Department of Health & Social Care

Technical Validation of Nonacus VirPath Sars-CoV-2 Multiplex qRT-PCR Kit

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Contents

Assay description and intended purpose

1. The SARS-CoV-2 multiplex qRT-PCR kit is a qualitative real-time reverse transcription polymerase chain reaction (RT-PCR) assay intended to be used for the nucleic acid detection of Severe Acute Respiratory Syndrome Virus coronavirus-2 (SARS-CoV-2).

   VirPath Sars-CoV-2 Multiplex qRT-PCR kit IFU version v1.0.3.

2. The SARS-CoV-2 multiplex qRT-PCR kit is a qualitative real-time reverse transcription polymerase chain reaction (RT-PCR) assay intended to be used for the detection of Severe Acute Respiratory Syndrome Virus coronavirus-2 (SARS-CoV-2). The kit is compatible with genomic RNA extracted from biological samples derived from lower or upper respiratory tract specimens collected from individuals that meet the criteria for testing. The inhibitor tolerant qRT-PCR mix provided in the kit is a one-step solution designed for amplification of the SARS-CoV-2 RNA targets.

   Included in the kit is a multiplex of primer or probe assays which target the nucleocapsid gene (assay N1) and the envelope gene (assay E) of SARS-CoV-2; and the RPP30 gene (assay RP) used as internal RNA extraction control. Two controls are also included in the kit to confirm functionality of the assays and the qRT-PCR reaction: the RPP30 Negative Control and the combined 2019-nCoV nucleocapsid gene and 2019-nCoV envelope gene Positive Control. The kit also contains ROX reference dye at 10x concentration for use with real-time quantitative PCR platforms that require it.

   Performance has been verified using the BioRad CFX96, controlled by the CFX software. The assay should be compatible with other equivalent real-time PCR cyclers but validation should be performed prior to use.

   Testing is intended for use by trained laboratory technicians who are proficient in performing molecular based tests.

3. The VirPath RT-PCR assay is intended for use with extracted nucleic acid. Biological safety precautions are not required for the assay, standard SARS-CoV-2 biosafety precautions are required for pre-analytics and nucleic acid extraction of samples.

Type of sample to be used in validation

1. Whole non-extracted virus received in VTM. The assay has been validated on nasopharyngeal swabs, the manufacturers IFU also states that lower respiratory tract specimens (for example bronchoalveolar lavage, sputum, tracheal aspirate) and upper respiratory tract specimens (for example nasopharyngeal fluids, nasal swab). Local validation of these sample types will be required.

2. Extracted RNA is required.

3. Previously tested VTM samples were frozen post processing and residual sample tested. The samples have gone through at least one additional freeze thaw cycle prior to processing. To assess any impact of the freeze thaw cycle, the products were compared to both the original comparator results and processed in parallel using the QuantuMDx SARS-CoV-2 RT-PCR Detection Assay.

Equipment and reagents

1. Equipment required:

    - Class II Biological safety cabinet.
    - Single and/or multichannel pipettes (10, 100, 200, 1000 ul)
    - PCR-clean filtered tips
    - 1.5 or 2 ml cold block (or access to ice)
    - 96 well cold block (or access to ice)
    - qPCR Instrument (4 colour) e.g. BioRad CFX 96 (or other validated QPCR instrument)
    - 96 well plate and optical seal compatible with qPCR instrument
    - 96 well plate compatible vortexer
    - 96 well plate compatible minifuge or centrifuge

2. Reagents required:

    - Molecular biology grade water

Performance characteristics

Analytical Sensitivity and Linearity of SARS COV-2 targets

1. A dilution series was created using a pool of negative VTMs which was subsequently spiked with an inactivated enveloped control ATCC (VR-1986HK = 1.92 x 105 genome copies/µl), the concentration of the lot was verified by ATCC using ddPCR.

   Results summary:

Copies/ml	Log 10	Mean E Cq	E assay +ve replicates	Mean N Cq	N assay +ve replicates
250	2.40	35.33	2/3	34.36	3/3
500	2.70	35.76	3/3	33.91	3/3
750	2.88	34.88	3/3	32.87	3/3
1000	3	34.80	3/3	32.02	3/3
10000	4	31.47	3/3	29.07	3/3
100000	5	28.30	3/3	25.80	3/3

   Conclusions:

   The N gene assay detected 3/3 replicates as positive down to 250 copies / ml.

   The E gene assay detected 3/3 replicates as positive down to 500 copies / ml.

2. A dilution series was created using a pool of negative VTMs which was subsequently spiked with an inactivated enveloped control ATCC (VR-1986HK = 1.92 x 105 genome copies/µl), the concentration of the lot is verified by ATCC using ddPCR.

3. The combined multiplex limit of detection verified by the dilution series in 4.1.2 was determined as between 250 and 500 copies / ml.

Precision and robustness

1. In order to assess intra-assay variability, 24 replicates were processed on the same day. Below is a summary of the data for each viral gene target, the table compares Ct values.

-	**E gene Cq**	**N gene Cq**	**RP IC Cq**
Mean	20.43	17.97	21.73
Standard Deviation	0.26	0.15	0.18
Coefficient of variance %	1.28	0.82	0.82

2. In order to assess inter-assay variability, 12 replicates were processed over multiple plates, instruments and days. Below is a summary of the data for each viral target, the table compares Ct values.

-	**E gene Cq**	**N gene Cq**	**RP IC Cq**
Mean	19.17	17.35	24.39
Standard Deviation	0.10	0.20	3.37
Coefficient of variance %	0.55	1.17	13.83

3. 30 individual negative samples were spiked with an inactivated enveloped control ATCC (VR-1986HK = 1.92 x 105 genome copies/µl) to ~5x LOD. Below is a summary of the data for each viral target. RNA was detected in every replicate (100% detection)

-	**E gene Cq**	**N gene Cq**	**RP IC Cq**
Mean	32.42	29.82	26.18
Standard Deviation	0.56	0.49	1.59
Coefficient of variance %	1.73	1.63	6.09

4. 30 individual negative samples were spiked with a previously positive sample. Below is a summary of the data for each viral target, the table compares Ct. RNA was detected in every replicate (100% detection)

-	**E gene Cq**	**N gene Cq**	**RP IC Cq**
Mean	29.77	28.10	26.26
Standard Deviation	0.33	0.26	1.63
Coefficient of variance %	1.10	0.91	6.21

Analytical specificity (interferences and cross-reactions)

1. Cross reactivity was assessed using the Microbiologics 21 control panel.

Target	Source	Result
Adenovirus Type 6	Microbiologics	Negative
Bordetella parapertussis	Microbiologics	Negative
Bordetella pertussis	Microbiologics	Negative
Chlamydia pneumoniae	Microbiologics	Negative
Coronavirus 229E	Microbiologics	Negative
Recombinant Coronavirus HKU1	Microbiologics	Negative
Recombinant Coronavirus NL63	Microbiologics	Negative
Recombinant Coronavirus OC43 Strain 1 and Recombinant Coronavirus OC43 Strain 2	Microbiologics	Negative
Recombinant Human Metapneumovirus	Microbiologics	Negative
Human Rhinovirus	Microbiologics	Negative
Influenza A	Microbiologics	Negative
Influenza A subtype H1	Microbiologics	Negative
Influenza A subtype H1-2009	Microbiologics	Negative
Influenza A subtype H3	Microbiologics	Negative
Influenza B	Microbiologics	Negative
Mycoplasma pneumoniae	Microbiologics	Negative
Parainfluenza Virus 1	Microbiologics	Negative
Parainfluenza Virus 2	Microbiologics	Negative
Parainfluenza Virus 3	Microbiologics	Negative
Recombinant Parainfluenza Virus 4a	Microbiologics	Negative
Respiratory Syncytial Virus	Microbiologics	Negative

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

1. 534 samples were analysed using blinded samples that had been tested using the current standard of care assays in two clinical ISO15189/UKAS accredited laboratories. The samples were processed Nonacus - VirPath Sars-CoV-2 Multiplex qRT-PCR kit C3COV191 v1.0.2 and results were verified using the QuantuMDX qRT-PCR assay, the latter is a CE marked qRT-PCR assay that meets the specification of the MHRA TPP for laboratory testing. In the final data, 12 samples were discounted. All discounted samples have CTs of >35-40 and were negative in 2 of 3 assays. 522 samples were included in the final analysis and included samples across the dynamic range of the assay

   Below provides a categorisation of a subset* of positive samples by CT

Ct Value	**<25**	**25-<30**	**30-<35**	**>35**
(N) in category	50	43	60	41

   *All CTs included except for those tests performed on a qualitative SoC

2. 534 samples were analysed using blinded samples that had been tested using the current standard of care assays in two clinical ISO15189/UKAS accredited laboratories. From the sum of data, 12 samples were discounted. All discounted samples had CTs of >35-<40 and were negative in 2 out of 3 assays. 522 samples were included in the final analysis and included samples across the dynamic range of the assay.

   Contingency table, VirPath compared to the standard of care rt-PCR method

Virpath	**Comparator SoC Assay**	**Comparator assay**
-	Positive	Negative
Positive	215	3
Negative	3	301
Total	218	304

   Sensitivity, specificity and predictive values of the VirPath assay compared to standard of care assay

-	-	**95% CI**
Sensitivity %	98.62	96.03 to 99.53
Specificity %	99.02	97.17 to 99.67
PPV	98.62	-
NPV	98.01	-

3. Diagnostic specificity: Confirmed clinical samples from patients (negative RT-qPCR result) were tested. 304 samples were included to align with MHRA TPP. The CT values or equivalent for positive samples in both the assessed and comparator assays are summarised in the validation report.

Diagnostic specificity was established at 99.02%

4. Conclusion

   The VirPath RT-PCR assay validation results fall within the required range for laboratory-based PCR assays as set out in the MHRA TPP (Version 1.0). The limitation of the validation included the sample types (Nasopharyngeal swabs only), the extraction amplification platform. Laboratories using the VirPath assay would need to locally validate and verify extraction and amplification platforms other than those stated in the manufacturers IFU (version 1.0.3).

   The validation was performed on samples that were stored frozen and subjected to 2 x freeze thaw cycles, therefore the CT values obtained in the VirPath assay may be artificially later (higher) than those that would have been obtained using freshly collected materials.

   TVG uses a wide range of sites in order to validate new technologies/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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