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Semi-Automated and Miniaturized SARS-CoV-2 Detection using TaqPath COVID-19 Multiplex Real-Time RT-PCR Assay

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Coronavirus Method Development Community

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ABSTRACT

The purpose of this procedure is to describe the setup of a semi-automated and miniaturized workflow for SARS-CoV-2 detection using the TaqPath COVID-19 Multiplex Real-Time RT-PCR Assay.

This procedure applies to specimens received for SARS-CoV-2 testing in a clinical setting. Samples are collected, processed, and RNA is extracted as described elsewhere, using the MagMax Viral Pathogen (MVPII) 96 Kit for SARS-CoV-2 Detection protocol or the Omega Bio-tek Mag-Bind Viral DNA/RNA 96 Kit for SARS-CoV-2 Detection protocol. The TaqPath COVID-19 Multiplex Real-Time RT-PCR Assay is a multiplex reverse transcription quantitative polymerase chain reaction (RT-qPCR) test intended for the qualitative detection of nucleic acid from SARS-CoV-2, the virus that causes Coronavirus Disease 2019 (COVID-19). The procedure has been modified to accommodate **3.0 µl** reactions as opposed to the standard **25.0 µl** reactions, significantly reducing per-reaction cost. This is achieved by using the SPT LabTech Mosquito HV and the SPT LabTech Mosquito X1 liquid handling robots.

Purified viral ribonucleic acid (RNA) is reverse-transcribed into cDNA and amplified using the TaqPath™ COVID-19 Combo Kit and the QuantStudio 7 Pro 384 Real-Time PCR Systems. In the process, the probe oligos anneal to four (4) specific target sequences located between four (4) unique forward and reverse primers for the following genes: ORF1ab, N Gene, S Gene, and the MS2 bacteriophage RNA extraction positive control. During the extension phase of the qPCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each qPCR cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored during each PCR cycle by the QuantStudio 7 Pro 384 Real-Time PCR System.

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PROTOCOL CITATION

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KEYWORDS

SARS-CoV-2, COVID-19, PCR, qPCR, RT-PCR, Virus, RNA

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GUIDELINES

Anterior nares swabs are obtained via self-collection: individuals are given an individually-wrapped and sterilized swab (Affordable IHC Solutions, PN: 5cmTH-SB) and instructed to insert the swab approximately 1.5 cm into the nostril, twist the swab inside the nostril three (3) times, and then hold the swab against the septum for a total of 15 seconds, before repeating these steps on the other nostril with the same swab. The swab is then inserted into a into a 5 mL sample tube containing 1 mL PrimeStore® Molecular Transport Medium (MTM), Mawi DNA Technologies iSWAB Microbiome buffer (Mawi), or 5% sodium dodecyl sulfate (SDS). If required (depending on the length of the swab), the handle of the swab is broken off at a pre-scored area directly above the tip so that the tip remains in the solution. MTM, Mawi, and SDS are all able to inactivate the SARS-CoV-2 virus while preserving the viral RNA needed for detection.

Nasopharyngeal swabs are collected by licensed personnel by sampling the posterior nasopharynx through both nares using an individually wrapped, sterilized swab, according to CDC instructions for nasopharyngeal swab collection. The swab is then inserted into a 5 mL sample tube containing 3 mL viral transport media (VTM). The handle of the swab is broken off at a pre-scored area directly above the tip so that the tip remains in the solution.

Table 1. Acceptable sample storage temperatures and durations.

A	B	C
Specimen Type	**Preservation Solution**	**Stability and Storage**
Anterior Nares Swab	Mawi	Store at Room Temperature up to 1 month. Store at -90 C to -70 C indefinitely.
Anterior Nares Swab	MTM	Store at Room Temperature up to 7 days. Store in Refrigerator (2 C to 8 C) up to 28 days. Store at -90 C to -70 C indefinitely.
Anterior Nares Swab	SDS	Store at Room Temperature up to 1 year. 5% concentration.
Nasopharyngeal Swab	VTM	Store in Refrigerator (2 C to 8 C) for up to 1 year.

MATERIALS TEXT

Definitions and Acronyms

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A	B
Term	**Definition**
AN Swab	Anterior nares swab.
cDNA	Complementary DNA: DNA synthesized from a single-stranded RNA template in a reaction catalyzed by the enzyme reverse transcriptase.
CSV	Document in the format of Comma Separated Values.
DNA	Deoxyribonucleic acid - carrier of genetic information.
Equilibrate	Bring to equilibrium to ambient room temperature.
EtOH	Ethanol. (CAS Number: 64-17-5), or equivalent.
Freezer	Temperature range of -25°C to -15°C.
HV	SPT LabTech Mosquito high volume (HV Genomics) 16-channel robotic liquid handler.
Internal Control	MS2 Phage control used to monitor RNA extraction. Provided with TaqPathTM COVID-19 Combo Kit.
Mawi	Mawi DNA Technologies iSwab collection media.
MM	Master Mix.
NP Swab	Nasopharyngeal swab.
Negative Control	Nuclease-free water. Used to monitor cross-contamination during nucleic acid extraction and reaction setup.
PCR	Polymerase Chain Reaction.
Positive Control	RNA control that contains targets specific to the SARS-CoV-2 genomic regions targeted by the assay. Provided with TaqPathTM COVID-19 Combo Kit. Used to monitor RT-qPCR reaction setup and reagent integrity.
PPE	Personal Protective Equipment.
QC	Quality Control.
Real Time (RT) PCR/qPCR	Quantitative Polymerase Chain Reaction. Used to quantify the amount of starting material in a sample.
RT-qPCR Reaction Plate (QRP)	384-well plate containing RT-qPCR master mix, to which viral RNA will be added prior to performing RT-qPCR.
Refrigerator	Temperature range of 2°C to 8°C.
RNA	Ribonucleic acid - carrier of genetic information. Makes up the genome of the SARS-CoV-2 virus.
RNA Extraction/Elution Plate (REP)	384-well plate containing viral RNA extracted from patient sample material. Used for viral RNA storage.
RNA Working Plate (RWP)	384-well plate containing aliquots of extracted RNA from the RNA Extraction Plate - used to run the COVID-19 RT-qPCR assay.
Room Temperature (RT)	Temperature range of 15°C to 25°C.
RT-PCR	Reverse Transcription Polymerase Chain Reaction.
RT-qPCR/qRT-PCR	Reverse Transcription Quantitative Polymerase Chain Reaction.
Ultra-Low Freezer	Temperature Range of -90°C to -70°C.
VTM	Viral Transport Media.
X1	SPT LabTech Mosquito X1 hit/cherry picker single-channel robotic liquid handler.

Equipment

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A
QuantStudio 7 Pro Real-Tim PCR System (Thermo Fisher, PN: A43183)
Mosquito HV (High Volume) 16-channel robotic liquid handler (SPT LabTech, PN: 3097-01057)
Mosquito X1 (X1 hit/cherry picker) single-channel robotic liquid handler (SPT LabTech, PN: 3019-0039)
Centrifuge (Eppendorf, PN: 5910R)
Benchtop Centrifuge - PicoFuge Benchtop Microcentrifuge (Stratagene, PN: 400550)
Barcode Scanner
384-well PCR Plate Cooling Block (SPT LabTech, PN: 3268-02004)
384-well Magnetic Bead Clean-Up Block (SPT LabTech, PN: 3268-02008)
Rainin Pipettes - P2, P20, P200, P1000 (or equivalent)
Barcode Scanner

Supplies

1. DNA LoBind 1.5 mL Microcentrifuge Tubes (PN: 022431021, Eppendorf)
2. CoolRack XT PCR 384 Cooling Block, 1 x 384-well PCR Plate; Gray (PN: 432055, Corning), or equivalent
3. Hard-Shell 384-Well PCR Plates, thin wall, skirted, clear/clear (PN: HSP3901 and HSP3905, Bio-Rad), or equivalent
4. MicroAmp EnduraPlate Optical 384 Well Reaction Plate with Barcode (PN: 4483273, Applied Biosystems), or equivalent
5. MicroAmp Optical Adhesive Film (PN: 4360954, Applied Biosystems), or equivalent
6. Microseal 'B' (PN: MSB-1001, Bio-Rad), or equivalent
7. BrandTech sealing film (PN: 701390ES, BrandTech Scientific Inc.), or equivalent
8. Pipette tips, filtered, various manufacturers
9. RNaseZap spray bottle (PN: R2020-250mL, Sigma Life Science), or equivalent
10. 70% Ethanol spray bottle
11. Crushed ice
12. Gamma Ray Sterilized Pipettes 4.5mm Pitch HV, Spool of 18500 (PN: 4150-03033, SPT LabTech)
13. Gamma Ray Sterilized Pipettes 9.0mm Pitch HV, Spool of 135000 (PN: 4150-03032, SPT LabTech)

Reagents

1. TaqPath™ COVID-19 Combo Kit, 1000 Reactions (PN: A47814, Thermo Fisher Scientific)
   1.1. TaqPath RT-qPCR COVID-19 Kit
      1.1.1. COVID-19 Real Time PCR Assay Multiplex, which contains three primer/probe sets targeting three regions of the SARS-CoV-2 genome, and one primer/probe set targeting the MS2 phage internal control
      1.1.2. MS2 phage internal control - internal process control for nucleic acid extraction
   1.2. TaqPath COVID-19 Control - positive RNA control that contains targets specific to the three regions of the SARS-CoV-2 genome targeted by this assay
   1.3. TaqPath COVID-19 Control Dilution Buffer
2. 4x Single Step RT-qPCR enzyme mix
   2.1. TaqPath™1-Step Multiplex Master Mix (No ROX™), 10mL (PN: A28523, Thermo Fisher), or
   2.2. or Luna Probe One-Step RT-qPCR 4X Mix, or
   2.3. Reliance One-Step Multiplex Supermix 4X
3. HyPure Molecular Biology Grade Water (PN: HS40003.01, GE Healthcare - HyClone), or equivalent

SAFETY WARNINGS

For sample collections, ensure appropriate personal protective equipment (PPE) are worn. If interacting with patients, appropriate PPE includes gowns, two layers of gloves, N95 respirators, eye protection, and face shields.

For identification of SARS-CoV-2 in the lab, face coverings (masks) are to be worn at all times. Lab coats/disposable lab gowns, eye protection, and gloves must be worn at all times when when handling samples containing SARS-CoV-2. Gloves should extend to cover wrists of lab coat/gown. Equipment and surfaces must be decontaminated regularly, especially before and after performing this procedure.

BEFORE STARTING

Read the guidelines for sample collection.

Workstation Preparation

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1 **Set up:**

- Turn on equipment (Mosquito HV, Mosquito X1, QuantStudio 7, Centrifuge) and ensure all equipment and programs start up correctly.

QuantStudio 7
Pro Real-Time PCR System
Applied Biosystems **A43183**

Mosquito HV
High Volume 16-Channel Robotic Liquid Handler
SPT LabTech **3097-01057**

Mosquito X1
X1 Hit/Cherry Picker Single-Channel Robotic Liquid Handler
SPT LabTech **3019-0039**

Centrifuge 5910 R
Centrifuge
Eppendorf **2231000772**

- Ensure the centrifuge is set to **4 °C**
- Decontaminate workspace, pipettes, and equipment using RNASEZap or equivalent.
- Fill two (2) ice trays with crushed ice.
- For rapid thawing of frozen reagents, add 2-3cm deep worth of **Room temperature** to a third ice tray, then add crushed ice (enough so ice is still floating) and a floating tube holder into the water.

1.1 **Consumable Preparation:**

- Scan the barcodes for the following supplies (Table 1) into the INSPECT application:

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A	B	C	D
Item	**Barcode**	**Consumable**	**# Needed Per Run**
RNA Working Plate (RWP)	######	Hard-Shell 384-Well PCR Plate	1
qPCR Reaction Plate (QRP)	######	Hard-Shell 384-Well PCR Plate	1
Master Mix (MM) Tube	No Barcode	1.5 mL Microcentrifuge Tube	1
Intermediate MM Plate	######	Hard-Shell 384-Well PCR Plate (one column used per run, plate re-useable up to 24 times – one time for each column)	1
Positive Control Plate	######	Hard-Shell 384-Well PCR Plate (re-useable)	1

Table 1. Consumables.

Steps involving the INSPECT application are optional and are not essential for performing RT-qPCR detection of SARS-CoV-2 viral RNA.

1.2 Reagent Preparation:

- Gather one (1) aliquot of each of the reagents listed below from their storage locations and follow handling conditions (Table 2):

TaqPath COVID-19 Combo Kit **Thermo Fisher Scientific Catalog #A47814**

A	B	C	D	E
Reagent Name	Quantity	Storage Container	Storage Conditions	Handling Conditions
TaqPath™ 1-Step Multiplex Master Mix	1	2 mL tube	-20C freezer	Thaw in ice water tray and then transfer to other ice tray.
COVID-19 Real Time PCR Assay Multiplex	1	2 mL tube	-20C freezer	Thaw in ice water tray and then transfer to other ice tray. Cover at all times to protect from light.
TaqPathTM COVID-19 Control Dilution Buffer	1	2 mL tube	-20C freezer	Thaw in ice water tray and then transfer to other ice tray.
Positive Control: TaqPathTM COVID-19 Control	1	1 mL tube	-80C freezer	Thaw in ice water tray and then transfer to other ice tray.
Nuclease-free water	1	100 mL Container	Benchtop	Keep at RT

Table 2. Reagents.

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Assay Controls

2 **Internal Control:**

- The MS2 Phage Internal Control is used to monitor RNA extraction and was added at the RNA extraction step - see related RNA-extraction protocols using the MagMax Viral Pathogen (MVPII) kit or the Omega Bio-tek Mag-Bind Viral DNA/RNA kit. The MS2 Phage Internal Control is added to every sample/well of the reaction plate except for the Positive Control well(s).

Negative Control:

- The Negative Control (nuclease-free water with MS2 Phage Internal Control) is used to monitor efficiency of RNA extraction process. One Negative Control is included in every 96-well block (i.e. one Negative control per 94 samples) and is added after patient and mock material is added to the RNA extraction reagent during the RNA extraction process.

Positive Control:

- The Positive Control (TaqPath™ COVID-19 Control) is used to validate the operation of ther RT-qPCR reaction process. One Positive Control is included in every 96-well block (i.e. one Positive Control per 94 samples) and is added at the RT-qPCR reaction step.

*For data analysis and results interpretation, refer to Step 9 below: Data Analysis and Interpretation.

Sample Preparation 3m

3

- If not proceeding directly from the Semi-Automated Extraction of Viral RNA using the MagMax Viral Pathogen (MVPII) 96 Kit for SARS-CoV-2 Detection RNA extraction protocol OR the Semi-Automated Extraction of Viral RNA using the Omega Bio-tek Mag-Bind Viral DNA/RNA 96 Kit for SARS-CoV-2 Detection protocol, locate and remove the appropriate 384-well RNA Working Plates (RWPs) from their storage location (ultra-low freezer). Place on a 384-well aluminum block at **Room temperature** until thawed.
- Once thawed, place RWP on a prechilled aluminum block and keep **On ice** .

Master Mix Preparation 10m

4

This process involves making a qPCR Master Mix (MM) in a 1.5 mL microcentrifuge tube, then aliquoting this mixture into the Intermediate Master Mix Plate (IMP) by hand, before it is transferred to the qPCR Working Plate (QRP) using the Mosquito HV (Step 5 below).

Samples will ideally be organized in multiples of 96 (94 samples + Positive and Negative Controls).

Preparation of the MM and the IMP should ideally be performed in a dead air PCR hood that has been prepared for work with RNA.

Prepare the Master Mix (MM):

- Pipette the correct volume of Nuclease-Free Water, Probe Mix, and Water from Table 3 below, in the order listed, into a 1.5 mL microcentrifuge tube labelled 'MM.' The correct volume will depend on how many sets of 94 samples are being processed.
- Keep MM tube **On ice** when creating the master mix. Once all reagents have been added, close the lid, vortex gently to mix, and centrifuge briefly to collect all liquid at the bottom of the tube.

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A	B	C	D	E	F
Reagents	**Per 1 reaction**	**For 96 reactions (+ error)**	**For 192 reactions (+ error)**	**For 288 reactions (+ error)**	For 384 reactions (+ error)
Nuclease-Free Water	0.10 µL	13.6 µL	23.5 µL	33.5 µL	43.5 µL
Probe Mix	0.15 µL	20.4 µL	35.2 µL	50.2 µL	65.2 µL
4x Single Step RT-qPCR Enzyme Mix	0.75 µL	101.8 µL	176.0 µL	251.0 µL	326.0 µL
MM Total:	**1.0 µL**	**136.5 µL**	**236.4 µL**	**337.2 µL**	438.1 µL
MM Volume to add to each well of IMP:	*N/A*	*8.2 µL*	*14.2 µL*	*20.3 µL*	26.3 µL

Table 3. TaqPath qPCR reagent volumes for Master Mix.

4.1 Transfer MM to IMP:

- Place the IMP on a chilled 384-well aluminum block and keep **On ice** .
- Mark the position of an unused column on the IMP, and to each well of that column, add the appropriate amount of master mix based on the last row of Table 3 above.
- Seal the IMP with a temporary plate seal and centrifuge

  2000 x g, 4°C, 00:00:30 , Purpose is to remove air bubbles from well .
- Check that each well has an equal volume of liquid, and double check that there are not bubbles in any well, which could interfere with reagent transfer.
- Place the IMP on a 384-well aluminum block **On ice** and cover to shield from light.

If there are bubbles, centrifuge again, coarsely flick the well(s) that contain bubbles, or pop the bubbles with a fine pipette tip.

Transfer Master Mix to qPCR Reaction Plate 5m

5

This process transfers the MM from the Intermediate Master Mix Plate (IMP) to the qPCR Reaction Plate (QRP) using the Mosquito HV. To each well of the QRP, 1 µL MM is added.

Set up Mosquito HV Computer:

- On the Mosquito-HV-associated computer, open the appropriate program (provided programs are designed for either 16 reactions/one column or 384 reactions/full plate).
- 16 reactions/one column: **MicroAmp_Stamp_1.protocol**
- 384 reactions/full plate: **MicroAmp_Stamp_24.protocol**

5.1 Set up Mosquito HV Plate Deck:

- On the Mosquito HV Computer, select the appropriate source column from the IMP source plate (the column containing the MM) and begin the program.
- Push the “Move Left” button on the computer and the plate deck will move to the left, allowing access to Positions 2 and 3 on the plate deck.

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- Place the QRP in Position 2 of the plate deck.
- Place the IMP in Position 3 of the plate deck.

When adding the plates to the Mosquito HV plate deck, ensure the lower right corner of each plate is resting in front of, and not on top of, the diagonal (spring-loaded) plate securing bar.

5.2

As this is a 1uL dry dispense, the Mosquito HV Genomics requires accurate tip spool calibration and accurate plate definition parameters.

Transfer Master Mix to qPCR Reaction Plate 5m

5.3 **Run the Program:**

- When the plates have been loaded onto the plate deck and the program is correct, select "Run" on the Mosquito HV computer.

Observe the pipette tips in action: if the tips are not entering the wells properly, hit the "Pause" or "Abort" buttons and troubleshoot (troubleshooting not included in this protocol).

- After the run is complete, remove the QRP, tap the plate gently to drive large drops towards the bottom of the wells, seal with a temporary plate seal, and centrifuge **2000 x g, 4°C, 00:00:30** to move all liquid to the bottom of each well.
- Hold the plate to the light and verify that all wells have an equal volume of liquid.

If there are wells without any MM, manually add with a P2 pipette.

5.4 **Post-Run:**

- Store the QRP **On ice** and cover to shield from light.
- Clearly mark on the IMP which column was used by marking the top and bottom of the column with an "X". Seal with a temporary plate seal and place on ice.

Only one column of the IMP is used at a time: the IMP can be re-used until all columns have been used before being discarded.

Positive Control Addition to qPCR Reaction Plate 5m

6

The result of this process is the addition of the COVID-19 Positive Control viral RNA material into the appropriate wells of the QRP using the Mosquito X1. One Positive Control is included for each block of 96 reactions (i.e. one Positive Control per 94 samples).

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If RNA was extracted using the MagMax protocol, the Positive Controls are placed in wells *B2, B8, B14,* and *B20* of the 384-well QRP.

If RNA was extracted using Omega protocol, the Positive Controls are placed in wells *A1, A7, A13,* and *A19* of the 384-well QRP.

One (1) Positive Control must be added for each block of 96 samples, or portion thereof. Therefore, 10 samples require one (1) Positive Control, and 100 samples require two (2) Positive Controls.

Prepare the Positive Control Plate (PCP):

- Place one aliquot of diluted RT-qPCR Positive Control RNA in a tube holder floating in ice water until thawed, then place **On ice** .
- Add an aliquot of the Positive Control to an empty well of the Positive Control Plate (PCP): add **0.5 µl** Positive Control RNA for each Positive Control reaction on the QRP + 2uL of this RNA for instrument dead volume. (2.5uL total control RNA for a single well, 4uL total for four wells.)

6.1 Set up Mosquito X1 Computer:

- On the Mosquito-X1-associated computer, open the appropriate program (provided programs are designed for either one (1) Positive Control or four (4) Positive Controls).
- Addition of one Positive Control: **Positive_Add_1x96.protocol**
- Addition of four Positive Controls: **Positive_Add_1x384.protocol**

Ensure that the X1 program properly references the correct source well on the PCP.

6.2 Set up Mosquito X1 Plate Deck:

- Place the PCP in Position 1 (left position) of the X1 plate deck.
- Remove the temporary seal from the QRP and place the plate in Position 2 (right position) of the X1 plate deck.

When adding the plates to the Mosquito X1 plate deck, ensure the lower right corner of each plate is resting in front of, and not on top of, the diagonal (spring-loaded) plate securing bar.

6.3 Run the Program:

- When everything is correctly positioned, select "Run". The X1 will add **0.5 µl** of the COVID-19 Positive Control to the appropriate well(s) of the QRP.
- When the run has finished, remove the QRP, seal with a temporary plate seal, and centrifuge **2000 x g, 4°C, 00:00:30** . Check the appropriate wells for volume change to confirm the Positive Control was added.
- Place QRP on the 384-well aluminum block **On ice** and cover to shield from light.
- Seal the PCP and place on benchtop.

Mark the used column of the PCP with an "X" so it is not accidentally used again - can re-use this plate up to 24 separate times (once per column on the plate).

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RNA Addition to qPCR Reaction Plate 10m

7 3m

In this process, **2.0 µl** aliquots of viral RNA are transferred from the RWP to the QRP using the Mosquito HV.

Prepare RNA Working Plate (RWP):

- At this time, RWP is thawed, and sitting **On ice** (see Step 3 above).
- Centrifuge **2200 x g, 4°C, 00:03:00 , Max speed to pellet any magnetic beads** **On ice**
- Place a 384-well magnetic plate onto a 384-well aluminum plate **On ice** to chill the magnetic plate without it getting wet.
- Place the RWP onto the magnetic plate to cool.

7.1 Set up the Mosquito HV Computer:

- On the computer associated with the Mosquito HV Genomics liquid handler, open the appropriate program (provided programs are designed for either 16 reactions/one column or 384 reactions/full plate).
- For 16 reactions/one column: **MicroAmp_RNA_Add_1.protocol**
- For 384 reactions/full plate: **MicroAmp_RNA_Add_24.protocol**

7.2 Set up the Mosquito HV Plate Deck:

- Remove the seals from the RWP and QRP.
- Place the RWP (and the magnetic block it sits on) onto Position 4 of the Mosquito HV plate deck. Ensure the plate is properly oriented, with will A1 in the upper left corner.
- Place the QRP onto Position 2 of the Mosquito HV plate deck, ensuring the plate is properly oriented.

The empty deck position between the RWP and the QRP is used for tip changing and reduces the potential for cross contamination of samples.

7.3 Run the Program:

- When everything is correctly positioned, select "Run".
- When the run is complete, remove the RWP and seal completely with a Bio-Rad Microseal 'B' plate seal. Store at **-80 °C** .
- Remove the QRP and seal completely with a MicroAmp Optical Adhesive Film plate seal. Properly center the plate seal on the plate, and seal completely around the edges and around each well, using a speedball soft rubber roller and a plastic wedge sealer.
- Centrifuge the QRP

  2200 x g, 4°C, 00:01:00 , or Max Speed. It is essential to remove air bubbles from reaction wells

  and ensure there are no bubbles in any of the wells.
- Transfer the QRP to a chilled 384-well aluminum block on ice and transport to the QuantStudio 7 PCR Machine.

When sealing the QRP, be careful to ensure no wrinkles are present in the plate seal, as miniaturized reactions can have a very small surface area for fluorescence, and thus any distortions in the light path can dramatically impact performance.

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Perform RT-qPCR 1h 45m

1h 35m

8

Set up the QuantStudio 7 (or QuantStudio 5) and Run:

- Turn on the QuantStudio 7 Pro (or QuantStudio 5) qPCR machine
- Enter the reaction plate barcode into the "Filename" portion.
- Insert the plate into the machine, ensuring well A1 is in the upper left corner.
- Run the following RT-qPCR program (Table 4):

A	B	C	D
Cycle Step	**Temperature**	**Time**	**Cycles**
Incubation	25 C	2 min	1
Reverse Transcription	53 C	10 min	1
Activation	95 C	2 min	1
Denaturation	95 C	3 sec	55
Anneal/Extension	60 C	30 sec	
Hold	4 C	holding	1

Table 4. RT-qPCR program.

Data Analysis and Interpretation

9

The purpose of this step is to analyze, interpret, and report the results of the RT-qPCR assay for the detection of SARS-CoV-2.

The individual gene target calls/results interpretations (Tables 5 and 6) are used in the subsequent steps to provide assessment calls for each sample. A target call of "Positive" signifies that the gene has successfully amplified. A target call of "Negative" signifies that there is no signal of gene amplification.

Results interpretation: ORF1ab, N gene, and S gene targets

A	B	C
Cq Cutoff	Cq Confidence	Target Call
< 37	> 0.7	Positive (+)
< 37	< 0.7	Negative (-)
> 37	< 0.7	Negative (-)
> 37	> 0.7	Negative (-)

Table 5. Results interpretation for ORF1ab, N gene, and S gene targets.

Results interpretation: MS2 Internal Positive Control

A	B	C
Cq Cutoff	Cq Confidence	Target Call
< 37	> 0.3	Positive (+)
< 37	< 0.3	Negative (-)
> 37	< 0.3	Negative (-)
> 37	> 0.3	Negative (-)

Table 6. Results interpretation for MS2 Internal Positive Control.

Citation: Peter De Hoff, Sydney C Morgan, Louise C Laurent (05/26/2021). Semi-Automated and Miniaturized SARS-CoV-2 Detection using TaqPath COVID-19 Multiplex Real-Time RT-PCR Assay. https://dx.doi.org/10.17504/protocols.io.brhvm366

9.1 Assessment calls: Controls

A	B	C	D	E	F
Control Sample	**ORF1ab**	**N Gene**	**S Gene**	**MS2 Internal Control**	**Assessment Call**
Negative Control	-	-	-	+	Negative (-)
Positive Control	+	+	+	-	Positive (+)

Table 7. Assessment calls for Control samples.

9.2 Assessment calls: Samples

For overall sample assessment:

- **Positive result:** At least 2/3 gene targets (ORF1ab, N gene, S gene) are positive (MS2 internal control can be positive or negative)
- **Inconclusive result:** 1/3 gene targets are positive (MS2 internal control can be positive or negative)
- **Negative result:** 0/3 gene targets are positive + MS2 internal control is positive
- **Invalid result:** 0/3 gene targets are positive + MS2 internal control is negative

If a sample is Inconclusive or Invalid, re-extract RNA and re-perform RT-qPCR.

A	B	C	D	E
ORF1ab	**N Gene**	**S Gene**	**MS2 Internal Control**	**Assessment Call**
+	+	+	-	Positive (+)
-	+	+	-	Positive (+)
+	-	+	-	Positive (+)
+	+	-	-	Positive (+)
+	-	-	-	Inconclusive
-	+	-	-	Inconclusive
-	-	+	-	Inconclusive
-	-	-	+	Negative (-)
-	-	-	-	Invalid

Table 8. Assessment calls for samples.

Validation of Reaction Miniaturization

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This step describes the validation of the miniaturization of the TaqPath COVID-19 Combo Kit **Thermo Fisher Scientific Catalog #A47814** testing kit that was granted Emergency Use Authorization by the U.S. Food and Drug Administration (FDA). This step does not need to be repeated.

Citation: Peter De Hoff, Sydney C Morgan, Louise C Laurent (05/26/2021). Semi-Automated and Miniaturized SARS-CoV-2 Detection using TaqPath COVID-19 Multiplex Real-Time RT-PCR Assay. https://dx.doi.org/10.17504/protocols.io.brhvm366

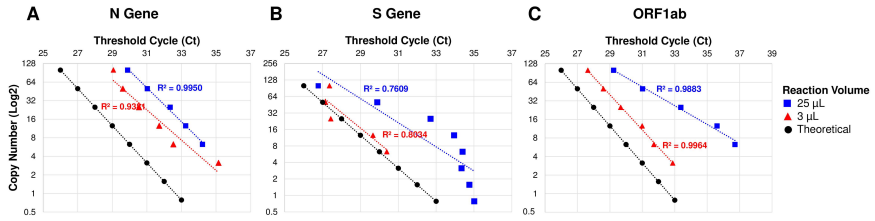

Figure 1. RT-qPCR performance of miniaturized (3 µL) and full-scale (25 µL) reactions as compared to theoretical results, for three targeted regions of the SARS-CoV-2 genome: (A) N Gene; (B) S Gene; (C) ORF1ab. The averages of 3 replicates per reaction are shown.

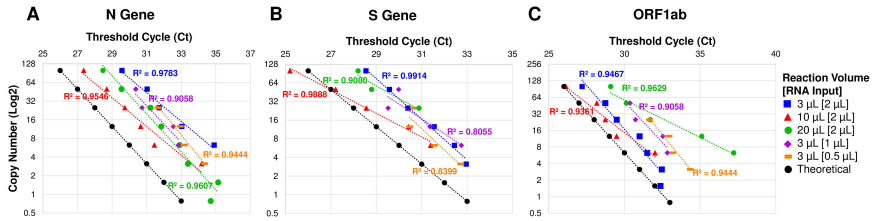

Figure 2. RT-qPCR performance of different reactions (varied total reaction volume and RNA input volume) as compared to theoretical results, for three targeted regions of the SARS-CoV-2 genome: (A) N Gene; (B) S Gene; (C) ORF1ab. The averages of 3 replicates per reaction are shown.

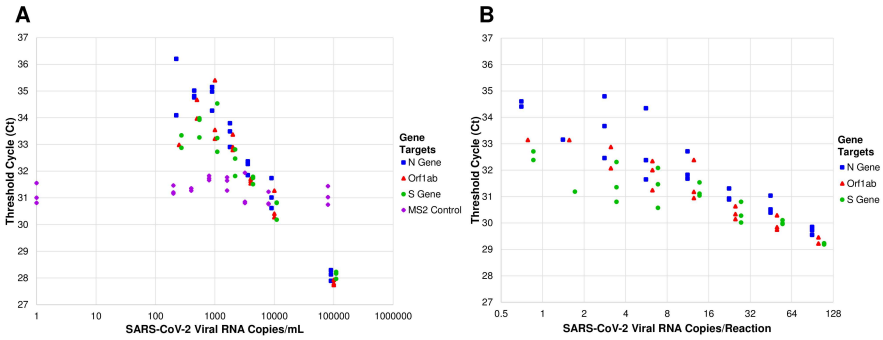

Figure 3. Limit of detection of SARS-CoV-2 viral RNA at different concentrations. (A) Viral RNA spiked into RT-qPCR reactions along with RNA from negative nasopharyngeal control samples. (B) Viral RNA spiked into negative nasopharyngeal control samples prior to RNA extraction. Each point represents a single replicate reaction. MS2 indicates positive internal control for each sample. Limit of detection was found to be 500 viral RNA copies/mL input sample with > 4 viral RNA copies per RT-qPCR reaction.

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