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Singleplex qPCR for SARS-CoV-2 N1 and BRSV

Chloe Svezia[1], Anh Nguyen[1], Jamie VanTassell[1], Julia Raymond[1], Marlene K Wolfe[1], Pengbo Liu[1], Christine Moe[1]

[1]Center for Global Safe WASH, Rollins School of Public Health, Emory University, Atlanta Georgia USA

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Emory University

Jamie VanTassell

This protocol describes the procedure to perform RT-qPCR for the detection of the SARS-CoV-2 N gene and a processing control (BRSV) in RNA extracted from wastewater samples. For samples obtained with a Moore swab, results will indicate the presence or absence of each target. For grab or composite wastewater samples, results may be used to estimate the concentration of the target in the original sample.

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wastewater, SARS-CoV-2, real-time, qPCR, RT-qPCR, Singleplex, N1, N1 target

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Equipment:

- Autoclave - Amsco Lab 240 Steam Sterilizer
- CFX96 Touch Real-Time PCR System
- LabGard Biological Safety Cabinet - Class 2 A2 Biosafety Cabinet
- Ice bucket or tray
- -80°C sample storage freezer
- 4 sample storage freezer
- Eppendorf Research Plus Single Channel Pipette
- Eppendorf Repeater Pipette or Multichannel Pipette
- Disposable micropipette tips (10 µL, 100 µL, 1250 µL)
- GENIE® SI-0236 Vortex-Genie 2 Mixer
- PlateFuge MicroCentrifuge

Materials:

- Molecular grade water
- IDT N1 Primer and Probe Mix
- 4X TaqPath Master Mix
- BRSV Primer and Probe Mix
- 1-96 well Plate
- 1-96 well Plate sealant cover
- 2-1.7 microcentrifuge tubes
- 70% ethanol
- 10% bleach
- Ice
- KimWipes
- Positive control material

Primer and probe sequences:

A	B	C
Target	**Component**	**Sequence**
N1	Forward Primer	5'-GAC CCC AAA ATC AGC GAA AT-3'
N1	Reverse Primer	5'-TCT GGT TAC TGC CAG TTG AAT CTG-3'
N1	Probe	ACC CCG CAT TAC GTT TGG TGG ACC (5' FAM/ZEN/3' IBFQ)
BRSV	Forward Primer	ACTCAGGACTTGTTCTTACCT
BRSV	Reverse Primer	TGGTAGGACAGGGTTATCAAAC
BRSV	Probe	ATGCTATCTCTGGGACCAAT (5' FAM or Cy5/ZEN/3' IBFQ)

Software Preparation

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1 Open BioRad CFX Manager Program.

2 Setup the proper thermocycler conditions:

Step	Time	Temperature	Cycles
	2 min	25°C	1
Reverse Transcription	15 min	50°C	1
PCR initial heat activation	2 min	95°C	1
Denaturation	3 sec	95°C	45
Annealing/Extension	30 sec	55°C	

Thermocycling Conditions

3 Set up well plate template:
- Load the appropriate fluorophores into the appropriate wells (FAM is used for this assay).
- Select for designated controls (no template controls, negative, and positive), standards (with concentrations), and unknowns (samples).
- Name wells for samples accordingly.

Preparation

4 Ensure that assay mix is prepared in advance for both N1 and BRSV assays (primers and probes mixed at appropriate concentrations and stored for future use at **-20 °C** , see Appendix I for details).

5 Ensure that standard curve aliquots have been prepared for SARS-CoV-2 (see Appendix II for details).

- Standards should be made for list target cp/well and used for grab or composite samples.
- Swab samples require only a positive control.

6 Spray surface of hood and wipe pipettes with 10% bleach. Let dry, and repeat with 70% ethanol. Wipe off carefully.

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7 Retrieve RNA from wastewater samples and thaw on ice.

8 Thaw the reagents from Invitrogen™, primers, and probes at **Room temperature** .

9 Prepare spreadsheet list of all samples to run to set up PCR template and to establish volumes needed for each reagent.

- For grab/composite samples, include standard curve for SARS-CoV-2 and two "no template controls" (NTCs).
- If only using swab samples, include only a positive control and and two no template controls (NTCs)

Procedure

10 Place all reagents on ice, including molecular biological grade water aliquot.

11 Confirm hood light is off to prevent denaturation of probes before starting mixture preparation.

12 Prepare two 1.7 mL microcentrifuge tubes labeled "N" and "B". Create the master mixes for each assay by mixing components under a clean hood in the order written in the tables below. Utilize the spreadsheet template to calculate the total volume of each component needed for the number of samples to be run.

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A	B	C	D
N1	*N (µL/well)*	*Initial Concentration*	*Final Concentration / Well*
Molecular biology grade water	8.5 µL	–	–
4X TaqPath Master Mix	5.0 µL	4X	1X
IDT N1 Primer and Probe Mix	1.5 µL	13.3X	1X
Primers	–	4 µM	300 nM
Probe	–	2 µM	150 nM
Template RNA	5 µL	–	–
Total volume	20.0 µL	–	–

N Master Mix

A	B	C	D
BRSV	*B (µL/well)*	*Initial Concentration*	*Final Concentration / Well*
Molecular biology grade water	8.0 µL	–	–
4X TaqPath Master Mix	5.0 µL	4X	1X
BRSV Primer and Probe Mix	2.0 µL	10X	1X
Primers	–	4 µM	400 nM
Probe	–	2 µM	200 nM
Template RNA	5 µL	–	–
Total volume	20.0 µL	–	–

B Master Mix

13 Vortex both N and B mixes for 5 seconds, then spin down for 5 seconds.

14 Aliquot **15 µL** of the master mix to each designated well on the PCR plate. Change pipette tips between wells and dispose used pipette tips into trash.

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15 Move the PCR plate to the lab's PCR bench for adding template. Do not add template in the hood.

16 Spray surface of PCR bench and wipe pipettes with 10% bleach and then repeat with 70% ethanol. Wipe off carefully.

17 Retrieve thawed RNA from wastewater samples. Vortex briefly and spin down.

18 Add **5 µL** of RNA template into the appropriate well on the PCR template, changing pipette tips in between each well.

19 For the PCR no template control (NTC), add **5 µL** of molecular grade water.

20 For the extraction negative control, add **5 µL** of control produced each time when extracting the RNA.

21 For positive control for Moore swab samples, add **5 µL** of diluted inactivated SARS-CoV-2 sample stored in **-80 °C** freezer.

22 For the standard curve for grab or composite samples, add **5 µL** of standards to designated wells in order, starting from the number of lowest to highest genomic copies (gc).

Std G	Std F	Std E	Std D	Std C
10 gc	100 gc	1,000 gc	10,000 gc	100,000 gc

gc = genomic copies

23 Once plate is completed, seal the wells with adhesive sealing film and spin down for **00:05:00** on a balanced 96-well plate centrifuge. 5m

Place samples in Bio-Rad Detection System and start PCR run with the appropriate

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24 thermocycling conditions as listed above.

25 Wipe down the pipette and surface with 10% bleach and then 70% ethanol.

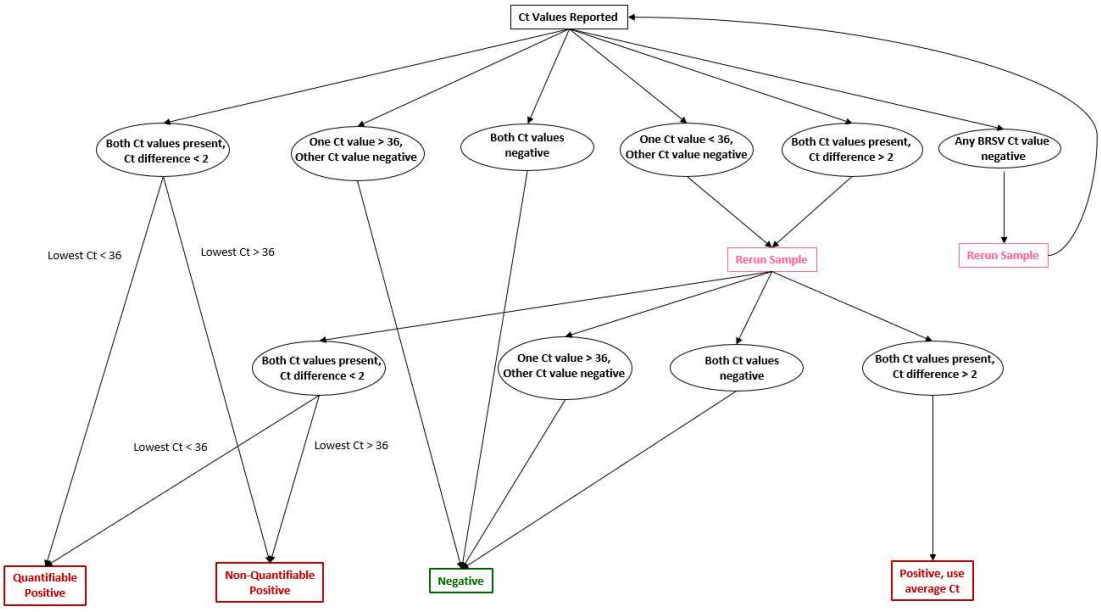
26 Store leftover RNA samples in the freezer at **-20 °C** .

Viewing and Saving Data

27 Export data as an Excel file or CSV file for viewing and analysis.

Interpreting Results

28 After viewing results, utilize the following decision tree to determine if results pass QA/QC.



Decision tree for interpreting qPCR results

- If both Ct values are present and the Ct difference is less than 2 --> Positive

1. If the lower Ct value is less than 36 --> Quantifiable positive
2. If the lower Ct value is greater than 36 --> Non-quantifiable positive

- If one Ct value is greater than 36 while the other is negative --> Negative
- If one Ct value is less than 36 while the other is negative --> Rerun sample
- If both Ct values are less than 36 and the Ct difference is greater than 2 --> Rerun sample
- If any Ct value of BRSV is negative --> Rerun sample

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- For re-rerun results, if both Ct values are present and the difference is greater than 2 --> Positive, average the Ct values. Otherwise, follow the same aforementioned guideline

Appendix I - Primer/Probe Mix

29 For each assay, combine primers and probes to create a stock mix with a concentration of 4 µM for each primer and 2µM for the probe.

29.1 Example to create 250 µL primer probe mix with 100 µM starting solutions:
- 10 µL Forward Primer (100 µM stock)
- 10 µL Reverse Primer (100 µM stock)
- 5 µL Probe (100 µM stock)
- 225 µL of microbiological grade H_2O

30 Vortex the mixture on a benchtop vortex and place into a **-20 °C** freezer until needed for PCR.

Appendix II - Standard Curve Preparation

31 The standard curve should be composed of the following and generated as a dilution series:

A	B	C	D	E	F	G
Dilution #	**Name**	**RNA (µL)**	**Molecular Water (µL)**	**Final Volume (µL)**	**gc/µL**	**gc/5µL**
1	B	27	73	100	200000	1000000
2	C	30	270	300	20000	100000
3	D	30	270	300	2000	10000
4	E	30	270	300	200	1000
5	F	30	270	300	20	100
6	G	30	270	300	2	10

gc = genomic copies

32 Thaw Quantitative Synthetic SARS-CoV-2 RNA on ice. Add **27 µL** of RNA into **73 µL** of molecular water to create 100 µL dilution B.

ATCC provides the concentration of RNA according to the LOT#. The RNA concentration of LOT# 70034420 is 7.4x 10^5 gc/µL.

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33 Add **30 µL** of dilution B into **270 µL** of molecular water to create 300 µL dilution C. Vortex the mix.

34 Add **30 µL** of dilution C into **270 µL** of molecular water to create 300 µL dilution D. Vortex the mix.

35 Add **30 µL** of dilution D into **270 µL** of molecular water to create 300 µL dilution E. Vortex the mix.

36 Add **30 µL** of dilution E into **270 µL** of molecular water to create 300 µL dilution F. Vortex the mix.

37 Add **30 µL** of dilution F into **270 µL** of molecular water to create 300 µL dilution G. Vortex the mix.

38 Make 14 µL aliquots for each dilution and save at **-20 °C** freezer. Keep the remaining dilution B in the **-70 °C** freezer for future use.

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