VIASURE

Real Time PCR Detection Kit

bestbiondx

smarter diagnostics

SARS-CoV-2 Variant II

CE IVD

These instructions for use apply to the following references / *Estas instrucciones de uso aplican para las siguientes referencias:*

OPEN FORMAT (SEE ANNEX 1) / *OPEN FORMAT (VER ANEXO 1)*

PRODUCT / PRODUCTO	REFERENCE / REFERENCIAS
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 6 x 8-well strips, low profile	03B51397
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 6 x 8-well strips, high profile	03B51398
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 12 x 8-well strips, low profile	03B51399
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 12 x 8-well strips, high profile	03B51400
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 96-well plate, low profile	03B51401
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 96-well plate, high profile	03B51402
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 1 x 8-well strips, low profile	Muster
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 1 x 8-well strips, high profile	Muster

Table A 1. References for Open format products. / *Referencias para productos Open Format.*

TUBE FORMAT (SEE ANNEX 2) / *FORMATO TUBO (VER ANEXO 2)*

PRODUCT / PRODUCTO	REFERENCE / REFERENCIAS
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit, 4 tubes x 24 reactions	03B51403

Table A 2. References for Tube format control products. / *Referencias para productos formato Tubo.*

Content

ENGLISH

1. Intended use

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit is a real-time RT-PCR test designed for the qualitative detection of RNA from genetic mutations in the S gene (P681R, L452R and E484Q) from positive SARS-CoV-2 nasopharyngeal samples. This test is intended for use as an aid to monitor the prevalence of genetic mutations in the S gene (P681R, L452R and E484Q) and to assist in control measures. RNA is extracted from respiratory specimens, complementary DNA (cDNA) is synthetised and amplified using RT-qPCR and detected using fluorescent reporter dye probes specific for genetic mutations in the S gene (P681R, L452R and E484Q).

2. Summary and Explanation

All viruses, including SARS-CoV-2, mutates over time. some changes may affect the virus's properties, such as how easily it spreads, the associated disease severity, or the performance of vaccines, therapeutic medicines, diagnostic tools, or other public health and social measures.

The appearance of genetic mutations is a natural and expected event within the evolution process of a virus. In fact, some specific mutations define the viral genetic groups currently circulating globally. Besides, thanks to the genetic sequencing of the pathogen worldwide, it has been possible to establish patterns of dispersal and evolution of the virus.

At the end of 2020, the appearance of variants with a higher risk for public health prompted the characterization of Variants of Interest (VOI) and Variants of Concern (VOC), in order to facilitate epidemiological control. Some of these SARS-CoV-2 variants are:

Delta (B.1.617.2 lineage) and Kappa (B.1.617.1 lineage) variants were closely associated with a huge COVID-19 increase in India during Spring, 2021. Delta variant has multiple mutations in the Spike protein, including P681R and L452R. Kappa variant has also genetic mutations in the Spike protein, including P681R, L452R and E484Q.

Epsilon variant, first detected in California (USA), consists of two distinct lineages B.1.427 and B.1.429 in clade 20C. This variant has multiple mutations in the spike protein, including L452R. An estimated increase in transmission rate of the B.1.427/B.1.429 lineages related to circulating non- B.1.427/B.1.429 lineages was 20%.

The B.1.526.1 linage was first detected in USA in October 2020. This variant has multiple mutations in the spike protein, including L452R.

All these mutations described above show potential reduction in neutralization by some immunotherapies and reduction of expected effects of vaccines or has been identified to cause community transmission.

That is why, the appearance of variants that increase the transmissibility of the virus, its virulence or that escape the action of the neutralizing antibodies generated after natural infection or the vaccine, constitute a first-order public health problem that can have an important impact on control of the pandemic.

A concern regarding the new variants is that their detection by molecular techniques (RT-PCR) could be affected. For this reason, VIASURE SARS-CoV-2 Variant II Real Time PCR Detection Kit has been designed to allow the detection of the main mutation associated with the variant under surveillance.

3. Principle of the procedure

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit is designed for the qualitative detection of RNA from genetic mutations in the S gene (P681R, L452R and E484Q) from positive SARS-CoV-2 respiratory samples. The detection is done in one step real time RT format where the reverse transcription and the subsequent amplification of specific target sequence occur in the same reaction well. The isolated RNA target is transcribed generating complementary DNA by reverse transcriptase which is followed by the amplification of a conserved region of the *S* gene for SARS-CoV-2 P681R, L452R and E484Q using specific primers and a fluorescent-labeled probe.

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit is based on the 5´ exonuclease activity of DNA polymerase. During DNA amplification, this enzyme cleaves the probe bounded to the complementary DNA sequence, separating the quencher dye from the reporter. This reaction generates an increase in the fluorescent signal which is proportional to the quantity of target template. This fluorescence can be measured on Real Time PCR platforms.

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit contains in each well all the components necessary for real time PCR assay (specific primers/probes, dNTPS, buffer, polymerase and retrotranscriptase) in a stabilized format, as well as an **endogenous internal control** to monitor the extraction process and/or discard the inhibition of the polymerase activity. The assay uses a human housekeeping gene as an **endogenous Internal Control (IC)** (human *RNase P* gene). Human housekeeping genes are involved in basic cell maintenance and, therefore, are expected to be present in all nucleated human cells and maintain relatively constant expression levels.

4. Reagents provided

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit includes the materials and reagents detailed in Annex 1 for open format products and Annex 2 for tube format products.

5. Reagents and equipment to be supplied by the user

The following list includes the materials that are required for use but not included in the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit.

- Real Time PCR instrument (thermocycler).
- Real Time PCR compatible plastic consumables (i.e. individual tubes, well-strips and/or microplates). Only for Tubes format (Annex 2).
- RNA extraction kit.
- Collection and transport system.
- Laboratory freezers: - 30°C to - 10°C and/or ≤ –70°C.
- Centrifuge for 1.5 mL tubes and PCR-well strips or 96-well plate (if available).
- Vortex.
- Micropipettes (0.5-20 µL, 20-200 µL).
- Filter tips.
- Powder-free disposable gloves.

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit has been validated on the following equipments: Applied Biosystems 7500 Fast Real-Time PCR System, Bio-Rad CFX96™ Real-Time PCR Detection System, Agilent

Technologies AriaMx Real-Time PCR System, DNA-Technology DTprime Real-time Detection Thermal Cycler, DNA-Technology DTlite Real-Time PCR System, Roche Molecular Diagnostics Cobas z480 Analyzer, Roche LightCycler 480 Instrument, Linear NEOS-96 Real Time PCR System, ThermoFisher QuantStudio™ 5 Real-Time PCR System and Rotor-Gene® Q. When using the Applied Biosystems 7500 Fast with strips it is recommend to place a plate holder to reduce the risk of crushed tube (Ref. PN 4388506). To check thermocycler compatibility and most common detection channels consult website (www.certest.es).

Optical measurement parameters of some thermocyclers must be adjusted to be suitable for operation with "VIASURE Real Time PCR Detection Kits". This assay has been validated with the following set exposition values:

- DTprime Real-time Detection Thermal Cycler (DNA-Technology): FAM channel -500*, HEX channel – 1000, ROX channel – 1000 and Cy5 channel - 1000.

- DTlite Real-Time PCR System (DNA-Technology): FAM channel - 500, HEX channel - 500, ROX channel – 500 and Cy5 channel - 500.

*If the result in channel FAM is not as expected, there are no amplifications or high background noise is observed, please lower the exposure values indicated above to 150.

6. Transport and storage conditions

- The kits can be shipped and stored at 2-40°C until the expiration date which is stated on the label.
- Once the positive control has been re-suspended, store it at -20°C. It is recommended to separate it in aliquots to minimize freeze and thaw cycles. Positive control has been validated as still being stable after 6 freeze-thaw cycles.
- Keep components away from light.
- For Tube format kits: Once the *SARS-CoV-2 Variant II* Reaction-Mix tube has been reconstituted, it may be kept it at 25°C±5°C or 2°C to 8°C for up to 4 hours. For a longer period of time, it is recommended store at –20°C and to separate in aliquots to minimize freeze and thaw cycles (up to 6 times).

7. Precautions for users

- The product is indented for use by qualified and trained clinical laboratory personnel specifically instructed and trained in the techniques of real-time PCR and *in vitro* diagnostic procedures (including training on the Real Time PCR instrument (thermocycler) and Nucleic acid extraction system).
- For *in vitro* diagnostic use.
- Do not use expired reagents and/or materials.
- Do not use the kit if the label that seals the outer box is broken.
- Do not use reagents if the protective box is open or broken upon arrival.
- Do not use reagents if the protective pouches are open or broken upon arrival.
- Do not use reagents if desiccant is not present or broken inside reagent pouches.
- Do not remove desiccant from reagent pouches once is open.
- Close protective pouches of reagents promptly with the zip seal after each use (for references: VS-VAI113L, and VS-VAI113H). Remove any air excess in the pouches prior to closing.
- Do not use reagents if the foil has been broken or damaged.
- Do not mix reagents from different pouches and / or kits and / or lots and / or another supplier.

- Protect reagents against from humidity. Prolonged exposure to humidity may affect product performance.
- An appearance of the reaction mixture in stabilized format, normally found at the bottom of the tube, different from the usual one (without conical shape, inhomogeneous, smaller/larger in size and/or color different from whitish) does not alter the functionality of the test.
- Design a unidirectional workflow. It should begin in the Extraction Area and then move to the Amplification and Detection Area. Do not return samples, equipment and reagents to the area in which the previous step was performed. Use separate areas for the preparation of patient samples and controls to prevent false positive results.
- In cases where other PCR tests are conducted in the same general area of the laboratory, care must be taken to ensure that the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit and any additional reagents or equipment required for testing are not contaminated. Always avoid microbial and ribonuclease (RNase)/deoxyribonuclease (DNase) contamination of reagents at all times. The use of sterile RNase/DNase-free disposable aerosol resistant or positive displacement pipette tips is recommended. Use a new tip for each specimen. Gloves must be changed before manipulating reagents.
- Follow Good Laboratory Practices. Wear protective clothing, use disposable gloves, goggles and mask. Do not eat, drink, smoke or apply cosmetic products in the working area. Wash your hands after finishing the test.
- Specimens must be treated as potentially infectious and/or biohazardous, as well as all the reagents and materials that have been exposed to the samples and they must be handled according to the national safety regulations. Take necessary precautions during the collection, transport, storage, handling, and disposal of samples.
- Samples and reagents must be handled in a biological safety cabinet. Use personal protective equipment (PPE) consistent with current guidelines for the handling of potentially infectious samples. Dispose of waste in compliance with local and state regulations.
- Regular decontamination of commonly used equipment is recommended, especially micropipettes and work surfaces.
- In accordance with Regulation (EC) No 1907/2006 (REACH), VIASURE Real Time PCR Detection Kits do not require Material Safety Data Sheets on account of their classification as non-hazardous to health and the environment, because they do not contain substances and/or mixtures which meet the hazard classification criteria available in Regulation (EC) No 1272/2008 (CLP), or which are in concentrations higher than the value established in the mentioned regulation for their declaration.
- Consult each Real Time PCR instrument's reference manual for additional warnings, precautions and procedures.

8. Test procedure

Please see Annex 1 for Open format products Test Procedure and Annex 2 for Tube format products Test Procedure.

8.1. Specimen collection, transport and storage

The VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection kit has been tested on nasopharyngeal specimens collected with synthetic fiber swabs with plastic and placed immediately into a sterile transport tube containing both, Viral transport medium (VTM) or Universal transport medium (UTM). Other types of samples must be validated by the user.

It is recommended to use initial samples characterized as positive for SARS-CoV-2 by an RT-qPCR assay that present Ct values less than or equal to 30.

Collection, storage, and transport specimens should be maintained per the conditions validated by the user. Overall, respiratory samples should be collected and labelled appropriately in clean containers with or without transport media (depending on sample type) and processed as soon as possible to guarantee the quality of the test. The specimens should be transported at 2 to 8°C for up to 72 hours, following the local and national regulations for the transport of pathogen material. For long term transport (more than 72 hours), It is recommended shipping at -20°C or lower. It is recommended to use fresh specimens for the test. The samples can be stored at 2 to 8°C for up to 72 hours or frozen at -20°C or ideally at -70°C for conservation. Repeated freeze-thaw cycles should be avoided in order to prevent degradation of the sample and nucleic acids.

The respiratory specimens must be collected, transport and storage according to appropriate laboratory guidelines. For details, refer to the CDC guideline (Specimen collection guidelines. Website https://www.cdc.gov/urdo/downloads/SpecCollectionGuidelines.pdf and Interim Guidelines for Collecting, Handling, and Testing Clinical Specimens for COVID-19. Website https:// www.cdc.gov/coronavirus/2019-ncov/lab/guidelines-clinical-specimens.html) and the IDSA guideline (Miller, J. M., Binnicker, M. J., Campbell, S., ... & Pritt, B. S. (2018). A guide to utilization of the microbiology laboratory for diagnosis of infectious diseases: 2018 update by the Infectious Diseases Society of America and the American Society for Microbiology. *Clinical Infectious Diseases*, 67(6), e1-e94).

8.2. RNA extraction

Perform the sample preparation according to the recommendations appearing in the instructions for use of the extraction kit used.

For RNA extraction from respiratory samples, you can use your manual or automatic routine optimized system, or any commercially available RNA extraction kit and follow the manufacturer´s instructions. The following extraction kits have been validated:

- MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit using the KingFisher Flex System instrument (ThermoFisher).
- MagDEA Dx SV kit, using the magLEAD® 12gC instrument (Precision System Science Co).
- Maxwell ® RSC 48 instrument using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega).

9. Result interpretation

All the result of the test should be evaluated by a healthcare professional in the context of medical history, clinical symptoms, and other diagnostic tests. Check Endogenous Internal Control (IC) signal to verify the extraction procedure and/or correct functioning of the amplification mix. The analysis of the controls and samples is done by the software of the used real time PCR equipment itself according to manufacturer´s instructions.

It is recommended to set the threshold values for each channel (target) independently by the end-user. Use the Positive Control amplification curve as a starting point during the run validation (before than interpretation of patient sample results), in order to ensure that thresholds fall within the exponential phase of the fluorescence curves

and above any background signal. The threshold value for different instruments may vary due to different signal intensities.

The use of positive and negative controls in each run, validate the reaction by checking the absence of signal in the negative control well and the presence of signal in the positive control well.

For a valid diagnostic test run, the following control conditions must be met:

Controls	P681R (*S* gene) (FAM)[1]	L452R (*S* gene) (HEX)[1]	E484Q (*S* gene) (ROX)[1]	Endogenous Internal Control (Cy5)[2]	Interpretation of Controls
Positive Control (PC)	≤40	≤40	≤40	≤40	**Valid**
Negative Control (NC)	≥40 or no signal	≥40 or no signal	≥40 or no signal	≥40 or no signal	**Valid**

Table 1. Expected Performance of Controls

1 In cases where one or more controls fail (an amplification signal is observed in the negative control and/or signals absence in the positive control well for any target channel), all results are reported as 'Invalid' and retesting is required.

2 The positive template control includes human housekeeping RNase P gene target; therefore, amplification signals are observed in all target channels, including the Endogenous Internal Control.

Assessment of clinical samples test results should be performed after the positive and negative controls have been examined and determined to be valid and acceptable. If one or more controls are not valid, the patient results cannot be interpreted.

For interpretation of patient sample results, use the following table, read and analyze the results:

P681R (*S* gene) (FAM)	L452R (*S* gene) (HEX)	E484Q (*S* gene) (ROX)	Endogenous Internal Control (Cy5)	Interpretation for patients' individual samples	
≤40	≥40 or no signal	≥40 or no signal	≤40 or no signal [1]	**Valid**	**P681R mutation detected**
≥40 or no signal	≤40	≥40 or no signal	≤40 or no signal [1]	**Valid**	**L452R mutation detected**
≥40 or no signal	≥40 or no signal	≤40	≤40 or no signal [1]	**Valid**	**E484Q mutation detected**
≤40	≤40	≥40 or no signal	≤40 or no signal [1]	**Valid**	**P681R and L452R mutations detected**
≤40	≥40 or no signal	≤40	≤40 or no signal [1]	**Valid**	**P681R and E484Q mutations detected**
≥40 or no signal	≤40	≤40	≤40 or no signal [1]	**Valid**	**L452R and E484Q mutations detected**
≤40	≤40	≤40	≤40 or no signal [1]	**Valid**	**P681R, L452R and E484Q mutations detected**
≥40 or no signal	≥40 or no signal	≥40 or no signal	≤ 35 [2]	**Valid**	**Targets not Detected [2]**
≥40 or no signal	≥40 or no signal	≥40 or no signal	≥ 35 or no signal [2]	**Invalid**	**Test Failure – Repeat Testing [2]**

Table 2. Interpretation of individual patient sample results. Ct values. no signal = no amplification curve.

1 The endogenous Internal Control (IC) shows or not an amplification signal (Ct ≤40 or no signal). Sometimes, its detection is not necessary because a high copy number of the target can cause preferential amplification of target-specific nucleic acids.

2 In the case of SARS-CoV-2 target genes negative, IC must show an amplification signal with Ct less than 35. The Ct value could be very variable due to the Endogenous Internal Control is a human housekeeping gene that should be present in all human nucleated cells in the original sample. If there is an absence of signal or Ct value ≥ 35 of the endogenous Internal Control, the result is considered as 'Invalid', and retesting is required. It is recommended to repeat the RT-qPCR diluting the RNA sample 1:10 and/or 1:100, or re-extract and retest to check for possible failure in the extraction procedure and/or inhibition issues.

Summary of mutations associated with the following lineages present in the most known Variants of Concern (VOC) and Variants of Interest (VOI) until date:

Who label	Pango lineage	Earliest documented samples	Mutations in *S* gene		
			P681R	L452R	E484Q
Delta	B.1.617.2	India, Oct-2020	x	x	
N.A	B.1.526.1	United States (New York) Oct-2020		x	
Epsilon	B.1.427/ B.1.429	United States (California) Mar-2020		x	
Kappa	B.1.617.1	India, Oct-2020	x	x	x

Table 3. Summary of mutations associated with known Variants of Concern (VOC)[1,2].

[1]Tracking SARS-CoV-2 variants: https://www.who.int/en/activities/tracking-SARS-CoV-2-variants/ (data up to 22th June 2021).

[2] Overview of Variants/Mutations https://covariants.org/variants (data up to 22th June 2021).

Other variants can present the mutations P681R, L452R and E484Q because they are not specific for the variants mentioned.

Final assignment to a lineage must be done by sequencing.

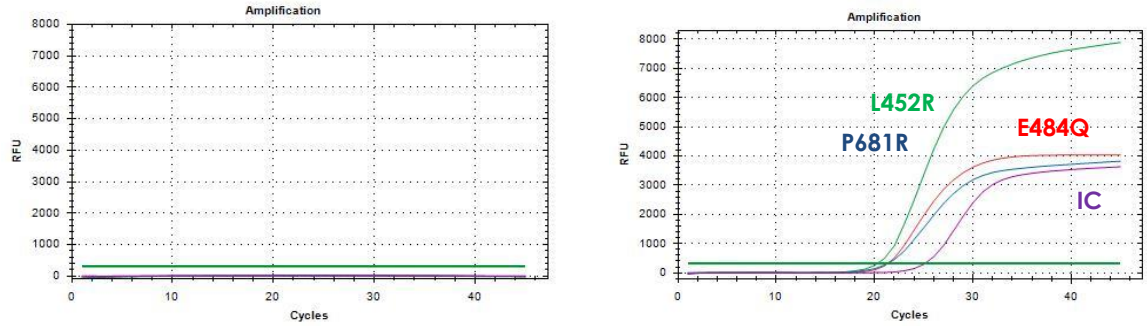
In case of a continued ambiguous result, it is recommended to review the instructions for use; the extraction process used by the user; to verify the correct performance of each RT-qPCR steps and review the parameters; and to check the sigmoid shape of the curve and the intensity of fluorescence. It is also recommended to repeat the assay, preferably in duplicate. Depending on the available material:

a) repeat RT-qPCR with the same isolated RNA sample, or

b) re-extract and retest another aliquot of the same specimen or,

c) obtain a new specimen and retest.

Figure 1. Correct run of negative and positive control run on the Bio-Rad CFX96™ Real-Time PCR Detection System.



Negative control **Positive control**

10. Limitations of the test

- For professional *in vitro* use.
- Although this assay can be used with other types of samples it has been validated only with RNA extracted from respiratory samples (nasopharyngeal swab). It is recommended to use initial samples characterized as positive for SARS-CoV-2 by an RT-qPCR assay that present Ct values less than or equal to 30.
- The quality of the test depends on the quality of the sample; properly extracted nucleic acid from clinical samples must be extracted.
- This test is a qualitative test and does not provide quantitative values or indicate the number of organisms present.
- Extremely low levels of target below the limit of detection might be detected, but results may not be reproducible.
- There is a possibility of false positive results due to cross-contamination by SARS-CoV-2 RNA from genetic mutations, either the high number of cDNA template copies which contains each *SARS-CoV-2 Variant II* Positive Control vial, samples containing high concentrations of target RNA or contamination due to PCR products from previous reactions.
- False Negative results may arise from several factors and their combinations, including:
    - Improper specimens' collection, transport, storage, and/or handling methods.
    - Improper processing procedures (including RNA extraction).
    - Degradation of the viral RNA during sample shipping/storage and/or processing.
    - Mutations or polymorphisms in primer or probe binding regions may affect detection of new or unknown SARS-CoV-2 variants.
    - A viral load in the specimen below the limit of detection for the assay.
    - The presence of RT-qPCR inhibitors or other types of interfering substances. The impacts of vaccines, antiviral therapeutics, antibiotics, chemotherapeutics or immunosuppressant drugs used to prevent COVID-19 or used during the treatment of the infection have not been evaluated.
    - Failure to follow instructions for use and the assay procedure.

    If in doubt, refer to section 9 to check the correct interpretation of the results.

- A positive test result does not necessarily indicate the presence of viable viruses and does not imply that these viruses are infectious or are the causative agents for clinical symptoms. However, a positive result is indicative of the presence of targets viral sequences.

- The presence of the P681R mutation in the S gene has been first detected in the following lineages: B.1.617.1 and B.1.617.2, the presence of the L452R mutation in the S gene in the following lineages: B.1.617.2, B.1.526.1, B.1.427/B.1.429 and B.1.617.1, the presence of the E484Q mutation in the S gene in the following lineages B.1.617.1, however, final assignment to a lineage must be done by sequencing.
- Negative results do not preclude presence of SARS-CoV-2 RNA due to this assay is intended to use with positive SARS-CoV-2 samples.
- Fluorescence values may vary due to multiple factors such as: PCR equipment, extraction system, type of sample, previous treatment of the sample, etc, among others.

11. Quality control

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit contains a positive and a negative control that must be included in each run to correctly interpret the results. Also, the endogenous internal control (IC) in each well confirms the correct performance of the technique.

12. Performance characteristics

12.1. Clinical sensitivity and specificity

The clinical performance of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit was tested using respiratory clinical samples (nasopharyngeal swabs) from patients diagnosed with COVID-19 disease.
In order to determine the clinical diagnostic accuracy, different multicenter evaluations have been conducted through collaboration with national and international entities. A summary of the sites, sample type and workflow is included in the following table:

	Site	Sample type	Workflow	Target
1	Addenbrooke's hospital (Cambridge University hospitals NHS Foundation Trust)	nasopharyngeal swab	KingFisher™ Flex system (Thermo-Fisher SCIENTIFIC) instrument using Omega BIO-TEK kit + Rotor-Gene®Q(QIAGEN)	Mutation P681R Mutation L452R Mutation E484Q
2	Norfolk and Norwich University Hospitals (NHS Foundation Trust, UK)	nasopharyngeal swab	MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit using the KingFisher™ Flex System instrument (ThermoFisher) + QuantStudio™ 5 Real-Time PCR System (ThermoFisher)	Mutation P681R Mutation L452R Mutation E484Q
3	A NHS Foundation Trust Hospital (UK)	nasopharyngeal swab	Maxwell ® RSC 48 instrument using the Maxwell® RSC Viral Total Nucleic Acid Purification Kit (Promega)+ CFX96 RT-PCR instrument (BioRad)	Mutation P681R Mutation L452R Mutation E484Q
4.1	CerTest Biotec S.L	nasopharyngeal swab (from IACS and the Biobanco del Sistema de Salud de Aragón, Spain)	MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit using the KingFisher™ Flex System instrument (ThermoFisher) +)+ CFX96 RT-PCR instrument (BioRad)	Mutation P681R Mutation L452R Mutation E484Q

4.2	CerTest Biotec S.L	Nasopharyngeal swab (Biobanco Vasco,from the Fundación Vasca de Innovación e Investigación Sanitarias, Spain)	MagMAX™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation Kit using the KingFisher™ Flex System instrument (ThermoFisher) +)+ CFX96 RT-PCR instrument (BioRad)	Mutation P681R
				Mutation L452R
				Mutation E484Q

Table 4. Site, sample type, workflow and target

True positive and negative values, false positive and negative values, sensitivity, specificity, PPV, NPV values for VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit were calculated in relation to each comparator assay as shown in the following table:

Site	Comparator assay	Target	TP	TN	FP	FN	Sensitivity	Specificity	PPV	NPV
1	Whole genome sequencing (WGS)	Mutation P681R	20	40	0	0	1 (0.83-1)	1 (0.91-1)	1 (0.83-1)	1 (0.91-1)
		Mutation L452R	20	40	0	0	1 (0.83-1)	1 (0.91-1)	1 (0.83-1)	1 (0.91-1)
		Mutation E484Q	0	60	0	0	n.a*	1 (0.94-1)	n.a*	1 (0.94-1)
2	Whole genome sequencing (WGS)	Mutation P681R	2	68	0	1	0.66 (0.94-0.99)	1 (0.94-1)	0.66 (0.94-0.99)	1 (0.94-1)
		Mutation L452R	2	68	0	1	0.66 (0.94-0.99)	1 (0.94-1)	0.66 (0.94-0.99)	1 (0.94-1)
		Mutation E484Q	0	71	0	0	n.a*	1 (0.94-1)	n.a*	1 (0.94-1)
3	Whole genome sequencing (WGS)	Mutation P681R	12	3	0	0	1 (0.73-1)	1 (0.29-1)	1 (0.73-1)	1 (0.29-1)
		Mutation L452R	12	3	0	0	1 (0.73-1)	1 (0.29-4)	1 (0.73-1)	1 (0.29-4)
		Mutation E484Q	0	15	0	0	n.a*	1 (0.78-1)	n.a*	1 (0.78-1)
4.1	Whole genome sequencing (WGS)	Mutation P681R	1	158	0	0	1 (0.02-1)	1 (0.97-1)	1 (0.02-1)	1 (0.97-1)
		Mutation L452R	1	158	0	0	1 (0.02-1)	1 (0.97-1)	1 (0.02-1)	1 (0.97-1)
		Mutation E484Q	0	159	0	0	n.a*	1 (0.97-1)	n.a*	1 (0.97-1)
4.2	Whole genome sequencing (WGS)	Mutation P681R	0	62	0	0	n.a*	1 (0.94-1)	n.a*	1 (0.94-1)
		Mutation L452R	0	62	0	0	n.a*	1 (0.94-1)	n.a*	1 (0.94-1)
		Mutation E484Q	0	62	0	0	n.a*	1 (0.94-1)	n.a*	1 (0.94-1)

Table 5. True positive and negative values, false positive and negative values, sensitivity, specificity, PPV, NPV values for VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit.

*** Due to all analyzed samples were negative for E484Q genetic mutation, the analytical sensitivity of the test could not be performed.**

Results show high agreement to detect P681R, L452R and E484Q mutations using VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit in nasopharyngeal samples previously characterized as SARS-CoV-2 positive.

12.2. Analytical sensitivity

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit has a detection limit (LoD) of 320 genome copies/rxn for P618R mutaton and 160 genome copies/rxn for L452R and E484Q mutations.

The limit of detection has been measured using the SARS-CoV-2 B.1.617.1 lineage.

Figure 2. Dilution series of mutation P618R (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (FAM channel).

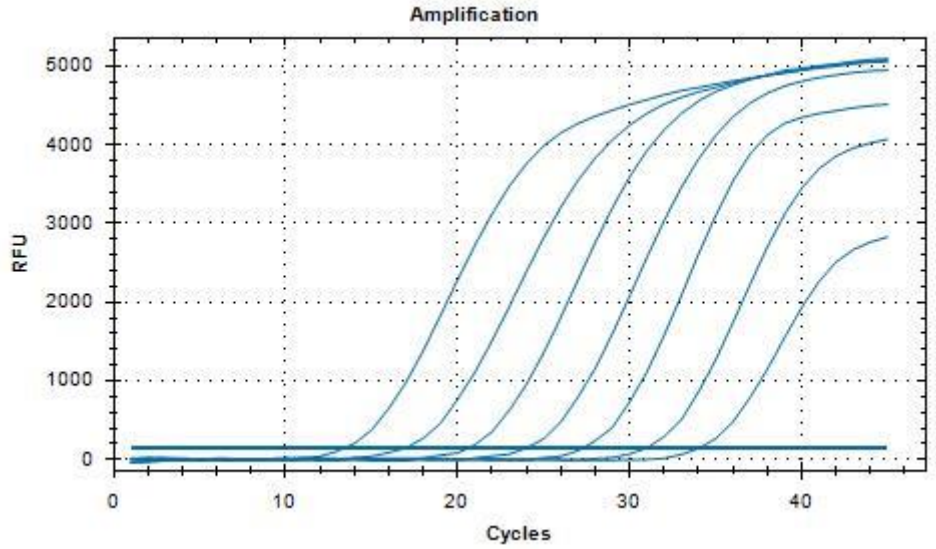


Figure 3. Dilution series of mutation L452R (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (HEX channel).

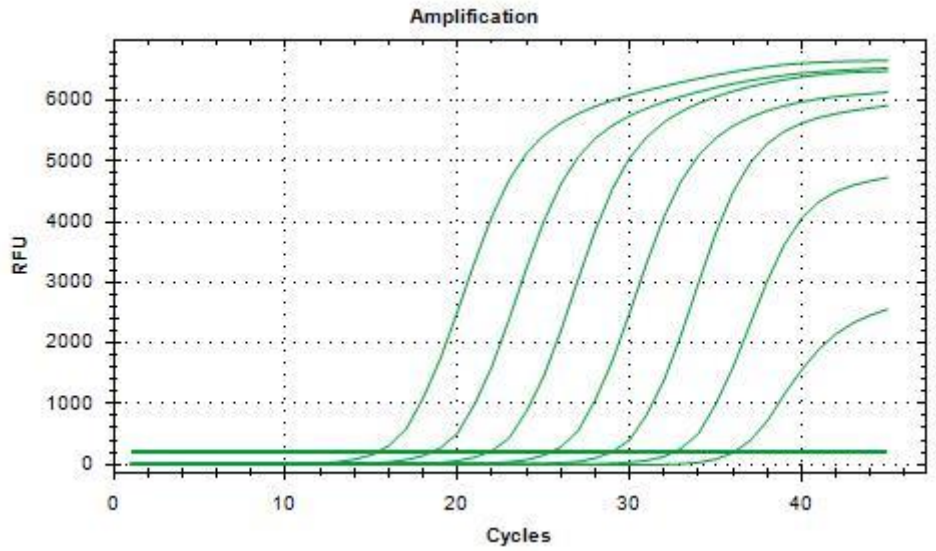
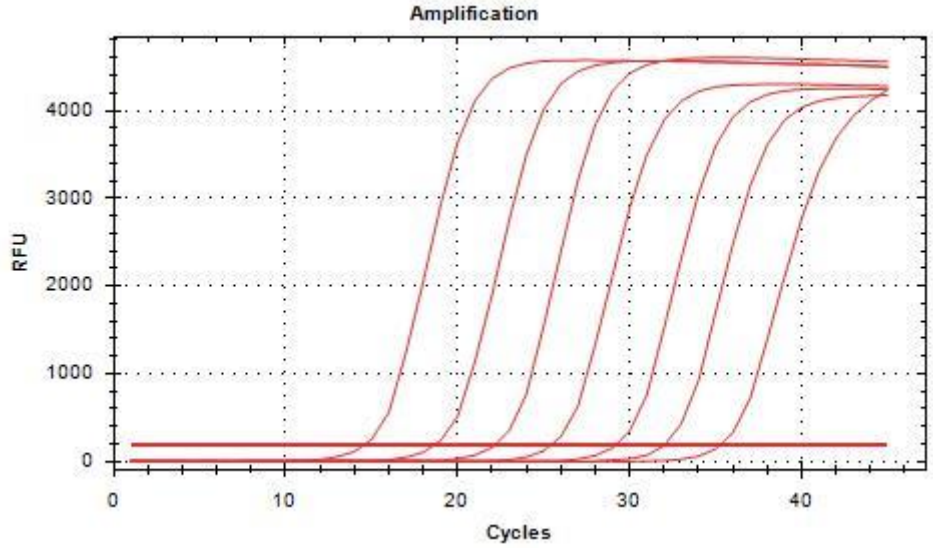


Figure 4. Dilution series of mutation E484Q (10^7-10^1 copies/rxn) template run on the Bio-Rad CFX96™ Real-Time PCR Detection System (ROX channel).



12.3. Analytical specificity

The specificity of the SARS-CoV-2 assay was confirmed by testing a panel consisting of different microorganisms representing the most common respiratory pathogens. No cross-reactivity was detected between any of the following microorganisms tested, except the targeted pathogens of each assay.

Cross-reactivity testing					
Human Adenovirus types 1-5, 8, 15, 31, 40 and 41	-	Influenza A/Thüringen/5/17 (H3N2) virus	-	Human rhinovirus type C	-
Human Bocavirus	-	Influenza A/Switzerland/9715293/2013 (H3N2) virus	-	*Staphylococcus aureus* subsp. *aureus*	-
Bordetella bronchiseptica	-	Influenza A/Hong Kong/4801/2014, NYMC X-263B (H3N2) virus	-	*Staphylococcus epidermidis*	-
Bordetella holmesii	-	Influenza A/South Australia/55/2014, IVR-175 (H3N2) virus	-	*Streptococcus pneumoniae* Z022	-
Bordetella parapertussis	-	Influenza A/DE-SH/Reiherente/AR8444/ 2016 (H5N8) virus	-	*Streptococcus pyogenes*	-
Bordetella pertussis	-	Influenza A/Anhui/1/2013 (H7N9) virus	-	*Streptococcus salivarius*	-
Chlamydia caviae	-	Influenza B/Brisbane/60/2008	-	Respiratory syncytial virus (RSV) A and B	-
Chlamydia psittaci genotype A and C	-	Influenza B/Florida/04/06 virus	-	SARS Coronavirus Strain Frankfurt 1	-
Chlamydophila pneumoniae CM-1	-	Influenza B/Phuket/3073/2013 virus	-	Human 2019-nCoV strain BetaCoV/Germany/BavPat1/2020 p.1 *	-
Human coronavirus 229E, OC43, NL63 and HKU1	-	*Legionella bozemanii*	-	Human 2019-nCoV strain 2019-nCoV/Italy-INMI1 *	-
MERS Coronavirus	-	*Legionella dumoffii*	-	SARS-CoV-2 strain 2019nCoV/USA-WA1/2020 *	-
Enterovirus 68 and 71	-	*Legionella longbeachae*	-	SARS-CoV-2 BetaCoV/Berlin/ChVir1670/2020_IsolatBER*	-
Enterovirus Echovirus 30	-	*Legionella micdadei*	-	SARS-CoV-2 BetaCoV/Munich/ChVir984/2020*	-
Enterovirus Coxsackievirus A24, A9 and B3	-	*Legionella pneumophila*	-	SARS-CoV-2 BetaCoV/Baden-Wuerttemberg/1/ChVir1577/2020_IsolatBER*	-
Haemophilus influenzae MinnA	-	Human metapneumovirus A and B	-	MT007544.1 (SARS-CoV2 isolate Australia/VIC01/2020) *	-
Influenza A/New Caledonia/20/99(H1N1) virus	-	*Moraxella catarrhalis*	-	MN908947.3 (SARS-CoV-2 isolate Wuhan-Hu-1) *	-
Influenza A/California/7/2009(H1N1)pdm09	-	*Mycoplasma pneumoniae*	-	SARS-CoV-2 B.1.1.7_710528 and SARS-CoV-2 B.1.1.7_601443 lineages (Alpha Variant) *	-
Influenza A/Michigan/45/2015 (H1N1)pdm09 virus	-	*Mycobacterium tuberculosis* not rifampin resistant	-	SARS-CoV-2 B.1.351 lineage (Beta Variant) *	-
Influenza A/Singapore/GP1908/2015, IVR-180 (H1N1)pdm09 virus	-	Human parainfluenza 1, 2, 3 and 4 viruses	-	SARS-CoV-2 P.1 lineage (Gamma Variant) *	-
Influenza A/Victoria/210/2009 (H3N2)	-	*Pneumocytis jirovecii* Type A1 and g885652	-		

Table 6. Reference pathogenic microorganisms used in this study.

* Please note that the detection of these SARS-CoV-2 strains is not considered in this assay. This test is designed for the qualitative detection of RNA from specific genetic mutations in the *S* gene (P681R, L452R and E484Q) present in several SARS-CoV-2 variants.

12.4. Analytical reactivity

The reactivity of the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit for P681R, L452R and E484Q mutations was evaluated against RNA from B.1.617.1(Twist Synthetic SARS-CoV-2 RNA Control 18 (EPI_ISL_1662307)/B.1.617.1), showing positive results.

ANNEX 1

OPEN FORMAT

Annex for the following references:

PRODUCT	REFERENCE
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 6 x 8-well strips, low profile	VS-VAI106L
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 6 x 8-well strips, high profile	VS-VAI106H
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 12 x 8-well strips, low profile	VS-VAI112L
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 12 x 8-well strips, high profile	VS-VAI112H
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 96-well plate, low profile	VS-VAI113L
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 96-well plate, high profile	VS-VAI113H
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 1 x 8-well strips, low profile	VS-VAI101L
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit 1 x 8-well strips, high profile	VS-VAI101H

Table A1 1. References

A1.1 Principle of the procedure

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit contains in each well all the components necessary for real time PCR assay (specific primers/probes, dNTPS, buffer, polymerase and retrotranscriptase) in a stabilized format, as well as an endogenous internal control to monitor the extraction process and/or discard the inhibition of the polymerase activity. The assay uses a human housekeeping gene as an endogenous Internal Control (IC) (human *RNase P* gene). Human housekeeping genes are involved in basic cell maintenance and, therefore, are expected to be present in all nucleated human cells and maintain relatively constant expression levels.

Target	Channel	Gene
P681R	FAM	*S* gene
L452R	HEX, VIC or JOE *	*S* gene
E484Q	ROX	*S* gene
Endogenous Internal control (IC)	Cy5	human *RNase P* gene

Table A1 2. Target, channel and genes.

***Depending on the equipment used select the proper detection channel, to check most common detection channels consult the website www.certest.es.**

A1.2 Reagents provided

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit includes the following materials and reagents detailed in Tables A1.3 and A1.4. Based on the commercial presentation and the Real Time PCR platform used, the stabilized PCR reaction mix could be placed inside different wells and could be marketed on multiple formats. Table A1.3 includes materials and reagents to be used with 8-well strips compatible devices. Table A1.4 includes materials and reagents to be used with 96-well plate compatible devices. (Consult the thermocycler compatibility on CerTest´s website (www.certest.es).

Reagent/Material	Description	Colour	Amount
SARS-CoV-2 Variant II 8-well strips	A mix of enzymes, primers probes, buffer, dNTPs, stabilizers in stabilized format	White	6/12 x 8-well strip
Rehydration Buffer	Solution to reconstitute the stabilized product	Blue	1 vial x 1.8 mL
SARS-CoV-2 Variant II Positive Control	Non-infectious synthetic lyophilized cDNA	Red	1 vial
Negative control	Non template control	Violet	1 vial x 1 mL
Water RNAse/DNAse free	RNAse/DNAse free water	White	1 vial x 1 mL
Tear-off 8-cap strips	Optical caps for sealing wells during thermal cycling	Transparent	6/12 x 8-cap strip

Table A1 3. Reagents and materials provided in VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit with Ref. VS-VAI106L, VS-VAI106H, VS-VAI112L and VS-VAI112H.

Reagent/Material	Description	Color	Amount
SARS-CoV-2 Variant II 96-well plate	A mix of enzymes, primers probes, buffer, dNTPs, stabilizers in stabilized format	White	1 plate
Rehydration Buffer	Solution to reconstitute the stabilized product	Blue	1 vial x 1.8 mL
SARS-CoV-2 Variant II Positive Control	Non-infectious synthetic lyophilized cDNA	Red	1 vial
Negative control	Non template control	Violet	1 vial x 1 mL
Water RNAse/DNAse free	RNAse/DNAse free water	White	1 vial x 1 mL
Tear-off 8-cap strips	Optical caps for sealing plate during thermal cycling	Transparent	12 x 8-cap strip

Table A1 4. Reagents and materials provided in VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit with Ref VS-VAI113L and VS-VAI113H.

A1.3 Test procedure

A1.3.1 Lyophilized positive control

SARS-CoV-2 Variant II Positive Control contains high copies of the template, the recommendation is to open and manipulate it in a separate laboratory area away from the other components. Reconstitute the lyophilized *SARS-CoV-2 Variant II* Positive Control (red vial) by adding 100 µL of the supplied Water RNAse/DNAse free (white vial) and vortex thoroughly.

Once the positive control has been re-suspended, store it at -20°C. It is recommended to separate it in aliquots to minimize freeze and thaw cycles.

A1.3.2 PCR protocol

Determine and separate the number of required reactions including samples and controls. One positive and negative control must be included in each run for each assay. Peel off protective aluminium seal from plates or strips.

1) Reconstitute the number of wells you need.

Add 15 µL of Rehydration Buffer (blue vial) into each well.

2) Adding samples and controls.

Add 5 µL of RNA sample, reconstituted *SARS-CoV-2 Variant II* Positive Control (red vial) or Negative Control (violet vial) in different wells and close them with the provided caps. It is recommended to briefly centrifuge the 8-well strips or 96-well plate.

Load the plate or the strips in the thermocycler.

3) Set up the thermocycler (consult thermocycler compatibility on CerTest´s website (www.certest.es).

Program the thermocycler following the conditions listed below and start the run:

Cycles	Step	Time	Temperature
1	Reverse transcription	15 min	45ºC
1	Initial denaturation	2 min	95ºC
45	Denaturation	10 sec	95ºC
	Annealing/Extension (Data collection*)	50 sec	63ºC

Table A1 5. PCR protocol

Fluorogenic data should be collected during the extension step (*) through the FAM (P681R), HEX, JOE or VIC (L452R), ROX (E484Q), and Cy5 (Endogenous Internal Control (IC)). Depending on the equipment used select the proper detection channel (to check most common detection channels consult website www.certest.es). In Applied Biosystems 7500 Fast Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System check that passive reference option ROX is none. In the Applied Biosystems 7500 Fast Real-Time PCR System select Ramp Speed Standard in Select New Experiment/Advanced Setup/Experiment Properties.

ANNEX 2

TUBE FORMAT

Annex for the following references:

PRODUCT	REFERENCE
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit, 4 tubes x 24 reactions	VS-VAI196T

Table A2. 1.References.

A2.1 Principle of the procedure

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit contains in each Reaction-Mix tube all the components necessary for 24 real time PCR reactions (specific primers/probes, dNTPS, buffer, polymerase and retrotranscriptase) in a stabilized format, as well as an endogenous internal control to monitor the extraction process and/or discard the inhibition of the polymerase activity. The assay uses a human housekeeping gene as an endogenous Internal Control (IC) (human *RNase P* gene). Human housekeeping genes are involved in basic cell maintenance and, therefore, are expected to be present in all nucleated human cells and maintain relatively constant expression levels.

Target	Channel	Gene
P681R	FAM	*S* gene
L452R	HEX, VIC or JOE *	*S* gene
E484Q	ROX	*S* gene
Endogenous Internal control (IC)	Cy5	human *RNase P* gene

Table A2. 2.Target, channel and genes.

***Depending on the equipment used select the proper detection channel, channel, to check most common detection channels consult website www.certest.es.**

A2.2 Reagents provided

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit includes the following materials and reagents detailed in Table A2.3.

Reagent/Material	Description	Colour	Amount
SARS-CoV-2 Variant II Reaction-Mix tube	A mix of enzymes, primers probes, buffer, dNTPs, stabilizers in stabilized format	White	4 vials
Rehydration Buffer	Solution to reconstitute the stabilized product	Blue	1 vial x 1.8 mL
SARS-CoV-2 Variant II Positive Control	Non-infectious synthetic lyophilized cDNA	Red	1 vial
Negative control	Non template control	Violet	1 vial x 1 mL
Water RNAse/DNAse free	RNAse/DNAse free water	White	1 vial x 1 mL

Table A2. 3. Reagents and materials provided in VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit with Ref. VS-VAI196T.

A2.3 Test procedure

A2.3.1 Lyophilized positive control

SARS-CoV-2 Variant II Positive Control contains high copies of the template, the recommendation is to open and manipulate it in a separate laboratory area away from the other components. Reconstitute the lyophilized *SARS-CoV-2 Variant II* Positive Control (red vial) by adding 100 µL of the supplied Water RNAse/DNAse free (white vial) and vortex thoroughly.

Once the positive control has been re-suspended, store it at -20ºC. It is recommended to separate it in aliquots to minimize freeze and thaw cycles.

A2.3.2 Lyophilized reaction mix tube

Determine the number of required reactions including samples and controls (one positive and negative control must be included in each run). Obtain the correct number of lyophilized Reaction-Mix vials (24-reactions each one) for testing.

Recommendation is to open and manipulate the *SARS-CoV-2 Variant II* Reaction-Mix tube in pre-PCR laboratory area. Open lyophilized Reaction-mix tube (white vial) carefully to avoid disruption of the pellet and add 390 µL of Rehydration Buffer (blue vial) supplied. Mix gently by pipetting up and down. Spin down briefly to remove bubbles generated during mixing.

Once the Reaction-Mix tube has been re-suspended, return unused reagents to the appropriate storage conditions at -20ºC. Recommendation is to separate it in aliquots to minimize freeze and thaw cycles.

Note: The volume of the rehydrated Reaction-Mix is sufficient for 24 reactions. The rehydrated Reaction-Mix may be kept at 25ºC±5ºC or 2-8ºC for up to 4-hours (see Transport and storage conditions section for additional storage options).

A2.3.3 PCR protocol

1) Adding rehydrated Reaction-Mix to the number of required wells.

Add 15 µL of rehydrated *SARS-CoV-2 Variant II* Reaction-Mix (white vial) into each tube.

2) Adding samples and controls.

Add 5 µL of RNA sample, reconstituted *SARS-CoV-2 Variant II* Positive Control (red vial) or Negative Control (violet vial) in different wells and close the tubes with caps or seal the plate. Centrifuge briefly.

Load the plate, the strips, or tube in the thermocycler.

3) Set up the thermocycler (consult thermocycler compatibility on CerTest´s website www.certest.es).

Program the thermocycler following the conditions listed below and start the run:

Cycles	Step	Time	Temperature
1	Reverse transcription	15 min	45ºC
1	Initial denaturation	2 min	95ºC
45	Denaturation	10 sec	95ºC
	Annealing/Extension (Data collection*)	50 sec	63ºC

Table A2. 4. PCR protocol.

Fluorogenic data should be collected during the extension step (*) through the FAM (P681R), HEX, JOE or VIC (L452R), ROX (E484Q), and Cy5 (Endogenous Internal Control (IC)). Depending on the equipment used select the proper detection channel (to check most common detection channels consult website www.certest.es). In Applied Biosystems 7500 Fast Real-Time PCR System and Stratagene Mx3005P™ Real Time PCR System check that passive reference option ROX is none. In the Applied Biosystems 7500 Fast Real-Time PCR System select Ramp Speed Standard in Select New Experiment/Advanced Setup/Experiment Properties.

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Symbols for IVD components and reagents/Símbolos para reactivos y productos para diagnóstico *in vitro*

IVD	*In vitro* diagnostic device Producto para diagnóstico *in vitro*		Keep dry Almacenar en lugar seco		Use by Fecha de caducidad		Manufacturer Fabricante
LOT	Batch code Número de lote		Consult instructions for use Consultar las instrucciones de uso		Temperature limitation Limitación de temperatura	Σ	Contains sufficient for <n> test Contiene <n> test
UDI	Unique Device Identification Identificación única de dispositivo	REF	Catalogue number Número de referencia				

Trademarks

Control de Cambios / Change Control		
Versión / Version nº	**Cambios / Changes**	**Fecha / Date**
00	Versión Original / Original Version	28/06/2021

Table A 3. Tabla de Control de Cambios / Control change table.

Revision: 28th June 2021

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