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Multiplex real-time PCR assay for the detection of SARS-CoV-2 variants

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We use this protocol and it's working

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Disclaimer

Reference to any commercial materials, equipment, or process does not in any way constitute approval, endorsement, or recommendation by the Food and Drug Administration.

Abstract

This document describes a real-time 4-plex, TaqMan based RT-PCR assay used for the detection of the majority of SARS-CoV-2 strains, and differentiation of both Delta and Omicron variants. 18S ribosomal RNA gene is included as an internal control for the assay quality control. The Bio-Rad CFX 96, Bio-Rad CFX Opus 96 RT-PCR systems, or other real-time PCR systems that have similar function and can generate similar results, can be used for this assay.

Method validation/evaluation/verification:
This method was evaluated through Single Randomized Method Test (SRMT) by Kansas State Veterinary Diagnostic Laboratory and Vet-LIRN. The SRMT report summarizing method performance evaluation data is available upon request.

Guidelines

- Always wear gloves.
- Put samples on ice until ready for use.
- Use aerosol pipette tips only.
- The master mix should be prepared ONLY in a clean hood designated for this purpose. Always prepare enough master mix to cover the number of samples and an additional 10-20%.

Materials

Materials

A. Reagents

- **4X TaqPath™ 1-Step RT-qPCR Master Mix, CG (Cat# A15299 or A15300)**
- Nuclease-free water
- Tris-EDTA (TE) buffer
- Dulbecco's Modified Eagle's Medium (DMEM)

Table 1: Primers and probes used in this assay.

Primer/probe name	Target Genotype	Sequence (5'-3')	Tm (°C)	Amplicon Size (bp)
Real-time PCR target primers and probes				
SARS2-dF;	Common primers for Delta (dPr) and non-Delta (wPr) probes	CCACAAAAACAACAAAAGTTGG	59.4	78 for Delta, 84 for non-Delta strains
SARS2-dR;		TGAGAGACATATTCAAAAGTGCAA	58.9	
SARS2-dPr	Delta variant	FAM-ATAAACTCCACTTTCCA	66.0	
SARS2-wPr	Non-Delta wildtype	VIC-ATAAACTCTGAACTCACTTT	65.0	
OmN-F	Common primers for Omicron (OmNm) and non-Omicron (OmNw) probes	GGACCCTCAGATTCAACTGG	59.5	86 for Omicron, 95 for non-Omicron strains
OmN-Fb		GGCCCTCAGATTCAACTGG	60.6	
OmN-R		GCAGTATTATTGGGTAAACCTTGG	60.0	
OmNm-Pr	Omicron variant	TexasRed-ATCGCGCCCCACCATTCT	66.1	
OmNw-Pr	Non-Omicron wildtype	VIC-CGCCCCACTGCGTTCTCC	67.6	
Real-time PCR internal control primers and probe				
18S-F	Human 18S ribosomal RNA gene	GGAGTATGGTTGCAAAGCTGA	60.2	100bp
18S-R		GGTGAGGTTTCCCGTGTTG	61.4	
18S-Pr		Cy5-AAGGAATTGACGGAAGGGCA	64.0	
Cloning and sequencing primers				
SARS2-cdF	Delta variant and non-Delta strains	TGGGACCAATGGTACTAAGAGG	60.2	440 for Delta/446 for non-Delta strains
SARS2-cdR		AACCCTGAGGGAGATCACG	60.1	
OmN-cF	Omicron variant and non-Omicron strains	CGTTGTTCGTTCTATGAAGACTTT	58.9	375 for Omicron/384 for non-Omicron strains
OmN-cR		TCATTTTACCGTCACCACCA	59.8	

B. Equipment/miscellaneous

- KingFisher 96 magnetic particle processor, or equivalent (Thermo Electron Corp. Cat#5400050)
- Benchtop centrifuge
- BioRad CFX96, BioRad Opus real-time PCR machine or equivalent
- Level 2 Biological Safety Cabinet or equivalent
- AirClean 600 PCR Workstation-AirClean Systems (Model# AC648LFUVC) or equivalent
- Vortex Mixer
- Mini Plate Spinner – Labnet (Model# MPS 1000) or equivalent, or larger centrifuge capable of centrifuging 96-well plates
- Seward Stomacher® 80 Biomaster, or equivalent
- QIAvac 24 Plus vacuum manifold, or equivalent
- Freezer (-15 °C - -25 °C)

C. Supplies

- 1000 µl, 200 µl, and 20 µl pipets
- 1000 µl, 200 µl and 20 µl aerosol pipet tips
- Serological pipettor
- 50 ml reservoirs
- Sterile 1.5 ml microcentrifuge tubes
- Sterile PCR reaction tubes, strip tubes, or 96-well plates
- Sterile PCR strip caps
- Optically clear adhesive seals for plates
- Gloves
- 10 ml, 5 ml serological pipets

Before start

Specimen Preparation:
Nucleic Acid can be extracted using the ThermoFisher MagMax™ Viral RNA Isolation Kit or the Qiagen QIAamp Viral RNA Mini Kit (Cat #52906). DMEM (ThermoFisher Cat. #: 11965-092) may be used to homogenize tissue prior to NA extraction.

Probe preparation

1 Prepare 100 µM individual **probe stock solution** with 1 × TE buffer.

1.1 Centrifuge the lyophilized probe tube at 6000 × g (8,000 rpm) for 00:05:00 . 5m

1.2 Dissolve lyophilized probe based on its molarity, and not molecular weight.
i.e., add 455 µL of 1× TE buffer to a tube containing 45.5 nM probe.

1.3 Mix by vortexing at half-full speed 3-4 times, 4-5 sec each time and centrifuge at 6000 × g for 10 sec. Do not vortex at high speed or for prolonged periods of time.

1.4 Label the tube with probe name and concentration, you initials and date of preparation. Store the stock solution in a designated -20 °C freezer.

2 Prepare 100 µL of **probe working solution** (10 µM) for each probe

2.1 Add 90 µL of nuclease-free water to a dark 1.5 ml sterile tube labeled with probe name and working solution concentration (10 µM).

2.2 Add 10 µL of the 100 µM probe stock solution and pipette up-and-down 2-3 times.

2.3 Invert the tube 3-5 times. Do not vortex.

2.4 Briefly centrifuge. This is the probe working solutions to be used in PCR reaction described below.

2.5 Store in designated -20 °C freezer.

Primer preparation

3 Prepare 100 µM individual **primer stock solution** with 1 × TE buffer.

3.1 Centrifuge the lyophilized primer tube at 6000 × g (8,000 rpm) for 00:05:00 . 5m

3.2 Dissolve lyophilized primer based on its molarity, and not molecular weight.
i.e., add 455 µL of 1 × TE buffer to a tube containing 45.5 nM primer.

3.3 Mix by vortexing at half-full speed 3-4 times, 4-5 sec each time and centrifuge at 6000 × g for 10 sec. Do not vortex at high speed or for prolonged periods of time.

3.4 Label the tube with primer name and concentration, your initial and date of preparation. Store the stock solution in a designated -20 °C freezer.

4 Prepare 100 µL of 18S (10 µM) **primer working solution**:

4.1 Add 80 µL of nuclease-free water into a 1.5 ml sterile tube labeled with primer name and working solution concentration (10 µM).

4.2 Add 10 µL of each 100 µM primer stock solution into the 80 µL water. Pipette up-and-down 2-3 times.

4.3 Invert the tube 3-5 times.

4.4 Briefly centrifuge.

4.5 Store in a designated -20 °C freezer.

5 Prepare 100 µL of SARS dF/R & Omicron Primer Mix (10 µM) **primer working solution**:

5.1 Add 50 µL of nuclease-free water into a 1.5 ml sterile tube labeled with primer name and working solution concentration (10 µM).

5.2 Add 10 µL of each 100 µM primer stock solution into the 50 µL water. Pipette up-and-down 2-3 times.

5.3 Invert the tube 3-5 times.

5.4 Briefly centrifuge

5.5 Store in a designated -20 °C freezer.

Note

All probe and primer working solutions can be prepared in different volumes if proportions of each component are kept in the same ratio. For example, use half volume of each component for 50 µl preparation, or use 2 times of each component for 200 µl preparation, etc.

Real-time PCR reaction preparation

6 **Master mix preparation**
Prepare the master mix in a 1.5 ml microcentrifuge tube using the volumes of reagents listed. DO NOT add the nucleic acid (template) at this time. Ice or cool blocks should be used for reaction preparation. Mix the master mix by low-speed vortexing, inverting, and/or flicking the tube, and then briefly centrifuge. Always prepare enough master mix for your samples, controls and an additional 10-20%.

6.1 Master mix

	A	B
	COVID19 Delta Omicron qPCR_TaqPath	
	Component	**Per rxn (µl)**
	4X TaqPath™ 1-Step RT-qPCR Master Mix, CG	5
	Nuclease-Free Water	3.75

	A	B
	SARS dF/R & Omicron Primer Mix (10 µM)	1
	18S Primer Mix (10 µM)	1
	SARS-dPr (10 µM; FAM)	0.5
	OmNm-Pr (10 µM; TxRed)	0.75
	SARS-wPr (10 µM; VIC)	1
	OmNw-Pr (10 µM; VIC)	1
	18S-Pr (10 µM; Cy5)	1
	Template	5
	Total reaction volume	**20**

6.2 Label PCR tubes, strips, worksheet or a plate map with sample IDs, negative controls, and positive controls. Add 15 µL **of master mix** to each well or tube.

6.3 Transfer plate or tubes with master mix to a DNA cabinet, or a clean working bench. Add 5 µL **of sample (Template)** to each corresponding PCR tube or well and mix by pipetting up and down several times. If the sample NA is in a plate, use extra caution when handling the uncovered NA plate to prevent any splashing. A multichannel pipet may be used to add 5µl of each sample NA to the reaction plate or tubes. Re-cover the NA plate immediately after all sample NA and control aliquots have been removed.

6.4 Cover the reaction tubes with optically clear caps or cap strips. If using a plate, cover the plate with optically clear PCR sealing film.

6.5 Briefly centrifuge, and put the tubes or plate onto the machine with correct orientation.

7 Machine run for the real-time PCR reactions

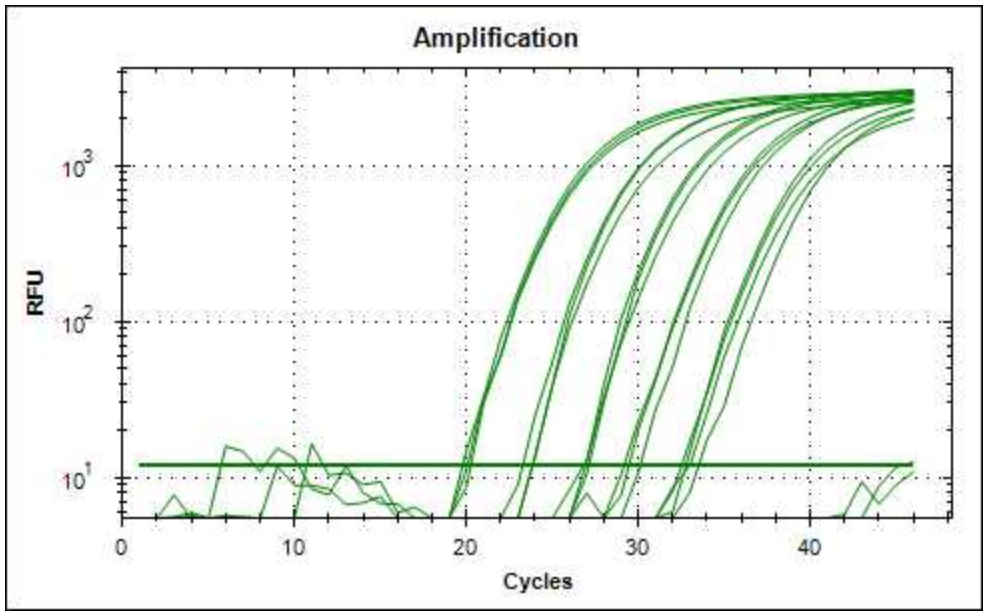
Run the samples, negative controls, and positive controls on a PCR machine using the protocol below

***RT*-qPCR Protocol:**

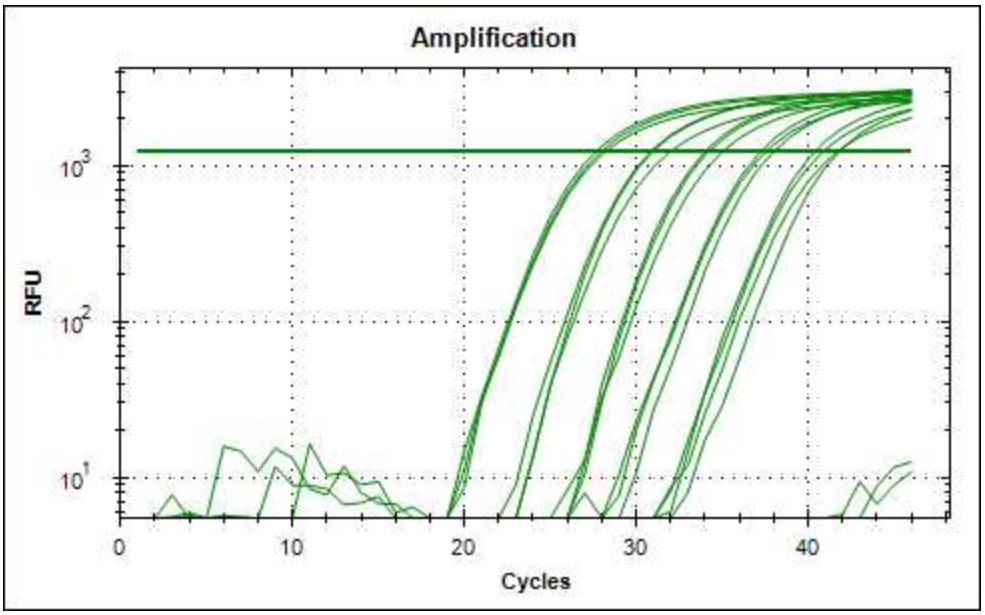
	A	B
	48°C	10 min
	95°C	5 min
	45 cycles of:	
	95°C	20 sec
	60°C	40 sec

8 Reaction Analysis

Threshold checking and data generation: After a run, the threshold levels need to be checked for each channel for accuracy. If it is off (Fig. 1), data could be misinterpreted.

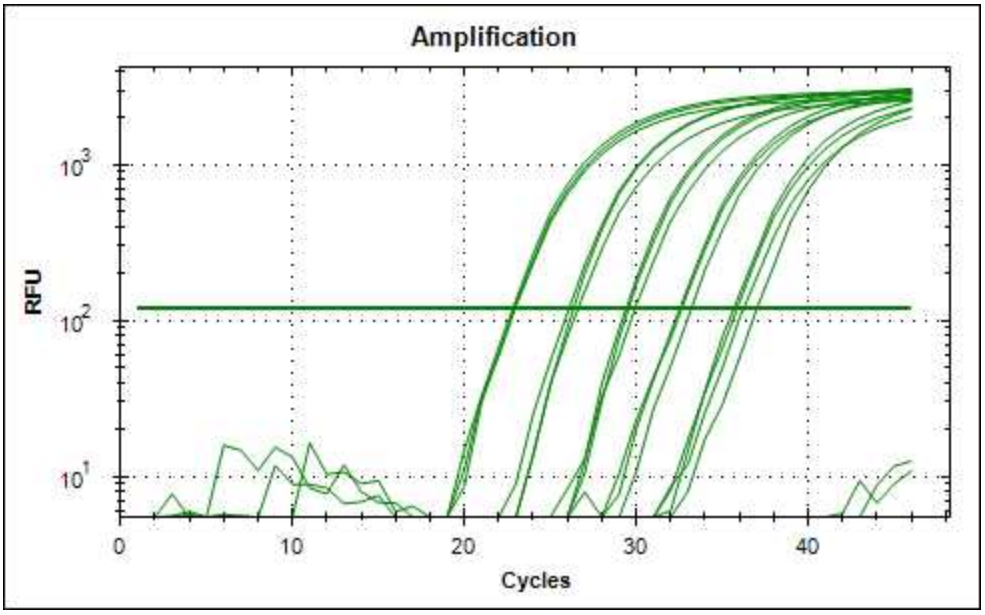


Incorrect threshold settings



Incorrect threshold settings

In these cases, switch to “log view”, and slide the threshold bar to the middle of the linear range (Fig. 2).



Correct threshold settings

9 Data to be Recorded/Interpretation

9.1 **Positive Result:** A positive sample is identified if Ct value is ≤37, and 18S Ct is ≤31.

9.2 **Negative Result:** A negative sample is defined if there is no Ct, or Ct >39, and 18S Ct is ≤31

9.3 **Other Possible Results (if applicable):** A weak positive/suspect/inconclusive sample is defined if Ct is >37 and ≤39, and 18S Ct is ≤31.