
N1/N2, BCoV, HepG	50°C 60 min	95°C 10 min	94°C 30s	55°C 1 min	98°C 10 min	4°C 30 mins and hold
PMMoV	50°C 60 min	95°C 10 min	94°C 30s	60°C 1 min	98°C 10 min	4°C 30 mins and hold

6 List of assay primers and probes.

A	B	C	D
CDC N1	GACCCCAAATCAGCGAAAT	FAM- ACCCCGCATTACGTTTGGTGGACC- BHQ1	TCTGGTTACTGCCAGTTGAATCTG
CDC N2	TTACAAACATTGGCCGCAAA	HEX-ACAATTTGCCCCAGCGCTTCAG- IowaBHQ	GCGCGACATTCCGAAGAA
BCoV	CTGGAAGTTGGTGGAGTT	FAM - CCTTCATATCTATACACATCAAGTTGTT- BHQ1	ATTATCGGCCTAACATACATC
HepG	CGGCCAAAAGGTGGTGGATG	HEX- AGGTCCCTCTGGCGCTTGTGGCGAG- BHQ1	CGACGAGCCTGACGTCGGG
PMMoV	GAGTGGTTTGACCTTAACGTTGA	FAM-CCTACCGAAGCAAATG- BHQ1	TTGTCGGTTGCAATG CAA GT