Department of Health & Social Care

Technical Validation for SARS-CoV-2 Nucleic Acid Detection

(SARS-CoV-2 RT-PCR and diagnosis in end-point detection configuration, using high-capacity PCR instrumentation and consumables (LGC Group Ltd, V1.0) and Diagnostic determination software (Ugen TecNV, v1.0)

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Assay description

1. End-point PCR (EPCR) diagnosis of SARS-CoV-2 (and other human disease statuses) leverages established, classical methods of RT-PCR target amplification, but detection and diagnosis are performed in a configuration enabling higher specimen processivity compared to quantitative RT-PCR (qPCR). While qPCR requires real-time kinetic reaction monitoring, EPCR can attain the comparable diagnostic outcome through monitoring the abundance of amplification product upon completion of the PCR process. Coupled with decision-support software that uses intelligent algorithm to automatically deliver a diagnostic determination, EPCR allows for higher batch capacity, lower in-life processing times, and assay miniaturisation that enhance the speed, cost and attainable test-capacity of the classical RT-PCR diagnostic methods.

2. A performance evaluation has established high assay concordance and suitable diagnostic outcomes using EPCR to detect the presenceSARS-CoV-2 infection, by directly comparing the method of qPCR detection using a paired study design and specimens run in parallel. The study was conducted using combined nose and throat swab analytes from nearly 50,000 individuals.

3. In a first test-site implementation, EPCR technology was established as a parallel detection method, suitable for integration with standard RNA extraction and purification methods already used for qPCR detection of SARS-CoV-2. While the EPCR performance evaluation was conducted in a Biosafety Level 2 facility, EPCR instrumentation, personnel and diagnostic procedures need not be performed under highest biosafety level precautions.

Type of sample to be used in validation

1. In parallel tests using an established qPCR workflow, EPCR performance evaluation utilised nasopharyngeal/oronasal swab analytes in standard viral transport media matrices. Viral antigen RNA was extracted from whole, live-virus analytes, using existing magnetic bead capture and physical isolation methods (Thermo Scientific).

2. EPCR uses the identical extracted RNA input material as existing qPCR methods, by aliquoting from analytes presented in a stabilised post-extraction elution buffer(Thermo). Specimens are typically extracted from 0.2mL of viral transport media, and all analyte resuspended in 50uL elution buffer. Reaction volumes for RT-PCR amplification and detection are smaller for EPCR compared to qPCR (Thermo Taqpath, 96-well detection on ABI7500 devices).In EPCR, a5uL total reaction volume is dispensed by the Nexar instrument (LGC Ltd), comprising 3.8uL of elution buffer RNA analyte which is added to 1.2uL of the reaction and detection assay mixture (LGC Ltd).

3. Specimen presentation parameters were derived from historical specifications previously established at the performance evaluation test site (UK Biocentre Ltd). A single NP swab specimen per testis sufficient: the new EPCR test requires no separate or additional steps for specimen extraction or storage. While not studied directly under idealised test conditions, viral RNA antigen specimens in the performance evaluation were intermittently subjected to temperature deviations typical and suitable to the qPCR workflow (including freeze-thaw for a small minority of specimens stored for archival and test repeat procedures). Specific studies for interfering or storage substances were not conducted: rather, the in-life workflow and concordance EPCR study established diagnostic precision using real-world specimen diluents and storage matrices, and compared directly to aqPCR method (Thermo Taqpath) where interfering and storage substances parameters are established.

Equipment and reagents

1. The evaluation test site employs an RNA extraction workflow using a variety of laboratory automation and standard equipment. Specimens are received and archived via a high-containment level procedure conducted in biosafety cabinets, using either manual pipettes (Gilson or comparable) or partial automation via Tecan robotic liquid handling instrumentation. The RNA extraction procedures are performed following viral inactivation and outside highest biosafety containment areas. To automate RNA extraction reagent dispensing and specimen processing, Thermo Kingfisher instrumentation is employed, along with Multidrop and Tecan instrumentation. A complete inventory and instrumentation asset register is available upon request: over 15 biosafety cabinets, 10 Tecan systems, 20 Thermofisher Kingfisher and 3 Multidrop devices were in use during the evaluation testing. All the equipment required to perform the test is supplied by the manufacturer except for laboratory consumables, saliva collection pots and nasopharyngeal/oropharyngeal swabs.

2. Two process controls were studied as known positives, extracted separately and in-parallel to the test specimens. An inactivated whole-virus SARS-CoV-2 was used as an established comparator standard (Qnostics), and the study also piloted use of an encapsidated whole pseudo-virus containing SARS-CoV-2 RNA genomic equivalents (Accuplex). The ubiquitous RNase P transcript present in specimen NP swab was used as an internal sample control(EPCR), or MS2 RNA analyte introduced as a bacteriophage whole virus-like particle prior to RNA extraction (qPCR).Negative process control was RNAse-free deionised water (Thermo), added in separate wells for parallel extraction.

Performance characteristics

1. Analytical Sensitivity and Linearity of SARS COV-2 targets: known positive control outcomes are only reported for the Qnostics whole-virus control. The Accuplex pseudo-viral control was studied only in exploratory assessments, with further studies intended prior to its inclusion in the instructions for use

2. Dilution series: the Qnostics control was utilised to generate a known-positive molecular detection range. Using input reference ranges established by digital-PCR quantitation, a 6-log dilution series was tested, with a top concentration of 1,000,000 SARS-CoV-2 genome equivalents per millilitre, and lowest concentration of 50 copies per millilitre. Specimens were introduced prior to RNA extraction, using a 0.2mL input volume of the control standard, presented in the manufacturer diluent with no further changes.

3. Linear range response and amplification efficiency: EPCR testing showed suitable dynamic response ranges over the entire dilution series tested. The precision and signal-to-noise characteristic remained high for all viral input concentrations, suggesting the majority of all plausible biological range concentrations will result in a discernible and accurately high signal range for endpoint detection.

Superplex Assay Result

Estimated Viral RNA Concentration as per the manufacturer (QNostics) (copies / millilitre)	N1/N2 Signal (normalised fluorescence)	Standard Deviation	Coefficient of Variation	Signal-to-Noise (Mean (pos)* Mean (neg)/SD (neg)
1,000,000	0.935	0.011	1.2%	224.59
100,000	0.932	0.023	2.4%	223.90
10,000	0.930	0.006	0.7%	223.45
5,000	0.919	0.006	0.7%	220.62
1,000	0.946	0.009	0.9%	227.33
500	0.956	0.014	1.4%	230.00
100	0.948	0.011	1.1%	228.01
50	0.586	0.199	34.1%	137.61
Blank	0.033	0.004	12.1%	N/A

4. As EPCR measures signal fluorescence after completion of the PCR amplification process, measures of signal amplification efficiency per se were not assessed. The general large signal-to-noise range observed across the test dilution range suggests that EPCR reaction efficiency is suitable for the assay purpose.

5. The lowest assay limit-of-detection(LLOD) for EPCR was observed to be 50 copies / mL genomic equivalents of SARS-CoV2 RNA (the lowest input concentration tested).As0.2mLof input specimen is added at the pre-extraction step, and a 1/10 volume of eluted RNA added to the RT-PCR reaction volume, this level of sensitivity appears consistent with assay performance for RNA analyte equivalents present at or near the single-molecule range within the final reaction detection volume.

Superplex Assay Result

Estimated Viral RNA Concentration as per the manufacturer (QNostics) (copies / millilitre)	**Rep 1**	**Rep 2**	**Rep 3**
1,000,000	Pos	Pos	Pos
100,000	Pos	Pos	Pos
10,000	Pos	Pos	Pos
5,000	Pos	Pos	Pos
1,000	Pos	Pos	Pos
500	Pos	Pos	Pos
100	Pos	Pos	Pos
50	Pos	Pos	Pos
Blank	Neg	Neg	Neg

Precision and robustness

1. Assay precision: Qnostics positive control (500 copies / mL genome equivalents) was tested four times in replicate on each assay plate, with multiple assay plates run each day. Per day, intra-assay precision exhibited coefficient of variation of <20% across at least 40 samples per day. An endpoint assay discrimination is more influenced by the deviation of positive samples relative to the assay background levels, and we observed <10% of positive control samples showed signal in marginal fluorescence ranges (>1 standard deviation below the daily mean).

2. Day-to-day variation in assay signal appeared highly concordant, with mean fluorescence of positive samples exhibiting significantly much less than 1 standard deviation of bias across days. Endpoint detection precision relates more closely to signal determination compared background levels, and assay signal-to-noise appeared suitable across days, showing a S/N ratio of greater than 3 on all days tested. As the signal levels for positive control specimens remained consistently high across all testing days, the elevation in S/N on 27 Sept indicated an unusually high-precision sampling of negative control specimens (CV of 0.051%), versus a more typical and expected variability in background levels on other days (CV of 1.15 and 1.18%).

Date	Total Samples	Mean N1/2 Adjusted Fluorescence (Log2)	Standard Deviation	Coefficient of Variation	Percent of Wells <1S.D from Mean (per day)	Assay Signal-to-Noise (Mean (pos)-Mean (neg) / SD (neg)
25/09/2020	40	10.443	1.599	15.3%	6.95%	3.54
26/09/2020	64	10.648	0.936	8.8%	8.92%	3.65
27/09/2020	92	10.591	2.103	19.9%	4.34%	136.93

Analytical specificity (Interferences and cross-reactions)

1. The EPCR assay configuration employs amplification primers for SARS-CoV-2 that were previously authorised in early 2020 for diagnostic use under FDA Emergency Use Authorisation, with analytical specificity and cross-reactivity studied and reported by the US Centers for Disease Control (Lu et al, 2020). The EPCR "N1/N2" primer set has and continues extensive use throughout global surveillance testing regimes, including in the intended current use jurisdiction of the United Kingdom.

2. While cross-reactivity was not under formal direct study during the performance evaluation, there are limited to no material differences in the assay constituents and reaction conditions between the EPCR method, and those reported in CDC procedures and other authorised uses. This includes the parallel concordance tests that Thermo Taqpath qPCR assays system, which detects the same viral gene as ECPR, with cross-reactivity and analytical sensitivity performance set forth in the manufacturer instructions (EUA and CE-IVD marked). The EPCR reaction master mix reagent and instrumentation and detection workstream are used routinely in high-capacity genotype testing for allelic discrimination (LGC Ltd). A similar detection calling scheme and software are utilised in qPCR testing: the EPCR detection rules are varied to establish positive signal levels and exclude negatives at the lower-detection bounds, but the fundamental principle of assaying monitoring using fluorescence detection for analyte discrimination are unchanged between qPCR and EPCR assays.

3. At the time of this application, no investigations have reported molecular changes requiring discontinuation or restrictions to the use of the N1 and N2 primer sets employed in EPCR

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

1. The performance evaluation utilised a fully parallel concordance testing study design. In blinded fashion, samples underwent qPCR diagnostic tests using standard procedures, and were then assayed separately using EPCR methods, prior to the qPCR test result being known. The approximately 50,000 input specimen set was comprised of unselected samples, receipted, extracted and tested under routine processing workflows, without interruption to ongoing qPCR testing. As they were unselected and blinded at receipt, the biologic and analytical diversity of test specimens reflects the full spectrum of specimen types that would be anticipated under routine testing, according to the instructions for use Samples selected for the validation were appropriate to the assay. Low medium and high viral load samples were tested d to avoid increasing or lowering diagnostic sensitivity and specificity.

2. Diagnostic sensitivity: the performance evaluation ascertained several measures of test sensitivity concordance between EPCR, using multiple comparison datasets derived from a fixed set of test results. Findings are summarised as follows:

   EPCR showed 98.68% test sensitivity compared to qPCR [97.86% to 99.24%; confidence bounds at the 95% level], when studied on a test set of 48,673 specimens available for concordance assessments. The specimen set was selected from 52,186 total tests performed and allowing for exclusion of 3,513 tests due to process QC failures with an attributable and remediable fault.

   A positive predictive value of 73.90% was observed.

Real Time PCR Output

EPCR Output	Positive	Negative	Void
EPCR Positive	1,192	421	0
EPCR Negative	16	46,892	0
EPCR Inconclusive	4	148	0

Test sensitivity of 98.56% was established for EPCR and qPCR as a standard [97.55% to 99.23%; confidence bounds at the 95% level], when a second comparison set of 39,909 test results was assessed. In this dataset, test results were

excluded due to failure of analytical QC criteria that assess known test prevalence and process control outcomes. This analytical QC step excluded a further 8,687 provisional tests were excluded from the set described

A positive predictive value of 90.62% was observed.

Real Time PCR Output

EPCR Output	Positive	Negative
EPCR Positive	869	92
EPCR Negative	13	38,915

3. Diagnostic specificity: using the test sets described above, the following test specificity was observed.

4. EPCR showed 99.11% test specificity in the process QC only dataset [99.02% to 99.19%; confidence bounds at the 95% level], and a 99.97% negative predictive value.

5. EPCR showed 99.76% test specificity [99.71% to 99.81%%; confidence bounds at the 95% level] when analytical filters were introduced, and a 99.97% negative predictive value.

6. Further, we tabulated and report a “Cohen’s Kappa” concordance measure. Using performance criteria above, the Cohen’s metric describes the level of agreement between EPCR and qPCR methods as two different “observers”, scoring the same result outcomes over the same specimen set. Unlike simple sensitivity and specificity measures, Cohen’s Kappa corrects for random effects influencing concordance, and reports results on a standardised scale, with hypothesis testing available (Kwiecienet al, 2011).

7. In the trial set prior to analytical QC, Cohen’s kappa is 0.84, placing the concordance level of EPCR in a category of “Very Good”.

8. In the comparator data where analytical QC filters were use, Cohen’s kappa was 0.94 — this level indicates “Excellent” or “Near Perfect” agreement between EPCR and qPCR.

9. As the Cohen's kappa measure integrates predicative performance corrected joint test trial size, it is encouraging that our test level above 0.8. This predicts testing agreement performance between EPCR and qPCR should be robust to higher sample capacity and life-time testing regimen performance. Alongside confidence level

boundaries for sensitivity and specificity, Cohen's measure indicates that test trial size and random effects did not materially impact our assessment of overall concordance and test agreement between the two technologies.

10. To better understand any source of test discordance, we inspected qPCR amplification thresholds of positive qPCR samples that were negative by EPCR. All tests showing positive discordance showed amplification near the qPCR limit-of-detection (Ct values greater than 30). As such, lack of agreement between qPCR and EPCR likely reflects variability in diagnostic precision at the lower assay bounds of detection.

11. Similarly, we re-tested qPCR samples that were negative but indicated as positive upon EPCR testing. For positive discordants where qPCR re-testing confirmed the EPCR finding (27 out of the 92 discordant samples), we similarly observed that only 3 of 27 such specimens exhibited a Ct lower than 30. This further indicates that discrepancies between the EPCR and qPCR assay concordance is driven by imprecision and stochastic variability near the limits-of-detection in each assay, as opposed to true biologically significant discordance that is captured only by one assay technology or another.

References

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