WORLD JOURNAL OF PHARMACEUTICAL RESEARCH
SJIF Impact Factor 8.084
Volume 12, Issue 15, 1137-1151. Review Article ISSN 2277–7105

INTERPRETING PCR CYCLE THRESHOLD (Ct) VALUES IN CLINICAL DIAGNOSIS BEYOND THE SCOPE OF COVID-19

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Article Received on
21 July 2023,

Revised on 11 August 2023,
Accepted on 31 August 2023

DOI: 10.20959/wjpr202315-29570

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ABSTRACT

The emergence of technology to rapidly and specifically detect early clinical prognosis of diseases, causative pathogens and the rate of infection result in timely therapeutic intervention, driving decisions in patient care and outcomes. This detection is facilitated by reliable, accurate diagnostic tools; of which, reverse transcriptase polymerase chain reaction (RT-PCR) has become prominent. RT-PCR reports cycle threshold (Ct), a numerical value, which is inversely related to the quantity of target genetic material detected in the patient specimen. Even though PCR testing and Ct values provide a deeper clinical understanding of disease prognosis, various limitations surround its interpretation. Ct value being dependent on multiple factors including the sample type and its quality, careful interpretation in relation to clinical findings is required. This review discusses the complexity, challenges and considerations involved in interpreting Ct value at the laboratory setting and its associated use in patient management decisions.

KEYWORDS: Polymerase Chain Reaction, Cycle threshold, Ct Value, Cycle quantification, RT PCR, Clinical diagnostics.

1. INTRODUCTION

Delivery of potent interventions for managing different clinical patient conditions depends on the time and specificity of detection by accurate and reliable clinical diagnostic tools. Molecular detection techniques like polymerase chain reaction (PCR) are currently becoming

more dominant. Of which, numerous advanced and efficient reverse transcriptase polymerase chain reaction (RT-PCR) tests are commercially available for a wide range of clinical applications.[1] The RTPCR method combines amplification and detection in a single step, allowing direct detection of PCR product during the exponential phase of the reaction. The interpretation of Ct value discussed in this article is primarily based on the principle of TaqMan chemistry.[2] The results obtained using RT-PCR tests are analysed using cycle threshold (Ct) values on a basic software incorporated within the RT-PCR instrument.[3] Generally, Ct values represent the number of PCR amplification cycles required for a target gene's fluorescent signal to exceed the threshold limit.[4] In this context, in RT-PCR tests, the Ct values obtained for a target gene and its relation to the actual gene expression levels is considered to be inversely proportional to each other; i.e., the lower the Ct value, the higher will be the expression of the targeted gene and vice versa. Thus, the significance of Ct value in RT-PCR mediated clinical diagnostics is said to be high in relation to early disease prognosis for better clinical decisions.[4,5]

However, it is important to understand that certain factors can play a crucial role in tampering the Ct values generated, leading to false positive results. Few of these factors include: the master mix reagents prepared for use in the PCR and the reference dyes used for fluorescence detection.[6] False positive results may also arise due to factors such as sample contamination, gene transcriptional errors, operational mislabelling and non-specific target product amplification.[7] Accordingly, interpreting Ct values in correlation to clinical findings is important for effective clinical diagnosis, better disease prognosis and patient management. In this review, we attempted to examine available global literature in order to understand the evidences, challenges and recommendations put forth in the correlations of Ct values with clinical outcomes.

2. METHODOLOGY

The current review was conducted through a high-quality comprehensive literature search on the cycle quantification (Cq) or cycle threshold (Ct) values and its role in clinical diagnostics. The literature search was performed using NCBI, PUBMED, NEJM, Research Gate, Science Direct, Hindawi, Elsevier databases ranging from 1980-2021 using search strategies like "Cycle threshold values in Polymerase chain reaction", "Cycle threshold values and clinical diagnostics", "Limitations and challenges of Ct in PCR" and "Ct value interpretation". The

search was also done from the reference lists of the selected articles for further understanding. Additional information was collected from reputed websites like FDA.

3. Ct VALUES IN REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

Cycle threshold (Ct) values signify the number of amplification cycles required for the fluorescent signal of the targeted gene to rise beyond threshold, i.e. crossing beyond the background level[8] as depicted in **Figure 1**. It is said to be the most convenient and frequently used method in the data analysis of RT-PCR.[5] It is also important to note that Ct values are said to be inversely proportional to the relative gene expression and provides a semi quantitative data.[9] Lower the Ct value higher the expression of targeted gene and higher the Ct values lower the expression of targeted gene.

The detection limit for an analytical procedure in clinical diagnosis also plays a major role in relating the Ct values, The limit of detection (LOD) can be defined as the point where one can be assured that the signal owing to the measure with a specified probability, and can be differentiated from the instrumental background signal. The LOD may alternatively be defined as the smallest quantity of an analyte in a sample that can be detected but not necessarily quantified as an accurate value for a certain analytical process. The LOD is defined as the analyte concentration at which the analytical technique detects the presence of the analyte at least 95% of the time.[10]

However, Ct values can also exceed the normal limit due to some pitfalls that include biological and manual errors[6], as detailed in the section below.

4. FACTORS AFFECTING Ct VALUE

Though RT-PCR technique is a sensitive and sophisticated molecular genetic technique having 80 to 90% accuracy, numerous factors influence the Ct values which include both biological events of the up and down regulation of the targeted gene as well as manual errors.[6]

4.1 AMOUNT OF TEMPLATE

The quality of ribonucleic acid (RNA) template used for a RT-PCR reaction is a critical factor for the detection of Ct value.[11] If the amount of RNA template added for each test is of poor quality or is too low than the amount required for setting up the reaction, chances for

accurate quantification of the target gene's Ct value is low. Addition of appropriate amounts of the template is thus important for accurate interpretation of the Ct values.[6]

4.2 NUCLEIC ACID EXTRACTION

Another important factor that can affect the cycle threshold value is the nucleic acid extraction process. Nucleic acid extraction (NAE) process includes 3 steps such as isolation, purification and concentration. The process of extraction should be rapid, simple, specific and sensitive. In case of clinical samples where specific nucleic acids are targeted, an appropriate extraction technique will depend on the specimen characteristics[12] which differ from the other cultural isolates of bacteria and fungi. The extraction method influences the performance of the diagnostic tests and the efficiency of nucleic acid extraction directly impacts the efficiency to the sensitivity of the final results.[13] Poorly performed isolations results in a lesser nucleic acid yield. This results in less specificity of the target gene generating inaccurate Ct values.[6] False-negative results due to poor quality and quantity of nucleic acid can thus be a concern.[14] Large amounts should be avoided because they may inhibit PCR amplification. All samples from a single experiment are reverse transcribed at the same time to reduce variability in reverse transcriptase efficiency.[2] It is hence essential to analyse the quality of nucleic acid post NAE for best results.

4.3 REVERSE TRANSCRIPTASE ACTIVITY

Reverse Transcriptase activity is said to be a fundamental component in the technology of RTPCR. Major factors like the amount of reverse transcribed RNA, volume of the RNA, temperature used for the process of transcription and its duration depends on the activity of reverse transcription which can lead to the poor transcriptase action. Poor reverse transcriptase activity during the process of complementary DNA(cDNA) synthesis eventually leads to the degradation of the cDNA This is a major factor that can contribute to less specific interpretation of the cycle threshold values for the targeted gene expression.[6]

4.4 DEGRADATION OF NUCLEIC ACIDS

During or after the isolation of nucleic acids from the specimen, it is important to ensure proper handling to avoid its degradation. Multiple freeze thaw cycles during the operational process can lead to its degradation. This in turn affects accuracy of the actual cycle threshold value.[6] Addition of the enzyme Proteinase K, can inactivates nucleases which degrade the nucleic acid from the sample at an elevated temperature; optimal temperature of 50-65°C is

required to maintain the samples from degradation. Temperatures beyond 65°C causes nuclease inactivation and quick degradation of the nucleic acid.[15]

4.5 PCR INHIBITION

Polymerase chain reaction being an enzymatic reaction, it is highly sensitive to inhibitions. The presence of PCR inhibitors, which include a heterogeneous group of chemical substances, can negatively impact the PCR through decreased sensitivity of the test sample or false negative results. PCR inhibitors can arise from processes involved during the extraction of sample, sample processing, or nucleic acid extraction.[16] PCR inhibitions can be avoided by preventing the inhibitor from being processed within the samples. Additionally, DNA purification is a major step for resisting the PCR inhibition by using commercially available kits. These types of kits are proven for eliminating such inhibitors. The DNA polymerase which chooses for the reaction largely impacts on the resistance to inhibition; Increasing certain DNA polymerases resists the PCR inhibitors and thus prevents the PCR inhibition.[17]

4.6 DRIFTING BASELINE

Drifting baseline in a PCR is caused due to the incorrect setting of the background cycle range where the range specifies the threshold fluorescence levels. It is considered to be one of the most important factors which affects the cycle threshold value and hence its interpretation.[5] This can be avoided by setting the baseline subtraction in the software along with removal of dithiothreitol (DTT) from the reverse transcription step.[18]

4.7 MASTER MIX SOLUTION

The master mix solution, when prepared at optimal conditions delivers the fluorescence emission depending on its pH and salt concentration. Changes in the master mix preparation can result in false positive Ct results.[6] Thus, good laboratory practices need to be followed to control the pH and to avoid unnecessary contaminations from external working conditions.

4.8 REFERENCE DYE

Reference dyes in polymerase chain reaction are used for normalization of the reference genes used in a reaction. It is one of the major factors involved in obtaining cycle threshold values. Any changes to the reference dye can result in the poor specificity and poor interpretation of the cycle threshold value resulting in a false positive result.[6] Precautions to be taken for avoiding the false positive results when using reference dyes include preparing fresh dilutions of the reference dye prior to the reaction and to maintain the reference dyes

away from light, as some of them are sensitive to light. Always make sure to make dilutions with nuclease free water. This prevents the reference dyes from external contamination too.

4.9 PRIMER PROBE PCR REACTION EFFICIENCY

The reaction efficiency is based on the performance of the prepared master mix, specificity of primer, primer annealing temperature along with the sample quality. The melting temperature (Tm) of the primers and probe, as well as the amplicon length, are the most important factors in PCR reactions. To anneal to the target during the extension phase of the PCR reaction, the Tm of the primers should be 58–60°C, while the Tm of the probe should be at least 10°C higher (thus around 68–70°C). The rules for amplicon lengths is "the shorter, the more efficient" along with suggested amplicon lengths of 50 and 150 bp. Primers are constructed on distinct exons or intron–exon boundaries to avoid co-amplification of contaminated genomic DNA.[2] So the changes in any of these may affects the reaction efficiency and thus interrupts the cycle threshold values.[6]

5. Ct VALUE CUTOFFS IN CLINICAL DIAGNOSIS

In Clinical diagnosis the implementation of Ct cut-off should be justifiable and reasonable based on proper clinical evidence. Diagnostic laboratories frequently select a subjective cut off value for real-time amplification assays, above which a threshold cycle (Ct) value is deemed false. There exist different analytical criteria for determining the cut-off range which include fluorescence threshold, reaction end-cycle, limit of detection, and artifact investigation.[19] However, in clinical diagnosis, epidemiologic cut offs are to be considered keeping in mind that they are population dependent, and their rationality is directly related with the targeted population only.[19] Moreover, as RT-PCR amplification efficiency varies according to the laboratory conditions in which they are being processed, Ct value cut-off cannot be standardized as a constant value. Studies thus recommend to normalize the cut-off Ct values for diagnostic purposes based on relative or absolute quantification approaches.[19] It is thus advocated that cut-off values must be established individually in respective laboratories based on their individual assay performance, and that consolidation of these data obtained from several such laboratories would pave way to better understanding of clinical diagnosis based on targeted gene of interest.

6. RELATING Ct VALUES TO CLINICAL DIAGNOSIS

An increasing volume of published clinical studies demonstrate the utility of real-time PCR for diagnosing microbial pathogens and disease prognosis. The high sensitivity and high

specificity along with a short turnaround time for results and ease of performance make real-time PCR an attractive replacement method for conventional culture and antigen-based assays. RT-PCR derived Ct values play an important role in test-based detection in clinical diagnostics.[20] For patients with co-infections, the Ct values from a multiplex target gene pane helps to identify which pathogen is likely to be the causative agent for the illness. For patients undergoing an antimicrobial therapy, comparison of Ct values over serial testing can serve as an indicator of the therapeutic response. While PCR testing and Ct values offer a deeper insight during the diagnostic process, the most important limitation is in its inability to distinguish between live and dead organisms. The keen sensitivity of PCR allows for the detection of minute quantities of nucleic acid; which does not always correlate with the presence of live organisms. The assay can amplify the nucleic acid from dead microbes as well as from live organism. Moreover, dead organisms can lean up to weeks even after recovery.[21] Hence, a recovering patient might possess the same positive test result just like an active infected patient. Here, it calls for distinguishing between these two types of patients as the positive test from the recovering patient doesn't possess any clinical relevance, while for the actively infected patient a timely treatment is required. Thus, relative quantification of the Ct value of the pathogen is required to help the clinician to distinguish between such two patients.[22] Additionally, interpretation of the sigmoidal curve pattern which denotes the quality of amplification is required to further validate the obtained results. Confirmation of expressed sigmoidal curve is important in PCR analysis as there will be an unusual level of fluorescence signals produced during the initial phase of the run which can result in the false positive Ct values.[23,24] Generally, the test samples are subjected to 40 cycles of PCR along with the positive control. If the Ct value obtained is less than or equal to 37, the test sample is considered to be highly expressed, where as if the Ct values is greater than or equal to 40 the test is considered as weakly expressed.[23] The appropriate range of cycle threshold value remains unclear, but several studies recommends the Ct range to be 40 cycles.[25–27] The value greater than 40 is subjected to be non-confirmatory and hence it requires a thorough confirmation using a retest.[26]

Nevertheless, studies report a direct relation between the lower cycle gene quantification value with the severity of a particular disease and vice versa as an important connection for clinical diagnostics[28,29] along with the limit of detection in RTPCR . Accordingly, for the interest of the readers, examples on the utility of Ct values in assessing disease prognosis are explained below.

6.1. UTILITY OF Ct VALUES IN ASSESSING COVID-19 DISEASE PROGRESSION

COVID-19 has brought on great level of interest in the clinical utility of Ct values. Ct holds a major role in determining the severity of disease and transmission rate. The infection rate and the Ct value are highly associated with each other as mentioned earlier before; Lower Ct value is associated with higher probability of the positivity of viral progression. Several related data show that the lower Ct values are correlated with higher mortality rate. Ct values were also found to relate with the numerous other clinical markers used in the associated disease.[4] Lower Ct values were also relatable to the observed lower lymphocyte levels that eventually causes lymphopenia resulting in severity in COVID -19 patients.[30,31]

6.2. Ct VALUES IN ASSESSING DISEASE PROGRESSION FOR OTHER RESPIRATORY DISEASES

RT-PCR is one of the most common diagnostic methods for detection and identification of respiratory pathogens. There are numerous studies that report the clinical utility of Ct values for respiratory pathogens. Ct values of RT-PCR and the disease severity / progression in respiratory disease patients largely depends on the virus dependency and its factors that influence the disease severity which are still unclear. Amount of the virus present in the human body per millilitre is known as the viral load which is inversely corelated with the cycle threshold values obtained from RTPCR. I.e.; Lower the Ct value higher the viral load.

A recent report on influenza study acknowledged that patients with low Ct values were more likely to have moderate to high disease severity and fever.[29] Many more studies on patients with respiratory syncytial virus reported correlation between low Ct values with clinically meaningful outcomes such as hospitalization, radiographical evidence of pneumonia or length of hospital stay, while others have failed to identify such associations.[32–34] Additionally, in most cases of respiratory infection, multiple co-infected pathogens could be responsible for disease progression making it difficult to find the exact causative pathogen behind severity. However, gene specific Ct values can acts as a useful tool for measuring the disease severity based on serial testing.[34] A standard reverse transcriptase RTPCR assays could be useful to determine the amount of known pathogen present in the subsequent respiratory infections. Some of the specific factors which affects these values and the illness severity are said to be the timing of the specimen collection, age of the patient and the amount of the pathogen which causes the infection.[29,35]

6.3. Ct VALUES FOR ASSESSING DISEASE PROGRESSION IN HEPATITIS VIRUS INFECTION

The quantification detection of hepatitis B virus (HBV) using Ct value with reverse transcriptase PCR includes high degree of precision with clinically relevant detection.[36] Similarly, in hepatitis C viral (HCV) infections, the precision and reproducibility of cycle threshold values are reported to be reliable and possess a wider application in the diagnostics and research of HCV.[37] In the case of hepatitis A virus (HAV) infection, it is reported that Ct values derived from the viral RNA are specific and can be clinically used in quantifying viral load from the clinical samples that determine disease progression.[38]

6.4 Ct VALUES IN ASSESSING OTHER DISEASES

Interpretation of Ct values in relation to disease progression of other diseases have also been reported. For example, Inflammatory bowel disease (IBD) with *Clostridioides difficile* infection (CDI) is considered to be a major hospital associated diarrhoea. Clinically, Ct values have been successful in providing relevant information regarding the test positivity rate and in identifying patients who are prone to IBD.[39] Correlation of Ct values for the assessment of CDI in inflammatory bowel disease have also been successful in identifying patients who fall in the high risk category.[40]

7. MAJOR CHALLENGES IN CLINICAL UTILITY OF CT VALUES

Usage of diverse specimen collection devices, specimen types, nucleic acid extraction methods, genomic targets, and RT-PCR chemistries present considerable variability in the Ct values that are reported.[41] Cycle threshold values are said to be very fragile requiring accurate data interpretation which is a difficult process.[5,42] This could be a major reason in its less popularity and acceptance across the medical community. Another major challenge is in identifying false positive results that occurs due to contamination, handling errors or in use of poor quality reagents.[43] Moreover, despite its high sensitivity and wider application, Ct values do not distinguish between infectious and non-infectious viruses. Different studies regarding the mild and severe viral shedding of COVID-19 patients during the time of hospitalization showed that SARS-CoV-2 RNA may well be detected in the respiratory tract for up to 21-, 32- and 34-days post recovery.[44] Thus, the complexity in understanding and interpreting Ct values may limit a broader use of this technique, narrowing its use to only those personnel's who have been trained to understand and appropriately interpret data from RT-PCR tests.

8. FUTURE PERSPECTIVES.

Despite the recent attention and use of RT-PCR as a molecular diagnostic tool, its clinical use and interpretation in and beyond COVID-19, remains a challenge. Reporting and successive use of Ct values in disease diagnosis, prognosis and in therapeutic decision making without robust assessment on its validation can affect misinterpretation and poor patient outcomes. This calls for evidence generated analytical and clinical data to justify the intended use of Ct values obtained from a test. Furthermore, supporting data to derive accurate standardization of the Ct values with a reference range pertaining to a respective target gene of interest is currently lacking for use at a clinical setting. It is also important to understand that Ct values interpretation should consider a variety of factors such as; whether the patient is symptomatic, their age, co-morbidities, symptom onset to sample collection time and epidemiological details in order to add clinical value. This challenges microbiologists to address and express the need for interpretation clearly to clinicians.

FIGURES

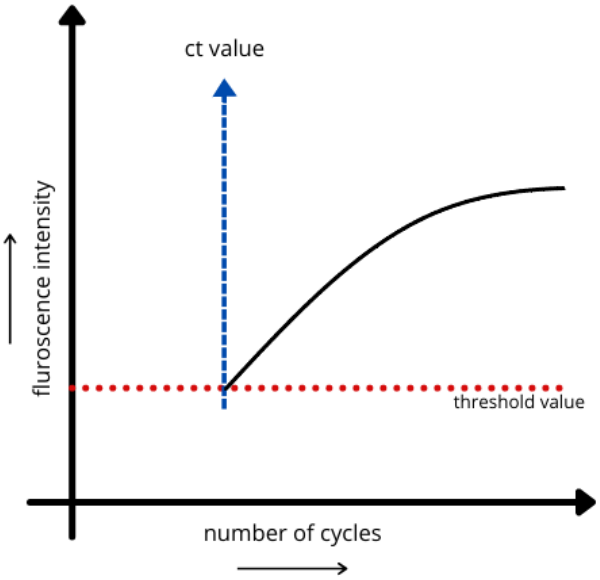

Figure 1: Cycle Threshold Value: Diagrammatic representation of the amplification cycle of a targeted gene crossing the fluorescent signal beyond its threshold (background level), whereby the crossing point signifies its Ct value.

CONCLUSION

Ct values offer a possibly easy and extensively available tool to envisage disease diagnosis and prognosis. Accurate interpretation of the Ct values also offers enormous benefits to both

clinicians and patients through appropriate decision management. We urge scientists and clinicians to undertake more studies at a global scale that will address challenges, encourage its use, bring out meaningful approaches to interpretation and add clinical value.

AUTHOR DECLARATION STATEMENT

We confirm that the manuscript has been read and approved by all named authors and that there are no other persons who satisfied the criteria for authorship but are not listed.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

AVAILABILITY OF DATA AND MATERIALS

All data generated or analysed during this study are included in this published article.

COMPETING INTERESTS

No conflict of interest has been declared by the authors; the present review paper is intended for educational purposes only.

FUNDING

The author(s) received no financial support for the research, authorship, and/or publication of this article.

AUTHOR CONTRIBUTION

Aparna J: Investigation, Methodology, Resources; Writing - original drafting.

Amita Ajit: Conceptualization; Methodology; Investigation; Resources; Supervision; Project administration; Validation; Visualization; original drafting; review & editing.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Prashanth Varkey, Zum Heilen Diagnostic & Therapeutics Pvt. Ltd. for providing facilities and support during the project tenure of this work.

REFERENCES

1. The role of cycle threshold values in infectious disease diagnostics [Internet]. Medical Laboratory Observer. 2021 [cited 2021 Feb 15]. Available from: https://www.mlo-

online.com/continuing-education/article/21206319/the-role-of-cycle-threshold-values-in-infectious-disease-diagnostics

2. Overbergh L, Giulietti A, Valckx D, Decallonne B, Bouillon R, Mathieu C. The Use of Real-Time Reverse Transcriptase PCR for the Quantification of Cytokine Gene Expression. J Biomol Tech., 2003 Mar; 14(1): 33–43.
3. Green MR, Sambrook J. Analysis and Normalization of Real-Time Polymerase Chain Reaction (PCR) Experimental Data. Cold Spring Harb Protoc., 2018 Oct; 2018(10): pdb.top095000.
4. Rao SN, Manissero D, Steele VR, Pareja J. A Narrative Systematic Review of the Clinical Utility of Cycle Threshold Values in the Context of COVID-19. Infect Dis Ther., 2020 Jul 28; 1–14.
5. Bustin SA, Nolan T. Pitfalls of Quantitative Real-Time Reverse-Transcription Polymerase Chain Reaction. J Biomol Tech., 2004 Sep; 15(3): 155–66.
6. Bustin SA, Benes V, Garson JA, Hellemans J, Huggett J, Kubista M, et al. The MIQE Guidelines: Minimum Information for Publication of Quantitative Real-Time PCR Experiments. Clinical Chemistry, 2009 Apr 1; 55(4): 611–22.
7. Johnston C, Healy B. Interpretation of COVID-19 PCR testing- what surgeons need to know. BJS (British Journal of Surgery), 2020; 107(10): e367–e367.
8. Rao SN, Manissero D, Steele V, Pareja J. Clinical utility of cycle threshold values in the context of COVID-19 [Internet]. In Review; 2020 Jul [cited 2021 Feb 17]. Available from: https://www.researchsquare.com/article/rs-41867/v1
9. Gaston DC, Malinis M, Osborn R, Peaper DR, Landry M, Juthani-Mehta M, et al. Clinical implications of SARS-CoV-2 cycle threshold values in solid organ transplant recipients. American Journal of Transplantation, 2021; 21(3): 1304–11.
10. Burns M, Valdivia H. Modelling the limit of detection in real-time quantitative PCR. Eur Food Res Technol, 2008 Apr 1; 226(6): 1513–24.
11. Dahdouh E, Lázaro-Perona F, Romero-Gómez MP, Mingorance J, García-Rodriguez J. Ct values from SARS-CoV-2 diagnostic PCR assays should not be used as direct estimates of viral load. Journal of Infection, 2021 Mar 1; 82(3): 414–51.
12. Shin JH. Nucleic Acid Extraction Techniques. In: Tang Y-W, Stratton CW, editors. Advanced Techniques in Diagnostic Microbiology [Internet]. Boston, MA: Springer US; 2013 [cited 2021 May 23]. p. 209–25. Available from: https://doi.org/10.1007/978-1-4614-3970-7_11

13. Smith K, Diggle MA, Clarke SC. Comparison of Commercial DNA Extraction Kits for Extraction of Bacterial Genomic DNA from Whole-Blood Samples. Journal of Clinical Microbiology, 2003 Jun 1; 41(6): 2440–3.
14. Emery SL, Erdman DD, Bowen MD, Newton BR, Winchell JM, Meyer RF, et al. Real-Time Reverse Transcription–Polymerase Chain Reaction Assay for SARS-associated Coronavirus. Emerg Infect Dis., 2004 Feb; 10(2): 311–6.
15. Tullis RH, Rubin H. Calcium protects DNase I from proteinase K: A new method for the removal of contaminating RNase from DNase I. Analytical Biochemistry, 1980 Sep 1; 107(1): 260–4.
16. Schrader C, Schielke A, Ellerbroek L, Johne R. PCR inhibitors – occurrence, properties and removal. Journal of Applied Microbiology, 2012; 113(5): 1014–26.
17. An Introduction to PCR Inhibitors [Internet]. [cited 2021 May 28]. Available from: https://www.promega.in/resources/profiles-in-dna/2007/an-introduction-to-pcr-inhibitors/
18. qPCR Troubleshooting [Internet]. [cited 2021 May 27]. Available from: http://blog.biosearchtech.com/TheBiosearchTechBlog/bid/63622/qPCR-Troubleshooting
19. Caraguel CGB, Stryhn H, Gagné N, Dohoo IR, Hammell KL. Selection of a Cutoff Value for Real-Time Polymerase Chain Reaction Results to Fit a Diagnostic Purpose: Analytical and Epidemiologic Approaches. J VET Diagn Invest, 2011 Jan 1; 23(1): 2–15.
20. Kralik P, Ricchi M. A Basic Guide to Real Time PCR in Microbial Diagnostics: Definitions, Parameters, and Everything. Front Microbiol [Internet]. 2017 Feb 2 [cited 2021 May 26]; 8. Available from: http://journal.frontiersin.org/article/10.3389/fmicb.2017.00108/full
21. Tom MR, Mina MJ. To Interpret the SARS-CoV-2 Test, Consider the Cycle Threshold Value. Clinical Infectious Diseases, 2020 Nov 19; 71(16): 2252–4.
22. Xiao AT, Tong YX, Zhang S. Profile of RT-PCR for SARS-CoV-2: A Preliminary Study From 56 COVID-19 Patients. Clinical Infectious Diseases, 2020 Nov 19; 71(16): 2249–51.
23. Syal K. Guidelines on newly identified limitations of diagnostic tools for COVID-19 and consequences. Journal of Medical Virology [Internet]. [cited 2021 Jan 19]; n/a(n/a). Available from: https://onlinelibrary.wiley.com/doi/abs/10.1002/jmv.26673
24. Bordon J. The Importance of Cycle Threshold Values in the Evaluation of Patients with Persistent Positive PCR for SARS-CoV-2: Case Study and Brief Review. JRI [Internet]. 2020 [cited 2021 Jan 19]; 4(1). Available from: https://ir.library.louisville.edu/jri/vol4/iss1/54

25. Cheng MP, Papenburg J, Desjardins M, Kanjilal S, Quach C, Libman M, et al. Diagnostic Testing for Severe Acute Respiratory Syndrome-Related Coronavirus 2: A Narrative Review. Ann Intern Med., 2020 Jun 2; 172(11): 726–34.
26. Loeffelholz MJ, Tang Y-W. Laboratory diagnosis of emerging human coronavirus infections – the state of the art. Emerging Microbes & Infections, 2020 Jan 1; 9(1): 747–56.
27. Woloshin S, Patel N, Kesselheim AS. False Negative Tests for SARS-CoV-2 Infection — Challenges and Implications. New England Journal of Medicine [Internet]. 2020 Jun 5 [cited 2021 Jan 19]; Available from: https://www.nejm.org/doi/10.1056/NEJMp2015897
28. Zhao B, Yu X, Wang C, Teng Z, Wang C, Shen J, et al. High Human Bocavirus Viral Load Is Associated with Disease Severity in Children under Five Years of Age. PLOS ONE., 2013 Apr 30; 8(4): e62318.
29. Spencer S, Chung J, Thompson M, Piedra PA, Jewell A, Avadhanula V, et al. Factors associated with real-time RT-PCR cycle threshold values among medically attended influenza episodes. Journal of Medical Virology, 2016; 88(4): 719–23.
30. Kermali M, Khalsa RK, Pillai K, Ismail Z, Harky A. The role of biomarkers in diagnosis of COVID-19 – A systematic review. Life Sciences, 2020 Aug 1; 254: 117788.
31. Yan L, Zhang H-T, Goncalves J, Xiao Y, Wang M, Guo Y, et al. An interpretable mortality prediction model for COVID-19 patients. Nat Mach Intell, 2020 May; 2(5): 283–8.
32. Feikin DR, Alraddadi B, Qutub M, Shabouni O, Curns A, Oboho IK, et al. Association of Higher MERS-CoV Virus Load with Severe Disease and Death, Saudi Arabia, 2014. Emerg Infect Dis., 2015 Nov; 21(11): 2029–35.
33. Hasegawa K, Jartti T, Mansbach JM, Laham FR, Jewell AM, Espinola JA, et al. Respiratory Syncytial Virus Genomic Load and Disease Severity Among Children Hospitalized With Bronchiolitis: Multicenter Cohort Studies in the United States and Finland. The Journal of Infectious Diseases, 2015 May 15; 211(10): 1550–9.
34. Wishaupt JO, Ploeg T van der, Smeets LC, Groot R de, Versteegh FGA, Hartwig NG. Pitfalls in interpretation of CT-values of RT-PCR in children with acute respiratory tract infections. J Clin Virol, 2017 May; 90: 1–6.
35. Ebell M. AHRQ White Paper: Use of Clinical Decision Rules for Point-of-Care Decision Support. Med Decis Making, 2010 Nov; 30(6): 712–21.
36. Wang Q, Wang X, Zhang J, Song G. LNA real-time PCR probe quantification of hepatitis B virus DNA. Experimental and Therapeutic Medicine, 2012 Mar; 3(3): 503–8.

37. Yang J-H, Lai J-P, Douglas SD, Metzger D, Zhu X-H, Ho W-Z. Real-time RT-PCR for quantitation of hepatitis C virus RNA. Journal of Virological Methods, 2002 Apr; 102(1–2): 119–28.
38. Costa-Mattioli M, Monpoeho S, Nicand E, Aleman M-H, Billaudel S, Ferré V. Quantification and duration of viraemia during hepatitis A infection as determined by real-time RT-PCR. Journal of Viral Hepatitis, 2002 Mar; 9(2): 101–6.
39. Jonica ER, Sulis CA, Soni K, Hughes M, Jones E, Weinberg J, et al. Role of Cycle Threshold in Clostridioides difficile Polymerase Chain Reaction Testing as a Predictor of Clinical Outcomes in Patients With Inflammatory Bowel Disease. Crohn's & Colitis 360 [Internet]. 2019 Oct 1 [cited 2021 May 27]; 1(otz036). Available from: https://doi.org/10.1093/crocol/otz036
40. Reigadas E, Alcalá L, Valerio M, Marín M, Martin A, Bouza E. Toxin B PCR cycle threshold as a predictor of poor outcome of *Clostridium difficile* infection: a derivation and validation cohort study. J Antimicrob Chemother, 2016 May; 71(5): 1380–5.
41. Rhoads D, Peaper DR, She RC, Nolte FS, Wojewoda CM, Anderson NW, et al. College of American Pathologists (CAP) Microbiology Committee Perspective: Caution Must Be Used in Interpreting the Cycle Threshold (Ct) Value. Clin Infect Dis., 2021 May 18; 72(10): e685–6.
42. Chan JF-W, Yip CC-Y, To KK-W, Tang TH-C, Wong SC-Y, Leung K-H, et al. Improved Molecular Diagnosis of COVID-19 by the Novel, Highly Sensitive and Specific COVID-19-RdRp/Hel Real-Time Reverse Transcription-PCR Assay Validated In Vitro and with Clinical Specimens. J Clin Microbiol, 2020 Apr 23; 58(5).
43. Freeman WM, Walker SJ, Vrana KE. Quantitative RT-PCR: Pitfalls and Potential. BioTechniques, 1999 Jan; 26(1): 112–25.
44. Xu K, Chen Y, Yuan J, Yi P, Ding C, Wu W, et al. Factors Associated With Prolonged Viral RNA Shedding in Patients with Coronavirus Disease 2019 (COVID-19). Clinical Infectious Diseases, 2020 Jul 28; 71(15): 799–806.