Department of Health & Social Care

Desktop validation report for SARS-CoV-2 Nucleic Acid Detection Certest VIASURE SARS CoV-2 Real Time PCR Detection Kit

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Contents

Assay Description and Intended Purpose

1. A real time RT-PCR assay intended for the qualitative detection of SARS CoV-2 RNA. The detection is carried out in a 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 ORF1ab and N genes for SARS-CoV-2 using specific primers and a fluorescent-labelled probe.

2. Qualitative detection of SARS CoV-2 RNA in respiratory samples (nasopharyngeal swab, nasal swab and oropharyngeal swab) and saliva samples from individuals suspected of COVID-19 infection by their healthcare provider. This test is intended for use as an aid in the diagnosis of COVID-19 in combination with clinical and epidemiological risk factors.

3. Laboratory test kit intended for use with standard real time PCR instruments. The assay requires other laboratory equipment to be provided including RNA extraction platform/kits, centrifuge, vortex and micropipettes. Samples should be handled in a biosafety cabinet before extraction.

Type of sample used in the validation

1. For clinical validation, nasopharyngeal swabs placed into sterile Vircell transport medium or Biocomma sterile virus transport medium and preservation medium were used.

   For analytical sensitivity:

   - linearity: known concentration of specific and synthetic cDNA from SARS CoV-2 was used

   - limit of detection: Negative oropharyngeal swabs in viral transport medium, VTM-Vircell, were spiked with inactivated with a known concentration of frozen quantified heat-inactivated culture 2019 Novel Coronavirus, Strain:2019-nCoV/USA-WA-1/2020 (ATCC-VR-1986HK) (which were at the detection limit)

   Precision, intra-assay and inter-assay testing were performed using nasopharyngeal swabs collected in viral transport material which were spiked with a known concentration of synthetic cDNA for each gene target.

For analytical specificity and cross-reactivity, wet testing was performed on either direct clinical samples or spiked samples that are known positives for the most common respiratory pathogens, control cultures and DNA/RNA of known concentration acquired from ATCC or Zeptometrix. For clinical validation, nasopharyngeal swabs placed into sterile Vircell transport medium or Biocomma sterile virus transport medium and preservation medium were used.

2. The assay uses extracted RNA. In the IFU, the manufacturer states that the sample should be extracted according to the IFU of the chosen extraction method.

3. The kits should be stored at 2 to 4°C until the expiration date on the kit. The lyophilised positive control sample once it has been re-constituted, should be stored at -20°C. The manufacturer recommends aliquoting the control to minimise freeze-thaw cycles. The positive control has been validated as still being stable after 6 freeze-thaw cycles.

   For Tube format kits: Once the SARS-CoV-2 Reaction-Mix tube has been reconstituted, it may be kept it at 25ºC±5ºC 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).

Equipment and reagents

Equipment

- real-time PCR instrument (thermocycler)
- RNA extraction kit
- centrifuge for 1.5 mL tubes and PCR-well strips or 96-well plate (if available)
- vortex
- micropipettes (0.5 to 20 µL, 20 to 200 µL)
- filter tips
- powder-free disposable gloves
- loading block (for use with Qiagen/Corbett Rotor-Gene® instruments).

Reagents

All reagents to perform the assay are included in the kit.

Performance characteristics

From the VIASURE Technical Report on NCO2 1120 rev.07 provided by the manufacturer: The analytical sensitivity (linearity of the assay and tentative limit of detection or LoD) was determined by testing a series of ten-fold dilutions containing a known concentration (ranging from 107 to 101 copies per reaction) of specific and synthetic cDNA belonging to SARS-CoV-2. Every tenfold dilution was tested in triplicate as well as the last dilution around the detection limit which was tested 20 replicates. The arithmetic mean (X), the standard deviation (σ) and the coefficient of variation (CV%) were calculated and detailed in Tables 1 and 2. The assays were run on the Biorad CFX96 instrument.

	Copies/rxn							
Synthetic Orf1ab gene cDNA	10^7	10^6	10^5	10^4	10^3	10^2	10^1	0
X (Ct)	12.78	16.47	19.64	23.08	26.56	29.48	33.49	Neg.
σ	0.11	0.17	0.29	0.08	0.14	0.07	0.76	n.a.
CV %	0.87	1.02	1.48	1.34	0.51	0.24	2.27	n.a.

Table 1. Analytical sensitivity was evaluated with synthetic ORF1ab gene specific cDNA and VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº: NCO2XL-001, expiry date 2022-02), rxn = reaction, (Ct) = threshold cycle, (X) = arithmetic mean Ct value, (σ) = standard deviation, (CV %) = coefficient of variation, Neg = negative, n.a.= not applicable.

VIASURE SARS-CoV-2 Real Time PCR Detection Kit showed a tentative detection limit of ≥10 cDNA copies per reaction for ORF1ab gene. cDNA molecules can be detected with a concentration of ≥10 copies/rxn (positive rate of ≥95%). Initially, until quantitative culture or genomic RNA of SARS-CoV-2 was available, the lowest concentration of cDNA that yielded positive test results was considered the LoD.

	Copies/rxn							
Synthetic Orf1ab gene cDNA	10^7	10^6	10^5	10^4	10^3	10^2	10^1	0
X (Ct)	15.78	19.31	22.84	26.09	29.19	32.65	36.08	Neg.
σ	0.04	0.08	0.09	0.06	0.06	0.34	1.01	n.a.
CV %	0.26	0.43	0.39	0.23	0.19	1.03	2.81	n.a.

Table 2. Analytical sensitivity was evaluated with synthetic N gene specific cDNA and VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº: NCO2XL-001, expiry date 2022-02), rxn = reaction, (Ct) = threshold cycle, (X) = arithmetic mean Ct value,

(σ) = standard deviation, (CV %) = coefficient of variation, Neg = negative, n.a.= not applicable.

VIASURE SARS-CoV-2 Real Time PCR Detection Kit showed a tentative detection limit of ≥10 cDNA copies per reaction for N gene. cDNA molecules can be detected with a concentration of ≥50 copies/rxn and ≥10 copies/rxn (positive rate of ≥95% and ≥85%, respectively). Initially, until quantitative culture or genomic RNA of SARS-CoV-2 was available, the lowest concentration of cDNA that yielded positive test results was considered the LoD.

The qPCR efficiency was estimated at >97.2% (Slope -3.392). Linear regression showed R2 value of 0.990.

The analytical sensitivity of VIASURE SARS-CoV-2 Real Time PCR Detection kit was also tested on Cobas z480 Analyzer (Roche Molecular Diagnostics), DTprime Real-time Detection Thermal Cycler (DNA-Technology), DTLite Real-time Detection Thermal Cycler (DNA-Technology), Mic Real Time PCR Cycler bms and Applied Biosystems 7500 Fast Real-Time PCR System (Batch nº: NCO2XL-001, expiry date 2022-02), and RotorGene®Q (Qiagen) (Batch nº: NCO2RG-001, expiry date 2022-02). The results for all targets match with the LoD which a positive rate of 95% (≥10 copies/rxn).

Data from Basingstoke and North Hampshire Hospital: NIBSC inactivated virus was diluted from 106/mL to 103/mL and extracted using a Promega Maxwell.

		Viasure Orf1ab	**Viasure N**
NIBSC Inactivated virus	10^6/ml	20.69	23.53
NIBSC Inactivated virus	10^5/ml	25.3	27.98
NIBSC Inactivated virus	10^4/ml	27.11	29.72
NIBSC Inactivated virus	10^3/ml	30.19	32.76

Table 3: LLOD data from Basingstoke and North Hampshire Hospital.

There was no 102/mL sample dilution tested but the assay detected the 103/mL diluted virus, suggesting an LOD of >1000 copies/mL.

4. Manufacturer’s data on linearity from the VIASURE Technical Report on NCO2 1120 rev.07.

   Orf1Ab gene: The qPCR efficiency was estimated at >95.6% (Slope -3.433). Linear regression showed R2 value of 0.994.

N gene: The qPCR efficiency was estimated at >97.2% (Slope -3.392). Linear regression showed R2 value of 0.990.

In conclusion, all real-time PCR assays showed an acceptable efficiency and linearity, (R2) were >0.98 in all the target reactions tested.

5. Lowest Limits of Detection (LLOD): Where a validated standard dilution series was used LLOD should be calculated, using data from 4.1.2, in copies/ml (to align with the relevant MHRA) TPP). Where clinical positive material is used, copies/ml cannot be calculated; median CT value or dPCR should be given for the lowest dilution detected from the samples used in 4.1.1.

   An analysis was performed to determine the LoD of SARS-CoV-2 (ORF1ab and N gene targets) in genome copies per reaction (genome copies/rxn). The LoD was determined by testing five times four negative clinical oropharyngeal (throat) swabs (viral transport medium, VTM- Vircell-) (total twenty times) spiked with a known concentration of frozen quantified heat-inactivated culture 2019 Novel Coronavirus, Strain:2019-nCoV/USA-WA-1/2020 (ATCC-VR-1986HK) (which were at the detection limit). The dilutions contained a known concentration of heat-inactivated culture (2.0 genome copies per µL and 1.0 genome copies per µL). The four spiked samples were extracted with MagDEA Dx SV kit (Batch nº18M010, expiry date 2022-04), using the magLEAD® 12gC instrument (Precision System Science Co.) and analysed with VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº NCO2XL-055, expiry date 2022-04) in quintupled on BioRad CFX96™ Real-Time PCR Detection System. The arithmetic mean (X), standard deviation (σ) and coefficient of variation (CV%) were calculated and are detailed in Tables 4 and 5.

	Genome copies/rxn (VTM, Vircell)	
Negative samples spiked with quantified heat-inactivated culture 2019 Novel Coronavirus (ATCC-VR-1986HK) –ORF1ab gene	**20**	**10**
X (Ct)	33.97	35.41
σ	1.16	1.75
CV %	3.40	4.93
n	20/20	16/20

Table 4: The LoD was determined with quantified heat-inactivated culture 2019 Novel Coronavirus and VIASURE SARSCoV-2 Real Time PCR Detection Kit -FAM channel- ORF1ab gene (Batch nº: NCO2XL-055, expiry date 2022-04). rxn = reaction, (Ct) = threshold cycle, (X) = arithmetic mean Ct value, (σ) = standard deviation, (CV %) = coefficient of variation, (n) = number of samples amplified.

VIASURE SARS-CoV-2 Real Time PCR Detection Kit showed a detection limit of 20 genome copies per reaction for SARS-CoV-2 (ORF1ab gene) with a positive rate of ≥95%. This concentration was finally considered the LoD for SARS-CoV-2 (ORF1ab gene).

	Genome copies/rxn (VTM, Vircell)	
Negative samples spiked with quantified heat-inactivated culture 2019 Novel Coronavirus (ATCC-VR-1986HK) –ORF1ab gene	**20**	**10**
X (Ct)	34.73	35.37
σ	0.79	0.9
CV %	2.26	2.54
n	20/20	15/20

Table 5: The LoD was determined with quantified heat-inactivated culture 2019 Novel Coronavirus and VIASURE SARSCoV-2 Real Time PCR Detection Kit -ROX channel- N gene (Batch nº: NCO2XL-055, expiry date 2022-04). rxn = reaction, (Ct) = threshold cycle, (X) = arithmetic mean Ct value, (σ) = standard deviation, (CV %) = coefficient of variation, (n) = number of samples amplified.

VIASURE SARS-CoV-2 Real Time PCR Detection Kit showed a detection limit of 20 genome copies per reaction SARS-CoV-2 (N gene) with a positive rate of ≥95%. This concentration was finally considered the LoD for SARS-CoV-2 (N gene).

Precision and robustness

1. To determine the precision, intra-assay (repeatability) and inter-assay (reproducibility) were performed with a spiked nasopharyngeal swab collected in Viral Transport Medium (VTM) 2 ml (Ref: TM011(which includes 1 sterile Rayon swab), Vircell S.L., Spain), with a known concentration of synthetic cDNA of each target gene. For all the assays, three positive samples for at least one of the target genes encoding different fragments of SARS-CoV-2 genome and a negative sample for the pathogen were tested. The panel of samples were extracted with MagDEA Dx SV kit (Batch nº98M020, expiry date 2020-09), using the magLEAD® 12gC instrument (Precision System Science Co.) following manufacturer's instructions and were analyzed with VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº: NCO2XL-002, expiry date 2022-02). These assays were carried out with the Product variation with IC in the master mix as indicated in the “POC-43 Validation procedure for qPCR products”. Spiked specimens were stored frozen at -20 or -80ºC and were totally thawed, brought to room temperature and homogenised before testing. RNA/DNA samples were stored

at -20 or -80ºC until used for molecular analyses. The arithmetic mean (X), the standard deviation (σ) and the coefficient of variation (CV%) were calculated and the results were showed in Tables 6 and 7.

2. Intra-assay precision: Use the data for 5 replicate values from a single day from 4.1.1 to calculate Standard Deviation & Coefficient of Variation measurement, with the values for the latter to be <10%. To include the use of inhibition controls

   To carry out the intra-assay analysis, eight replicates of all samples were tested in the same run using VIASURE SARS-CoV-2 Real Time PCR Detection Kit and Bio-Rad CFX96TM Real-Time PCR Detection System. In addition, in the same run, the Positive and Negative Controls (PC and NC, respectively) were also analysed 8 times, as well as the Internal Control (IC). Ct values were obtained from the Negative sample and the Negative Control. Table 6 shows the results obtained in this assay.

Sample	**Target**	**Channel**	**X (Ct)**	**σ**	**CV %**
Positive 1	SARS CoV-2 (Orf1ab gene)	FAM	30.64	0.41	1.35
		ROX	Neg	n.a.	n.a.
Positive 3	SARS CoV-2 (N gene)	FAM	Neg	n.a.	n.a.
		ROX	31.98	0.40	1.26
Pool positive	Orf 1ab gene	FAM	31.31	0.59	1.87
	N gene	ROX	32.58	0.76	2.35
Negative sample	Orf 1ab and N genes	FAM/ROX	Neg	n.a.	n.a.
	Internal control	HEX	23.45	0.27	1.17
Positive control	SARS CoV-2 (Orf1ab gene)	FAM	21.89	0.11	0.50
	SARS CoV-2 (N gene)	ROX	23.51	0.18	0.76
Negative control	Orf1ab and N genes	FAM	Neg	n.a.	n.a.
	Internal control	HEX	23.62	0.26	1.12

Table 6. Intra-assay reproducibility of VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº: NCO2XL-002, expiry date 2022-02). (Ct) = threshold cycle. (X) = arithmetic mean Ct value, (σ) = standard deviation, (CV %) = coefficient of variation, Neg = negative, n.a.= not applicable.

3. Inter-assay precision: Use the data for 5 replicate values from multiple days from 4.1.1 for Standard Deviation & Coefficient of Variation with the values for the latter to be <15%.

The inter-assay values were determined by testing the different samples on three different days by three different operators with the VIASURE SARS-CoV-2 Real Time PCR Detection Kit run on Bio-Rad CFX96TM RealTime PCR Detection System. In a similar way, the Positive and Negative controls (PC and NC, respectively) were also analysed (Table 7).

Sample	Target	Channel	X (Ct)	σ	CV %
Positive 1	SARS CoV-2 (Orf1ab gene)	FAM	30.58	0.51	1.67
		ROX	Neg	n.a.	n.a.
Positive 3	SARS CoV-2 (N gene)	FAM	Neg	n.a.	n.a.
		ROX	32.20	0.42	1.30
Pool positive	Orf 1ab gene	FAM	31.36	0.50	1.61
	N gene	ROX	32.55	0.79	2.43
Negative sample	Orf 1ab and N genes	FAM/ROX	Neg	n.a.	n.a.
	Internal control	HEX	23.42	0.26	1.11
Positive control	SARS CoV-2 (Orf1ab gene)	FAM	22.00	0.08	0.34
	SARS CoV-2 (N gene)	ROX	23.66	0.24	1.02
Negative control	Orf1ab and N genes	FAM	Neg	n.a.	n.a.
	Internal control	HEX	23.61	0.32	1.34

Table 7. Inter-assay reproducibility of VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº: NCO2XL-002, expiry date 2022-02). (Ct) = threshold cycle. (X) = arithmetic mean Ct value, (σ) = standard deviation, (CV %) = coefficient of variation, Neg= negative, n.a.= not applicable.

4. No information on repeatability was provided by the manufacturer.

Analytical specificity (Interferences and cross-reactions)

1. Cross-reactivity to non-target samples/organisms. A range of samples either direct clinical samples or spiked samples that are known positives for other diseases, both closely related (, other coronaviruses), syndromic diseases (for example, other

respiratory viruses and bacteria) and common diseases (such as HIV, HBV, HCV, VZV, EBV, CMV) should be tested.

The Analytical Reactivity (Inclusivity) and Analytical Specificity (Cross-reactivity) of VIASURE SARS-CoV-2 ORF1ab and N targets were evaluated using both wet testing and in silico analysis.

The Analytical Reactivity (Inclusivity) and Analytical Specificity (Cross-reactivity) were in silico assessed by using publicly available nucleotide sequence database as NCBI Genbank (https://www.ncbi.nlm.nih.gov/genbank/), in particular NCBI Virus Severe acute respiratory syndrome coronavirus 2 data hub (https://www.ncbi.nlm.nih.gov/labs/virus/vssi/#/virus?SeqType_s=Nucleotide&VirusLineage_ss=SARS-CoV2,%20taxid:2697049), Global Initiative on Sharing All Influenza Data (GISAID EpiCoV database (https://www.gisaid.org/) and/or search and/or alignment tools as BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast), and an in-house bioinformatic analysis software.

The analytical specificity for this assay was confirmed by testing a panel of different microorganisms which represents the most common respiratory pathogens. No cross-reactivity of the VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batches nº: NCO2XL-00, NCO2XL-055 and NCO2XL-261, expiry dates 2022-02, 2022-04 and 2022-10) with genomic RNA/DNA of the selected pathogens was observed These assays were run on Bio-Rad CFX96™ Real-Time PCR Detection System. Please refer to the manufacturers IFU for full details.

No cross-reactivity of the VIASURE SARS-CoV-2 Real Time PCR Detection Kit (Batch nº NCO212HERUO-017 and -018, expiry date 2022-05) with genomic RNA/DNA of the selected pathogens and with nucleic acid extracted from pooled nasal wash was observed Extraction Control showed amplification in all the samples tested. The microorganism SARS Coronavirus, Parainfluenza virus 1, 2 and 4, Mycoplasma pneumoniae, and Pneumocystis jirovecii, were not available for wet testing at USA Laboratory, but they were analysed at CerTest facility and found negative.

In addition to microorganism wet testing, in silico analysis was performed to assess the specificity of the assay in relation to the microorganisms listed. BLAST (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and Primer-BLAST (http://www.ncbi.nlm.nih.gov/tools/primer-blast) analyses over each primer and/or probe against the sequences from NCBI Genbank Nucleotide Database (https://www.ncbi.nlm.nih.gov/genbank/) available as of May 7th, 2020, was performed.

Excepting SARS Coronavirus and Bat and Pangolin Coronaviruses, all the analysed organisms and sequences showed:

- ≤80% homology between one of the full-length primers or probes and any sequence present in the targeted microorganism

- homologous regions split in several sequence fragments spanning along the organism sequence, making PCR product amplifications unlikely

- no amplification product resulting in Primer-BLAST analysis or too lengthy (>800 nt) to hinder SARS-CoV-2 amplification and detection. Particularly, Leptospira (1) sequences CP021412.1 and CP000348.1 (GenBank ID) align with N gene forward primer with 5 mismatches and in resulting product length of 3326 nt and Staphylococcus aureus (2) sequence LS483317.1 (GenBank ID) aligns with ORF1ab gene reverse primer with 4 mismatches and in resulting product length of 828 nt

Based on the in silico analysis, it is predicted that the assay may cross-react with SARS-CoV, SARS-like coronaviruses, and animal coronaviruses (Bat and Pangolin Coronaviruses) (3), however, the aligned sequences show several mismatches. Besides, these animal coronaviruses have either not been identified in humans before or are considered eradicated, dating the last SARS coronavirus official diagnosis back to 2004. Therefore, the homology of primers and probes sequences to these viruses should cause no interference in the SARS-CoV-2 detection.

Diagnostic sensitivity and specificity (Clinical validation with confirmed positives and negatives)

1. Samples selected for this validation will be appropriate to the assay. Low medium and high viral load samples will be equally distributed to avoid increasing or lowering diagnostic sensitivity and specificity.

2. Diagnostic sensitivity: Confirmed clinical samples from patients (positive RT-qPCR result) should be used. Preferably, depending on the availability of samples, ~150 samples should be included to align with MHRA TPP. Clinical sensitivity (95% CI) and positive predictive value (PPV) should be calculated in comparison with a CE marked reference method that itself has sensitivity and specificity and a limit of detection within the specifications of the MHRA TPP. The CT values or equivalent for both the assessed and comparator assays must be included in the validation report.

   Based on clinical study data (See below for combined sensitivity and specificity data), the diagnostic sensitivity based on 237 RT-PCR positive samples is 98.7% (95% CI 96.04-99.67).

3. Diagnostic specificity: Confirmed clinical samples from patients (negative RT-qPCR result) should be used. Preferably, depending on the availability of samples, ~250

samples should be included to align with MHRA TPP. Clinical specificity (95% CI) and negative predictive value (PPV) should be calculated in comparison with a CE marked reference method that itself has sensitivity and specificity in line with the MHRA TPP. The CT values or equivalent for both the assessed and comparator assays must be included in the validation report.

Based on information from a clinical study from the manufacturer (see below for combined sensitivity and specificity data), The diagnostic specificity based on 287 RT-PCR negative samples is 100% (95% CI 98.35-100.00).

237 RT-PCR positive samples by four different comparator assays (72 positive with Simplexa™ COVID-19, 9 with Cobas® SARS-CoV-2, 77 with Allplex™ SARS-CoV-2 and 78 with VIASURE SARS-CoV-2) and 287 RT-PCR negative samples (101 with Simplexa™ COVID-19, 55 with Cobas® SARS-CoV-2, 57 with VIASURE SARS-CoV-2 and 75 with Allplex™ SARS-CoV-2) were used in the analysis.

Five comparative analyses have been carried out:

- VIASURE SARS-CoV-2 Real time PCR detection kit vs all assays used in routine diagnosis
- VIASURE SARS-CoV-2 Real time PCR detection kit vs Simplexa™ COVID-19 Direct assay
- VIASURE SARS-CoV-2 Real time PCR detection kit vs Cobas® SARS-CoV-2 real time RT-PCR test
- VIASURE SARS-CoV-2 Real time PCR detection kit vs Allplex™2019-nCoV Assay
- VIASURE SARS-CoV-2 Real time PCR detection kit vs VIASURE SARS-CoV-2 (N1+N2) Real time PCR detection kit

VIASURE SARS-CoV-2 Real time PCR detection kit vs all assays used in routine diagnosis:

Results were concordant for 521 specimens (99.4% overall agreement), including 234 positive and 287 negative specimens. A total of 3 discordant samples (3 false negative) were found in VIASURE SARS-CoV-2 Real Time PCR Detection kit compared to the molecular assays used in routine diagnosis (Tables 8 and 9).

		Reference Diagnosis Molecular Methods Table – header text		
Viasure		+	-	Total

		Reference Diagnosis Molecular Methods Table – header text		
SARS CoV-2 Real Time PCR Kit	+	234	0	234
	-	3	287	290
	Total	237	287	524

Table 8. VIASURE SARS-CoV-2 Real Time PCR Detection Kit results compared to reference molecular assays.

ORA	TP	TN	FP	FN	PPA	NPA
99.05%	234	287	0	3	98.7%	>99%

Table 9. True positive and negative values, false positive and negative values, Positive Percent agreement (PPA), negative percent agreement (NPA) and overall rates of agreement (ORA) for SARS-CoV-2 Real time PCR kit compared with routine methods.

CT values of VIASURE SARS CoV-2 Real Time PCR detection kit v's all comparator assays:

The CT values of the positive samples for both kits were compared in the low (<25), mid (25 to 30), high (30 to 35) and >35 range. The CT values for 67 samples were not available.

CT range	Comparator assay positive (All assays)	Proportion (%)	VIASURE SARS CoV-2 real time RT-PCR assay positive	Proportion (%)	PPA
<25	77	45.6%	79	46.7%	97.5%
25-30	29	17.2%	30	17.8%	96.7%
30-35	39	23.1%	36	21.3%	92.3%
>35	24	14.2%	21	12.4%	87.5%

Table 10. Comparison of CT values at low, mid, high and LOD ranges for VIASURE SARS CoV-2 Real Time PCR Detection kit against reference molecular assays.

VIASURE SARS-CoV-2 Real Time PCR detection kit vs Simplexa COVID-19 Direct kit:

From the total of samples included in this study, PCR analysis of 173 nasopharyngeal specimens was performed with Simplexa™ COVID-19 Direct assay. All these samples were collected in Vircell Transport medium. Following this, the retrospective-

comparative analysis was performed with VIASURE SARS-CoV-2 Real time PCR kit. Results were concordant for the 173 specimens (>99% overall agreement), including 72 positive and 101 negative specimens (Tables 11 and 12).

		Simplexa COVID-19 Direct Assay		
Viasure SARS CoV-2 Real Time PCR Kit		+	-	Total
	+	72	0	72
	-	0	101	101
	Total	72	101	173

Table 11. VIASURE SARS-CoV-2 Real Time PCR Detection Kit results compared to Simplexa™ COVID-19 Direct assay

ORA	**TP**	**TN**	**FP**	**FN**	**PPA**	**NPA**
>99%	72	101	0	0	>99%	>99%

Table 12. True positive and negative values, false positive and negative values, Positive Percent agreement (PPA), negative percent agreement (NPA) and overall rates of agreement (ORA) for SARS-CoV-2 Real time PCR kit compared with Simplexa™ COVID19 Direct assay.

CT values of VIASURE SARS CoV-2 Real Time PCR detection kit v’s Simplexa COVID-19 Direct kit:

The CT values of the positive samples for both kits were compared in the low (<25), mid (25 to 30), high (30 to 35) and LOD (>35) range. The CT values for 16 samples were not available.

CT range	**Comparator assay positive (All assays)**	**Proportion (%)**	**VIASURE SARS CoV-2 real time RT-PCR assay positive**	**Proportion (%)**	**PPA**
<25	40	71.4%	38	67.9%	95%
25 to 30	10	17.9%	11	19.6%	90.9%
30 to 35	6	10.7%	7	12.5%	85.7%
>35	0	N/A	0	N/A	N/A

Table 13. Comparison of CT values at low, mid, high and LOD ranges for VIASURE SARS CoV-2 Real Time PCR Detection kit against Diasorin Simplexa COVID-19 Direct assay.

PCR analysis of 64 nasopharyngeal specimens (9 were positive and 55 were negative) was performed with Cobas® SARS-CoV-2 real time RT-PCR test. All these samples were collected in Vircell Transport medium. Following this, the retrospective analysis was performed with VIASURE SARS-CoV-2 Real time PCR kit. Results were concordant in the 64 specimens (>99% overall agreement), including 9 positive and 55 negative specimens (Table 14 and 15).

		COBAS SARS CoV-2 Real Time RT-PCR Test		
Viasure SARS CoV-2 Real Time PCR Kit		+	-	Total
	+	9	0	9
	-	0	55	55
	Total	9	55	64

Table 14. VIASURE SARS-CoV-2 Real Time PCR Detection Kit results compared to Cobas® SARS-CoV-2 real time RT-PCR test.

ORA	**TP**	**TN**	**FP**	**FN**	**PPA**	**NPA**
>99%	9	55	0	0	>99%	>99%

Table 15. True positive and negative values, false positive and negative values, Positive Percent agreement (PPA), negative percent agreement (NPA) and overall rates of agreement (ORA) for SARS-CoV-2 Real time PCR kit compared with Cobas® SARS-CoV2 real time RT-PCR test.

CT values of VIASURE SARS CoV-2 Real Time PCR detection kit v's COBAS SARS CoV-2 Real Time RT-PCR test:

The CT values of the positive samples for both kits were compared in the low (<25), mid (25 to 30), high (30 to 35) and LOD (>35) range.

CT range	**Comparator assay positive (All assays)**	**Proportion (%)**	**VIASURE SARS CoV-2 real time RT-PCR assay positive**	**Proportion (%)**	**PPA**
<25	3	33.3%	3	33.3%	100%
25 to 30	2	22.2%	2	22.2%	100%
30 to 35	3	33.3%	2	22.2%	66.7%

CT range	Comparator assay positive (All assays)	Proportion (%)	VIASURE SARS CoV-2 real time RT-PCR assay positive	Proportion (%)	PPA
>35	1	11.1%	2	22.2%	50%

Table 16. Comparison of CT values at low, mid, high and LOD ranges for VIASURE SARS CoV-2 Real Time PCR Detection kit against COBAS SARS CoV-2 Real time RT-PCR kit.

VIASURE SARS-CoV-2 Real Time PCR detection kit vs Allplex™2019-nCoV Assay

PCR analysis of 152 nasopharyngeal specimens (77 were positive and 75 were negative) was performed with Allplex™2019-nCoV Assay. Seven positive samples were in Vircell® transport medium and the rest were collected in Virus transport and preservation medium (Biocomma®). These last samples were inactivated before initial clinical diagnosis. Following this, the retrospective analysis was performed with VIASURE SARS-CoV-2 Real time PCR kit. As observed in Table 8, a total of 3 false negative (FN) were obtained using VIASURE SARS-CoV-2 compared to Allplex™2019-nCoV Assay. These 3 samples were on the LoD of the reference method and they presented amplification only in one (N gene) of the three targets (E, RdRP and N genes), of the reference assay. The N Ct values of these samples were between 35 to 39.5 on the reference assay. Both gene targets (N and Orf1ab) of the VIASURE SARS-CoV-2 Real Time PCR Detection Kit were negative with these samples. The overall agreement of both assays was 98.02% (Tables 17 and 18).

The incongruent results were analysed with the validated diagnostic workflow for 2019-nCoV published by Drosten C et al (known as the Charité protocol) [Detection of 2019 novel coronavirus (2019-nCoV) by real-time RT-PCR. Christian Drosten et al. Eurosurveillance. V. 25, 2020.] The 3 positive samples for Allplex™2019-nCoV Assay were also positive for Charité assay indicating that VIASURE SARS-CoV-2 Real Time PCR Detection Kit is reporting 3 false negative samples.

		Allplex™2019-nCoV Assay		
Viasure SARS CoV-2 Real Time PCR Kit		+	-	Total
	+	74	0	74
	-	3	75	78
	Total	77	75	152

Table 17. VIASURE SARS-CoV-2 Real Time PCR Detection Kit results compared to Allplex™2019-nCoV Assay.

ORA	TP	TN	FP	FN	PPA	NPA
98.02%	74	75	0	3	>96.1%	>99%

Table 18. True positive and negative values, false positive and negative values, Positive Percent agreement (PPA), negative percent agreement (NPA) and overall rates of agreement (ORA) for SARS-CoV-2 Real time PCR kit compared with Allplex™2019-nCoV Assay.

CT values of VIASURE SARS CoV-2 Real Time PCR detection kit v's Seegene Allplex 2019-nCoV assay:

The CT values of the positive samples for both kits were compared in the low (<25), mid (25 to 30), high (30 to 35) and LOD (>35) range.

CT range	Comparator assay positive (All assays)	Proportion (%)	VIASURE SARS CoV-2 real time RT-PCR assay positive	Proportion (%)	PPA
<25	28	36.4%	30	39%	93.3%
25 to 30	5	6.5%	5	6.5%	100%
30 to 35	21	27.3%	20	26%	95.2%
>35	23	29.9%	19	24.7%	82.6%

Table 19. Comparison of CT values at low, mid, high and LOD ranges for VIASURE SARS CoV-2 Real Time PCR Detection kit against Seegene Allplex 2019-nCoV assay.

VIASURE SARS-CoV-2 Real Time PCR detection kit vs VIASURE SARSCoV-2 (N1+N2) Real Time PCR assay

PCR analysis of 135 nasopharyngeal specimens (78 were positive and 57 were negative) was performed with the VIASURE SARS-CoV-2 (N1+N2) Real Time PCR assay open format. All these samples were collected in Vircell® transport medium. Following this, the retrospective analysis was performed with VIASURE SARS-CoV-2 Real time PCR kit. Results were concordant for the 135 specimens (100% overall agreement), tables 23 and 24.

		VIASURE SARS-CoV-2 (N1+N2) Real Time PCR assay		
Viasure SARS CoV-2 Real Time PCR Kit		+	-	Total
	+	78	0	78
	-	0	57	57
	Total	78	57	135

Table 20. VIASURE SARS-CoV-2 Real Time PCR Detection Kit results compared to VIASURE SARS-CoV-2 (N1 + N2) Real Time PCR assay/

ORA	TP	TN	FP	FN	PPA	NPA
>99%	78	57	0	0	>99%	>99%

Table 21. True positive and negative values, false positive and negative values, Positive Percent agreement (PPA), negative percent agreement (NPA) and overall rates of agreement (ORA) for VIASURE SARS-CoV-2 Real time PCR kit compared with VIASURE SARS-CoV-2 (N1 + N2) Real Time PCR assay.

CT values of VIASURE SARS CoV-2 Real Time PCR detection kit v's VIASURE SARS-CoV-2 (N1+N2) Real Time PCR Assay:

The CT values of the positive samples for both kits were compared in the low (<25), mid (25 to 30), high (30 to 35) and LOD (>35) range. The CT values for 51 samples were not available.

CT range	Comparator assay positive (All assays)	Proportion (%)	VIASURE SARS CoV-2 real time RT-PCR assay positive	Proportion (%)	PPA
<25	6	22.2%	8	29.6%	75%
25 to 30	12	44.4%	12	44.4%	100%
30 to 35	9	33.3%	7	26%	77.8%
>35	0	N/A	0	N/A	N/A

Table 22. Comparison of CT values at low, mid, high and LOD ranges for VIASURE SARS CoV-2 Real Time PCR Detection kit against VIASURE SARS-CoV-2 (N1+N2) Real Time PCR Assay.

4. Evaluation data from NEQAS and NIBSC panels

Evaluation of the VIASURE SARS-CoV-2 Real Time PCR Detection Assay was undertaken at Basingstoke and North Hampshire Hospital using samples from 3 External Quality Assessment panels, 2 panels from NEQAS and one from NIBSC. The data on the evaluations are presented in Tables 23, 24, 25 and 26 below.

No. Samples in Panel	Positive	Negative	Equivocal
NEQAS (40 samples)	22	18	0
NEQAS (20 samples)	15	4	1
NIBSC (30 samples)	24	6	0
Total - 90	61	28	1

Table 23: Total number of samples in panels and results from evaluation using VIASURE SARS-CoV-2 Real Time PCR Detection Assay.

NEQAS 40

CT Range	No. Positive Samples	Proportion of Total Positive Samples (%)
<25	0	0
25 to 30	14	63.7
30 to 35	7	31.8
>30	1	4.5

Table 24: CT range for RT-PCR positive samples using NEQAS 40 sample panel

. NEQAS 20

CT Range	No. Positive Samples	Proportion of Total Positive Samples (%)
<25	0	0
25 to 30	4	26.7
30 to 35	9	60.0
>30	2	13.3

Table 25: CT range for RT-PCR positive samples using NEQAS 20 sample panel.

NIBSC

CT Range	No. Positive Samples	Proportion of Total Positive Samples (%)
<25	13	54.2
25 to 30	11	45.8
30 to 35	0	0
>30	0	0

Table 26: CT range for RT-PCR positive samples using NIBSC panel

Summary

This assay meets the desirable criteria for sensitivity and specificity of the MHRA TPP for Laboratory based SARS CoV-2 Viral Detection tests with a sensitivity of 98.7% (95% CI 96.04-99.67), and a specificity of 100% (95% CI 98.35-100.00). The data from the manufacturer showed a LLOD of 20 genome copies/rxn for the Orf1ab gene and 20 genome copies/rxn for the N gene with >95% samples tested. A separate study at Basingstoke and North Hampshire Hospital gave a LLOD of <1000 copies/mL.

TVG uses a wide range of sites in order to validate new technologies or tests. These independent sites use a range of RT-qPCR assays against different genomic regions and it is recognised that for some assay comparisons the sensitivity of RT-qPCR assay(s) may subtly differ from the true sensitivity of the test if compared to the same genomic region.

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