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

Description

The PrimePCR SARS-CoV-2 Multiple Mutation RT-qPCR Assay* is a single-well multiplexed panel which can be validated by researchers to detect and differentiate four key mutations identified in the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) genome. The identification of mutations targeted by the assay can be evaluated by researchers to assess the likelihood of a specific variant in the sample. The assay's high sensitivity can be validated by researchers to assess low-level samples with SARS-CoV-2 quantification cycle (Cq) values up to 40. The single tube assay is 20x concentrated and contains a primer set plus up to six probes, including one probe that is the multiplexed control (MPC). This probe has homology to a conserved region between the primers and serves as an internal RNA integrity and experimental setup control. The other probes are perfect matches to the four mutations of interest in the spike protein gene. The assay format is optimal for use with the Reliance One-Step Multiplex RT-qPCR Supermix and should be run on a real-time PCR detection system equipped with five fluorescence channels, such as the CFX96 Touch or CFX Opus 96 Real-Time PCR System. Additional assays will become available as the virus continues to evolve and the need to identify novel variants arises. Visit bio-rad.com/SC2Variants for a complete list of assays available.

* For research use only (RUO). Not for use in diagnostic procedures.
** The multiplexed control (MPC) is designed in a conserved region of the S gene. MPC functionality may be compromised if the virus acquires mutations in this region.

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, which can be validated for use with the Reliance One-Step Multiplex RT-qPCR Supermix for detection of four mutations in a single well. It is provided as 200, 1,000, or 2,500 µl volumes, sufficient for 200, 1,000, or 2,500 x 20 µl reactions.

Suggested Reagents and Equipment*
- Reliance One-Step Multiplex RT-qPCR Supermix (catalog #12010220, 12010221)
- Reference control: SARS-CoV-2 Standard (#COV019)
- SARS-CoV-2 Variant Controls:
    - 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)
- Negative control: SARS-CoV-2 Negative (#COV000)
- CFX Opus 96 or CFX96 Touch Real-Time PCR System (#12011319 or 1855195, respectively) or equivalent 5-color instrument
- Hard-Shell 96-Well PCR Plates (#HSP9655, HSP9955) or equivalent
- Microseal 'B' PCR Plate Sealing Film (#MSB1001)

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

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Sample Preparation

Sample Type

The optimal sample type is total RNA extracted from human specimen samples. Alternative sample types may be compatible but should be optimized and validated before use by the researcher. Assay performance depends on the amount and quality of template RNA validated. The PrimePCR SARS-CoV-2 Multiple Mutation Assay is for research use only and is not intended for patient diagnosis from clinical 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 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 Multiple Mutation Assay product, or the PrimePCR SARS-CoV-2 Multiple Mutation Assay product page. Dilute gBlocks Controls in 1x Tris-EDTA (TE) buffer to 10^5–10^6 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	Amplification	Template denaturation	95	10 sec	45
4		Annealing/extension and plate read	58	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 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, Texas Red, Cy5,* and Cy5.5 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: If samples contain very low amounts of viral RNA, consider adding a second set of control samples that contain SARS-CoV-2 Variant Controls and the SARS-CoV-2 Standard diluted in TE buffer at 1:100, and undiluted.

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. For example, if using PrimePCR SARS-CoV-2 Multiple Mutation Assay 1:

   FAM — 484K/Q
   HEX — MPC
   Texas Red — 501Y
   Cy5 — 417N
   Cy5.5 — 452R

9. Save the file by clicking **File** > **Save As**.

10. Name the plate file and click **Save**.

11. Click **OK** to exit the Plate Editor (Figure 1).

* Assays utilize an ATTO 647 fluorophore, which has a similar excitation and emission spectra to Cy5.

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 the PrimePCR SARS-CoV-2 Multiple Mutation Assay at 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. Thaw samples on ice. Place the SARS-CoV-2 Standard, Negative, and Variant Controls (stored at 4°C) on ice. Mix thoroughly by vortexing each tube to ensure homogeneity. Centrifuge briefly to ensure that all components are at the bottom of each reaction tube.
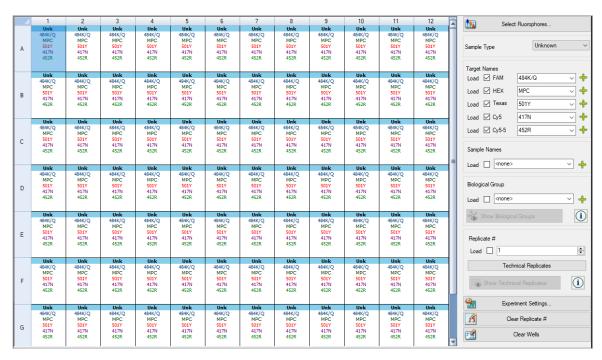

Fig. 1. Plate layout in CFX Maestro Software Plate Editor.

Note: The controls are supplied at 200,000 copies/ml and can be validated for use in these assays without dilution and without extraction. However, if samples contain very low amounts of viral RNA (<300 copies/ml or have Cq values ≥30), it is recommended to prepare diluted SARS-CoV-2 Variant Controls (1:100) so that the control wells cross the threshold at approximately the same cycle as the samples.

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.

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 Multiple 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. We recommend using 10 µl of sample. In each 20 µl reaction, the volume of sample plus water should equal 14 µl.

5. To obtain a final volume of 20 µl, pipet 10 µl of master mix into each well of a Hard-Shell 96-Well PCR 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.

7. 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.

8. Load the PCR plate into the CFX96 Touch Real-Time PCR System, select the plate and protocol files defined in earlier steps, and start the run.

Suggested Data Analysis

Analysis should be performed in a three-step process. First, adjust the baseline and threshold setting for each fluorophore. Second, confirm that reactions were set up properly and that all samples contain amplifiable RNA by assessing the MPC amplification curve in the HEX channel for each sample. Third, assess the presence or absence of each mutation by determining whether the amplification plot indicates exponential growth and crosses the threshold for each fluorophore.

Baseline-Threshold Setting

The baseline and threshold settings should be confirmed and manually defined, if necessary, in the Quantification tab of CFX Maestro Software.

1. All detection channels should be selected by default during plate setup; confirm before proceeding to step 2.

2. Select **Settings** > **Cycles to Analyze**.

3. Change lower cycles to 5 and upper cycles to 45.

4. Click **OK** to apply changes and exit.

5. Select **Settings** > **Baseline Setting** > **Apply Fluorescence Drift Correction**.

6. Deselect the check boxes for HEX, Texas Red, Cy5, and Cy5.5 below the amplification plot (only **FAM** should be selected).

7. Select **Log Scale** by checking the box in the lower right corner of the amplification plot. Change the log scale to a minimum value, such as 0.5, so that the baseline signals are visible.

8. Click and drag the bold horizontal threshold line until it lies in the exponential phase of the fluorescence curve and above any baseline signal.

9. Repeat steps 4–8, for each of the remaining fluorophores (HEX, Texas Red, Cy5, and Cy5.5).

Confirm Expression for Multiplexed Control

MPC (HEX) can be an internal sample control. The probe has homology to a highly conserved region between the primers. Failure to amplify MPC may suggest incorrect reaction setup, inadequate reagent quality, or insufficient intact RNA by the researcher's method. Alternatively, the sample may contain a new mutation that impacts the ability of the probe to bind.

1. Select **HEX** by unchecking all other fluorophores in the boxes under the amplification plot.

2. Confirm that all wells identified by researchers as unknowns cross the threshold before cycle 45 in the HEX channel.

3. Exclude from analysis any well containing an unknown that fails to cross the threshold before cycle 45 in the HEX channel.

Assess Presence or Absence of Mutations

1. Select **FAM** by unchecking all other fluorophores in the boxes under the amplification plot.

2. Identify unknowns that may contain the mutation associated with the FAM fluorophore by determining whether the amplification curves in the FAM channel display exponential growth and cross the threshold before cycle 40.

3. Repeat steps 1 and 2 for fluorophores Texas Red, Cy5 (ATTO 647), and Cy5.5 sequentially to identify unknowns that contain mutations that encode the other targeted mutations.

Data Interpretation

Researchers can determine the likelihood that a sample contains a variant of interest by considering which of the mutations are assessed as positive in the sample. The currently reported most common SARS-CoV-2 variants of interest or concern that can be identified using the PrimePCR SARS-CoV-2 Multiple Mutation Assay are listed in Table 3. To determine the likelihood that a sample contains a specific variant, researchers should identify which fluorophore(s) cross the threshold before cycle 45 in a sample, and then confirm the sample is positive for MPC. Data can

be compared to the information about currently reported variants of interest or concern in Table 3. For example, using PrimePCR SARS-CoV-2 Multiple Mutation Assay 1, if a sample is determined to be positive for FAM (E484K/Q), Texas Red (N501Y), ATTO 647 (K417N), and HEX (MPC), then the sample mutation profile may be consistent with the Beta variant (B.1.351).

Visit bio-rad.com/SC2Variants for a complete list of multiple mutation assays and data interpretation tables.

Table 3. Example of mutation fingerprint of SARS-CoV-2 variants of interest or concern for assay 1.

WHO Label	Pango Lineage	E484K/Q (FAM)	N501Y (Texas Red)	K417N (ATTO 647)	L452R (Cy5.5)	MPC (HEX)
Alpha*	B.1.1.7	–	•	–	–	•
Beta	B.1.351	•	•	•	–	•
Gamma	P.1	•	–	–	–	•
Delta**	B.1.617.2	–	–	–	•	•
Epsilon**	B.1.427/B.1.429	–	–	–	•	•
Kappa	B.1.617.1/B.1.617.3	•	–	–	•	•
Omicron	B.1.1.529	–	•***	•	–	•

WHO, World Health Organization.

* Some Alpha strains contain the E484K mutation, but the majority do not. Those that do contain the E484K mutation will have a mutation pattern similar to the Beta variant. In such cases, the PrimePCR SARS-CoV-2 Single Mutation Assay targeting the D80A or A701V assay for Beta or the HV69-70 deletion assay for Alpha can be used by researchers to further distinguish the viral strain present in the sample.

** In this panel, it is not possible to distinguish the Delta variant from the Epsilon variant. To differentiate between these strains, the singleplex T478K assay can be used by researchers.

*** Omicron has the N501Y mutation but it may not be detected due to other mutations in the region.

Additional PrimePCR SARS-CoV-2 Multiple Mutation Assays will become available as the virus undergoes additional mutations and a need to survey for novel mutations arises. Visit bio-rad.com/SC2Variants for a complete list of assays available and evaluation of results.

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
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**
1855195	**CFX96 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 Multiple Mutation Assays	

Refer to bio-rad.com/SC2Variants for assays and ordering information.

Visit bio-rad.com/SC2Variants for a complete list of PrimePCR SARS-CoV-2 Mutation Assays and ordering information.

Visit bio-rad.com/PCRPlastics for a full range of PCR plastics and seals.

Visit bio-rad.com/RelianceOneStep for more information.

Visit ExactDiagnostics.com for more information about these controls. 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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