CerTest BIOTEC

Report

Clinical evaluation and validation of the VIASURE *SARS-CoV-2 Variant I and SARS-CoV-2 Variant II* Real Time PCR Detection Kits

1. TITLE

Clinical evaluation and validation of the VIASURE *SARS-CoV-2 Variant I* and *SARS-CoV-2 Variant II* Real Time PCR Detection Kit

2. AUTHORS

This evaluation was carried out in the Bob Champion Research and Education building Covid Testing Cell (BCRE CTC), in the University of East Anglia Medical School, in partnership with the Microbiology department of the Norfolk and Norwich University Hospital, NHS Foundation Trust. This evaluation was coordinated by Dr. Orla Jupp (Orla.Jupp@nnuh.nhs.uk) and Dr. Rose Davidson (R.Davidson@uea.ac.uk) with the collaboration of Charlotte Duncan (cduncan@pro-lab.com), Technical Manager of Pro-Lab Diagnostics. The contact persons from CerTest Biotec S.L. were Natalia Bertran (nbertran@certest.es) and Cristina Escolar (cescolar@certest.es) (Technical coordinator). Clinical guidance and oversight were provided by Dr. Lindsay Coupland (Lindsay.Coupland@nnuh.nhs.uk) and Dr. Samir Dervisevic (Samir.Dervisevic@nnuh.nhs.uk).

3. OBJECTIVES

The objectives of this work were:

- To analytically validate the prototype VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit by assessing the analytical sensitivity and specificity using reference material.
- To analytically validate the prototype VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit by assessing the analytical sensitivity and specificity using reference material.
- To evaluate and clinically validate the prototype VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit using respiratory clinical samples already characterized as SARS-CoV-2 positive and sequenced to assign lineage (WGS).
- To evaluate and clinically validate the prototype VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit using respiratory clinical samples already characterized as SARS-CoV-2 positive and sequenced to assign lineage (WGS).

- To assess the technical performance of VIASURE SARS-CoV-2 Variant I and Variant II kits in the identification of Variants of Concern currently designated by Public Health England.

4. STUDY DESIGN

This study was divided into two parts. The first investigated analytical sensitivity and specificity and the second part focused on the analysis of SARS-CoV-2 positive clinical samples.

Analytical sensitivity was performed using a dilution series of the first WHO International Standard for SARS-CoV-2 RNA (20/146) for Nucleic acid Amplification Technique (NAT)-based assays consisting of acid-heat inactivated England/02/2020 isolate of SARS-CoV-2 (NIBSC, Hertfordshire, UK), as well as Alpha (VOC-20DEC-01, B.1.1.7), Beta (VOC-20DEC-02, B.1.351), Gamma (VOC-21JAN-02, P.1) and Delta (VOC-21APR-02, B.1.617.2) variant material provided by PHE Porton. The first WHO International Standard for SARS-CoV-2 RNA (20/146) was reconstituted in 0.5 mL of molecular grade water to provide a final concentration of 7.70 Log_{10} IU/mL prior to preparation of the dilution series. The dilution series of the first WHO International Standard for SARS-CoV-2 RNA (20/146) as well as the as well Alpha (VOC-20DEC-01, B.1.1.7), Beta (VOC-20DEC-02, B.1.351), Gamma (VOC-21JAN-02, P.1) and Delta (VOC-21APR-02, B.1.617.2) variant material was prepared in molecular grade water.

The analytical specificity was assessed using the Nucleic Acid Amplification Techniques (NAT) Panel for SARS-CoV-2 (20/266) (National Institute for Biological Standards and Controls (NIBSC), Hertfordshire, UK), which comprises of 30 members of which 24 were positive for acetic acid and heat inactivated SARS-CoV-2 virus Melbourne strain (BetaCoV/Australia/VIC01/2020) at six different unitages as well as two negative control panel members and the other four panel members were positive for one of Coronavirus 229E, Coronavirus NL63, Respiratory Syncytial Virus Strain A2 or Influenza virus B (B/Jiangsu/10/2003) and the NATtrol™ Respiratory Pathogen Panel-1 (Zeptometrix Corporation, New York, United States), which consists of 5 vials of the respiratory virus and bacteria NATtrol™ targets (1 vial of each pool) and 1 negative control vial (**Table 1**).

These controls were supplied in a purified protein matrix that mimics the composition of a true clinical specimen.

Table 1. NATtrol™ Respiratory Pathogen Panel-1 (Zeptometrix Corporation, New York, United States),

Panel Member (Strain)	Pool 1	Pool 2	Pool 3	Pool 4	Pool 5	Negative
Influenza A H1N1 (A/NY/02/09)	✓					
Parainfluenza Type 4A	✓					
Parainfluenza Type 4B	✓					
Rhinovirus (1A)	✓					
Adenovirus Type 3	✓					
Influenza A H1 (A/New Caledonia/20/99)		✓				
Respiratory Syncytial Virus A		✓				
Parainfluenza Type 1		✓				
Coronavirus NL63		✓				
Mycoplasma pneumoniae (M129)		✓				
Influenza A H3 (A/Brisbane/10/07)			✓			
Respiratory Syncytial Virus B (CH93(18)-18)			✓			
Coronavirus OC43			✓			
Coronavirus HKU-1			✓			
Influenza B (B/Florida/02/06)				✓		
Parainfluenza Type 3				✓		
Human Metapneumovirus (Peru6-2003)**				✓		
Legionella pneumophila (Philadelphia)				✓		
Parainfluenza Type 2					✓	
Coronavirus 229E					✓	
Human Bocavirus					✓	
Chlamydophila pneumoniae (CWL-029)					✓	
Negative						✓

The clinical analysis was performed by retrospective-comparative analysis conducted with the remnant of nasopharyngeal clinical samples collected in 1 mL of VTM of patients diagnosed with COVID-19 disease. The selected samples were characterized as SARS-CoV-2 positive by either the Respiratory Virus (16-

well) Panel (20620) (AusDiagnostics UK Ltd, Buckinghamshire, United Kingdom) or VIASURE *SARS-CoV-2* Real time PCR detection kit VS-NCO296TE (CerTest BioTech, Zaragoza, Spain) and fully characterised by WGS. Therefore, the information of the mutations that are associated with VoC (Variants of Concern) was known. After the routine diagnosis, the samples were stored in optimal conditions (-80 °C) until the completion of this analysis.

5. VIASURE *SARS-CoV-2 Variant I* and *SARS-CoV-2 Variant II* REAL TIME PCR DETECTION KITs

CerTest Biotec designed and analytically validated two real-time RT-PCR assays for the qualitative detection of genetic mutations in the *S* gene from SARS-CoV-2 RNA. The VIASURE *SARS-CoV-2 Variant I* detects the E484K, K417N, K417T and N501Y mutations and the VIASURE *SARS-CoV-2 Variant II* detects the P681R, E484Q and L452R mutations.

Both assays are intended for use for the identification of genetic mutations in the S gene (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R) in combination with clinical and epidemiological risk factors.

The detection is performed by a one-step real time RT-qPCR 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 E484K, K417N, K417T, N501Y, P681R, E484Q and L452R using specific primers and a fluorescent-labelled probes.

VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit channels			
E484K	K417N	K417T	N501Y
FAM	ROX	HEX	Cy5

VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit channels			
P681R	E484Q	L452R	RNASeP
FAM	ROX	HEX	Cy5

The VIASURE SARS-CoV-2 Variant II assay also uses a human reference gene as an endogenous internal control (human RNase P gene) and is detected in channel Cy5.

Mutation	Detected in known Lineages
E484K	B.1.1.28.1, B.1.1.33, B.1.351, B.1.525
K417T	B.1.1.28.1
K417N	B.1.351
N501Y	B.1.1.28.1, B.1.1.7, B.1.351

The presence of mutations in the *S* gene have been detected in the lineages summarised above[1].

Mutation	Detected in known Lineages
P681R	B.1.617.1, B.1.617.2, B.1.617.3, and A.23.1
L452R	A.27, B.1.427, B.1.429, B.1.526.1, B.1.617.1, B.1.617.2 and B.1.617.3
E484Q	B.1.617.1 and B.1.617.3

The presence of mutations in the S gene have been detected in the lineages summarised above.

[1] (https://www.gov.uk/government/publications/covid-19-variants-genomically-confirmed-case-numbers/variants-distribution-of-cases-data).

5.1 Method

SARS-CoV-2 RNA extraction from clinical samples was performed on the automated KingFisher™ Flex system (Thermo-Fisher SCIENTIFIC) instrument using MagMax™ Viral/Pathogen II (MVP II) Nucleic Acid Isolation kit (A48383). Total nucleic acid was extracted from 200 µl of SARS-CoV-2 positive archived clinical respiratory samples and eluted into a final volume of 50 µl of molecular grade water. The amplification and analysis of the VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit VS-VAR196TRUO (Batch: VAR196TRUO-Exp532D, expiry date: 04/2023) and the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit VS-VAI196TRUO (Batch: VAI196TRUO-Exp532, expiry date: 04/2023) was conducted using the QuantStudio™ 5 Real-Time PCR System (ThermoFisher Scientific).

6. DATA COLLECTION AND ANALYSIS

All raw data was analysed using DA2 Design and Analysis Software Version 2.5.1 (Thermo Fisher Scientific) and all data collated in an Excel file "Viasure_VOC_Results_all tests V6 and REPORT Detecting known variants of concern".

7. ANALYSIS OF INCONGRUENT RESULTS

In this case, as the selected samples were sequenced, the true value is that obtained by the WGS.

8. RESULTS

8.1. Analytical Sensitivity (LoD)

To assess the analytical sensitivity, Alpha, Beta, Gamma and Delta variant material, provided by PHE Porton was used. In addition, the reference strain First WHO International Standard for SARS-CoV-2 RNA (NIBSC 20/146) was also used.

Using this material, serial dilutions were prepared (from 1:100 to 1:640,000).

Results from testing the dilution series of the variants gave an estimated analytical sensitivity. The predicted concentrations for each dilute provided the Limit of Detection for each variant. Each dilution (apart from neat samples) was tested in triplicate on two separate occasions. Summary data for the two replicates are recorded in Tables 2 to 11. Due to limited availability of the Delta material, this variant was only assessed once (Tables 10 and 11).

No amplification results indicated that the end point of the genotyping assay was reached. The first no amplification result was used as an indication of the assay's analytical sensitivity. Amplification/detection of mutation (Mut) is reported where Ct ≤40. Where no amplification was observed or where Ct >40 'No amp' is reported.

Table 2. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit measured using NIBSC 20/146 (England/02/2020)

Dilution of Variant NIBSC 20/146	Predicted conc. In IU/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	N501Y	E484K	K417N	K417T
neat	$50x10^6$	18-20	Positive	20.89	17.51	No Amp	No Amp	No Amp	No Amp
1:100	$50x10^4$	24-26	Positive	29.06	26.51	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:1,000	$50x10^3$	27-29	Positive	32.51	30.62	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:10,000	5000	30-32	Positive	34.94	34.89	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:20,000	2500	31-33	Positive	36.02	37.39	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:40,000	1250	32-34	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:80,000	625	33-35	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:160,000	313	34-36	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:320,000	156	35-37	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	78	36-38	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed.

VIASURE SARS-CoV-2 Variant I Real Time PCR Detection Kit did not detect the SARS-CoV-2 "Wild type" (reference strain First WHO International Standard for SARS-CoV-2 RNA (NIBSC 20/146)) (Table 2).

Table 3. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit measured using NIBSC 20/146 (England/02/2020)

Dilution of Variant NIBSC 20/146	Predicted conc. In IU/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	P681R	E484Q	L452R	RNAseP
neat	$50x10^6$	18-20	Positive	20.89	17.51	No Amp	No Amp	No Amp	Amp
1:100	$50x10^4$	24-26	Positive	29.06	26.51	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:1,000	$50x10^3$	27-29	Positive	32.51	30.62	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:10,000	5000	30-32	Positive	34.94	34.89	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:20,000	2500	31-33	Positive	36.02	37.39	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:40,000	1250	32-34	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:80,000	625	33-35	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:160,000	313	34-36	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:320,000	156	35-37	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	78	36-38	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed.

VIASURE SARS-CoV-2 Variant II Real Time PCR Detection Kit did not detect, the SARS-CoV-2 "Wild type" (reference strain First WHO International Standard for SARS-CoV-2 RNA (NIBSC 20/146)) (Table 3). RNAseP detection was variable and may represent process in material preparation.

Table 4. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit measured using Alpha variant (VOC-20DEC-01, B.1.1.7)

Dilution of Alpha variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			Pos / Neg	Ct value *N* gene	Ct value *Orf1ab* gene	N501Y	E484K	K417N	K417T
neat	$3.2x10^7$	18-20	Positive	20.20	18.42	Mut	No Amp	No Amp	No Amp
1:100	$3.2x10^5$	24-26	Positive	27.71	25.86	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:1,000	$3.2x10^4$	27-29	Positive	30.81	29.45	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:10,000	3162	30-32	Positive	33.67	32.99	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:20,000	1581	31-33	Positive	34.45	34.12	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:40,000	791	32-34	Positive	34.98	36.21	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:80,000	395	33-35	Positive	35.93	40.18	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:160,000	198	34-36	Positive	36.90	No Amp	Mut	No Amp	No Amp	No Amp
						Mut	No Amp	No Amp	No Amp
1:320,000	99	35-37	Positive	38.47	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	49	36-38	Negative	No Amp	No Amp	Mut	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Results from testing of the dilution series of the Alpha VOC-20DEC-01 variant shows the expected mutant N501Y detected down to an estimated concentration of 198 copies/mL. This strain showed the expected no amplification result for the E484K, K417N and K417T mutant variants.

Taking all the results together the overall analytical sensitivity of the Alpha variant **LOD = 198 copies/mL**.

Table 5. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit measured using Alpha variant (VOC-20DEC-01, B.1.1.7)

Dilution of Alpha variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			Pos / Neg	Ct value *N* gene	Ct value *Orf1ab* gene	P681R	E484Q	L452R	RNAseP
neat	$3.2x10^7$	18-20	Positive	20.20	18.42	No Amp	No Amp	No Amp	Amp
1:100	$3.2x10^5$	24-26	Positive	27.71	25.86	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	Amp
1:1,000	$3.2x10^4$	27-29	Positive	30.81	29.45	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:10,000	3162	30-32	Positive	33.67	32.99	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:20,000	1581	31-33	Positive	34.45	34.12	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:40,000	791	32-34	Positive	34.98	36.21	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:80,000	395	33-35	Positive	35.93	40.18	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:160,000	198	34-36	Positive	36.90	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:320,000	99	35-37	Positive	38.47	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	49	36-38	Negative	No Amp	No Amp	No Amp	No Amp	No Amp	Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

No amplification was observed using the Alpha variant with the VIASURE SARS-CoV-2 Variant II Real Time PCR Detection Kit (Table 5).

Table 6. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit measured using Beta variant (VOC-20DEC-02, B.1.351)

Dilution of Beta variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	N501Y	E484K	K417N	K417T
neat	$3.2x10^7$	18-20	Positive	19.04	16.66	Mut	Mut	Mut	No Amp
1:100	$3.2x10^5$	24-26	Positive	26.17	23.21	Mut	Mut	Mut	No Amp
						Mut	Mut	Mut	No Amp
1:1,000	$3.2x10^4$	27-29	Positive	29.89	27.23	Mut	Mut	Mut	No Amp
						Mut	Mut	Mut	No Amp
1:10,000	3162	30-32	Positive	32.83	30.65	Mut	Mut	Mut	No Amp
						Mut	Mut	Mut	No Amp
1:20,000	1581	31-33	Positive	33.71	31.70	Mut	Mut	No Amp	No Amp
						Mut	Mut	Mut	No Amp
1:40,000	791	32-34	Positive	34.53	33.00	Mut	Mut	No Amp	No Amp
						Mut	Mut	No Amp	No Amp
1:80,000	395	33-35	Positive	35.35	33.75	Mut	No Amp	No Amp	No Amp
						No Amp	Mut	No Amp	No Amp
1:160,000	198	34-36	Positive	35.34	35.73	Mut	Mut	No Amp	No Amp
						No Amp	Mut	No Amp	No Amp
1:320,000	99	35-37	Positive	37.94	38.45	Mut	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	49	36-38	Positive	37.88	37.67	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Results from testing of the dilution series of the Beta VOC-20DEC-02 variant shows the expected mutant N501Y detected down to an estimated concentration of 395 copies / ml, E484K detected down to an estimated concentration of 198 copies/mL and K417N detected down to 1581 copies/mL.

Taking all the results together the overall analytical sensitivity of Beta variant

LOD = 1581 copies/mL.

Table 7. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit measured using Beta variant (VOC-20DEC-02, B.1.351).

Dilution of Beta variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	P681R	E484Q	L452R	RNAseP
neat	$3.2x10^7$	18-20	Positive	19.04	16.66	No Amp	No Amp	No Amp	No Amp
1:100	$3.2x10^5$	24-26	Positive	26.17	23.21	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:1,000	$3.2x10^4$	27-29	Positive	29.89	27.23	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:10,000	3162	30-32	Positive	32.83	30.65	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:20,000	1581	31-33	Positive	33.71	31.70	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:40,000	791	32-34	Positive	34.53	33.00	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	Amp
1:80,000	395	33-35	Positive	35.35	33.75	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:160,000	198	34-36	Positive	35.34	35.73	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:320,000	99	35-37	Positive	37.94	38.45	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	49	36-38	Positive	37.88	37.67	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

No amplification was observed using the Beta variant with the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit (Table 7).

Table 8. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit measured using Gamma variant (VOC-21JAN-02, P.1).

Dilution of Gamma variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	N501Y	E484K	K417N	K417T
neat	$1.25x10^8$	18-20	Positive	16.96	15.55	Mut	Mut	No Amp	Mut
1:100	$1.25x10^6$	24-26	Positive	23.86	22.53	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:1,000	$1.25x10^4$	27-29	Positive	27.89	26.70	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:10,000	12589	30-32	Positive	30.61	29.92	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:20,000	6295	31-33	Positive	31.51	31.02	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:40,000	3147	32-34	Positive	32.48	31.86	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:80,000	1574	33-35	Positive	33.19	33.58	Mut	Mut	No Amp	Mut
						Mut	Mut	No Amp	Mut
1:160,000	787	34-36	Positive	34.19	37.51	No Amp	Mut	No Amp	No Amp
						Mut	Mut	No Amp	Mut
1:320,000	393	35-37	Positive	35.15	35.60	No Amp	No Amp	No Amp	No Amp
						No Amp	Mut	No Amp	No Amp
1:640,000	197	36-38	Positive	35.59	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Testing the Gamma variant dilution series detected the expected mutants N501Y, E484K and K417T. The N501Y and K417T mutant assays detected down to an estimated concentration of 787 copies/mL. The E484K detected down to 393 copies / mL.

Taking all the results together the overall analytical sensitivity of the Gamma variant

LOD = 787 copies/mL.

Table 9. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit measured using Gamma variant.

Dilution of Gamma variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	P681R	E484Q	L452R	RNAseP
neat	$1.25x10^8$	18-20	Positive	16.96	15.55	No Amp	No Amp	No Amp	Amp
1:100	$1.25x10^6$	24-26	Positive	23.86	22.53	No Amp	No Amp	No Amp	Amp
						No Amp	No Amp	No Amp	Amp
1:1,000	$1.25x10^4$	27-29	Positive	27.89	26.70	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:10,000	12589	30-32	Positive	30.61	29.92	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:20,000	6295	31-33	Positive	31.51	31.02	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:40,000	3147	32-34	Positive	32.48	31.86	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:80,000	1574	33-35	Positive	33.19	33.58	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:160,000	787	34-36	Positive	34.19	37.51	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:320,000	393	35-37	Positive	35.15	35.60	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp
1:640,000	197	36-38	Positive	35.59	No Amp	No Amp	No Amp	No Amp	No Amp
						No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

No amplification was observed using the Gamma variant with the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit (Table 9).

Table 10. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit measured using Delta variant (VOC-21APR-02, B.1.617.2).

Dilution of Delta variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	N501Y	E484K	K417N	K417T
neat	$7x10^6$	18-20	Positive	17.56	18	No Amp	No Amp	No Amp	No Amp
1:100	$7x10^4$	24-26	Positive	24.47	24.79	No Amp	No Amp	No Amp	No Amp
1:1,000	$7x10^3$	27-29	Positive	27.42	27.89	No Amp	No Amp	No Amp	No Amp
1:10,000	700	30-32	Positive	31.41	32.46	No Amp	No Amp	No Amp	No Amp
1:20,000	350	31-33	Positive	32.30	34.07	No Amp	No Amp	No Amp	No Amp
1:40,000	175	32-34	Positive	32.66	34.14	No Amp	No Amp	No Amp	No Amp
1:80,000	88	33-35	Positive	34.58	36.75	No Amp	No Amp	No Amp	No Amp
1:160,000	44	34-36	Positive	34.39	37.50	No Amp	No Amp	No Amp	No Amp
1:320,000	22	35-37	Positive	36.56	38.94	No Amp	No Amp	No Amp	No Amp
1:640,000	11	36-38	Positive	35.89	No Amp	No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit did not detect the Delta variant (Table 10).

Table 11. Analytical sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit measured using Delta variant (VOC-21APR-02, B.1.617.2).

Dilution of Delta variant	Predicted conc. In Copies/mL	PHE expected Ct value	SARS-CoV-2 screening assay result			VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			Pos/Neg	Ct value *N* gene	Ct value *Orf1ab* gene	P681R	E484Q	L452R	RNAseP
neat	$7x10^6$	18-20	Positive	17.56	18	Mut	No Amp	Mut	No Amp
1:100	$7x10^4$	24-26	Positive	24.47	24.79	Mut	No Amp	Mut	No Amp
1:1,000	$7x10^3$	27-29	Positive	27.42	27.89	Mut	No Amp	Mut	No Amp
1:10,000	700	30-32	Positive	31.41	32.46	Mut	No Amp	Mut	No Amp
1:20,000	350	31-33	Positive	32.30	34.07	Mut	No Amp	No Amp	No Amp
1:40,000	175	32-34	Positive	32.66	34.14	Mut	No Amp	No Amp	No Amp
1:80,000	88	33-35	Positive	34.58	36.75	No Amp	No Amp	No Amp	No Amp
1:160,000	44	34-36	Positive	34.39	37.50	No Amp	No Amp	No Amp	No Amp
1:320,000	22	35-37	Positive	36.56	38.94	Mut	No Amp	No Amp	No Amp
1:640,000	11	36-38	Positive	35.89	No Amp	No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Using the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit with the Delta variant dilution series, expected mutants P681R and L452R were detected. The P681R mutant assays detected down to an estimated concentration of 175 copies/mL. The L452R detected down to 700 copies / mL.

Taking all the results together the overall analytical sensitivity of Delta variant **LOD = 700 copies/mL**.

Table 12. Summary of LOD results

	Mutations required for PHE reporting. LOD in copies/mL				Additional kit mutations LOD in copies/mL		
	E484K	**K417N**	**K417T**	**P681R**	N501Y	E484Q	L452R
Alpha	-	-	-	-	198	-	-
Beta	198	1581	-	-	395	-	-
Gamma	393	-	787	-	787	-	-
Delta	-	-	-	175	-	-	700

8.2. Analytical Specificity

To assess the specificity of the primers and probes in the genotyping assay it is important to test the assays with panel of samples that are positive for A) respiratory viruses other than SARS-CoV-2 and B) lineages of the SARS-CoV-2 virus that do not contain the mutations of interest (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

In this evaluation the following control materials were used:

- First WHO International Standard for SARS-CoV-2 RNA (NIBSC 20/146)
- Nucleic Acid Amplification Technique (NAT) Panel for SARS-CoV-2 (NIBSC 20/266)
- ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Catalog Number: NATRPP-1

Table 13. Analytical Specificity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit. Specificity panel for respiratory viruses other than SARS-CoV-2

Panel	Respiratory targets Assessed	Target screening Call	VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
			N501Y	E484K	K417N	K417T
NIBSC 20/266	Coronavirus 229E	Negative	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Coronavirus NL63	Negative	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Influenza-B (B/J]iangsu/10.2003)	Negative	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	RSV A	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 1	Influenza A H1N1 (A/NY/02/09); Parainfluenza Type 4A and 4B; Rhinovirus Type 1A; Adenovirus Type 3	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 2	Influenza A H1 (A/New Caledonia/20/99); RSV Type A; Parainfluenza Type 1; Coronavirus NL63; *M. pneumoniae* (M129)	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 3	Influenza A H3 (A/Brisbane/10/07; RSV type B; Coronavirus OC43; Coronavirus HKU-1	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 4	Influenza B (B/Florida/02/06); Parainfluenza Type 3; Human Metapneumovirus (Peru6-2003); *L. pneumophila*;	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 5	Parainfluenza Type 2; Coronavirus 229E; Human Bocavirus; C. pneumoniae (CWL-029)	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 negative	negative	Negative	No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit did not detect mutations N501Y, E484K, K417N, or K417T in samples containing respiratory viruses other than SARS-~CoV-2 (Table 13).

Table 14. Analytical Specificity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit. Specificity panel for respiratory viruses other than SARS-CoV-2

Panel	Respiratory targets Assessed	Target screening Call	VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit			
			P681R	E484Q	L452R	RP
NIBSC 20/266	Coronavirus 229E	Negative	No Amp	No Amp	No Amp	Amp
NIBSC 20/266	Coronavirus NL63	Negative	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Influenza-B (B/J]iangsu/10.2003)	Negative	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	RSV A	Negative	No Amp	No Amp	No Amp	Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 1	Influenza A H1N1 (A/NY/02/09); Parainfluenza Type 4A and 4B; Rhinovirus Type 1A; Adenovirus Type 3	Negative	No Amp	No Amp	No Amp	Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 2	Influenza A H1 (A/New Caledonia/20/99); RSV Type A; Parainfluenza Type 1; Coronavirus NL63; *M. pneumoniae* (M129)	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 3	Influenza A H3 (A/Brisbane/10/07; RSV type B; Coronavirus OC43; Coronavirus HKU-1	Negative	No Amp	No Amp	No Amp	No Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 4	Influenza B (B/Florida/02/06); Parainfluenza Type 3; Human Metapneumovirus (Peru6-2003); *L. pneumophila*;	Negative	No Amp	No Amp	No Amp	Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 Pool 5	Parainfluenza Type 2; Coronavirus 229E; Human Bocavirus; C. pneumoniae (CWL-029)	Negative	No Amp	No Amp	No Amp	Amp
ZeptoMetrix NATrol Respiratory Pathogen Panel -1 negative	negative	Negative	No Amp	No Amp	No Amp	Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Using the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit no amplification of P681R, E484Q or L452R was observed in samples containing respiratory viruses other than SARS-~CoV-2 (Table 14).

Table 15. Analytical Specificity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit. Specificity panel for SARS-CoV-2 that do not contain the mutations of interest (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

Panel	Respiratory targets Assessed	Target screening assay Ct value		VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
		N	ORF1ab	N501Y	E484K	K417N	K417T
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 2	31.75	32.29	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 3	32.10	31.99	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 4	21.39	19.90	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 5	24.80	23.71	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 6	24.42	23.18	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 7	24.37	22.90	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 8	27.90	26.76	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 9	29.18	28.39	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 10	22.35	20.66	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 11	28.01	26.46	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 12	29.17	28.13	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 14	21.76	20.45	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 15	32.03	32.13	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 17	24.81	23.52	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 18	27.55	26.65	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 19	29.39	28.64	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 20	32.27	32.82	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 22	29.63	28.03	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 24	21.23	20.15	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 25	21.20	19.92	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 26	28.10	27.28	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 27	26.56	25.61	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 28	24.51	23.47	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 30	31.75	32.29	No Amp	No Amp	No Amp	No Amp
NIBSC 20/146	England/02/2020 (neat)	20.89	17.51	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Negative control sample 1	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Negative control sample 23	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Using the VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit no amplification of N501Y, E484K, K417N, or K417T was observed in SARS-CoV-2 positive samples known not to contain the mutations of interest (Table 15).

Table 16. Analytical Specificity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit. Specificity panel for SARS-CoV-2 that do not contain the mutations of interest (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

Panel	Respiratory targets Assessed	Target screening assay Ct value		VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit			
		N	ORF1ab	P681R	E484Q	L452R	RP
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 2	31.75	32.29	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 3	32.10	31.99	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 4	21.39	19.90	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 5	24.80	23.71	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 6	24.42	23.18	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 7	24.37	22.90	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 8	27.90	26.76	No Amp	No Amp	No Amp	Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 9	29.18	28.39	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 10	22.35	20.66	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 11	28.01	26.46	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 12	29.17	28.13	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 14	21.76	20.45	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 15	32.03	32.13	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 17	24.81	23.52	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 18	27.55	26.65	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 19	29.39	28.64	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 20	32.27	32.82	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 22	29.63	28.03	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 24	21.23	20.15	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 25	21.20	19.92	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 26	28.10	27.28	No Amp	No Amp	No Amp	Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 27	26.56	25.61	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 28	24.51	23.47	No Amp	No Amp	No Amp	Amp
NIBSC 20/266	BetaCoV/Australia/VIC 01/2020 sample 30	31.75	32.29	No Amp	No Amp	No Amp	Amp
NIBSC 20/146	England/02/2020 (neat)	20.89	17.51	No Amp	No Amp	No Amp	Amp
NIBSC 20/266	Negative control sample 1	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp
NIBSC 20/266	Negative control sample 23	No Amp	No Amp	No Amp	No Amp	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

Using the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit no amplification of P681R, E484Q, or L452R was observed in SARS-CoV-2 positive samples known not to contain the mutations of interest (Table 16).

8.3. Clinical sensitivity and specificity evaluation

A total of 71 clinical samples (nasopharyngeal samples) were analysed retrospectively. All clinical samples had lineages characterised by whole genome sequencing from the COG-UK and included 11 specimens of the Alpha variant B.1.1.7, 4 specimens of Beta variant B.1.351, 1 specimen of the Gamma variantP2, 3 specimens of the Delta variant B.1.617.2, 51 specimens of B.1.177 lineage and 1 B.1.1.198 (Tables 20 to 27).

From the total of samples, 70 were correctly detected; therefore, one incongruent result was obtained. A sample of the Delta lineage was not detected with the VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit. Neither of the two expected mutations could be detected (Tables 20, 25 & 27).

Table 17. Diagnostic specificity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit. Clinical samples positive for SARS-CoV-2 that do not contain the mutations of interest as determined by WGS (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

SARS-CoV-2 lineage identified by WGS	Target Screening Assay result		Genotyping assay result (Mut/No Amplification)				Confirm absence of SNP mutations in WGS
	Call	Calculated Ct Value	N501Y	E484K	K417N	K417T	
B.1.1.198	positive	21.9	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	16.82	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	16.77	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	14.59	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	25.22	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	23.81	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	14.58	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	12.85	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	17.13	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	25.41	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	19.2	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	26.12	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	21.95	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	17.07	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	18.88	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	12.32	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.24	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.77	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	33.68	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.3	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	23.55	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.36	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	17.49	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	23.89	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	20.8	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	26.28	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	14.52	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	27.98	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	24.02	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	15.92	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	17.26	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.44	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	23.08	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	25.51	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	13.82	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	8.81	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	24.73	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	24.58	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	16.39	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	19.5	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	19.39	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	15.9	No amp	No amp	No amp	No amp	Yes

SARS-CoV-2 lineage identified by WGS	Target Screening Assay result			Genotyping assay result (Mut/No Amplification)				Confirm absence of SNP mutations in WGS
	Call	N gene	Orf1ab	N501Y	E484K	K417N	K417T	
B.1.177	positive	23.34	25.22	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	22.32	20.88	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	24.93	24.24	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	32.88	35.81	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	27.00	26.69	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	28.30	27.42	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	32.17	32.45	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	31.15	33.01	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	21.98	21.00	No amp	No amp	No amp	No amp	Yes
B.1.177	positive	27.60	26.95	No amp	No amp	No amp	No amp	Yes

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit did not detect N501Y, E484K, K417N, or K417T in SARS-CoV-2 positive samples shown by WGS not to contain the mutations of interest.

Table 18. Diagnostic specificity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit. Clinical samples positive for SARS-CoV-2 that do not contain the mutations of interest as determined by WGS (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

SARS-CoV-2 lineage identified by WGS	Target Screening Assay result		Genotyping assay result (Mut/No Amplification)				Confirm absence of SNP mutations in WGS
	Call	Calculated Ct Value	P618R	E484Q	L452R	RNaseP	
B.1.1.198	positive	21.9	NT	NT	NT	NT	Yes
B.1.177	positive	16.82	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	16.77	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	14.59	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	25.22	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	23.81	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	14.58	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	12.85	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	17.13	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	25.41	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	19.2	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	26.12	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	21.95	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	17.07	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	18.88	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	12.32	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.24	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.77	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	33.68	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.3	NT	NT	NT	NT	Yes
B.1.177	positive	23.55	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.36	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	17.49	NT	NT	NT	NT	Yes
B.1.177	positive	23.89	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	20.8	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	26.28	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	14.52	NT	NT	NT	NT	Yes
B.1.177	positive	27.98	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	24.02	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	15.92	NT	NT	NT	NT	Yes
B.1.177	positive	17.26	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.44	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	23.08	NT	NT	NT	NT	Yes
B.1.177	positive	25.51	NT	NT	NT	NT	Yes
B.1.177	positive	13.82	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	8.81	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	24.73	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	24.58	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	16.39	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	19.5	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	19.39	NT	NT	NT	NT	Yes
B.1.177	positive	15.9	NT	NT	NT	NT	Yes

SARS-CoV-2 lineage identified by WGS	Target Screening Assay result			Genotyping assay result (Mut/No Amplification/Amp)				Confirm absence of SNP mutations in WGS
	Call	N gene	Orf1ab	P618R	E484Q	L452R	RNaseP	
B.1.177	positive	23.34	25.22	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	22.32	20.88	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	24.93	24.24	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	32.88	35.81	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	27.00	26.69	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	28.30	27.42	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	32.17	32.45	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	31.15	33.01	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	21.98	21.00	No amp	No amp	No amp	Amp	Yes
B.1.177	positive	27.60	26.95	No amp	No amp	No amp	Amp	Yes

Abbreviations: NT = Not tested due to insufficient availability of test material.

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit no amplification of P618R, E484Q, or L452R was observed in SARS-CoV-2 positive samples shown by WGS not to contain the mutations of interest.

Table 19. Diagnostic sensitivity of VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit. Clinical samples positive for SARS-CoV-2 that contain one or more of the mutations of interest as determined by WGS (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

SARS-CoV-2 lineage identified by WGS	LAN	Target Screening Assay result		Genotyping assay result (Mut/No Amplification)			
		Call	Calculated Ct Value	N501Y	E484K	K417N	K417T
B.1.1.7	D21.0006762	Positive	31.31	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006793	Positive	22.13	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006821	Positive	15.78	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006846	Positive	21.25	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006859	Positive	19.49	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006884	Positive	16.61	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0006944	Positive	26.10	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0007057	Positive	25.10	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0007170	Positive	27.46	Mut	No Amp	No Amp	No Amp
B.1.1.7	D21.0007175	Positive	20.83	Mut	No Amp	No Amp	No Amp
B.1.1.7	K21.0082485	Positive	14.80	Mut	No Amp	No Amp	No Amp
B.1.351	D21.0000431	Positive	24.04	Mut	Mut	Mut	No Amp
B.1.351	D21.0003115	Positive	18.78	Mut	Mut	Mut	No Amp
B.1.351	SA001 C1	Positive	19.58	Mut	Mut	Mut	No Amp
B.1.351	VOI SA001	Positive	18.52	Mut	Mut	Mut	No Amp
B.1.617.2	K21.0123228	Positive	33.43	No Amp	No Amp	No Amp	No Amp
B.1.617.2	V-F21-10342	Positive	22.88	No Amp	No Amp	No Amp	No Amp
B.1.617.2	B.1.617.2 ALEX	Positive	24.01	No Amp	No Amp	No Amp	No Amp
B.1.1.248 (P.2)	K20.084188	Positive	25.30	No Amp	Mut	No Amp	No Amp

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit detected N501Y in B.1.1.7 samples, N501Y, E484K, and K417N in B.1.351 samples, and E484K in the P.2 sample (Table 19).

Table 20. Diagnostic sensitivity of VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit. Clinical samples positive for SARS-CoV-2 that contain one or more of the mutations of interest as determined by WGS (E484K, K417N, K417T, N501Y, P681R, E484Q and L452R).

SARS-CoV-2 lineage identified by WGS	LAN	Target Screening Assay result		Genotyping assay result (Mut/No Amplification/Amp)			
		Call	Calculated Ct Value	P618R	E484Q	L452R	RNaseP
B.1.1.7	D21.0006762	Positive	31.31	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006793	Positive	22.13	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006821	Positive	15.78	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006846	Positive	21.25	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006859	Positive	19.49	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006884	Positive	16.61	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0006944	Positive	26.10	NT	NT	NT	NT
B.1.1.7	D21.0007057	Positive	25.10	NT	NT	NT	NT
B.1.1.7	D21.0007170	Positive	27.46	No Amp	No Amp	No Amp	Amp
B.1.1.7	D21.0007175	Positive	20.83	NT	NT	NT	NT
B.1.1.7	K21.0082485	Positive	14.80	No Amp	No Amp	No Amp	Amp
B.1.351	D21.0000431	Positive	24.04	No Amp	No Amp	No Amp	Amp
B.1.351	D21.0003115	Positive	18.78	No Amp	No Amp	No Amp	Amp
B.1.351	SA001 C1	Positive	19.58	No Amp	No Amp	No Amp	No Amp
B.1.351	VOI SA001	Positive	18.52	No Amp	No Amp	No Amp	No Amp
B.1.617.2	K21.0123228	Positive	33.43	No Amp	No Amp	No Amp	Amp
B.1.617.2	V-F21-10342	Positive	22.88	Mut	No Amp	Mut	Amp
B.1.617.2	B.1.617.2 ALEX	Positive	24.01	Mut	No Amp	Mut	Amp
B.1.1.248 (P.2)	K20.084188	Positive	25.30	NT	NT	NT	NT

Abbreviations: NT = Not tested due to insufficient availability of test material.

Mut: Amplification/detection of mutation where Ct ≤40.

No Amp: no amplification is observed or Ct >40.

The VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit detected P618R and L452R in B.1.617.2 samples. There was one discordant result where P618R and L452R was not detected in a B.1.617.2 sample where a late calculated Ct value was observed in the screening assay (Table 20).

Table 21. VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit results compared to WGS for N501Y mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit		+	-	Total
	+	15	0	15
	-	0	56	56
	Total	15	56	71

Table 22. VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit results compared to WGS for E484K mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit		+	-	Total
	+	5	0	5
	-	0	66	66
	Total	5	66	71

Table 23. VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit results compared to WGS for K417N mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit		+	-	Total
	+	4	0	4
	-	0	67	67
	Total	4	67	71

Table 24. VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit results compared to WGS for K417T mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit		+	-	Total
	+	0	0	0
	-	0	71	71
	Total	0	71	71

Table 25. VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit results compared to WGS for P681R mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit		+	-	Total
	+	2	0	2
	-	1	68	69
	Total	3	68	71

Table 26. VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit results compared to WGS for E484Q mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit		+	-	Total
	+	0	0	0
	-	0	71	71
	Total	0	71	71

Table 27. VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit results compared to WGS for L452R mutation.

		whole genome sequencing result		
VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit		+	-	Total
	+	2	0	2
	-	1	68	69
	Total	3	68	71

Table 28. True positive (TP), true negative (TN), false positive (FP) and false negative (FN), Sensitivity (SE), specificity (SP), PPV and NPV of the VIASURE *SARS-CoV-2 Variant I* and VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kits

Target	Overall agreement	TP	TN	FP	FN	SE	SP	PPV	NPV
Global	0.98 (0.92-0.99)	18	52	0	1	0.94 (0.74-0.99)	1 (0.93-1)	0.94 (0.74-0.99)	1 (0.93-1)
N501Y	1 (0.94-1)	15	56	0	0	1 (0.78-1)	1 (0.93-1)	1 (0.78-1)	1 (0.93-1)
E484K	1 (0.94-1)	5	66	0	0	1 (0.47-1)	1 (0.94-1)	1 (0.47-1)	1 (0.94-1)
K417N	1 (0.94-1)	4	67	0	0	1 (0.39-1)	1 (0.94-1)	1 (0.39-1)	1 (0.94-1)
K417T	1 (0.94-1)	0	71	0	0	NA	NA	NA	NA
P681R	0.98 (0.92-0.99)	2	68	0	1	0.66 (0.094-0.99)	1 (0.94-1)	1 (0.19-1)	1 (0.94-1)
E484Q	1 (0.94-1)	0	71	0	0	NA	NA	NA	NA
L452R	0.98 (0.92-0.99)	2	68	0	1	0.66 (0.094-0.99)	1 (0.94-1)	1 (0.19-1)	1 (0.94-1)

9. CONCLUSIONS

With this study it was possible to observe that the VIASURE *SARS-CoV-2 Variant I* Real Time PCR Detection Kit and VIASURE *SARS-CoV-2 Variant II* Real Time PCR Detection Kit present good analytical and clinical sensitivity and specificity compared to sequencing results.

No false reactivity was associated with the mutations when tested against a wide range of viral and bacterial respiratory pathogens.

Determination of the estimated sensitivity and specificity was limited by the availability of samples and specific linages containing all the mutations.

A turnaround time of 24 hours was achieved during validation of the assays using clinical samples that were previously tested using the VIASURE SARS-CoV-2 real time PCR assay.

These assays can be used combined as a tool for the detection of the mutations E484K, K417N, K417T, N501Y, P681R, E484Q and L452R in clinical samples or RNA material previously characterized as SARS-CoV-2 positive.

These assays can detect K417N, K417T, E484K and P681R mutations to aid identification of the VoC currently designated by Public Health England within the routine diagnostic setting as an alternative to WGS.

10. SIGNATURE

Orla Jupp 

Rose Davidson