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# Laboratory considerations for reporting cycle threshold value in COVID-19

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ABSTRACT

The Coronavirus Disease 2019 (COVID-19) pandemic is caused by the SARS-CoV-2 RNA virus. Nucleic acid amplification testing (NAAT) is the mainstay to confirm infection. A large number of reverse transcriptase polymerase chain reaction (RT-PCR) assays are currently available for qualitatively assessing SARS-CoV-2 infection. Although these assays show variation in cycle threshold values (Ct), advocacy for reporting Ct values (in addition to the qualitative result) is tabled to guide patient clinical management decisions. This article provides critical commentary on qualitative RT-PCR laboratory and clinical considerations for Ct value reporting. Factors contributing to Ct variation are discussed by considering relevant viral life-cycle factors, patient factors and the laboratory total testing processes that contribute to the Ct variation and mitigate against the reporting of Ct values by qualitative NAAT.

## 1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) is an enveloped RNA Betacoronavirus identified amongst patients with pneumonia in Wuhan City in China in December 2019 (1). SARS-CoV-2 is responsible for causing coronavirus disease 2019 (COVID-19), categorised as a pandemic by the World Health Organisation (2). Current statistics estimate that the pandemic has resulted in 518,368,648 globally confirmed cases, with global mortality estimated at 6,266,459 (3), with new variants of concern continuously emerging to date (4).

Nucleic acid amplification tests (NAAT) testing is an essential tool in detecting SARS-CoV-2 viral RNA in infected persons and is the reference standard for diagnosing infection and screening for viral variants of concern. The qualitative reverse transcriptase polymerase chain reaction (RT-PCR) molecular methodology is commonly employed to identify viral infection and is considered the gold standard for diagnosing positive cases of COVID-19. RT-PCR amplifies genomic structural and non-structural targets of SARS-CoV-2. The method is highly sensitive and specific at identifying viral gene targets. The various NAAT assays utilise automated and manual sample steps to improve analysis throughput. The proliferation of new assays shows variable assay characteristics and regulatory subscription (5).

RT-PCR tests for viral RNA detection can be reported qualitatively (positive or negative or equivocal) or quantitatively using the cycle threshold (Ct) value. Some assays also use a semi-quantitative output, for example, stratifying positive results as high or medium or low. The Ct value is the measurable number of output cycles that describe DNA amplification of the viral nucleic acid target (with background assay noise removed) (6) and thus functions as a cut-off point to identify positive viral nucleic acid present in the sample. The amplification is detected in the exponential phase, with no limitation of reagents, and the viral cDNA doubles with each PCR cycle. A threshold value can be manually or automatically inserted in the analysis of the result to identify the point at which exponential amplification is achieved. In the context of SARS-CoV-2, it identifies the presence of viral RNA for particular gene targets present in the viral RNA. Not all molecular techniques utilised to quantify SARS-CoV-2 produce Ct values; however, the RT-PCR method is the most ubiquitous assay methodology utilised in diagnosing SARS-CoV-2 infection and generates a Ct value. RT-PCR does not distinguish between detecting viable live virus shed in the sample from viral fragments of non-viable (non-infectious) virus present in the sample.

The reporting of the Ct value to indicate a proxy measure of the amount of virus (viral load) in qualitative RT-PCR analysis for SARS-CoV-2 for the diagnosis and care of patients infected with SARS-CoV-2 is controversial, with the majority of leading international guidelines recommending against reporting Ct values. This review will discuss the viral dynamics of SARS-CoV-2, the general design of the qualitative RT-PCR assay used to measure SARS-CoV-2 infection (highlighting important laboratory factors relevant to the interpretation of Ct values), and then examine the potential clinical and laboratory factors that impact the Ct value and its interpretation.

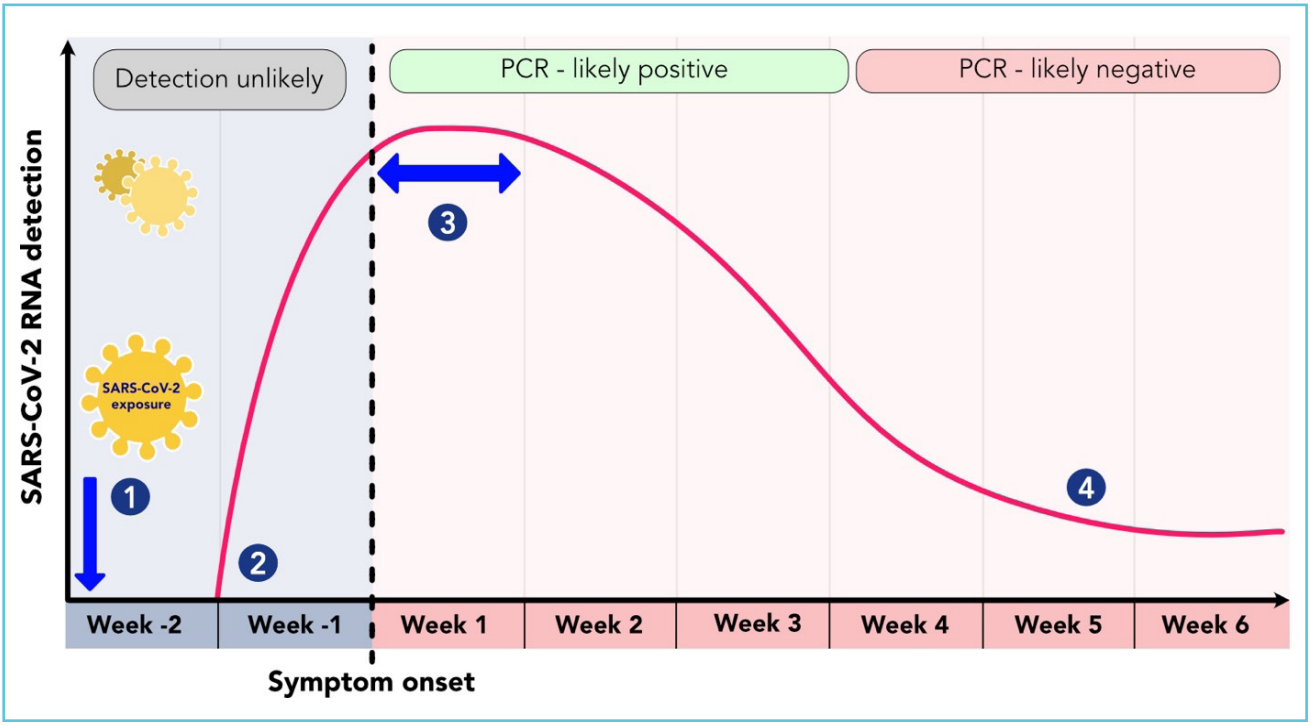
## 2. SARS-COV-2 VIRUS

SARS-CoV-2 is a single-stranded enveloped RNA virus belonging to the genus Coronavirus and the family Coronaviridae. The SARS-CoV-2 virion ranges in size from 70-90 nm, as evidenced by ultrastructural studies of virus-infected cells. The virus's genome is ± 30kb (26-32 kb) and compromises 6-11 open reading frames (ORF), which encode 9680 amino acid polyprotein. ORF one

constitutes 67% of the genome and encodes 16 non-structural proteins (nsps) compared to the remainder of accessory and structural proteins. The nsps include two viral cysteine proteases, including papain-like protease (nsp3), chymotrypsin-like, 3C-like, or main protease (nsp5), RNA-dependent RNA polymerase (nsp12), and helicase (nsp13). The four structural proteins include spike surface glycoprotein (S), membrane, nucleocapsid protein (N), envelope (E) and accessory proteins like ORFs (2,7). Specific structural and non-structural genes form RNA targets for molecular-based nucleic acid tests to identify SARS-CoV-2 viral infection in humans. Different molecular-based assays amplify single or multiple gene targets for SARS-CoV-2.

The life cycle of SARS-CoV-2 has been reviewed in human infection and is incompletely understood. In Figure 1, a summary of generalised findings in nasopharyngeal swab PCR is indicated (8-10). Systematic reviews and metanalysis indicate that the mean incubation is five to six days (range: 2-14 days). A definitive Ct cut-point that defines potential infectivity is unknown. Infectiousness factors include viral strain and the varying degrees of infectivity based on the characteristics of the virus variant of concern properties.

**Figure 1** **Generalised schema of viral RNA detection of SARS-CoV-2 in nasopharyngeal specimens of infected individuals. The viral kinetic dynamic of SARS-CoV-2 is adapted from (8-10).**




*Once infected individuals can progress through an asymptomatic incubation period followed by increased viral load; symptomatic phase and propensity to spread the virus, followed by symptom recovery. Four key points are essential in the viral kinetics: (1) Point of infection by SARS-CoV-2; (2) Virus detection starts to emerge and is dependent on patient factors and variant viral strain; (3) Symptom onset and period around symptom onset marks a highly infectious state; although asymptomatic spread is also evidenced; and (4) Late infection is marked by low transmission potential and high Ct values. This period is variable and dependent on patient factors, vaccination status and analytical considerations. The final temporal ranges and Ct values in infection are dependent on patient factors, specimen type and NAAT assay utilised in viral detection.*

Host factors may also affect the persistence of the virus, and pre-existing immune status via vaccination or prior infection or disease severity may also affect the duration of infectivity (9). In general, patients with severe disease or immunocompromised status remain infectious for longer, in contrast to non-severe patients with viral infectiousness shown to be present for up to ten days from symptom onset.

Many studies have shown that viraemia achieves peak levels around the symptomatic phase of COVID-19 and then gradually tapers over weeks. Asymptomatic infection at laboratory analysis is documented in various settings, and the potential for transmission is high in communities (11, 12). Patient factors can influence absolute viraemia, and the degree of viral load is higher (lower Ct values) in severe disease and age greater than 60 years. The persistence of viraemia may affect patient outcomes and possibly the degree of infectiousness. Also, variants of concern have been reported to have shorter incubation periods than other variants to potentially affect transmission (4, 9, 10). Viral transmission is affected by viral load and viral shedding factors, amongst others. Viral load is very important in transmission, and higher viral loads are associated with increased secondary attack rates and also symptomatic disease progression (4, 13). The life-cycle and transmission potential highlight the importance of considering Ct values more holistically, considering clinical factors, symptomatology and time point post-infection. In addition, it is important to note that the detection of positive Ct values does not indicate definitive infectious potential.

The specimen type used for detecting SARS-CoV-2 can affect the Ct value. In a systematic review and metanalysis of viral shedding behaviour, the mean duration of SARS-CoV-2 RNA shedding was 17.0 days (95% CI 15.5-18.6; 43 studies, 3229 individuals) in the upper respiratory tract, 14.6 days (9.3-20.0; seven studies, 260 individuals) in the lower respiratory tract, 17.2 days (14.4-20.1; 13 studies, 586 individuals) in stool, and 16.6 days (3.6-29.7; two studies, 108 individuals) in serum samples. The maximum shedding duration was 83 days in the upper respiratory tract, 59 days in the lower respiratory tract, 126 days in stools, and 60 days in serum (14). Ct values thus are influenced by specimen matrix and this pre-analytical factor can determine spurious early diagnostic results, and persistent viral clearance in late infection or resolved infection.

## 3. GENERAL DESIGN OF THE SARS-COV-2 RT-PCR QUALITATIVE ASSAY

The SARS-CoV-2 virus is measured in the laboratory by NAAT, most commonly, the reverse transcription polymerase chain reaction (RT-PCR), which measures a cycle threshold (Ct) value that identifies viral infection. The Ct value quantifies the amount of viral cDNA present in the specimen, which is detected from the assay background. Ct represents a PCR cycle number point on the PCR amplification plot where viral cDNA is exponentially amplified under optimal assay conditions where reagent, temperatures and incubation times are non-limiting. This enables the viral cDNA to double with each cycle and increase by a factor of 10 for every 3.3 cycles (15).

The SARS-CoV-2 qPCR assay consists of three vital analytical steps: (1) viral RNA isolation, (2) cDNA synthesis and (3) amplification of target viral genes in the cDNA. The steps can be performed in a single tube (one-step reaction) or split into two steps where viral RNA is first transcribed to cDNA and then transferred to the amplification phase of the analysis (two-step reaction) (16).

Firstly, viral SARS-CoV-2 RNA is extracted from the sample. Viral RNA is detected in upper, lower and gastrointestinal specimens, with various viral RNA shedding patterns observed

(14). There is uncertainty regarding the optimal upper respiratory tract specimen type for RT-PCT testing. The Infectious Diseases Society of America Guidelines (IDSA) suggests a nasopharyngeal swab, a mid-turbinate swab, an anterior nasal swab, saliva, or a combined anterior nasal/oropharyngeal swab rather than an oropharyngeal swab because of limited data suggesting lower sensitivity with oropharyngeal specimens (17). Notably, the Ct value will be influenced by the sample type, where it may be challenging to identify the infectious potential and glean an estimate of high viral RNA expression in the sample.

Extraction of the RNA is then followed by the reverse transcription step which copies the RNA viral genome to form complementary DNA (cDNA) catalysed by reverse transcriptase. And, thirdly, specific viral genes for the SARS-CoV-2 are then amplified using the cDNA input template in the qPCR reaction to identify the presence or absence of viral genome expression in the sample. Higher amounts of viral cDNA in the sample produce lower Ct values.

The quantitative polymerase chain reaction is based on real-time monitoring of DNA or cDNA amplification from input viral nucleic RNA. The RT-PCR amplification is detected by intercalating double-strand dye or probe-based emission of fluorescence (which is released by digestion of the attached probe to the newly amplified DNA strand). The fluorescence signal is detected by detectors in the instrument on a cycle-by-cycle basis in real-time (usually across 40 cycles) - the fluorescence output signal increases in each cycle. The measurable fluorescent signal is proportional to the viral cDNA present in the sample. Thus quantification cycle (Cq) or the cycle threshold (Ct) value of an amplification reaction is defined as the fractional number of cycles required for the fluorescence to reach a quantification threshold (15, 18).

The generation of an interpretative signal from RT-PCR produces a fluoresce emitted signal, which is detected by the instrument and proportional to the number of viral genes in the specimen. Thus, individuals with high viraemia produce a signal that results in a rapid increase in fluorescence output than low viraemia, requiring more amplification cycles to emit a detectable quantifiable signal. The Ct value is inversely proportional to viral gene expression and thus may serve as a surrogate marker of viral load for SARS-CoV-2. Current qualitative assays do not enumerate a viral load as the assays do not run calibration curves using reference samples to derive a viral count in the specimen objectively. These tests are thus able to produce a qualitative result that laboratories report (positive, negative, indeterminate) guided by manufacturer or laboratory-based guidelines on interpreting the results and the Ct values.

In contrast, in quantitative RT-PCR, reference samples spanning a range of known genome copies are simultaneously run alongside patient samples for each RT-PCR batch of tests, and the Ct value measured for the patient is used to calculate viral load by comparing the Ct value of the patient to the reference sample curve. The raw Ct value thus is not reported, but the laboratory issues a quantitative genome copy number.

The targets for the viral genes include structural or non-structural genes and in different combinations. The assays demonstrate various detection limits and analytical sensitivity (Table 1).

The targets for different nucleic acid amplification testing methods can produce false-negative results for variant SARS-CoV-2 virus. For example, S gene target failure with the recent Omicron variant in some RT-PCR tests can occur. However, other targets will amplify, and the result can be reported as positive for infection, guided by the assay manufacturer's interpretative recommendations. Also, as not all RT-PCR

**Table 1** Commonly utilised SARS-CoV-2 molecular tests target various viral genes and demonstrate variable limit of detection (LOD) and analytical performance (19-24)

| SARS-CoV-2 test | Company | Genes targeted; Limit of detect (LOD) | Analytical performance |
|---|---|---|---|
| **Alinity m** | Abbott Laboratories, Abbott Park, IL, USA | N, RdRp; 100 copies/ml | 100% Sensitivity 100% Specificity |
| **Abbott RealTime** | Abbott Laboratories, Abbott Park, IL, USA | N, RdRp; 100 copies/ml | 100% Sensitivity 100% Specificity |
| **Xpert® Xpress** | Cepheid, Sunnyvale, CA, USA | E, N; 8.26 copies/mL | 97.80% Sensitivity 95.60% Specificity |
| **Cobas®** | Roche Molecular Systems Inc, Branchburg, NJ, USA | ORF1, E; 25-32 copies/mL | 96.10% Sensitivity 96.80% Specificity |
| **TaqPath™** | Thermofisher Scientific, Waltham, MA, USA | S, E, N; 10 GCE/reaction | 93.50% Sensitivity 93.30% Specificity |
| **Allplex™** | Seegene Inc, Seoul, South Korea | E, N, RdRp 4; 167 copies/mL | 100% Sensitivity 96.70% Specificity |

*Abbreviations of RNA viral targets: N, nucleocapsid; RdRp, RNA-dependent RNA polymerase; E, envelope; ORF1, open reading frame; S, spike protein.*

assays will result in failed S gene target amplification in Omicron variant infection, interpretation of a positive Ct value for the S gene cannot rule out Omicron variant infection (25).

## 4. CLINICAL AND LABORATORY CONSIDERATIONS FOR REPORTING CT VALUES DERIVED FROM QUALITATIVE NUCLEIC ASSAY AMPLIFICATION TESTING

The potential use of Ct values in clinical applications to predict disease severity, assess individuals' infectious potential and determine re-infection is not clearly understood. Many studies have demonstrated higher expression of SARS-CoV-2 biomarkers of infection by RT- PCR with disease severity. For example, a systematic review of 18 studies concluded that lower SARS-CoV-2 Ct values were associated with worse clinical outcomes. In 57 % of studies (n=8), Ct values were correlated with disease severity. The authors concluded that Ct values might help predict patients' clinical course and mortality with COVID-19, pending further confirmatory studies (26). In another systemic review of RT-PCR analysis for SARS-CoV-2, 29 moderate quality studies were identified. Twelve studies identified a significant inverse relationship between Ct values

and positive viral culture. Also, symptom onset was related to Ct value and disease severity. Two studies also showed that viral culture positivity reduced by 33% for every increase in one Ct value unit (27). Clinical studies also support the quantitative evaluation of Ct values for the prognostication of adverse patient outcomes. SARS-CoV-2 viral load (as assessed by CT values) can predict patients' adverse clinical outcomes and more invasive management (28, 29).

In contrast, other clinical studies have not found an association between Ct values and patient outcomes and management. Shah et al. (30) observed that patients admitted with positive SARS-CoV-2 RT-PCR diagnosis failed to evidence a correlation between COVID-19 disease severity and mortality. Patients with mild disease showed lower Ct values than patients with severe disease. Furthermore, patients who died had significantly lower Ct values than patient survivors (30). Additionally, a systematic review and meta-analysis of seven clinical studies identified no significant association between hospitalisation and Ct value. This metanalysis showed an association between Ct value <25 and severe disease and mortality in comparison to Ct values >30; however, increased disease severity and mortality were less pronounced at Ct values of 25-30 compared with >30 (31).

Some studies have demonstrated that the utility of Ct values in patient prognosis is limited. Ct values have not been found to support the prognosis of COVID-19 disease in community patients and were insignificantly associated with worse outcomes (32). The administration of oxygen treatment to positive SARS-CoV-2 patients was not associated with Ct values. The investigators concluded that Ct values should not be used as an isolated indicator of patient prognosis (33). The utility of Ct values in solid organ transplant patients did not help predict COVID-19 disease severity (34). Repeated Ct value analysis at initial and nadir levels found no differences in prognosticating patient survival and disease severity and suggested that Ct values have limited use in managing COVID-19 disease (35).

The Food and Drug Administration (FDA) has acknowledged the use of serial testing of asymptomatic individuals to decrease false-negative results and has attempted to improve screening pathway tools (36, 37). The utility of sequential testing may afford some positive benefit in patient diagnosis, management and risk-stratification. Sequential testing of SARS-CoV-2 in a retrospective cohort analysis showed that a three-fold increase in Ct value correlated with a 0.15 improvement of the disease severity index score: Sequential Organ Failure Assessment (SOFA). This finding implicates the potential utility of sequential measurement of Ct values for prognosis in specific patient populations with COVID-19 (38). Serial testing has also been found to help diagnose individuals living in shared quarters who would be misdiagnosed if symptom screening or testing at only one timepoint were used. Therefore, serial testing can reduce transmission in congregated settings such as correctional facilities (39). Serial testing can also be valuable in identifying new infections and curbing SARS-CoV-2 spread in hospital settings (40). Interestingly, the utility of Ct values at a population level to identify changing trends in virus infectivity and the evolution of new viral strains by extracting Ct values from population surveillance data can inform the trajectory of the SARS-CoV-2 pandemic. For example, an increase in aggregated population Ct values indicates a decline in clinical SARS-CoV-2 cases (41).

Various clinical studies have identified that Ct values from RT-PCR can assist as a proxy for infectious virus detection. The probability of viral growth in cell culture declines to approximately 6% after ten days from symptom onset (Public Health England, 2020). A large study that analysed 754 upper respiratory samples from 425 symptomatic cases that tested positive for SARS-CoV-2 by

Rt-PCR targeting the RNA-dependent RNA polymerase (RdRp) gene showed that the estimated odds ratio of infectious viral recovery decreased by 0.67 for each unit increase in Ct value (95% CI: 0.58–0.77) with 8.3% (95% CI: 2.8%–18.4%) recovery of virus from samples with Ct > 35. Regression analysis also indicated that pre-symptomatic samples were at least as likely to be culture-positive as samples taken during symptomatic phases. (42). Other studies have tried to link Ct values with infectiousness; for example, in a small cross-sectional study, the viral infectivity by cell culture was significantly reduced for SARS-CoV-2 E-gene Ct > 24, with the odds ratio for infection decreasing by 32% for every increase of 1 Ct unit above 24. (43).

Although higher expression of viral biomarkers usually correlates with culture positivity, inter-assay Ct variability for SARS-CoV-2 is significant (6). Thus attribution of a single Ct cut-off point that predicts cell culture positivity is not available using the current qualitative RT-PCR assays. In addition, positive culture specimens have also been identified with high Ct values (44, 45). It would also be erroneous to tailor clinical management decisions based exclusively on low Ct value test results. Furthermore, it is important to re-iterate that the amplification of viral RNA by qualitative RT-PCR may not be consistent with live virus detection. The use of viral cell culture is thus an important adjunctive, although non-routine, tool to identify the infectious replicative potential of virus in samples. Viral culture, however, is itself limited by non-standardised methodology and interpretation of the cytopathic viral cell features. Therefore, the utility of viral cell culture as a gold standard to determine the infectious potential of samples requires standardisation and studies comparing SARS-CoV-2 Ct values should consider this limitation. Standardising cell culture procedures and interpretation of results and using internal and external quality control to improve overall quality assurance can reduce analytical and post-analytical test variation (46-49). Improved standardisation of cell culture can tie in with standardising Ct values and potentially identifying universal Ct threshold cut-off points that define viable virus and culture positivity.

Cevik *et al.* (14) also noted that many studies failed to identify positive viral cultures beyond day nine post-infection. Their review supports an association between viral load and virus viability. Therefore, the latter observation suggests that a particular threshold Ct value may support clinical practice points for the duration of infectiousness and isolation of index cases. Moreover, it does emphasise the importance of tying viral load dynamics to clinical presentation in SARS-CoV-2 infection. There are broad caveats that should be considered when Ct values are being utilised to inform the clinical management of patients. Assay-specific between-run variation and inter-assay variability hamper a single cut-off Ct threshold point derivation that demarcates disease severity and informs clinical risk stratification approaches and prognostication of infected individuals.

The development of fit-for-purpose quantitative qPCR assays may support clinical applications on the proviso that a definite clinical utility for Ct is demonstrated. In a joint consensus statement issued by the Infectious Diseases Society of America (IDSA) and the Association for Molecular Pathology (AMP), the use of Ct values in clinical decision-making is cautioned and not advised for the correlation with disease severity or in the prediction of active infection (and thence transmission of SARS-CoV-2) (6). The American Association of Clinical Chemistry also recommended against the reporting of SARS-CoV-2 Ct values. It supports the position by highlighting various points along the total testing process for SARS-CoV-2 RT-PCR testing, which considers pre-examination, examination and post-examination factors. Furthermore, it

impresses a standardised post-analytical comment for the release of Ct values that acknowledges limitations across the total testing process.

During the pre-examination (pre-analytical and pre-pre-analytical phases of viral testing), the lack of standardisation of patient preparation for obtaining specimens, such as removing mucous from respiratory passages or ingesting food and drinks, can cause inaccurate results. The efficiency of the specimen collection, specimen type and media utilised to collect the specimen also introduce potential variation (50). A stronger focus on clinical factors of time of onset and resolution of symptomatology also influences viral RNA, and integrating the Ct value with the evolving clinical history is essential (51). He *et al.* (51) studied temporal viral shedding patterns in 94 laboratory-confirmed patients of COVID-19 and further modelled infectiousness profiles from 77 infector-infectee transmission pairs. The investigators estimated that 44% (95% confidence interval, 30-57%) of secondary cases were infected at the pre-symptomatic level. The highest viral load was present in throat swabs at symptom onset.

Broader relevant clinical factors of vaccination status and immunisation also need consideration. Traditional pre-analytical sample stability factors of transport and age of specimen are essential to ensure intact viral RNA is preserved to avoid false-negative results. (52). The Centre for Disease Control (CDC) also does not support Ct values to determine the viral load to guide decisions on infectiousness and releasing patients from quarantine. They contend an imperfect relationship between the amount of virus present in samples and the Ct value. They note that factors which may affect the Ct value are improper collection and storage methods, processing of the specimen, and molecular assay sensitivity, which can cause the imperfect relationship (53). The pre-analytical phase of laboratory testing can affect the amount of detectable viral RNA present in the specimen and produce variable or false results. In summary, pre-examination processes that can affect COVID-19 viral RNA concentration include the specimen collection method, specimen matrix and collection methods, transport media volume, and type and time taken to arrive at the laboratory. Ultimately, the amount of RNA present in the specimen and the quality of whole RNA molecules will affect the assay's ability to amplify the RNA and provide high analytical sensitivity.

The limit of detection (LoD) for molecular tests indicates the lowest concentration of gene target that can be detected in ≥ 95% of repeat measurements and thus measures the analytical sensitivity of the molecular assay(54). This property is varied between RT-PCR assays; for example, LoD variation up to 10 000 fold was evidenced by ± 275 applications of new Emergency Use Authorisation in vitro diagnostic molecular assays to the United States Food and Drug Administration (37). This can affect the detection of low viral copy numbers and produce false-negative results and variation in Ct values between assays. The units of reporting LoD are also varied between assays, and comparisons of LoD are confusing. LoD unit of reports includes copies of genomic RNA per millilitre of transport media (copies/ml), copies/microliter, copies per reaction volume and molarity of assay target (54). In an extensive study of 27 500 patient test results by the Abbott RealTime SARS-CoV-2 assay (with a LoD of 100 copies viral RNA/ml of transport medium), each 10-fold increase in LoD increased the false-negative rate by 13%. The investigators showed that the highest LoDs could thus produce false-negative rates as high as 70% (54). The variability of LoD between assays (table 1) foregrounds low viral RNA copies may produce false-negative Ct values and potentially misclassify early disease where viral copies are low during the incubation (asymptomatic) period of SARS-CoV-2 infection.

Assay design features consisting of variable primer sets, probes and fluorescent labels also can potentially affect Ct values. These assay design features can affect the efficiency of the RT-PCR reaction, the specificity of the reaction to identify the true positive viral target and the optimal binding of primers and probes to target sequences in variants of SARS-CoV-2. In addition, the calculation of the threshold level by manual or automated selection modes can impact the Ct value and affect the accuracy of patient results. In addition, Ct range reliability should span values that permit the amplification of viral RNA in a clinically relevant range and considers the natural life cycle of the virus. As a general principle, values outside the assay's linear range should not be reported to avoid false results and misclassification of patients.

The Ct value variation has been demonstrated between assays in various studies. Cycle thresholds and diagnostic performance of clinical samples assessed by ten nucleic amplification techniques that included RT-PCR and loop-mediated isothermal amplification (LAMP) methodologies, utilising the LightMix E-gene test as the gold standard, showed excellent specificity of 100%. However, sensitivity ranged between 68.2% (95% CI 45.1% - 86.1%) to 95.5% (95% CI, 77.2% - 99.9%). Notably, all samples with viral loads >100 copies/µl showed positive results. Furthermore, Ct values that amplified the same gene targets for SARS-CoV-2 demonstrated significant variation (55). This study highlights the potential inter-assay variation of Ct values for SARS-CoV-2 RNA detection amongst current qualitative RT-PCR assays. Moreover, it suggests that analytical accuracy, the potential for misdiagnosis and assessment of infectious status may be adversely affected by Ct value reporting of qualitative nucleic acid-based testing for SARS-CoV-2 infection. Nalla *et al.* (56) assessed the diagnostic performance of seven RT-PCR assays by analysing clinical samples by different primer-probe RT-PCR designs for SARS-CoV-2. All assays were highly specific for SARS-CoV-2, with no cross-reactivity with other respiratory viruses tested. The assay's sensitivity to detect SARS-CoV-2 varied between assays, with Center for Disease Control (CDC) N2-gene and Corman E-gene primer-probe sets demonstrating the highest sensitivity (100%) with detection limit at six genomic equivalents of the the the viral RNA. Kasteren *et al.* (57) have also investigated PCR efficiency, LoD and diagnostic performance by seven commercial RT-PCR assays for SARS-CoV-2 detection using viral RNA isolated from cell culture. The efficiency varied for similar RNA targets between assays; for example, RdRp-gene efficiency varied between 104% - 118%. Also, where two targets were detected within an assay, the efficiencies were variable between the targets in the same assay; for example, the KH Medical assay demonstrated the efficiency of 118% by RdRp versus S-gene efficiency of 99%. The variation in test amplification efficiency invites rigorous validation of analytical test performance. Furthermore, even on the same molecular-based assay, longitudinal repeat testing may produce false results reflecting assay efficiency and multi-target variability within an assay.

External quality assurance schemes also evidence variation in Ct values for single and multiple viral RNA targets between laboratories, potentially impacting patient management. Although samples with lower Ct values correspond in general to higher levels of viral RNA, there is inconsistent data which demonstrates the quantitation and precision of the observed differences in Ct values. Therefore, the College of American Pathologists (CAP) cautions against the limitations of Ct values by scientists and healthcare providers (20). In an external quality assurance survey by CAP, same-batch quality assurance material was administered to 700 laboratories to analyse SARS-CoV-2 RNA. CAP identified that the median value for the analysed

samples showed variation by up to 14 cycles. In addition, a single sample analysis by the same instrument showed that the difference in median Ct values for different viral targets was three cycles. Furthermore, the survey also noted that for a single gene target analysed by the same instrument amongst all laboratory participants, a difference of up to 12 cycles was observed (20). Laboratory-specific practices of selecting multiple testing platforms or analysing specimens by multiplex nucleic acid testing assays with different viral targets can further promote variation in measured Ct values. Therefore, the reporting of instrument-specific identity for analysis of patients specimens potentially could be relevant for the interpretative analysis of patients' results. Qualitative reporting does not routinely distinguish Ct values for individual amplified viral targets. This may also be a valuable consideration as the variation between different targets observed by the CAP survey may erroneously suggest a higher viral load.

The Austrian EQA for SARS-CoV-2 analysed data for qualitative outcomes for nucleic acid extraction and detection of the virus by the 52 participant laboratories by utilising three positive (Ct values: S1, 28.4; S2, 33.6; S3, 38.5) and one negative sample. All laboratories scored a 100% for analytical specificity. However, 60% of the laboratories detected all positive samples correctly, 37% did not detect the weakest positive specimen and 3% of laboratories obtained false-negative results for S2 and S3 (58).

Furthermore, a national EQA programme in South Korea showed that 110 (93.2%) laboratories reported correct results for all qualitative molecular tests, and 29 (24.6%) laboratories had >1 outlier according to cycle threshold values. (59). Collectively, these EQA data show that Ct value sensitivity is variable and influenced by pre-analytical nucleic acid extraction procedures and the amplification step of the RT-PCR assay. Assays also positively demonstrate excellent analytical specificity.

Until recently, the lack of standardised reference material to quantify SARS-CoV-2 viral load has made comparability between assays challenging. The recent designation of the first WHO International Standard for SARS-CoV-2 RNA for nucleic acid amplification technique-based assays consists of acid-heat inactivated England/2020 isolate of SARS-CoV-2 (NIBSC code 20/146), was evaluated in a WHO international collaborative study (60, 61). The unit for the potency is 7.40 Log 10 IU/lyophilised ampoule which after reconstitution is 7.70Log10/ml. Using this material to develop quantifiable NAAT will improve harmonisation between assays and move to the development of consensus assay threshold Ct value points for the management of patients.

## 5. CONCLUSIONS

An examination of qualitative molecular-based testing for SARS-CoV-2 infection, especially by RT-PCR analysis, reveals variation in Ct values between assays that mitigate against reporting of Ct values for qualitative analysis by NAAT. The sources of Ct variation are a consequence of pre-assessment factors that affect the quality of viral specimen RNA and variation at the analytical level of the NAAT.

Furthermore, the interaction between patient factors, viral life-cycle and shedding kinetics make assigning Ct threshold cut-off points problematic in guiding patient management. Currently, Ct value reporting for SARS-CoV-2 is not supported by many international laboratory regulatory bodies. The derivation of a WHO preparation of standardised RNA reference material provides an avenue to move toward quantifiable viral load measurement, and harmonisation of NAAT with the potential of deriving cut-off points to guide clinical management decisions.

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