There has been growing interest in the possibility of using reverse transcriptase polymerase chain reaction (RT-PCR) cycle threshold (Ct) values as a surrogate measure of the amount of SARS-CoV-2 in clinical specimens. Ct value-based estimates of viral density have been used to predict progression to severe COVID-19 disease, infer transmissibility and differentiate active viral replication from prolonged virus shedding. However, definitive data to support the predictive value of Ct values in these situations are lacking. Ct values generated by qualitative PCR assays do not reliably correspond to specific RNA concentrations and are not consistent across platforms. In addition, multiple factors other than viral load are known to affect Ct values. Therefore, many clinical laboratories do not routinely include Ct values in laboratory reports, opting instead to share them on a case-by-case basis. This document summarizes important caveats that need to be considered when interpreting and applying Ct values in clinical practice.

**ANALYTICAL ISSUES**

**PCR cycle threshold (Ct) definition**
Current U.S. Food and Drug Administration (FDA) Emergency Use Authorized (EUA) real-time PCR tests for SARS-CoV-2 are designed as qualitative assays, which means that the test procedure generates a binary result of “positive” or “negative”. The threshold for defining a positive result is established using both analytical and clinical data specific to each individual assay. Some, but not all, real-time PCR tests also generate a Ct as a part of the test process and these values are available to laboratory staff. The Ct value refers to the number of PCR cycles required to amplify the targeted viral nucleic acid to a detectable level that is discernable from background. Certain EUA tests target multiple different SARS-CoV-2 genes simultaneously, with each target potentially generating a different Ct value from the same specimen. Whether certain gene target Ct values are more informative than others has not been established.

**Relationship between Ct values and SARS CoV-2 viral load**
There is an inverse relationship between Ct values and the amount of virus in the specimen. In general, low Ct values indicate a higher viral density (i.e., fewer amplification cycles are needed to detect a positive result) and high Ct values generally indicate a lower viral density (i.e., more amplification is needed to detect a positive result). Qualitative real-time PCR tests, however, are not designed to provide a quantitative or semi-quantitative measurement of nucleic acid in a sample. This is because qualitative test Ct values are not normalized to standardized controls of known concentration. Qualitative assays are also not optimized to have a linear relationship between the Ct value and the concentration of target nucleic acid in the specimen, which may
disproportionally impact the reliability of Ct values obtained from samples containing either high or low viral loads. Truly quantitative viral load tests are typically performed using blood specimens. Phlebotomy is an easily standardized and reproducible procedure. In contrast, respiratory specimen types are less amendable to quantitative PCR testing due to the variability inherent to collection process as well as difficulties associated with testing complex sample types like saliva or sputum.

Factors affecting Ct values
Multiple variables, other than the amount of nucleic acid present in the sample, may impact the determination of Ct values (see the TABLE below). As a result, Ct values may not be comparable for individual patients tested sequentially with the same method and are definitely not directly comparable across different real-time PCR tests or testing laboratories. The College of American Pathologists (CAP) recently reported the results of SARS CoV-2 PCR proficiency testing conducted across 700 laboratories in the United States [1]. The median Ct values reported by different FDA EUA test methods using identical control material varied by as many as 14 cycles (i.e., 4000-fold), while reproducibility of the control sample tested on the same instrument differed by a median of 3 cycles (i.e., 10-fold). It is also important to recognize that clinical laboratories commonly perform SARS-CoV-2 testing using a variety of different tests and testing platforms.

TABLE. Factors that impact Ct values

<table>
<thead>
<tr>
<th>Patient factors</th>
<th>Specimen factors</th>
<th>Test factors</th>
</tr>
</thead>
<tbody>
<tr>
<td>Presence or absence of symptoms</td>
<td>Adequacy of specimen collection</td>
<td>Volume of sample subjected to testing</td>
</tr>
<tr>
<td>Severity of symptoms</td>
<td>Reproducibility of the specimen collection method</td>
<td>Gene target</td>
</tr>
<tr>
<td>Time from symptom onset</td>
<td>Specimen type (e.g., nasal swab, saliva, BAL)</td>
<td>PCR primer and probe design, which may be variably affected by emerging viral variants</td>
</tr>
<tr>
<td>Immune status</td>
<td>Dilution of the sample in transport medium or other liquid</td>
<td>Nucleic acid extraction efficiency (note, not all tests include a nucleic acid purification and extraction step to remove potential PCR inhibitors in the specimen)</td>
</tr>
<tr>
<td>Age</td>
<td>Specimen transport and storage conditions</td>
<td>Gene target amplification efficiency</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PCR instrument parameters and settings</td>
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</tbody>
</table>

CLINICAL QUESTIONS

Do Ct values predict disease severity?
Multiple single center studies have attempted to correlate Ct values with symptom severity at presentation as well as with disease progression. A recent review noted that the majority of these reports observed an association between lower Ct values and worse clinical outcome [2]. However, given the wide variability of Ct values both across and within PCR platforms, it is not possible to identify a universal threshold of prognostic value.

Can Ct values identify which individuals are most infectious to others?
Growth of SARS CoV-2 in cell culture has been used as a proxy for the presence of replication competent virus in a clinical specimen. However, the relationship between viral culture positivity and infectiousness in humans has not been established. Viral culture is relatively insensitive and there are problems with reproducibility. Therefore, culture negativity may not equate with inability to transmit infection. Moreover, SARS COV-2 viral culture is a labor intensive procedure that requires biosafety level 3 (BSL3) containment not available in most clinical laboratories.

Several studies have correlated Ct values with the presence or absence of infectious virus detectable in culture [3]. One report from Canada’s National Microbiology Laboratory (CNML) observed that PCR positive respiratory specimens with Ct values >24 were viral culture negative [4]. In comparison, the United States Centers for Disease Control and Prevention (CDC) reported that attempts to recover SARS-CoV-2 in culture of upper airway samples was generally unsuccessful when their assay Ct values were >35 (unpublished). Although higher Ct values tend to correlate with culture negativity, the differences between the CNML and CDC studies reinforce the principle that Ct values are not directly comparable across platforms or laboratories. Furthermore, there are occasional SARS-CoV-2 culture positive specimens with high Ct values (e.g. >35 using the CDC assay) [5, 6].

Higher range Ct values have also been used to suggest that antigen negative, PCR positive samples are not infectious because they are less likely to be culture positive. In a recent report, 58% (85/147) of concordant antigen plus PCR positive upper respiratory tract swab specimens were culture positive, while 9% (11/124) of PCR positive only samples contained cultivable virus [7]. It must be emphasized that not all antigen negative, PCR positive samples are likely to be culture negative. At this time, additional data are needed regarding when an individual is no longer infectious before Ct values (or negative antigen results) alone can be used to inform decision making.

Are Ct values useful for assessing potential cases of reinfection?
Like other respiratory viruses, SARS-CoV-2 RNA may remain persistently detectable in respiratory secretions for weeks or months following resolution of symptoms. Prolonged PCR positivity also occurs in some asymptomatic individuals. Differentiating persistent viral shedding from cases of relapse or reinfection requires genome sequencing of paired specimens sampled across distinct time periods. In clinical practice, genome sequencing may not be readily available and clinical laboratories do not typically retain PCR positive specimens for future testing due to storage space constraints. In lieu of sequencing, comparing Ct values across time points is tempting; the assumption might be that rising Ct values over time are more suggestive of residual viral shedding than new infection. It is plausible, however, that cases of reinfection could present with high Ct values as a result of partial immunity or if the second infection is identified very early in the course of infection. Given these potential issues combined with the variability intrinsic to qualitative PCR methods, even comparisons using the same specimen type analyzed on the same instrument may lead to inaccurate conclusions.

Take home messages
Although there is a relative relationship between Ct values and the amount of virus in a clinical specimen, Ct values generated by qualitative PCR tests should not be considered quantitative measures of viral load. Due to the myriad of analytical and clinical factors known to impact Ct values, caution is advised when applying published correlations of Ct values with disease
severity or as a predictor of active infection and hence transmissibility. At the current time, routine use of Ct values to inform clinical decision making is not advised. Development of a quantitative SARS-CoV-2 real-time PCR assay may overcome some of the limitations outlined above. The development of such tests may eventually occur if clinical utility for measurement of SARS-CoV-2 viral load is shown.

References

4. Ontário PH. An overview of cycle threshold values and their role in SARS-CoV-2 real-time PCR test interpretation. Accessed March