

# SARS-CoV-2 Variant RT-qPCR Assay Using Reliance One-Step Multiplex RT-qPCR Supermix

## Protocol

### Description

The SARS-CoV-2 Variant Reverse Transcription Quantitative PCR (RT-qPCR) Assays\* are designed to target specific mutations identified in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome that are believed to confer an evolutionary advantage to the virus in terms of transmissibility and ability to escape the immune response. These assays have been expertly designed against the original SARS-CoV-2 genome derived from the strain first identified in Wuhan, China, using proprietary computational algorithms. They are intended to be validated by researchers for use in an allelic discrimination assay, an endpoint assay that detects down to a single nucleic acid sequence variation. Assays contain a primer set plus two probes: one probe is a perfect match to the wild type (HEX), and the other probe is a perfect match to the mutation (FAM). This single-tube format is 20x concentrated. Each assay contains 18  $\mu$ M primers and 5  $\mu$ M of each probe in a ready-to-use primer and probe mix optimized for use with the Reliance One-Step Multiplex RT-qPCR Supermix. Because it is an allelic discrimination assay, the actual quantity of the target sequence is not determined. Instead, the presence of wild-type or mutant variant is determined by comparing the endpoint fluorescence values. These assays can be validated by researchers for use in Droplet Digital PCR (ddPCR) using One-Step RT-ddPCR Supermix.

\* For research use only. Not for use in diagnostic procedures.

### Storage and Stability

The assays are stable for 12 months when stored at 4°C, protected from light. The 20x assay mix can be kept at -20°C for long-term storage.

### Product Contents

This product is a 20x concentrated, ready-to-use primer/probe mix optimized for use with Reliance One-Step Multiplex RT-qPCR Supermix. It is provided as 200, 1,000, or 2,500  $\mu$ l of the 20x assay, sufficient for 200, 1,000, or 2,500 x 20  $\mu$ l reactions, respectively.

### Suggested Reagents and Equipment\*

- Reliance One-Step Multiplex RT-qPCR Supermix (catalog #12010220, 12010221)
- RNase P Reference Assay (Unique Assay ID #dHsaCPE67329290: RPP40 Cy5 is orderable through [bio-rad.com/digital-assays](https://www.bio-rad.com/digital-assays))

- SARS-CoV-2, Flu, RSV Run Control Positive (#COVFLU)
- CFX Opus 96, CFX Opus 384, CFX 96 Touch, or CFX 384 Touch Real-Time PCR System (#12011319, 12011452, 1855195, or 1855485, respectively) or equivalent
- Hard-Shell 96-Well PCR Plates (#HSP9655, HSP9955), Hard-Shell 384-Well PCR Plates (#HSP3805), or equivalent
- Microseal 'B' Adhesive Seals (#MSB1001)
- Positive control consisting of synthetic SARS-CoV-2 RNA or gBlocks Gene Fragments that contain the mutation of interest. The recommended sequence for a gBlock is defined on the Positive Control tab of each Variant Assay product page in the Droplet Digital PCR Assay portal ([bio-rad.com/digital-assays](https://www.bio-rad.com/digital-assays)) and can be ordered directly from Integrated DNA Technologies, Inc.

\* Bio-Rad reagents and equipment, unless otherwise noted.

## Sample Preparation

### Sample Type

Total RNA is extracted from specimen samples. The variant assays' performance depends on the amount and quality of template RNA purified from human specimens.

### Positive Controls

**Positive mutant control** can be either gBlocks Gene Fragments or synthetic SARS-CoV-2 RNA controls that include the mutation of interest. gBlocks should be diluted in 1x Tris-EDTA (TE) buffer to  $10^5$ – $10^6$  copies/ $\mu$ l (50–500 fM). Use 1–5  $\mu$ l per reaction. Synthetic SARS-CoV-2 RNA controls should be diluted to 1,000 copies/ $\mu$ l in TE buffer. Use 5–10  $\mu$ l per reaction. No extraction is necessary.

**Note:** Synthetic, unprotected RNA cannot be extracted in a background sample that is negative for SARS-CoV-2 unless a lysis buffer is first added.

**Positive wild-type SARS-CoV-2 control** (SARS-CoV-2, Flu, RSV Run Control Positive, #COVFLU) contains whole inactivated SARS-CoV-2 (2019-nCoV/USA-WA1/2020), and can be considered a wild-type strain for the variant assays. This standard can be used as an extraction control by spiking 5  $\mu$ l of the standard into a tube containing 995  $\mu$ l of phosphate buffered saline and processing for nucleic acid extraction together with other samples, according to the extraction kit instructions. Alternatively, 5–10  $\mu$ l of the standard can be added directly to an allelic discrimination reaction.

**Important:** To prevent sample contamination, do not dilute or store high-copy synthetic template positive controls in reaction preparation areas. Change gloves after preparing control samples and use aerosol barrier pipet tips to prevent contamination.

### No Template Control (NTC)

NTC should be included on each plate to detect reagent or environmental contamination. Allelic discrimination analysis requires a minimum of two NTC wells to identify background fluorescence of the two fluorophores. This control uses nuclease-free water in place of a specimen sample.

### Bio-Rad Instrument Setup Example

The following instructions are suggested for researchers to use as a guide to develop a protocol to validate for use in their laboratory. The example shown here is for research use instruments, such as the CFX Opus and CFX Touch Real-Time PCR Systems, or equivalent systems. For more detailed information, refer to the instrument manual.

### Cycling Protocol Setup

1. Click **File > New > Protocol** in the menu bar to open the Protocol Editor.
2. Change Sample Volume to 20  $\mu$ l.
3. Modify the Cycling Protocol according to the guidelines in Table 1.

**Table 1. Example of thermal cycling protocol.\***

Step	Description	Temperature, °C	Time	Number of Cycles	
1	Reverse transcription	50	10 min	1	
2	DNA polymerase activation and template denaturation	95	10 min	1	
3	Amplification	Template denaturation	95	10 sec	35–40
4		Annealing/extension and plate read	60	30 sec	

\* Set the lid temperature to 105°C.

4. Confirm step 4 includes a plate read, as indicated by a camera symbol in the step.
5. To add a plate read to step 4, click on the step to highlight, then click **Add Plate Read to Step**.
6. Save the protocol by clicking **File > Save As**, assign a name and click **Save**.
7. Click **OK** to exit the Protocol Editor.

### Plate Setup

1. Click **File > New > Plate** in the menu bar to open the Plate Editor.
2. Select **Settings > Plate Size** > choose 96 or 384 wells.
3. Select **Settings > Plate Type** > choose BR White.
4. Expand the dropdown menu to the right of Scan Mode and select **All Channels**.
5. Highlight the wells where samples and controls will be on the plate. To highlight all the wells, click the upper left corner of the plate graphic.
6. Click **Select Fluorophores** and choose FAM, HEX, and Cy5 by checking the box to the right of the selected fluorophore (uncheck SYBR). Click **OK** to apply changes.
7. Define the sample type for each well by highlighting the wells, then choosing the appropriate identifier from the Sample Type dropdown menu.

**Note:** A minimum of two wells must be identified as NTC. This well contains water instead of sample. Positive and negative samples should also be identified as such. Samples should be identified as Unknown.

8. Apply target names and fluorophores to all wells by highlighting the wells then checking the Load box to the left of each of the fluorophores listed in the Target Name section. To include the target name, replace <none> in the open text box to the right of the fluorophore with the following:  
 FAM — Mutant  
 HEX — WT  
 Cy5 — RP

- Group the wells into separate well groups if more than one variant assay is included on the plate.
- Save the file by clicking **File > Save As**.
- Name the plate file and click **Save**.
- Click **OK** to exit the Plate Editor (Figure 1).
- Prepare a master mix volume sufficient for the number of samples and controls to be tested plus 10% more to account for pipetting variability (Table 2). The volumes in Table 2 assume 5 µl of sample is added to each reaction. If samples contain a low amount of RNA, add 10 µl of sample and omit the water from the master mix.

### Suggested Reaction Setup

- Thaw the Reliance One-Step Multiplex RT-qPCR Supermix on ice. Mix thoroughly by vortexing the tube to ensure homogeneity and resuspend any precipitates that may have formed during storage. Centrifuge briefly to collect contents at the bottom of the tube. Store on ice protected from light until ready to use.

**Note:** Reliance One-Step Multiplex RT-qPCR Supermix is a 4x ready-to-use solution. The viscosity of this solution is greater than a 2x mix. If using an automated liquid handling system, allow the supermix to equilibrate at room temperature for 10 min to reduce its viscosity.

- Thaw all other components to room temperature. Mix thoroughly by vortexing the tube to ensure homogeneity. Centrifuge briefly to ensure that all components are at the bottom of the reaction tube. Store protected from light.
- Prepare samples and negative and positive controls before setting up the reaction mix.

**Table 2. Example of preparation of master mix.**

Component	Volume per Reaction, µl	Volume for n Samples, µl
Reliance One-Step Multiplex RT-qPCR Supermix	5 µl	= (5 x n) x 1.1
20x wild-type (HEX) and mutant (FAM) primer/probes	1 µl	= (1 x n) x 1.1
20x RPP40 primers/probe (Cy5)	1 µl	= (1 x n) x 1.1
Nuclease-free water*	3–8 µl	= (volume x n) x 1.1

\* Adjust volume to accommodate larger sample volume. The volume of sample plus water should equal 13 µl.

- To obtain a final volume of 20 µl, aliquot 10 or 15 µl of master mix (for 10 or 5 µl sample volume, respectively) into each well of a Hard-Shell 96- or 384-Well PCR Plate.
- Add the RNA sample or 5 µl of positive or negative control to each well to complete the reaction mix. If wells containing controls need adjustment to reach a final volume of 20 µl, add an appropriate amount of nuclease-free water.
- Seal the plate with a Microseal 'B' Adhesive Seal and vortex to mix reagents. Centrifuge the plate briefly to ensure that all components are at the bottom of the reaction tube.
- Load the PCR plate into the CFX System, select the plate and protocol files defined in earlier steps, and start the run.

**Fig. 1.** Plate layout in CFX Maestro Software Plate Editor.

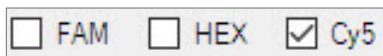
### Suggested Data Analysis

Analysis should be performed in a two-step process. First, confirm all samples contain amplifiable RNA and that the reactions were set up correctly. Second, determine which allele is present by using the allelic discrimination feature in CFX Maestro Software.

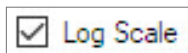
#### Confirm Expression of RNase P Control

RPP40 (Cy5) is an internal sample control. Failure to amplify this target suggests incorrect reaction setup, inadequate reagent quality, or insufficient intact RNA in the sample. Use the Quantification tab to confirm the amplification of the RPP40 target in each of the samples as follows.

1. Select **Settings > Cycles to Analyze** and enter 5 in the first cell.
2. Deselect FAM and HEX fluorophores by unchecking the corresponding boxes under the amplification plot. Only the Cy5 box should be selected.



3. Select **Log Scale** by checking the box in the lower right corner of the amplification plot.

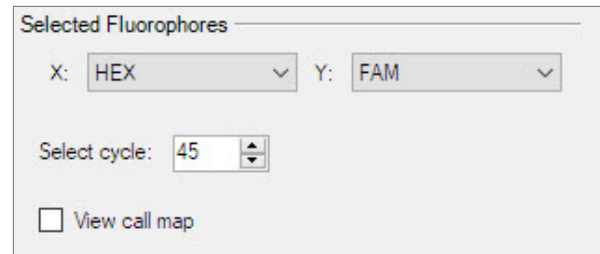


4. Visually inspect the traces. Any well with amplification in the Cy5 channel should show an exponential increase in relative fluorescence units (RFU) values until the reaction plateaus.
5. Set the Cy5 threshold in the amplification plot by clicking and dragging the threshold line until it lies in the exponential phase of the fluorescence curves and above any background signal.
6. Confirm all wells identified as unknowns cross the threshold before cycle 40 in the Cy5 channel. If an amplification curve of an unknown fails to cross the threshold before cycle 40, the integrity or the amount of RNA in the sample may be insufficient for that sample.

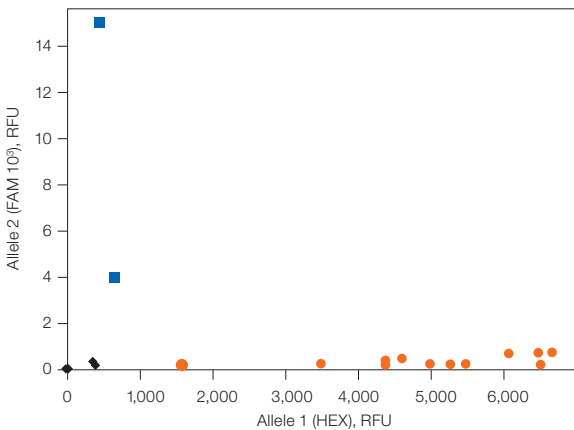
#### Determine Genotype Using Allelic Discrimination

The allelic discrimination function in CFX Maestro Software is used to determine which of the two variants tested are present in the sample. The HEX fluorescent dye detector is associated with the probe that is a perfect match to the wild-type allele. The FAM fluorescent dye detector is associated with the probe that is a perfect match to the mutant allele. The allelic discrimination function plots the endpoint fluorescence signal in each of the two channels for each well. Samples that contain the mutation will have a high FAM signal relative to the HEX signal. A dominant HEX signal in a well indicates a wild-type allele. If multiple variant assays are present on a plate, assess each assay individually by selecting specific well groups.

1. Click on the **Allelic Discrimination** tab.
2. Define axes using the dropdown menu in the Selected Fluorophores box located in the lower right quadrant of the allelic discrimination screen. Set X = HEX and Y = FAM. The selected cycle should be the last cycle of the thermal cycling protocol (endpoint).



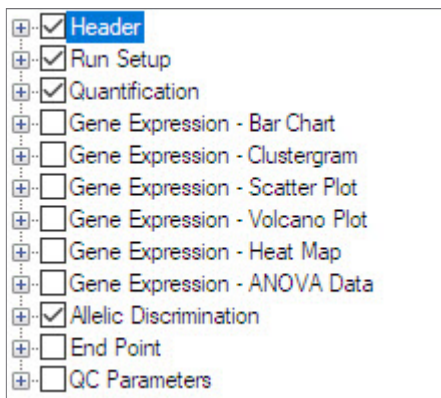
3. Confirm the NTC wells are correctly identified with black diamonds in the lower left corner of the plot. These wells should have only background fluorescence in both channels. Significant RFU in either channel indicates reagent or sample contamination.
4. Identify wells that have high HEX signal and low FAM signal (orange circles, Figure 2). These wells are identified in the chart as having an allele 2 call and contain the wild-type allele.
5. Identify wells that have a high FAM signal and low HEX signal (blue squares, Figure 2). These wells are identified in the chart as having an allele 1 call and contain the mutant allele.
6. If a sample has a signal above background signal in both FAM and HEX channels (NTC wells), then the well will be plotted as a green triangle and identified in the chart as having a heterozygote call. If a manual call is necessary, use the mouse to hover over the sample in the allelic discrimination plot, right click to access a pop-up menu, mouse over the well number, and select the desired call.
7. Click **View call map** in the Selected Fluorophores box for a color-coded view of the plate layout.



**Fig. 2. Allelic discrimination data plot.** Wild-type allele (●); mutant allele (■); NTC (◆).

### Generate a Printable Report

1. Select **Tools > Reports**.
2. Select the output report in the tree window located in the upper left quadrant of the report screen. The recommended output is shown.



3. Customize the report details by assigning a Title, Sub-Header Title, and alignment in the **Header** box located in the bottom left quadrant.

**Note:** If this section is grayed out, select the Notes option in the tree window under Header.

4. Click **Update Report**.
5. Select **File** → **Print** or **Save Report**.

### Optimization Recommendations

The SARS-CoV-2 Variant Assays and Reliance One-Step Multiplex RT-qPCR Supermix cycling protocols have been optimized for assays with a predicted primer melting temperature of 60°C. However, researchers can validate the protocol in their laboratory. For example, if the RFU differentiation between wild-type and mutant allele is suboptimal, adjusting the annealing/extension temperature is suggested.

1. Prepare a temperature gradient reaction plate using two samples: one positive for the mutant allele and the other sample with a wild-type allele. Prepare a reaction mix for at least 16 wells (two columns) according to Table 2. Dispense the reaction mix into two columns. Add wild-type allele sample to one column and the mutant allele sample to the other column.
2. Change the thermal cycling protocol defined in Table 1 by replacing the annealing/extension step with a thermal gradient. Set the thermal gradient temperature to span 55–65°C.
3. Run the plate set up in step 1.
4. Identify the optimal annealing temperature based on signal polarization: wells containing a sample with a mutant allele should have a strong FAM signal relative to HEX and wells containing wild-type samples should have a strong HEX signal relative to FAM.

### Bio-Rad Suggested Reagents and Equipment

Catalog #	Description
MSB1001	<b>Microseal 'B' Adhesive Seals</b> , optically clear seals, 100
HSP9655	<b>Hard-Shell 96-Well PCR Plates</b> , low profile, thin wall, skirted, white shell/white wells
HSP9955	<b>Hard-Shell 96-Well PCR Plates</b> , low profile, thin wall, skirted, white shell/white wells, barcoded
HSP3805	<b>Hard-Shell 384-Well PCR Plates</b> , thin wall, skirted, clear shell/white wells
12010220	<b>Reliance One-Step Multiplex RT-qPCR Supermix</b> , 1,000 x 20 µl reactions
12010221	<b>Reliance One-Step Multiplex RT-qPCR Supermix</b> , 2,000 x 20 µl reactions
12011319	<b>CFX Opus 96 Real-Time PCR System</b>
12011452	<b>CFX Opus 384 Real-Time PCR System</b>
1855195	<b>CFX96 Touch Real-Time PCR System</b>
1855485	<b>CFX384 Touch Real-Time PCR System</b>
COVFLU	<b>SARS-CoV-2, Flu, RSV Run Control Positive</b>



Visit [bio-rad.com/SC2VariantSurveillance](https://bio-rad.com/SC2VariantSurveillance) for predesigned SARS-CoV-2 genotyping assays.

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Hard-Shell Plates are covered by one or more of the following U.S. patents or their foreign counterparts owned by Eppendorf AG: U.S. Patent Numbers 7,347,977; 6,340,589; and 6,528,302.

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