

PrimePCR SARS-CoV-2 Single Mutation RT-qPCR Assay Using Reliance One-Step Multiplex RT-qPCR Supermix Protocol

Description

PrimePCR SARS-CoV-2 Single Mutation RT-qPCR Assays* can be validated by researchers to target specific individual mutations identified in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome believed to confer the evolutionary advantages of increased transmissibility and the ability to escape 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 include a primer set plus two probes: one probe (labeled with HEX) is a perfect match to the wild type, and the other probe (labeled with FAM) is a perfect match to the mutation. In addition, all assays contain primers and a probe to *RPP40* (Cy5) as an RNA integrity and amplification control. Each assay contains 18 μM primers and 5 μM of each probe in a ready-to-use single-tube format and is optimal 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 variant mutation is determined by the researcher in comparing the endpoint fluorescence values.

* For research use only (RUO). 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 optimal for use with the Reliance One-Step Multiplex RT-qPCR Supermix. It is provided as 200, 1,000, or 2,500 μl volumes, sufficient for 200, 1,000, or 2,500 x 20 μl reactions.

Optimal Reagents and Equipment*

- Reliance One-Step Multiplex RT-qPCR Supermix (catalog #12010220, 12010221)
- Reference control: SARS-CoV-2 Standard (#COV019)
- Negative control: SARS-CoV-2 Negative (#COV000)

* Bio-Rad reagents and equipment, unless otherwise noted. Reference, variant, and negative controls are from Exact Diagnostics, a Bio-Rad company.

- SARS-CoV-2 Variant Controls for assays targeting spike gene mutations:
 - SARS-CoV-2 S Gene Alpha Variant Control (B.1.1.7) (#COVA)
 - SARS-CoV-2 S Gene Beta Variant Control (B.1.351) (#COVB)
 - SARS-CoV-2 S Gene Epsilon Variant Control (B.1.427 and B.1.429) (#COVE)
 - SARS-CoV-2 S Gene Gamma Variant Control (P.1) (#COVG)
 - SARS-CoV-2 S Gene Delta-Kappa Variants Control (Delta B.1.617.2 and AY sublineages, Kappa B.1.617.1 and B.1.617.3) (#COVDK)
- gBlocks Gene Fragments can be validated for use as positive controls for assays that target genes other than the spike gene. The optimal gBlocks sequences can be found in the Assay Control tab of each PrimePCR SARS-CoV-2 Single Mutation Assay product, and can be ordered directly from Integrated DNA Technologies, Inc.

- 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' PCR Plate Sealing Film (#MSB1001)

Sample Preparation

Sample Type

The PrimePCR SARS-CoV-2 Single Mutation Assays amplify and detect specific variants in purified genomic RNA samples.

Reference Control

The SARS-CoV-2 Standard (#COV019) from Exact Diagnostics is manufactured with synthetic RNA transcripts containing five gene targets (the *E*, *N*, *ORF1ab*, *RdRp*, and *S* genes of SARS-CoV-2). The product is optimal to validate reverse transcription, amplification, and detection of SARS-CoV-2 RNA. This product also contains human genomic DNA, which can serve as an amplification control (*RPP40*). Add 10 µl per reference control reaction; extraction or dilution should not be required.

Positive Variant Controls

The SARS-CoV-2 *S* Gene Variant Controls from Exact Diagnostics are individually manufactured with synthetic RNA transcripts of the *S* gene from current SARS-CoV-2 variants of interest or concern. These controls contain human genomic DNA, which serves as an amplification control. These variant controls can be validated for use to monitor the reverse transcription, amplification, and detection steps in assays that target *S* gene mutations. Add 10 µl per variant control reaction; extraction or dilution should not be required.

gBlocks Gene Fragments from Integrated DNA Technologies, Inc. are DNA oligos, which are optimal as positive controls in assays that target mutations other than the *S* gene. Ordering information can be found in the Assay Control tab of each PrimePCR SARS-CoV-2 Single Mutation Assay product, or the PrimePCR SARS-CoV-2 Single Mutation Assay product page. Dilute gBlocks Controls in 1x Tris-EDTA (TE) buffer to 10⁵–10⁶ copies/µl (50–500 fM). Use 5 µl per variant control reaction.

Negative Control

The SARS-CoV-2 Negative (#COV000) from Exact Diagnostics is formulated in a synthetic matrix and contains genomic DNA. It should be used as negative reference material when testing for SARS-CoV-2. Add 10 µl per negative control reaction; extraction or dilution should not be required.

No Template Control (NTC)

This control can be validated by researchers to monitor for reagent and environmental contamination of the reactions. Use 10 µl of nuclease-free water per NTC reaction. A minimum of two NTC wells per plate is required to analyze data using the allelic discrimination module in CFX Maestro Software.

Bio-Rad Instrument Setup Example

The following instructions are suggested for researchers to develop a validation protocol in their laboratory. The example shown here is for the CFX Opus 96 and CFX96 Touch Real-Time PCR Systems,

which are research instruments equipped with five fluorescence channels. For more detailed information, refer to the respective 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 µl.
3. Modify the thermal 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 | | Template denaturation | 95 | 10 sec | 45 |
| 4 | Amplification | Annealing/extension and plate read | 60 | 30 sec | |

* Set the lid temperature to 105°C.

4. Confirm the annealing/extension step includes a plate read, as indicated by a camera symbol in the step.
5. To add a plate read, 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 per mutation detection assay must be identified as NTC. These wells contain water instead of sample. Reference, variant positive, and negative controls, 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 names, replace <none> in the open text box to the right of each fluorophore as follows:
 FAM — Mutant
 HEX — WT
 Cy5 — RP
9. Group the wells into separate well groups if more than one variant assay is included on the plate.
10. Save the file by clicking **File > Save As**.
11. Name the plate file and click **Save**.
12. Click **OK** to exit the Plate Editor (Figure 1).

Suggested Reaction Setup

1. 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.

2. 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.
3. Prepare samples and SARS-CoV-2 negative, reference, and variant positive controls before setting up the reaction mix.
4. 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 are based on 10 µl of sample being added to a final volume of 20 µl. Scale volumes appropriately if using a 384-well plate and a final volume of 10 µl.

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 PrimePCR SARS-CoV-2 Single Mutation Assay | 1 µl | = (1 x n) x 1.1 |
| Nuclease-free water* | 4 µl | = (volume x n) x 1.1 |

* Adjust volume to accommodate larger sample volume. The volume of sample plus water should equal 14 µl.

5. Pipet 10 µl of master mix into each well of a reaction plate. Adjust the volume if accommodating a larger sample volume or using a 384-well plate.
6. Add 10 µl of RNA sample, reference control, variant control, negative control, or nuclease-free water (no template control) to the appropriate well of the reaction plate for a final volume of 20 µl. Adjust volume, if necessary.

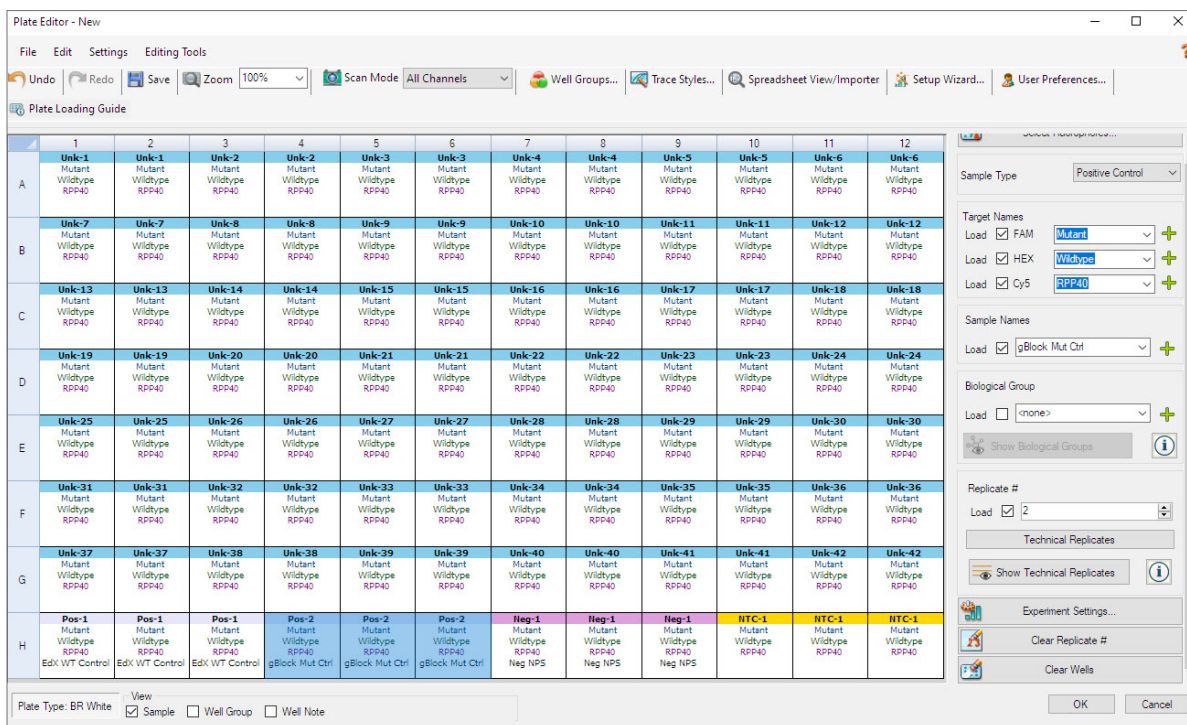


Fig. 1. Plate layout in CFX Maestro Software Plate Editor.

- Seal the plate with Microseal 'B' PCR Plate Sealing Film 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.
- 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.
- Confirm that 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.

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 by the researcher's method. Use the Quantification tab to confirm the amplification of the *RPP40* target in each of the samples as follows.

- Select **Settings > Cycles to Analyze**. Enter **5** in the first cell to replace the default setting of 1, and change the second cell to **45** as shown. Click **OK** to apply changes and exit.

- Select **Settings > Baseline Setting > Apply Fluorescence Drift Correction**.
- Deselect FAM and HEX fluorophores by unchecking the corresponding boxes under the amplification plot. Only the **Cy5** box should be selected.

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

- 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.
- Manual baseline adjustment may be necessary if amplification traces are not exponential. To manually define the baseline, select **Settings > Baseline Threshold**. Highlight the well to be adjusted, enter **2** in the Baseline Begin cell, and enter a cycle number that is two cycles before the amplification trace starts to rise in the Baseline End cell. Click **OK** to apply.

Determine Genotype Using Allelic Discrimination

The allelic discrimination function in CFX Maestro Software can be used by researchers 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 should have a high FAM signal relative to the HEX signal. A dominant HEX signal in a well likely indicates a wild-type allele. If multiple variant assays are present on a plate, assess each assay individually by selecting specific well groups.

- Click the Allelic Discrimination tab.
- Define axes using the dropdown menu in the Selected Fluorophores box located in the lower right quadrant of the allelic discrimination screen. Set X to FAM and Y to HEX to place the mutant allele on the x-axis and the wild-type allele on the y-axis. The selected cycle should be the last cycle of the thermal cycling protocol (endpoint).

- 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 may indicate reagent or sample contamination.
- Identify wells that have a high FAM and low HEX signal (orange circles, Figure 2). These wells can be identified in the chart as having an allele 1 call and contain the mutant allele.
- Identify wells that have a high HEX and low FAM signal (blue squares, Figure 2). These wells can be identified in the chart as having an allele 2 call and contain the wild-type allele.

- If a sample has a signal above background signal in both FAM and HEX channels (NTC wells), then the well may be plotted as a green triangle and can be 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.
- Click **View call map** in the Selected Fluorophores box for a color-coded view of the plate layout.

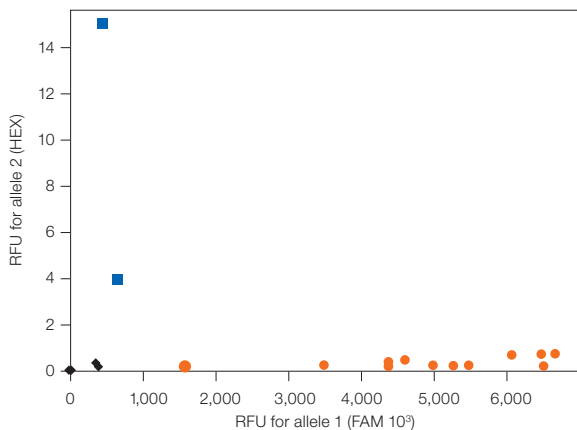
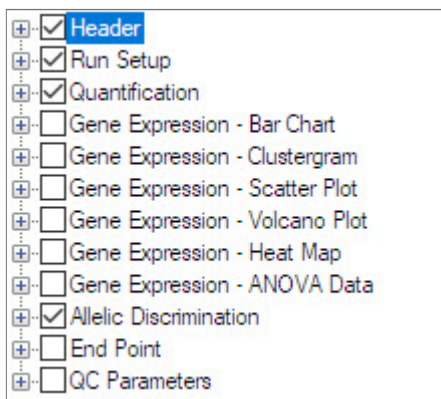


Fig. 2. Allelic discrimination data plot. Mutant allele (●); wild-type allele (■); NTC (◆). RFU, relative fluorescence units.

Generate a Printable Report

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



- 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.

- Click **Update Report**.
- Select **File > Print** or **Save Report**.

Optimization Recommendations

The PrimePCR SARS-CoV-2 Single Mutation Assays and Reliance One-Step Multiplex RT-qPCR Supermix cycling protocols are optimal for assays with a predicted primer melting temperature of 60°C. However, the protocol can be validated for use by researchers in their laboratories. For example, if the RFU differentiation between wild-type and mutant allele is suboptimal, adjusting the annealing/extension temperature is suggested.

- 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 the wild-type allele sample to one column and the mutant allele sample to the other column.
- 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.
- Run the plate set up in step 1.
- 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 PCR Plastics, Reagents, and Equipment

| Catalog # | Description |
|-----------|--|
| MSB1001 | Microseal 'B' PCR Plate Sealing Film , adhesive, optical, pkg of 100 |
| HSP9655 | Hard-Shell 96-Well PCR Plates , low profile, thin wall, skirted, white shell/white well |
| HSP9955 | Hard-Shell 96-Well PCR Plates , low profile, thin wall, skirted, white shell/white well, barcoded |
| HSP3805 | Hard-Shell 384-Well PCR Plates , low profile, thin wall, skirted, clear shell/white well |
| 12010220 | Reliance One-Step Multiplex RT-qPCR Supermix , 1,000 x 20 µl reactions |
| 12010221 | Reliance One-Step Multiplex RT-qPCR Supermix , 2,000 x 20 µl reactions |

Instruments

| | |
|----------|--|
| 12011319 | CFX Opus 96 Real-Time PCR System |
| 12011452 | CFX Opus 384 Real-Time PCR System |
| 1855195 | CFX96 Touch Real-Time PCR System |
| 1855485 | CFX384 Touch Real-Time PCR System |

Exact Diagnostics SARS-CoV-2 Reference Control (RUO)

COV019 SARS-CoV-2 Standard

Exact Diagnostics SARS-CoV-2 Variant Mutation Controls (RUO)

| | |
|-------|---|
| COVA | SARS-CoV-2 S Gene Alpha Variant Control (B.1.1.7) |
| COVB | SARS-CoV-2 S Gene Beta Variant Control (B.1.351) |
| COVE | SARS-CoV-2 S Gene Epsilon Variant Control (B.1.427 and B.1.429) |
| COVG | SARS-CoV-2 S Gene Gamma Variant Control (P.1) |
| COVDK | SARS-CoV-2 S Gene Delta-Kappa Variants Control (Delta B.1.617.2 and AY sublineages, Kappa B.1.617.1 and B.1.617.3) |

Exact Diagnostics SARS-CoV-2 Negative Control (RUO)

COV000 SARS-CoV-2 Negative

PrimePCR SARS-CoV-2 Single Mutation AssaysRefer to [bio-rad.com/SC2Variants](https://www.bio-rad.com/SC2Variants) for assays and ordering information.Visit [bio-rad.com/SC2Variants](https://www.bio-rad.com/SC2Variants) for a complete list of PrimePCR SARS-CoV-2 Mutation Assays and ordering information.Visit [bio-rad.com/PCRPlastics](https://www.bio-rad.com/PCRPlastics) for a full range of PCR plastics and seals.Visit [bio-rad.com/RelianceOneStep](https://www.bio-rad.com/RelianceOneStep) for more information.Visit [ExactDiagnostics.com](https://www.ExactDiagnostics.com) for more information about these controls. [Q-1678 COVID-19 Quality Control Solutions](https://www.ExactDiagnostics.com/Q-1678-COVID-19-Quality-Control-Solutions) contains a complete list of SARS-CoV-2 controls.**Bio-Rad Technical Support**

The Bio-Rad Technical Support department in the U.S. is open Monday through Friday, 5:00 AM to 5:00 PM, Pacific time.

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Email: Support@Bio-Rad.com (U.S./Canada only)For technical assistance outside the U.S. and Canada, contact your local technical support office or click the Contact us link at [bio-rad.com](https://www.bio-rad.com).

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