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

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Vet LIRN



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**Protocol status:** Working

**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')                    | T <sub>m</sub> (°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                |                                             |
| <u>O<sub>m</sub>N-F</u>                          | Common primers for Omicron ( <u>O<sub>m</sub>Nm</u> ) and non-Omicron ( <u>O<sub>m</sub>Nw</u> ) probes | GGACCCTCAGATTCAACTGG                | 59.5                | 86 for Omicron, 95 for non-Omicron strains  |
| <u>O<sub>m</sub>N-Fb</u>                         |                                                                                                         | GGCCCTCAGATTCAACTGG                 | 60.6                |                                             |
| <u>O<sub>m</sub>N-R</u>                          |                                                                                                         | GCAGTATTATTGGGTAAACCTTGG            | 60.0                |                                             |
| <u>O<sub>m</sub>Nm-Pr</u>                        | Omicron variant                                                                                         | <u>TexasRed-ATCGCGCCCCACCAT</u> TCT | 66.1                |                                             |
| <u>O<sub>m</sub>Nw-Pr</u>                        | 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                                            |                                                                                                         | GGTGAGGTTTCCCGTGTG                  | 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                |                                             |
| <u>O<sub>m</sub>N-cF</u>                         | Omicron variant and non-Omicron strains                                                                 | CGTTGTTCGTTCTATGAAGACTTT            | 58.9                | 375 for Omicron/384 for non-Omicron strains |
| <u>O<sub>m</sub>N-cR</u>                         |                                                                                                         | 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  $\mu\text{M}$  individual **probe stock solution** with 1  $\times$  TE buffer.
  - 1.1 Centrifuge the lyophilized probe tube at 6000  $\times$  g (8,000 rpm) for  00:05:00 . 
  - 1.2 Dissolve lyophilized probe based on its molarity, and not molecular weight.  
i.e., add  455  $\mu\text{L}$  of 1 $\times$  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  $\times$  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  $^{\circ}\text{C}$  freezer.
- 2 Prepare  100  $\mu\text{L}$  of **probe working solution** (10  $\mu\text{M}$ ) for each probe
  - 2.1 Add  90  $\mu\text{L}$  of nuclease-free water to a dark 1.5 ml sterile tube labeled with probe name and working solution concentration (10  $\mu\text{M}$ ). 
  - 2.2 Add  10  $\mu\text{L}$  of the 100  $\mu\text{M}$  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  $^{\circ}\text{C}$  freezer.

## Primer preparation

- 3 Prepare 100  $\mu\text{M}$  individual **primer stock solution** with 1  $\times$  TE buffer.



- 3.1 Centrifuge the lyophilized primer tube at  $6000 \times g$  (8,000 rpm) for 00:05:00 .
- 3.2 Dissolve lyophilized primer based on its molarity, and not molecular weight.  
i.e., add 455  $\mu\text{L}$  of  $1 \times$  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  $6000 \times g$  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  $-20^\circ\text{C}$  freezer.
- 4 Prepare 100  $\mu\text{L}$  of 18S (10  $\mu\text{M}$ ) **primer working solution**:
- 4.1 Add 80  $\mu\text{L}$  of nuclease-free water into a 1.5 ml sterile tube labeled with primer name and working solution concentration (10  $\mu\text{M}$ ).
- 4.2 Add 10  $\mu\text{L}$  of each 100  $\mu\text{M}$  primer stock solution into the 80  $\mu\text{L}$  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  $-20^\circ\text{C}$  freezer.
- 5 Prepare 100  $\mu\text{L}$  of SARS dF/R & Omicron Primer Mix (10  $\mu\text{M}$ ) **primer working solution**:

- 5.1 Add  50  $\mu\text{L}$  of nuclease-free water into a 1.5 ml sterile tube labeled with primer name and working solution concentration (10  $\mu\text{M}$ ).
- 5.2 Add  10  $\mu\text{L}$  of each 100  $\mu\text{M}$  primer stock solution into the  50  $\mu\text{L}$  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  $^{\circ}\text{C}$  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  $\mu\text{L}$  preparation, or use 2 times of each component for 200  $\mu\text{L}$  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                                         |
|-------------------------------------------|-------------------------------------------|
| <b>COVID19 Delta Omicron qPCR_TaqPath</b> |                                           |
| <b>Component</b>                          | <b>Per rxn (<math>\mu\text{L}</math>)</b> |

| A                                         | B         |
|-------------------------------------------|-----------|
| 4X TaqPath™ 1-Step RT-qPCR Master Mix, CG | 5         |
| Nuclease-Free Water                       | 3.75      |
| 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         |
| <b>Template</b>                           | 5         |
| <b>Total reaction volume</b>              | <b>20</b> |

- 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

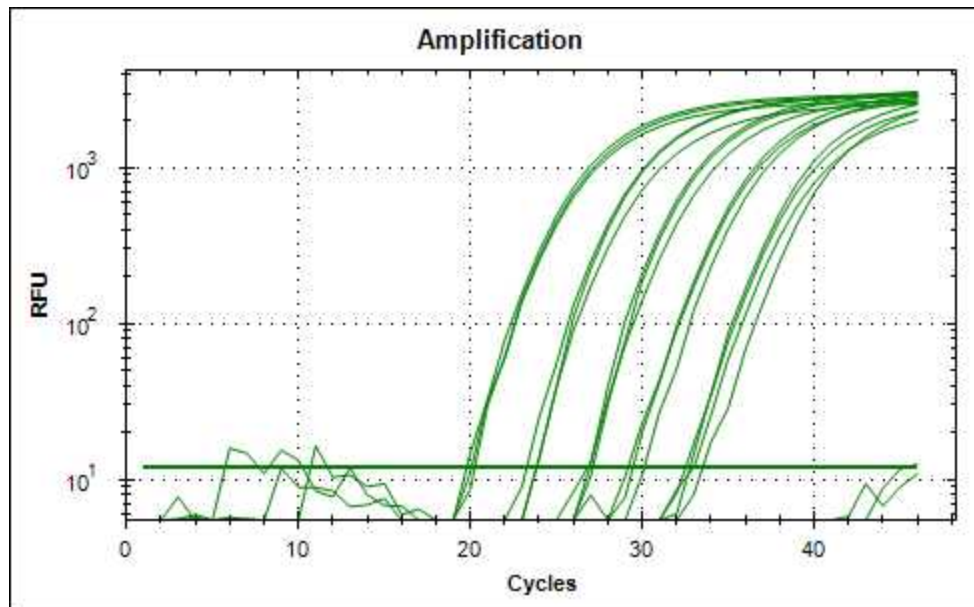
Run the samples, negative controls, and positive controls on a PCR machine using the protocol below

### **RT-qPCR Protocol:**

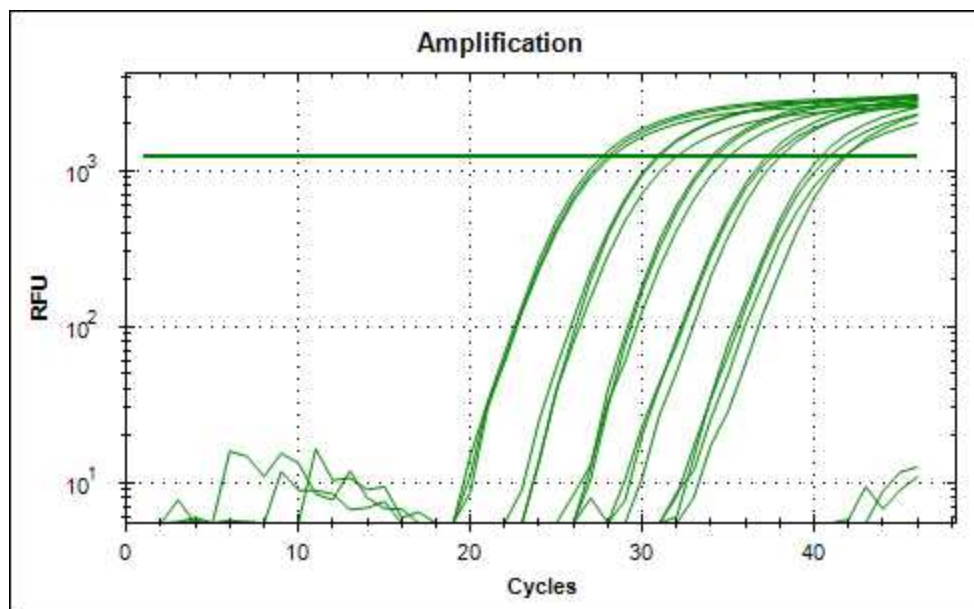
|  | A                | B      |
|--|------------------|--------|
|  | 48°C             | 10 min |
|  | 95°C             | 5 min  |
|  | 45 cycles<br>of: |        |
|  | 95°C             | 20 sec |
|  | 60°C             | 40 sec |

## 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.

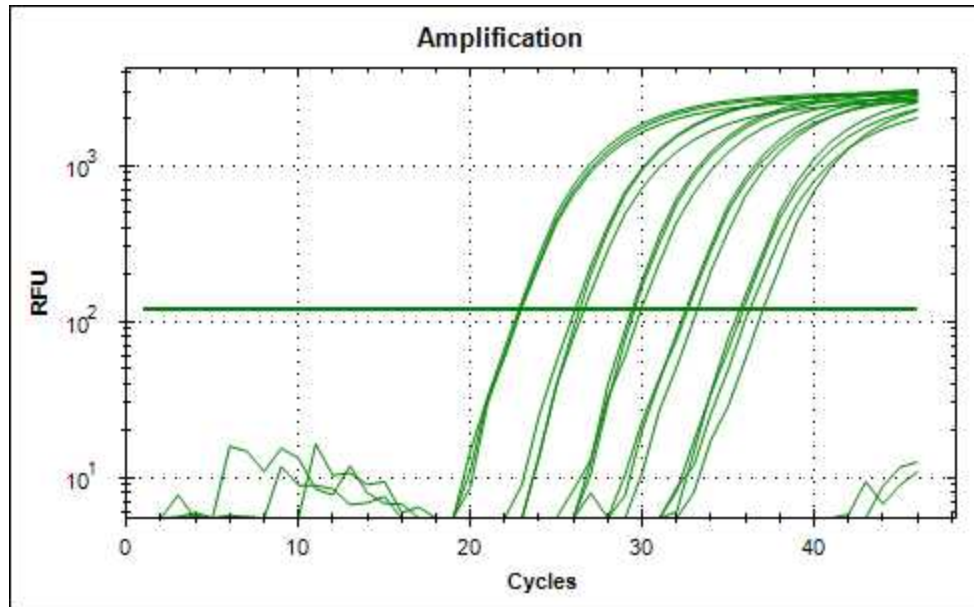


Incorrect threshold settings



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  $\leq 37$ , and 18S Ct is  $\leq 31$ .
- 9.2 **Negative Result:** A negative sample is defined if there is no Ct, or Ct  $> 39$ , and 18S Ct is  $\leq 31$ .
- 9.3 **Other Possible Results (if applicable):** A weak positive/suspect/inconclusive sample is defined if Ct is  $> 37$  and  $\leq 39$ , and 18S Ct is  $\leq 31$ .