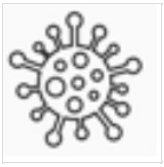


Aug 06, 2020

One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled Solids

Stephanie Loeb[1], Katy Graham[1], David Catoe[1], Marlene Wolfe[1], Alexandria B Boehm[1], Krista Wigginton[2]

[1]Stanford University; [2]University of Michigan

1 Works for me dx.doi.org/10.17504/protocols.io.bi6vkhe6

Wastewater-based epidemiology working group

Alexandria Boehm
Stanford University

ABSTRACT

Goal: Quantify concentration of N1, N2, BCoV, and PMMoV in RNA extractions using RT-ddPCR.

Estimated Time

8 hours per RT-ddPCR plate

DOI

dx.doi.org/10.17504/protocols.io.bi6vkhe6

PROTOCOL CITATION

Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton 2020. One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled Solids . **protocols.io** https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

KEYWORDS

digital PCR, SARS-CoV-2, wastewater, solids, Stanford, COVID-19, coronavirus, PMMoV, solids, sludge, Michigan

CREATED

Jul 30, 2020

LAST MODIFIED

Aug 06, 2020

PROTOCOL INTEGER ID

39861

GUIDELINES

Work-flow recommendation for running two plates in a day with two people:

1. Person 1 starts dilutions of samples
2. Person 2 start making the mastermix for two plates
3. Person 1 loads samples in plate 1
4. Person 2 starts making dilutions
5. Person 1 starts plate 1 in droplet generator

6. Person 2 loads samples in plate 2
7. Person 1 moves plate 1 to thermocycler
8. Person 2 starts plate 2 in droplet generator
9. Person 1 or 2 creates template in QuantaSoft for both plates
10. Person 2 starts plate 2 in other thermocycler
11. Both plates can sit overnight or you can wait to read both plates

*for first two rounds of cleaned samples, there were 4 aliquots- labeled 1-4. Aliquots 2, 3, and 4 have exactly 25 uL; aliquot 1 has slightly less since that is the tube that KG aliquots from and aliquot 1 is the tube used for Qubit-ing.
*for round 3 of extraction on: there are three 33 uL aliquots for each extract, labeled 1-3. Aliquots 2 and 3 have exactly 33 uL; aliquot 1 has slightly less since that is the tube that is aliquoted from and is used for Qubiting.

STEPS MATERIALS

NAME	CATALOG #	VENDOR
One-Step RT-ddPCR Advanced Kit for Probes	186-4021	BioRad Sciences

MATERIALS TEXT

Materials
●PMMoV/BCoV primers/probe mix
●SARS CoV-2 primers/probe mix
●One-Step RT-ddPCR Advanced Kit for Probes (catalog #1864022)
●*RAININ* pipet tips: Only use Rainin tips (Rainin UNV tips fit on eppendorf pipettes, Rainin specific pipettes need to be used/matched to LTE or XLS rainin tips)
●ddPCR plates (2x per run)
●Plate sealing film
●Automated droplet generator cartridges
●Racked pipette tips for droplet generation
●Droplet generation oil
Equipment
●P20 pipet
●P100 pipet
●P1000 pipet
●Droplet Generator
●Thermal cycler
●Plate Sealer

DISCLAIMER:

Plan Analysis - Plate Setup

1 The goal of this procedure is to quantify concentration of N1, N2, BCoV, and PMMoV in extractions using RT-ddPCR. Two duplexed assays will be run for each sample and are dexcribed in this protocol: 1) SARS-CoV-2 N1/N2, and 2) BCoV and PMMoV. Samples from a single plant should be run together on a plate, with the follow types of samples and controls run on each plate: NTC, Standards/Positive Controls, a Pooled Matrix Control, and a Spike Positive Pooled Matrix Control.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

NTC: No template control; molecular grade water is added to the reaction in place of the template. Double the number of wells run for each sample should be run as NTCs.

Standards/Positive Controls: While a standard curve is not constructed for ddPCR, a positive control confirms that the assay is working and can be used to gauge interplate variability. A positive control is used for each assay, in this case:

- **SARS-CoV-2 N1/N2:** The standard is pre-extracted clinical patient RNA diluted in molecular grade water.
- **BCoV**: The standard is a direct extraction of 200 µL of reconstituted BCoV vaccine used for spiking diluted in molecular grade water to 10^6 cp/mL.
- **PMMoV**: The standard is a synthetic DNA or RNA ultramer. The following sequence was used as an RNA PMMoV positive control template (Haramoto et al. 2013): tttcaaatgagagtggtttgaccttaacgtttgagaggcctaccgaagcaaatgtcgcacttgcattgcaaccgacaa.

Pooled Matrix Control: For the pooled matrix, several µL from each sample will be pooled and then used as the template for the reaction (for SARS-CoV-2 targets).

Spiked Positive Control Pooled Matrix: A second set of wells for the pooled matrix will be spiked with the positive control clinical RNA at a 1:10 concentration.

2 Plate Setup and Requirements: SARS CoV-2

In order to maximize the likelihood of detecting and quantifying SARS-CoV-2 targets, run 3 wells for each sample at 3 dilutions (undiluted, 1:10, and 1:50). Once several samples have been run for a given plant, it may be possible to reduce the number of wells or dilutions depending on the desired limits of detection and quantifiaction and experience with inhibition and anticipated SARS-CoV-2 concentrations at the plant.

Running 3 wells for each sample at 3 dilutions will require:

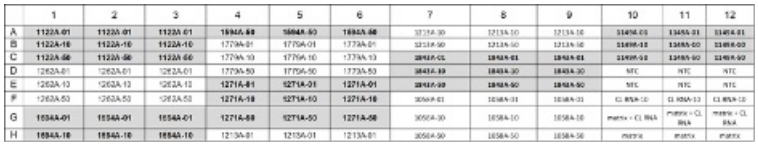
- 9 samples per plate
- **27 µl** of eluent for each sample

Run on each plate (for 3 wells for each sample at 3 dilutions):

- NTCs (x6)
- Clinical RNA (x3)
- Clinical RNA spiked into pooled matrix (x3)
- Pooled plant matrix (x3)

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

- 9 wells from the same plant:
  Undiluted (x3)
  Diluted 1:10 (x3)
  Diluted 1:50 (x3)



SARS CoV-2 N1-N2 example plate set-up

SARS-CoV2_Duplex_ddPCR_202007XX.xlsx

3 SARS-CoV-2 (N1/N2) Assay Chemistry and Parameters

The following primer and probe squences, reaction chemistry, and cycling conditions are used for the N1/N2 assays.

Primers and Probes

N1
Forward Primer N1-F: 5'-GAC CCC AAA ATC AGC GAA AT-3''
Reverse Primer N1-R: 5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
Probe N1-P-FAM: ACC CCG CAT TAC GTT TGG TGG ACC (5'**FAM**/ZEN/3' IBFQ)

N2
Forward Primer N2-F: 5'-TTA CAA ACA TTG GCC GCA AA-3'
Reverse Primer N2-R: 5'-GCG CGA CAT TCC GAA GAA-3'
Probe N2-P-HEX: ACA ATT TGC CCC CAG CGC TTC AG (5'**HEX**/ZEN/3' IBFQ)

Chemistry			
Stock Solution	Initial Conc.	Final Conc.	µL / rxn
Supermix	4 X	1 X	5.5
RT	10 X	1 X	2.2
DTT	300mM	15mM	1.1
N1/N2 Duplex Assay Mix	4 X	1 X	5.5
Template			5.5
Molecular-grade H20			2.2
Total			**22**

Reaction chemistry for duplex N1/N2 RT-ddPCR.

Note: 4X assay mix consists of 3.6 µM of each forward and reverse primers, and 1 µM of each probe. Instructions for preparation in appendix.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

Cycling Phase	*Temp °C*	*Time*	*# Cycles*
RT	50	60 min	1
Enzyme Activation	95	10 min	1
Denature	94	30 sec	40
Anneal/Extension	**55**	1 min	
Enzyme deactivation	98	10 min	1
Droplet Stabilization	4	30 min	1
Hold	4	∞	1

Cycling conditions for duplex N1/N2 RT-ddPCR.

4 Plate Setup and Requirements: BCoV/PMMoV

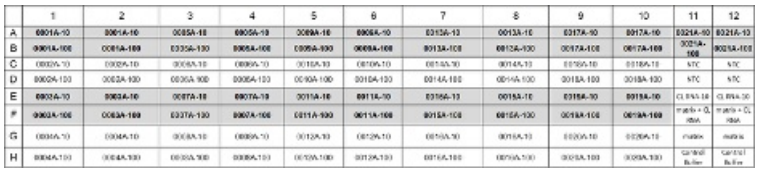
In order to maximize the likelihood of detecting and quantifying BCOV and PMMoV targets, running a 1:100 and 1:1000 dilution, respectively, is recommended. Both dilutions may be run with the duplex assays, or single assays can be run with each dilution. Run 2 wells for each sample at each dilution. Please note that once several samples have been run for a given plant, there may be a need to adjust the number of wells or dilutions.

Running 2 wells for each sample at 2 dilutions will require:

- 21 samples per plate
- **15 µl** of eluent for each sample

Run on each plate (for 2 wells for each sample at 2 dilutions):

- NTCs (x4)
- BCoV/PMMoV Standard (x2)
- 1:1 Control Buffer in MGW (x2)
- 22 samples from the same plant:
  Diluted 1:100 (x2)
  Diluted 1:1000 (x2)



BCoV-PMMoV example plate set-up

BCoV-PMMoV_Duplex_ddPCR_202007XX.xlsx

5 BCoV and PMMoV Assay Chemistry and Parameters

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

The following primer and probe squences, reaction chemistry, and cycling conditions are used for the BCoV/PMMoV assays.

Primers and Probes

BCoV
Forward Primer BCoV-F: 5'-CTGGAAGTTGGTGGAGTT-3''
Reverse Primer BCoV-R: 5'-ATTATCGGCCTAACATACATC-3'
Probe BCoV-P-FAM: CCTTCATATCTATACACATCAAGTTGTT (5'**FAM**/ZEN/3'IBFQ)

PMMoV
Forward Primer PMMoV-F: 5'-GAGTGGTTTGACCTTAACGTTTGA-3'
Reverse Primer PMMoV-R: 5'-TTGTCGGTTGCAATGCAAGT-3'
Probe PMMoV-P-HEX: CCTACCGAAGCAAATG(5'**HEX**/ZEN/3'IBFQ)

Chemistry			
Stock Solution	Initial Conc.	Final Conc.	µL / rxn
Supermix	4 X	1 X	5.5
RT	10 X	1 X	2.2
DTT	300mM	15mM	1.1
N1/N2 Duplex Assay Mix	4 X	1 X	5.5
Template			5.5
Molecular-grade H20			2.2
Total			**22**

Reaction chemistry for BCoV/PMMoV duplex assay.

Note: 4X assay mix consists of 3.6 µM of each forward and reverse primers, and 1 µM of each probe. Instructions for preparation in appendix.

Cycling Phase	***Temp °C***	***Time***	***# Cycles***
RT	50	60 min	1
Enzyme Activation	95	10 min	1
Denature	94	30 sec	40
Anneal/Extension	**60**	1 min	
Enzyme deactivation	98	10 min	1
Droplet Stabilization	4	30 min	1
Hold	4	∞	1

Cycling conditions for duplex BCoV/PMMoV RT-ddPCR.

Retrieve Samples and Prepare Workspace

6 Fill in ddPCR worksheet with samples and controls.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

7 Sterilize workspace and equipment: UV pipettes, tube racks and molecular grade water, bleach bench and ice buckets (let sit ~ 10 min), turn on PCR hood UV light, wipe bench and ice buckets with 70% EtOH.

8 Turn on plate sealer (times out after 2 hours, make sure block is not in machine) and put ice in small bucket for PCR reagents and large bucket for samples.

9 Set up tubes for dilutions according to worksheet (tubes will need **16 µl** , **18 µl** or **27 µl** of molecular grade water).

10 Retrieve samples for the day from the -80C freezer. Use a fresh aliquot; each aliquot should only undergo one freeze/thaw cycle before RT-ddPCR.

Reagents and mastermix preparation

11 Gather reagents and thaw all components on ice for at least **00:15:00** :

One Step RT-ddPCR Advanced Kit for Probes (stored in **-20 °C**)

- Supermix
- Reverse transcriptase (RT)
- 300 mM dithiothreitol (DTT) solution

- Duplex assay mix of primers and probes (pre-prepared and stored at **4 °C**)

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

12 Mix thoroughly by vortexing each tube to ensure homogeneity (concentration gradient can form when stored below freezing) and briefly spin down to collect contents at the bottom of the tube.

13 In the PCR hood, prepare reaction master mix according to ddPCR worksheet, listed for N1/N2 in step 3 **go to step #3** or BCoV-PMMoV from **go to step #5** .

14 Dispense equal aliquots of **16.5 µl** into each reaction well.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

Due to the autoDG process, all columns in use must be completely filled. If any wells in a column are empty, fill with 22 µL ddPCR Buffer Control for Probes (catalog. #1863052) diluted 1:1 with water.

Template preparation and dilution

15 Add 3 µL of sample to each of the 1:10 dilutions tubes containing 27 µL MGW. Vortex and spin down the 1:10 dilution.

To keep RNA cold through-out, plates/samples can be prepared on a cold block/on ice

15.1 For SARS-CoV-2 targets, add 4 µL of the 1:10 sample dilution to 1:50 dilution tubes that have 16 µL MGW. Vortex and spin down.

15.2 FOr BCoV/PMMoV targets, continue to dilute to 1:100 and 1:1000. Vortex and spin down.

16 For SARS-CoV-2 targets:

Create the pooled-matrix by combining 5 µL from each sample into a clean epi-tube. Ensure that the samples that you are working with are all from the same plant.

16.1 Add 2 µL of standard (CL-RNA or BCoV/PMMoV standards) to 18 µL matrix, leaving another aliquot without spiking.

17 Add 2 µL of standard (CL-RNA or BCoV/PMMoV standards) to 18 µL MGW

18 Load samples into the plate (5.5 µL template per well) according to ddPCR worksheet from part 1.

Droplet Generation

19 Prep the autoDG:
- Ensure the trash container is empty
- Ensure there is a ready cooling block that has been stored at -20°C for at least 2 hours

20 Use the plate sealer to seal the sample plate with a heat seal cover.

Place the red strip at top, line up with bottom there should be no overlap at the top or bottom, but the seal will slide up when the drawer shuts.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

21 Vortex plate for 30 seconds, then centrifuge plate at **1000 rpm 00:01:00**

22 Gather autoDG materials and load them:
- DG32 cartridges (required: one green strip per column, check directionality, will turn green when seated)
- AutoDG pipet tips in racks (required: two columns of pipets or sample column)
- Check the level of the AutoDG oil for probes
- Remove the cooling black accessory from the freezer and place in the droplet plate location
- **Empty droplet plate** (new ddPCR plate into which the generated droplets will be dispensed) loaded into the cooling block

23 Load the sample plate into the automated droplet generator in the sample plate position, making sure it is fully seated. The indicator light should turn green.

24 Touch the configure sample plate button on the AutoDG interface, select the columns in which the samples are located, and touch “OK”. Enter a plate name, and run the droplet generator.

25 Within 5 minutes of it finishing droplet generation, remove the droplet containing plate from the cold block and seal in the plate sealer. Transfer to the thermocycler.

Thermocycling

26 Run the appropriate protocol (N1/N2 from step 3 **go to step #3** or BCoV-PMMoV from step 5 **go to step #5**). Confirm the protocol matches the worksheet.

The protocols are identical, except N1/N2 has an annealing temperature of 55°C, while BCoV-PMMoV has an annealing temperature of 60°C.

27 The plate can be transferred to the droplet reader immediately or left at **4 °C** on the thermal cycler overnight and run on the droplet reader in the morning. While the thermocycler is running, prepare the droplet reader program as described below.

Droplet Reader

28 Open QuantaSoft software (NOT QuantaSoft Analysis).

29 Load template from previous plate of the same assay - using an old template ensures that all parameters (dye, absorbance etc.) are correct. Save as with a new plate name.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

30 Change sample names (NTCs and controls should be the same)

30.1 Double click on A1 - this highlights the name and brings you to the screen where you can change the names (and other parameters)

30.2 Change name and click apply or hit enter. Click on the next cell to change (use mouse to click on the next cell). Fill in the entire column, then cut and paste for triplicates (you have to use control C, control V, right clicking with the mouse won't work)

31 Make sure the Supermix is set to One-Step RT-ddPCR kit for probes.

This is the only parameter that cannot be changed after the run - if it is not set, the plate reader will fail and the samples will be wasted.

32 Click ok (next to apply) to get back to the save screen. Save as and double check the name

33 Open the droplet leader and remove the old plate if it is still inside

34 Prime the droplet reader before first plate of the day – "Prime" is in the Instrument Routines panel in the top right

35 Transfer the sample plate from the thermocycler to the droplet reader - it is inserted by sandwiching the plate inside the holder and holding the clips downward.

36 Hit run. The droplet reader takes 90 seconds per well, or almost 2 ½ hours for a full plate.

If the run won't start and you don't see Instrument Routines panel in top right, close program ensure QX200 is plugged in and reopen)

Exporting Data

37 When the droplet reader has finished, close QuantaSoft (the program used to run the droplet reader) and open QuantaSoft Analysis Pro.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6

38 Got to Browse - under QuantaLife>Data you will see folders containing the plate names. Select the folder for your plate and double click the QuantaSoft Plate file. This will open the file in QuantaSoft Analysis Pro.

39 In QuantaSoft Analysis Pro go to File>Save As and save to a USB key as a .ddpcr file. Data can now be managed and exported for downstream analyses.

Appendices

40 # Appendix

Preparing ddPCR assay mix

Primer and probes will arrive either lyophilized, or as 100 µM suspended in TE. Resuspend all primers and probe to 100 µM in molecular grade TE, and combine in the appropriate ratio, dilute with molecular grade water and store at -20°C. [NB: Currently, SARS CoV-2 primers and FAM labeled-probe are only available from IDT as 100 µM pre-suspended in TE.]

When plates are being frequently run, assay mix can be stored at 4°C for up to 2 weeks. Recommended to defrost on ice, or overnight at 4°C when possible.

40.1 Prepare the bench top area for use – wipe surface and equipment with 10% bleach, allow to dry for at least 10 min, and wipe with 70% ethanol.

40.2 Add molecular grade TE buffer to any lyophilized primers and probes according to the moles designated on the tube to make 100 µM solutions. (e.g. add 348 µL of TE to 34.8 nmol of primer).

40.3 **SARS CoV-2 4X assay mix** (**1 mL**): In a 2 mL LoBind epi-tubes combine **36 µl** N1-F, **36 µl** N1-R, **36 µl** N2-F, **36 µl** N2-R, **10 µl** N1-P-FAM, and **10 µl** N2-P-HEX. Add **836 µl** molecular grade water and store at -20°C until ready for use.

40.4 **BCoV-PMMoV 4X assay mix** (**1 mL**): In a 2 mL LoBind epi-tubes combine **36 µl** BCoV-F, **36 µl** BCoV-R, **36 µl** PMMoV-F, **36 µl** PMMoV-R, **10 µl** BCoV-P-FAM, and **10 µl** PMMoV-P-HEX. Add **836 µl** molecular grade water and store at **-20 °C** until ready for use.

Citation: Stephanie Loeb, Katy Graham, David Catoe, Marlene Wolfe, Alexandria B Boehm, Krista Wigginton (08/06/2020). One-Step RT-ddPCR for Detection of SARS-CoV-2, Bovine Coronavirus, and PMMoV RNA in RNA Derived from Wastewater or Primary Settled SolidsÃÂ . https://dx.doi.org/10.17504/protocols.io.bi6vkhe6