CoVi K417/E484K/N501Y Primer/Probe Mix

INSTRUCTIONS FOR USE

For Research Use Only

Protocol Version V1.0
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Document History – Important notes and updates

Protocol Version	Document Version	Date	Author	Summary of changes	Authorized by
1	v1	23/03/2021	Noémi Petrovicz	First version	Elmar Schilling

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1. Principle of the method

The test is a real-time reverse transcription polymerase chain reaction (rRT-PCR) test which is designed to detect RNA from regions of the SARS-CoV-2 S gene in nasopharyngeal (NP) and oropharyngeal (OP) swabs from patients with signs and symptoms of infection who are suspected of COVID-19. The kit primer and probe set also detects human RNase P (RP) in a clinical sample as an internal control.

Nucleic acids are isolated and purified from NP and OP swabs using well established nucleic acid extraction systems. Sample input and elution volumes are system dependent.

The purified nucleic acid is reverse transcribed into cDNA by combining nucleic acid with reagents of the AzureSeq qPCR Kit SARS-CoV-2 for 200 Reactions (CE/RUO) (further referred as AzureSeq – 200 CE/RUO) master mix containing the CoVi K417/E484K/N501Y Primer/Probe Mix which is then subsequently amplified in the real time PCR instrument. 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 by the real time PCR instrument. This test assesses polymorphisms for SARS-CoV-2 E484K (G23012A), N501Y (A23063T), and for wild type K417. RNase P is used as a positive control for sample integrity.

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2. Kit content (for 100 RXN)

Product Name	Number of tubes	Volume (µL)
20X K417 E484K N501Y Genotyping Mix	**1**	**100**
Mutant gBlock DNA Control	**1**	**150**
WT gBlock DNA Control	**1**	**150**

3. Shipping and Storage

The AzureSeq–200 CE/RUO kit, Controls and 20X K417 E484K N501Y Genotyping Mix are shipped on dry ice and should arrive frozen. Please contact azureseq.support@omixon.com if any components are not frozen upon receipt or are compromised during shipment.
To prevent the reagents from degradation, all components should be stored immediately at - 20°C.
It is recommended to have a back-up generator for your freezer as well as a temperature data log to ensure that the AzureSeq–200 CE/RUO kit components, Controls and 20X K417 E484K N501Y Genotyping Mix remain frozen at -20°C if you work in an area prone to power outages.
Expiry date is 12 months after date of manufacture. Do not use it after the expiration date.
Do not use the kit if it is defective.
Repeated freeze/thaw cycles must be avoided because it can cause reagent degradation that leads to decrease in sensitivity.
Dispose of unused reagents and waste according to country regulations.

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4. Specimen collection, transfer and storage

Inadequate or inappropriate specimen collection, transfer and storage can increase the probability of false negative results.

4.1 Collection

Refer to CDC's webpage for Interim Guidelines for Collecting, Handling and Testing Clinical Specimens from Patients Under Investigation (OUIs) for 2019 Novel Coronavirus (2019-nCoV).
https://www.cdc.gov/coronavirus/2019-nCoV/guidelines-clinical-specimens.html

Use only synthetic fiber swabs with plastic shafts. Do not use calcium alginate swabs or swabs with wooden shafts, as they may contain substances that inactivate some viruses and inhibit PCR testing.

Follow specimen collection devices manufacturer instructions for proper collection methods.

Oropharyngeal swab

Use a sterile swab to wipe the posterior pharynx, avoiding the tongue. Place swabs immediately into labeled sterile tubes containing viral transport medium. Break each applicator sticks off at the score line (flocked swabs) or near the tip or cut with sterile scissors to permit tightening of the cap. Ship sample immediately on cold packs.

Nasopharyngeal swab

Insert a sterile swab into nostril parallel to the palate. Swab should reach depth equal to distance from nostrils to outer opening of the ear. Leave swab in place for several seconds to absorb secretions. Slowly remove swab while rotating it. Place swabs immediately into labeled sterile tubes containing viral transport medium. Break each applicator sticks off at the score line (flocked swabs) or near the tip or cut with sterile scissors to permit tightening of the cap. Ship sample immediately on cold packs.

4.2 Transport

All specimen must be transported with ice cool / ice-gel box / dry ice and securely sealed and handled.
Transport of clinical samples must obey local regulations. The biosafety local regulations for SARS-CoV-2 must be followed.

4.3 Handling

During handling potentially infectious specimens, laboratory workers should wear appropriate personal protective equipment (PPE), which includes disposable gloves, laboratory coat/gown, and eye protection. For specific instructions on the handling of clinical specimens for coronavirus disease 2019, see also the CDC's webpage mentioned above.

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4.4 Storing

Specimens can be stored at 2-8°C for up to 48 hours after collection. For storage longer than 2 days, specimens should be frozen at -70°C.

If the sample is stored for more than 48 hours, extraction of the RNA using validated RNA isolation system is required.

If the sample is not frozen and stored for less than 48 hours, direct approach of workflow can be used.

Repeated freezing and thawing of a specimen should be avoided. If a specimen is kept for retesting, it should be aliquoted in different tubes to avoid freeze/thaw cycles.

Depending on the type of sample and the transport medium used, specific storage and, for RNA isolation, pre-treatment of the sample may be required. Please observe the instructions provided by the manufacturer.

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5. Materials and Equipment needed (not provided)

5.1. Equipment

- Real-time PCR thermal cycler able to detect FAM, HEX, CY5 and Texas Red (or equivalent) channels
- Thermoblock
- 100 µL and 1000 µL micropipettes
- 10 µL and 100 µL multichannel pipette
- Vortex mixer
- Centrifuge

5.2. Reagents

- Viral RNA/total RNA extraction kit

5.3. Materials

- Optical 96-well plates or 0.2 ml optical tubes
- Optical seal compatible with the qPCR instrument
- Disposable DNase/RNase free pipette tips with filters (10 µL, 20 µL, and 200 µL)
- DNase/RNase free 1.5 ml tubes
- Disposable powder-free gloves
- Surface decontaminant products such as "RNAse away"
- Material necessary for nucleic acid extraction

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6. Materials needed (provided separately)

6.1 For using AzureSeq – 200 CE

Product Code	Product Name	Number of tubes	Volume (μL)
OA-ITMP-MM-100	**2X InhibiTaq Multiplex HotStart MasterMix**	**2**	**1000**
OA-RT-200	**RTScript Reverse Transcriptase, 200U/uL**	**1**	**100**
OA-NFW-350uL	Nuclease Free Water	2	350
OA-CPPM-100uL	CoVi Primer/Probe Mix 3	2	100

6.2 For using AzureSeq – 200 RUO

Product Code	Product Name	Number of tubes	Volume (μL)
OA-ITMP-MM-100	**2X InhibiTaq Multiplex HotStart MasterMix**	**2**	**1000**
OA-RT-200	**RTScript Reverse Transcriptase, 200U/uL**	**1**	**100**
OA-NFW-350uL	**Nuclease Free Water**	**2**	**350**
OA-CPPM-100uL	CoVi Primer/Probe Mix 3	2	100
OA-CVNC-150	CoVi Negative Control	1	150
OA-CVPC-150	CoVi Positive Control	1	150

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7. Warning and Precaution

Good laboratory practices are essential to the proper performance of this assay. Due to the high sensitivity of the test, care should be taken while handling samples and materials while performing the assay to keep reagents and amplification mixtures free of contamination.

Users should pay attention to the following:

- Read the Instructions for Use carefully before processing samples. Any deviation from procedures written here may affect optimal performance.
- Use disinfectant to clean and disinfect the area around the sample
- Decontaminate and dispose of all specimens, reagents, and other potentially contaminated materials in accordance with local regulations
- Use universal precautions when performing the assay. Handle samples as if capable of transmitting infection.
- Wear personal protective equipment throughout the assay procedure.
- Thoroughly wash hands after removing gloves, and dispose of gloves as hazardous wastes
- Do not reconstitute or dilute the reagents in volumes other than described in this IFU. Do not use less volume of the reagents other than specified in this IFU. These activities can lead to performance errors.
- Omixon cannot provide support for any problems resulting from not following the protocol steps described in this IFU.
- Do not use the product in case of detectable damage to the components (broken vials, plate, loose caps etc.).
- Do not use reagents past their expiration date.
- Do not substitute or mix the AzureSeq–200 CE/RUO kit reagents with reagents from other manufacturers.
- All instruments must be calibrated, maintained and operated according to manufacturer's instructions.
- Every workplace must be equipped with its own set of variable-volume pipettes, necessary auxiliary materials, and equipment.
- Do not pool reagents from different lots or from different vials of the same lot.
- Do not smoke, drink, eat or apply cosmetics in areas in which specimens or Kit components are handled.

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8. Procedure

The AzureSeq–200 CE/RUO kit contains a control assay targeting the RNaseP. This is an internal control needed to confirm the presence of nucleic acid in every sample run with the AzureSeq–200 CE/RUO test kit and is used to generally confirm functionality of the test kit components.

NOTE: CoVi Primer/Probe Mix 3 (OA-CPPM-100ul) provided in AzureSeq – 200 CE/RUO kit and CoVi Negative Control (OA-CVNC-150) and CoVi Positive Control (OA-CVPC-150) provided in AzureSeq – 200 RUO must not be used for the reaction.

8.1. Instructions for setting up the reactions

1. Completely thaw the 2x InhibiTaq Multiplex qPCR Master Mix and the 20X K417 E484K N501Y Genotyping Mix by setting it on ice for ~30 minutes. Reagent 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 the tube.
3. Proceed to master mix setup as shown below in a clean room or designated setup area.
   NOTE: The assay is designed and warranted only for a 20µL reaction volume. Reducing reaction volume may decrease sensitivity, reduce specificity and/or impact detection.

Component	Volume/ reaction (µL)	Volume/100 reactions (µL)
Nuclease Free Water	2.75	275
20X K417 E484K N501Y Genotyping Mix	1	100
2x InhibiTaq Multiplex qPCR MasterMix	10	1000
RTScript Reverse Transcriptase, 200U/µL	0.5	50

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 the isolated RNA samples, 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.

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8. Place the plate into the designated real-time machine and run the following program.

Recommended cycling conditions:

Cycling step	Stage	No. of cycles	Temperature (°C)	Time
RT incubation	**1**	**1**	**50**	**15 minutes**
Enzyme activation	**2**	**1**	**95**	**2 minutes**
Amplification	**3**	**45**	**95**	**3 seconds**
			60**	**30 seconds**

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

- Texas Red can be replaced by ROX if not present on the program.

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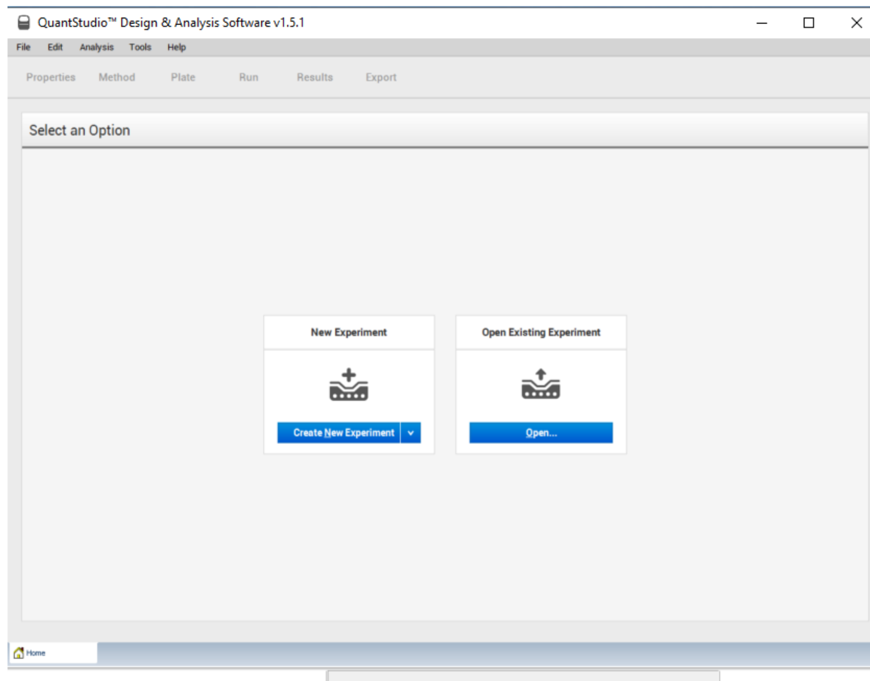
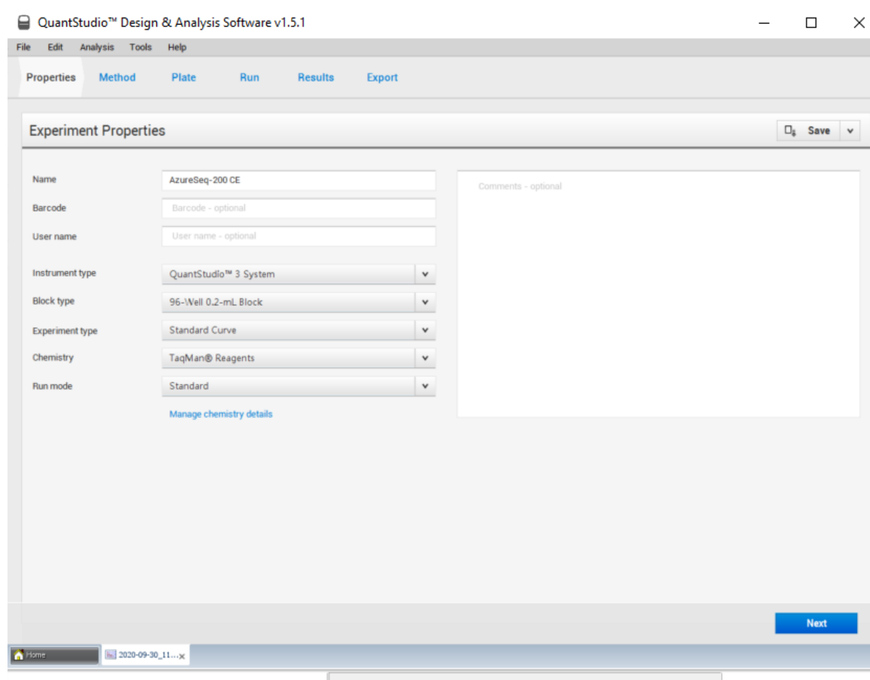
8.2 qPCR setup

8.2.1 QuantStudio 3, 5, 7 Pro

- Create a new experiment and then setup the Properties (Instrument Type, Block Type, Experiment Type, Chemistry, Run mode) according to the picture below.





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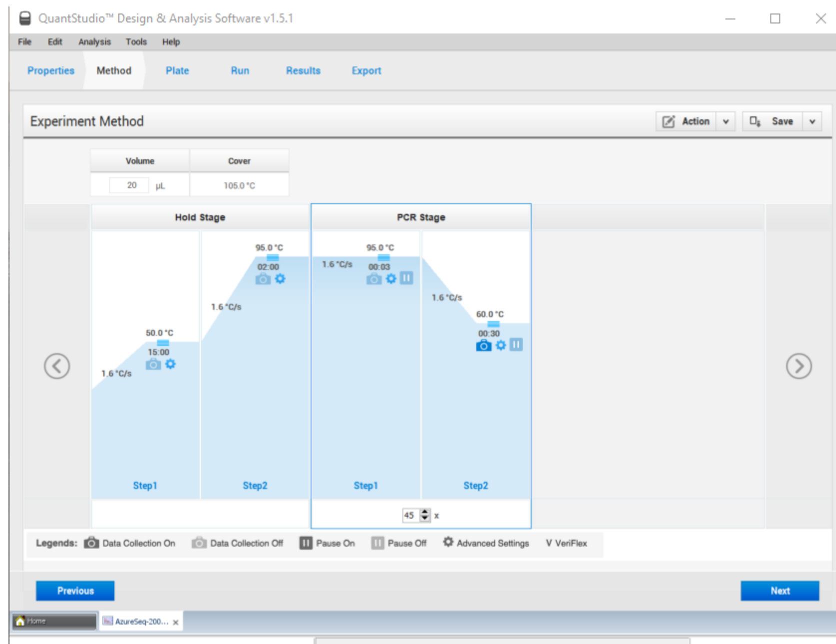
- Set the method according to the PCR program detailed above.



- Set the targets (E484K variant - HEX, N501Y variant - CY5, K417 wild type - FAM, Rnase P - Texas Red) and the samples on the Plate tab and assign the channels.
- Export the protocol to an USB drive.
- Load the Experiment from the USB drive on the qPCR instrument, insert the plate and start the Run.

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8.2.2 BioRAD CFX96 Touch

Please refer to the CFX96™ Touch Instruction Manual for additional information on using the instrument.

- Run the CFX Manager software on the computer connected to the CFX96.
- Create a New Protocol by selecting th 'User-defined' run type.
- Click 'Edit Selected' on 'Protocol' tab to make changes to the protocol. Set the parameters of the PCR program detailed in Section 8.1.
- Click 'Edit Selected' on 'Plate' tab to setup the Plate.
- Click on 'Select Fluorophores', check the cyberbox of the fluorophores (FAM, HEX, CY5, Texas Red – or equivalent).
- Specify the positive control well, set the sampe type to "Positive Control", and set the detection fluorescences (E484K variant - HEX, N501Y variant - CY5, K417 wild type - FAM, Rnase P - Texas Red).
- Specify the negative control well, set the sampe type to "Negative Control", and set the detection fluorescences (E484K variant - HEX, N501Y variant - CY5, K417 wild type - FAM, Rnase P - Texas Red).
- Wells with clinical specimens should be specified as Unknown, set the detection fluorescences (E484K variant - HEX, N501Y variant - CY5, K417 wild type - FAM, Rnase P - Texas Red).
- In the Setting menu set the plate type to BR white.
- Go to Start Run, select Block Name (PCR instrument) to use, close Lid and start the run.

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9. Result interpretation

All test controls should be examined prior to interpretation of patient results. If the controls are not valid, the patient results cannot be interpreted. Collect fluorescence during annealing/extension phase (60°C) step on HEX (E484K variant marker), CY5 (N501Y variant marker), FAM (K417 wild type marker) and Texas Red (RNaseP marker) channels (or equivalent channels). Ct values <40 are interpreted as positive by the user for the BioRad CFX96 and QuantStudio 3, 5, and 7 Pro. Use the tables below as a general guide for result interpretation.

SARS-CoV-2 K417 (FAM)	SARS-CoV-2 E484K variant (HEX)	SARS-CoV-2 N501Y variant (CY5)	RNase P (Texas Red)	Result Interpretation	Report	Action
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.

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Expected results for positive controls:

	SARS-CoV-2 K417 (FAM)	SARS-CoV-2 E484K variant (HEX)	SARS- CoV-2 N501Y variant (CY5)	RNase P (Texas Red)
Mutant gBlock DNA Control	-	+	+	-
WT gBlock DNA Control	+	-	-	-

RNase P (Extraction Control)

All clinical samples should exhibit fluorescence growth curves in the RNase P reaction that cross the threshold line within 40.00 cycles (< 40.00 Ct), thus indicating the presence of the human RNase P gene. Failure to detect RNase P in any clinical specimens may indicate:

- Improper extraction of nucleic acid/improper heat extraction from clinical materials resulting in loss of RNA and/or RNA degradation.
- Absence of sufficient human cellular material due to poor collection or loss of specimen integrity.
- Improper assay set up and execution.
- Reagent or equipment malfunction.

If the RNase P assay does not produce a positive result for human clinical specimens, interpret as follows:

- If the 2019-nCoV markers are positive even in the absence of a positive RNase P, the result should be considered valid. It is possible that some samples may fail to exhibit RNase P growth curves due to low cell numbers in the original clinical sample. A negative RNase P signal does not preclude the presence of 2019-nCoV virus RNA in a clinical specimen.
- If all 2019-nCoV markers AND RNase P are negative for the specimen, the result should be considered invalid for the specimen. If residual specimen is available, repeat the extraction procedure and repeat the test. If all markers remain negative after re-test, report the results as invalid and a new specimen should be collected if possible.

NOTE: Negative results do not preclude 2019-nCoV infection.

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10. Procedure Limitation

- The intended users must be trained with this technology prior to the use of this device.
- It is the intended user's responsibility to validate system performance for any procedures used in their laboratory which are not covered by Omixon Biocomputing Ltd.'s performance studies.
- Use this product only with following human biological samples: nasopharyngeal (NP) and oropharyngeal (OP) swabs
- The negative result does not exclude the possibility of infection, because results are dependent on appropriate specimen collection and absence of inhibitors. The presence of PCR inhibitors may cause invalid or inconclusive results which requires repetition.
- False Positive results may occur due to the several reasons, most of which relate to RNA contamination during specimen handling and preparation.

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11. Troubleshooting

<table>
<tr><th>Problems</th><th>Possible root cause</th><th>Recommendation</th></tr>
<tr><td>Fluorescent intensity is weak or does not appear in the positive control</td><td>Probe degradation</td><td>Use a new probe aliquot or repeat test with a new kit LOT</td></tr>
<tr><td>High inconsistency in the fluorescence signals in samples</td><td>Inaccurate pipetting</td><td>Use calibrated pipettes, make sure that an equal volume of reagents is added to each wells/tube</td></tr>
<tr><td rowspan="3">Fluorescent signal is detected in the negative control reaction</td><td>Carry over contamination</td><td>Always change tips between samples. Take care when dispensing samples, negative controls, and positive controls</td></tr>
<tr><td>Contamination of the amplification master mix</td><td>Use a new aliquot of amplification mix</td></tr>
<tr><td>Contamination of the extraction/preparation area</td><td>Use disinfectant to clean and disinfect the areas</td></tr>
<tr><td rowspan="3">No fluorescent signal is detected in all samples, including positive control</td><td>Probe degradation</td><td>Use new probe aliquot</td></tr>
<tr><td>Thermal cycler setting error</td><td>Verify the correct program setting of the real time PCR instrument</td></tr>
<tr><td>Wrongly prepared master mix, possible omitted component</td><td>Verify each component and repeat the PCR master mix preparation</td></tr>
<tr><td rowspan="4">False negative results</td><td>Presence of RT-PCR inhibitors</td><td>Validate the RNA extraction kit prior to use.</td></tr>
<tr><td>Improper sample collection</td><td>Follow validated sample collection method</td></tr>
<tr><td>Failure to follow instruction for use</td><td>Read the Instructions for Use carefully before processing samples. Any deviation from procedures written here may affect optimal performance.</td></tr>
<tr><td>Using unauthorized reagents</td><td>Do not substitute or mix the AzureSeq–200 CE/RUO kit reagents and 20X K417 E484K N501Y Genotyping Mix with reagents from other manufacturers.</td></tr>
<tr><td rowspan="2">False positive reaction</td><td>Cross contamination between samples</td><td rowspan="2">Take extra care when handle patient specimens, use straightforward and validated traceability processes in the laboratory</td></tr>
<tr><td>Sample mix-up</td></tr>
</table>

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12. Quality Control

Validating the whole assay procedure and amplification session by processing a negative tested sample and a positive tested sample or a calibrated reference material is recommended.

13. Contact information

For general assistance with this protocol contact:

Email Support:
azureseq.support@omixon.com

Telephone Support:
+36-70-672-7551

Manufacturer information:
Company name: SeqOnce BioSciences Inc
Visiting address: 2265 E Foothill Blvd, CA
City: Pasadena
Post code: 91107
Country: USA

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