

AzureSeq One-Step Universal RT-qPCR Kit

SARS-CoV-2 E484K N501Y K417 Variant Assay

Instructions for Use

Intended Use:

The AzureSeq One-Step Universal RT-qPCR Kit SARS-CoV-2 E484K N501Y K417 Variant Assay is an RT-qPCR test intended for the qualitative detection of nucleic acid from the 2019-nCoV in nasopharyngeal (NP) and oropharyngeal (OP) swabs from individuals with signs and symptoms of infection who are suspected of COVID-19. Testing is limited to laboratories - certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. §263a, to perform high complexity tests, or by similarly qualified non-U.S. laboratories.

Results are for the identification of 2019-nCoV RNA. The 2019-nCoV RNA is generally detectable in nasopharyngeal and oropharyngeal swabs during the acute phase of infection. Positive results are indicative of active infection. Laboratories within the United States and its territories are required to report all positive results to the appropriate public health authorities.

Negative results **do not** preclude 2019-nCoV infection.

The AzureSeq One-Step Universal RT-qPCR Kit SARS-CoV-2 E484K N501Y K417 Variant Assay is intended for use by qualified, trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and in vitro diagnostic procedures.

The AzureSeq One-Step Universal RT-qPCR Kit SARS-CoV-2 E484K N501Y K417 Variant Assay is for Research Use Only and requires an LDT for use by CLIA laboratories, or similar for qualified non-U.S. laboratories.

Test Principle:

Extracted viral RNA is reverse transcribed into cDNA by combining the nucleic acid with the AzureSeq One-Step Universal RT-qPCR Kit SARS-CoV-2 Variant Assay master mix. In the process, the probe anneals to a specific target sequence located between the forward and reverse primers. During the extension phase of the PCR cycle, the 5' nuclease activity of Taq polymerase degrades the probe, causing the reporter dye to separate from the quencher dye, generating a fluorescent signal. With each cycle, additional reporter dye molecules are cleaved from their respective probes, increasing the fluorescence intensity. Fluorescence intensity is monitored at each PCR cycle. This test assesses the variant polymorphisms for SARS-CoV-2 E484K (G23012A), N501Y (A23063T), and for wild type K417. RNaseP is used as a positive control for sample integrity.

Note 1: This assay does not identify variants of K417 (for example K417N, K417T). A sample previously tested positive for SARS-Cov-2 that tests negative for K417 in this assay may indicate a variant of K417.

Note 2: This assay will likely require customization for use on a Roche LightCycler. Please contact SeqOnce.



Materials Provide and Storage:

Concentration: 2X

Storage and Handling: Store at -20°C upon arrival

Ordering Information:

Item Number	Total Volume Received	Quantity Received
ASD-ENK-Variant-200	SB-ITMP-MM-100-PC SB-DRT-200-PC SB-CPPM-ENK-100-PC SB-NFW-350uL-PC SB-CVNC-150-PC SB-CVPC-150-PC	SB-ITMP-MM-100-PC: 2 x 1mL SB-DRT-200-PC: 1 x 250µL SB-CPPM-ENK-100-PC: 2 x 100µL SB-NFW-350uL-PC: 2 x 350µL SB-CVNC-150-PC: 1 x 150µL SB-CVPC-150-PC: 1 x 150µL

Workflow:

Protocol for One-Step SARS-nCoV-2 E484K N501Y K417 Variant Detection: The following reaction set up cycling conditions are recommended but can vary depending on the template and primers being used. Reaction volumes are to be determined by the end user. The recommended protocol for 20µL reaction is listed on the following pages.

Detection:

- Dyes in the Multiplex Primers/Probe: E484K variant (HEX), N501Y variant (Cy5), K417 Wildtype (FAM), RnaseP (Texas Red)
 - Texas Red can be replaced by ROX if not present on the program.
 - HEX can be replaced by VIC or JOE if not present on the program.
 - Cy5 can be replaced by MUSTANG PURPLE if not present on the program.
- Select all four dyes for each labeled well

Instructions for setting up 20µL reactions Using Previously Isolated RNA:

RT-qPCR Master Mix and Reagent Setup

1. Completely thaw the 2x InhibiTaq MM and CoVi Primer/Probe Mix (brown tube/cap) by setting on ice for ~30 minutes. Reagents can be thawed at room temperature for ~5 minutes. Once thawed, briefly centrifuge to collect at the bottom of the tube.
2. Vortex the tube at max speed for 10 seconds to mix, then spin down briefly to collect at bottom of tube.
3. Proceed to combine in a 1.5mL Eppendorf tube, in the order shown below, in a clean room or designated setup area:

Component	Volume 1 reaction (µl)	Volume 100 reactions (96-well plate (µl))
Sterile Water	2.75	275
CoVi Primer/Probe Mix: K417N E484K N501Y Variants	1	100
2X InhibiTaq Multiplex qPCR MM	10	1000
Direct RTScript™, 200U/µL	1.25	125

4. Mix the master mix by pipetting up and down repeatedly with pipette set to volume of 2X master mix added, or by capping the tube, vortexing briefly, and spinning down briefly to collect mix.
5. Distribute **15 µl** of the master mix using an appropriate pipette to all wells of a plate that will be used.
6. Add **5 µl** of sample, positive control, or negative control to appropriate wells.
7. Seal the plate, vortex briefly or flick to mix; spin down in a centrifuge to collect the mixed samples.
8. Place the plate into the designated real-time machine and run the following program:

Thermal cycling conditions:

Recommended Cycling Conditions

Cycling Step	Stage	No. of Cycles	Temperature	Holding Time
RT Incubation	1	1	50°C	15 minutes
Enzyme Activation	2	1	95°C	2 minutes
Amplification**	3	40-45	95°C	3 seconds
			60°C**	30 seconds

**Collect fluorescence during annealing/extension phase (60°C) step on FAM, HEX, CY5, and ROX channels.

Rapid Cycling Conditions- Under Review

Cycling Step	Stage	No. of Cycles	Temperature	Holding Time
RT Incubation	1	1	50°C	5 minutes
Enzyme Activation	2	1	95°C	1 minute
Amplification**	3	40 (Minimum)	95°C	1 second
			60°C**	1 second

**Collect fluorescence during annealing/extension phase (60°C) step on FAM, HEX, CY5, and ROX channels.

Interpretation of Results:

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted. Ct values <40 are interpreted as positive by the user for the BioRad CFX96 and QuantStudio 5 and 7 Pro.

SARS-CoV-2 K417 Wildtype (FAM)	SARS-CoV-2 E484K Variant (HEX)	SARS-CoV-2 N501Y Variant (Cy5)	RNaseP (Texas Red)	Result Interpretation	Report	Actions
Any Positive			+/-	2019-nCov detected	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
+	-	-	+/-	2019-nCov detected Presumed K417 Wildtype. E484K, N501Y Variants not detected.	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
+	+	-	+/-	2019-nCov detected Presumed E484K Variant	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
+	-	+	+/-	2019-nCov detected Presumed N501Y Variant	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
-	+	-	+/-	2019-nCov detected Presumed E484K Variant Potential K417 Variant	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
-	-	+	+/-	2019-nCov detected Presumed N501Y Variant Potential K417 Variant	Positive 2019-nCov	Report results to the healthcare provider and appropriate public health agencies
-	-	-	-	Invalid Result	Invalid	Report results to healthcare provider. Check sample quality
-	-	-	+	2019-nCov not detected OR Potential K417 Variant	Inconclusive	Report results to the healthcare provider and appropriate public health agencies

Warning: This assay is designed and warranted (only) for a **20 µl** reaction volume.

Reducing reaction volume may decrease sensitivity, reduce specificity, and/or impact detection.

Included Controls:

	SARS-CoV-2 N501Y Variant (Cy5)	SARS-CoV-2 E484K Variant (HEX)	SARS-CoV-2 K417 Wild Type (FAM)	RNAse P (Texas Red)
Mutant gBlock DNA control	+	+	-	-
WT gBlock Positive Control	-	-	+	-

QuantStudio 5 Instrument Setup

1. In the QuantStudio™ Design & Analysis Software v1.5.1 home screen, select **Create New Experiment -> Template**.
2. Browse to, then open the AzureSeq template file.
3. In the Properties tab, enter or confirm the following:
 - Experiment Name: **Enter a unique name (ex: Date of Run - Batch #)**
 - Instrument Type: (select instrument in use)
 - Block: (select block in use)
 - Experiment Type: **Standard Curve**
 - Chemistry: **Other**
 - Properties: **Standard**
4. In the toolbar, select File, then Import Plate Setup, and navigate to the .txt file that was saved from the Sample Setup sheet.
5. In the Method tab, confirm that the **Reaction Volume Per Well** is 20µL, then confirm the thermocycling protocol.

Step	Temperature	Time	Number of Cycles
RT Incubation	50°C	15 minutes	1
Enzyme Activation	95°C	2 minutes	1
Amplification	95°C	3 seconds	40-45
	60°C**	30 seconds	

**collect fluorescence during annealing/extension phase (60°C) step on all four channels.

6. In the Plate -> Advanced Setup, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter Dye	Quencher
K417 Wildtype	FAM	NFQ-MGB
E484K Variant	VIC	NFQ-MGB

RNaseP	ROX	NFQ-MGB
N501Y Variant	Cy5	NFQ-MGB

- In the Plate -> Quick Setup, confirm the Passive Reference is set to **None**.
- In the Plate -> Advanced Setup, in the Samples window, review the imported samples to ensure correct sample import and all three targets are assigned to all applicable wells.

QuantStudio 7 Instrument Setup

- In the QuantStudio™ Real-Time PCR Software v1.3 home screen, select Template.
- Browse to, then open the AzureSeq Direct template file.
- In the Experiment Properties tab, enter or confirm the following:
 - Experiment Name: **Enter a unique name (ex: Date of Run - Batch #)**
 - Instrument Type: (select instrument)
 - Block: (select block size)
 - Type of Experiment: **Standard Curve**
 - Reagents: **Other**
 - Properties: **Standard**
- In the toolbar, select File, then Import Plate Setup, and navigate to the .txt file that was saved from the Sample Setup sheet.
- In the Define tab for Targets, confirm that the targets, reporter dyes, and quenchers are listed correctly.

Target	Reporter Dye	Quencher
K417 Wildtype	FAM	NFQ-MGB
E484K Variant	VIC	NFQ-MGB
RNaseP	ROX	NFQ-MGB
N501Y Variant	Cy5	NFQ-MGB

- In the Define tab, confirm the Passive Reference is set to **None**.
- In the Assign tab, in the Samples window, review the imported samples to ensure correct sample import and all three targets are assigned to all applicable wells.
- In the Run Method tab, confirm that the **Reaction Volume Per Well** is 20µL, then confirm the thermocycling protocol.

Step	Temperature	Time	Number of Cycles
RT Incubation	50°C	15 minutes	1
Enzyme Activation	95°C	2 minutes	1
Amplification	95°C	3 seconds	40-45
	60°C**	30 seconds	

**collect fluorescence during annealing/extension phase (60°C) step on all four channels.

CFX Instrument Setup

- In the Bio-Rad CFX Maestro home screen, select the **Run Setup** tab. Enter or confirm instrument as the instrument to be used. Select run type as **User-defined**.
- In the Protocol tab, click **Select Existing...** and navigate to the AzureSeq protocol template.
 - For reference, see thermocycling protocol below (20µL reaction volume).

Step	Temperature	Time	Number of Cycles
RT Incubation	50°C	15 minutes	1
Enzyme Activation	95°C	2 minutes	1
Amplification	95°C	3 seconds	40-45
	60°C**	30 seconds	

**collect fluorescence during annealing/extension phase (60°C) step on all four channels.

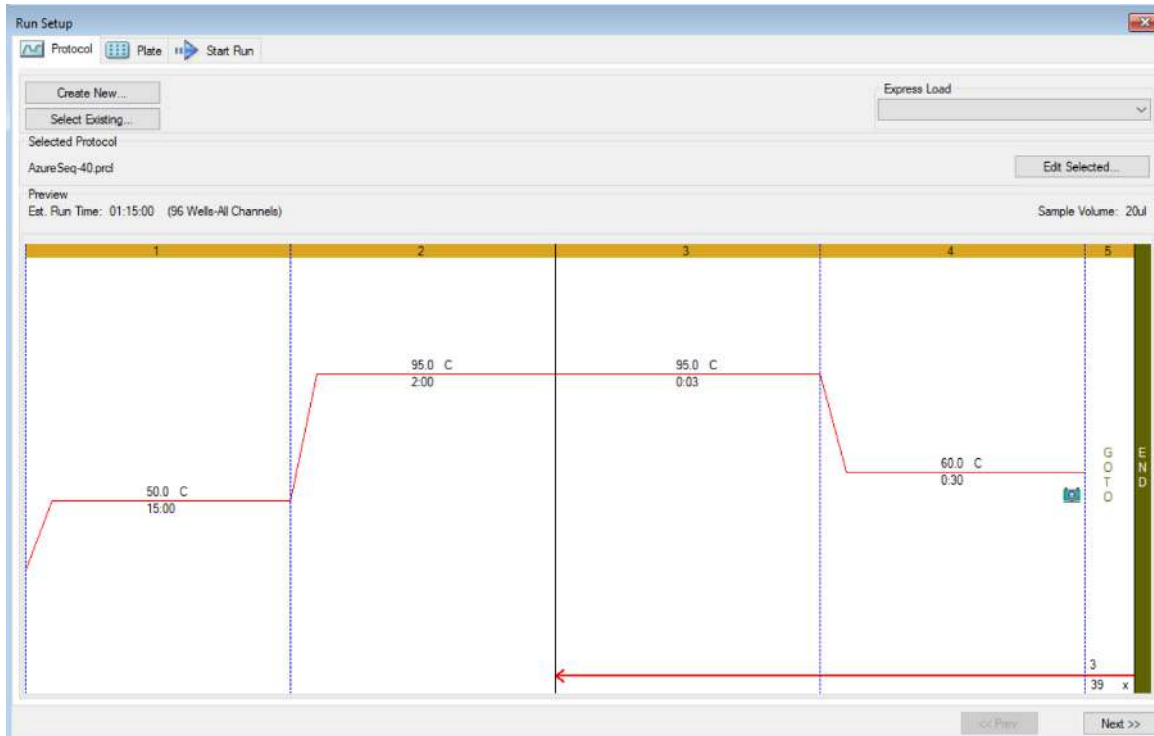


Figure 1: Run setup – protocol

- In the Plate tab, click **Select Existing...** and navigate to the AzureSeq plate template.
- In the toolbar, select **Spreadsheet View/Importer**, then **Import**, and navigate to the .txt file that was saved from the Sample Setup sheet.
- In the **Define** tab for Targets, confirm that the targets and reporter dyes are listed correctly.
 - Confirm all applicable wells' Sample Type as **Unknown** (except for each control), all with the following targets and dyes.

Target	Reporter Dye
K417 Wildtype	FAM
E484K Variant	HEX

RNaseP	Texas Red
N501Y Variant	Cy5

6. Verify plate layout has samples/wells designated for control samples. Positive control, negative control, and no template control (PCR blank) should be present for each run.

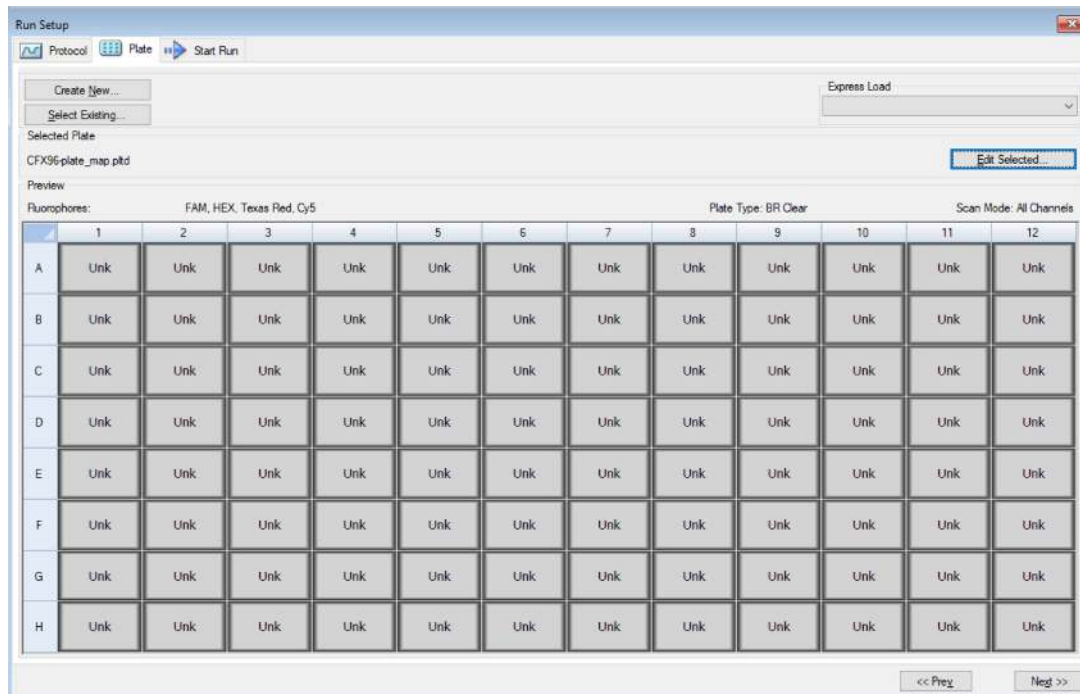


Figure 2: Run setup - plate



Figure 3: Detailed plate setup



Analysis Using QuantStudio Design and Analysis Software

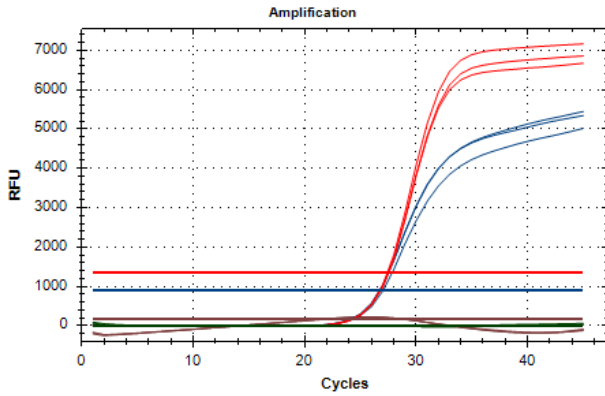
1. Using a USB drive or other method, transfer the EDS file from the computer connected to the instrument to the computer that will be performing the analysis.
2. Open the QuantStudio Design & Analysis Software 2.5.0 and open the EDS for the completed run.
3. In the **Quality Check** tab, select **Analyze** to perform initial analysis of the run.
4. Review each target's threshold by selecting the target on the dropdown box under **Search Targets**. Ensure each target's threshold is above the background while intersecting the reaction curves towards the beginning of the exponential phase.
 - a. To adjust, select Actions->Primary Analysis Setting... and for each target that needs to have an adjusted threshold, deselect the checkmarks for that target for Use Default and Auto Threshold and under Threshold, input value for the adjusted threshold.
5. Review positive/negative control samples to ensure their validity before continuing with the run analysis. If controls are not valid, the patient results cannot be interpreted.

Analysis Using BioRad Maestro Software: Interpretation of Results

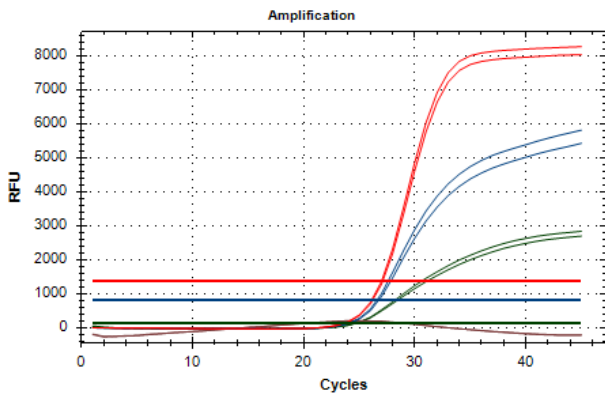
6. In the CFX Maestro software after the run is complete, select **File** and **Save As** and select a location to save the run's .pcrd file.
7. Using a USB drive or other method, open the run data file from the computer connected to the instrument to the computer that will be performing the analysis.
8. Open the CFX Maestro software and open the run data file for the completed run.
9. On the toolbar, select **Settings->Baseline Setting->Apply Fluorescence Drift Correction**.
10. Review each target's threshold by selecting the target with the checkboxes between the amplification plot and the plate layout. Ensure each target's threshold is above the background while intersecting the reaction curves towards the beginning of the exponential phase.
 - a. To modify, select the baseline and adjust as necessary.
11. Review positive/negative/no template control samples to ensure their validity before continuing with the run analysis. If controls are not valid, the patient results cannot be interpreted.

Analysis Using BioRad Maestro Software: Sample Data

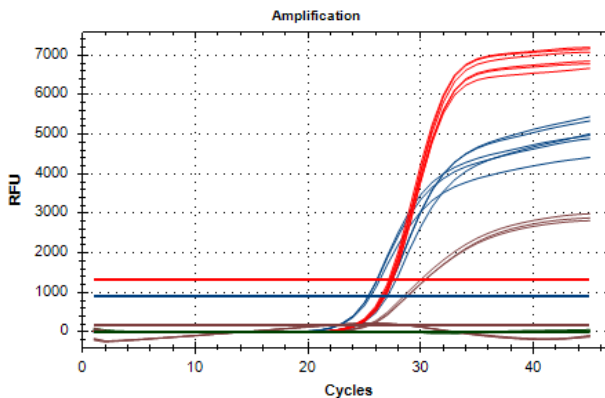
Review the Texas Red (RNaseP) fluorophores to confirm sample validity with the RNaseP IPC (internal PCR control) result. Review the WT positive control: K417 should have signal, N501Y and E484K should have no signal.



WT K417, WT N501, E484K variant result. FAM (K417) and HEX (E484K) shows amplification while Quasar670 (Cy5 N501Y variant) shows no amplification.



WT K417, N501Y variant result. HEX (E484K) shows no amplification while FAM (K417) and Quasar670 (Cy5 N501Y variant) shows amplification.





Risk and Safety Information:

Safety Data Sheets are available online at www.seqonce.com

The user should carefully read all warnings, instructions or Safety Data Sheets provided by the supplier for any additional materials or chemicals required for the use of the AzureSeq One-Step Universal RT-qPCR Kit SARS-CoV-2. The user should also follow general safety precautions when handling biohazards, chemicals and other materials.

General Precautions:

1. Treat all samples, materials and instrumentation as potentially infectious.
2. Avoid potential contamination by employing good laboratory practices, wearing proper personal protective equipment, and decontaminating workspaces before and after use.
3. All instruments must be maintained and operated according to manufacturer's instructions.
4. Dispose of waste according to state and local regulations.
5. Only use DNase and RNase free consumables.
6. Do not use reagents beyond their expiration.

Manufacturing Information:









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Support:

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Explanation of Symbols:

Symbol	Definition
	<i>In vitro</i> diagnostic medical device

	Lot Number
	Manufacturer
	Date of Manufacture
	Storage & Transport Conditions
	Consult instructions for use
	Use by Date