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# Ct Value from RT-qPCR Can Predict SARS-CoV-2 Virus Assembly and Lineage Assignment Success

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**Featured Application: The results of the study can be applied in choosing the correct RNA-sequencing strategy for the purpose of SARS-CoV-2 genome assembly.**

**Abstract:** During the recent pandemics of COVID-19, sequencing technics became a powerful tool for gaining information about the SARS-CoV-2 virus and using this knowledge to our advantage. Thanks to this advantage, scientists all over the world were able to search for emerging variations, watching the virus evolve in real time. Assembly of the virus genomes is a crucial part of obtaining this kind of useful information. In our study, we sequenced 79 samples from nasopharyngeal swabs of COVID-19 patients. Positivity to disease was evaluated using RT-qPCR. In this work, we described the relationship between RT-qPCR Ct value and genome construction success (plus genome lineage assignment). The specific value of this study is that this relationship was described for data from metatranscriptomic sequencing of human tissue, while there was no step of viral RNA isolation (usual for genome assembly procedure). RT-qPCR Ct value and assembly quality metric NG50 were correlated. We observed that the RT-qPCR Ct value threshold of the certain success of genome assembly (Ct value < 25) and certain failure (Ct value > 30) could be drawn, while results varied for values between (with completed, completed with lower quality, and failed assemblies).

**Keywords:** SARS-CoV-2; RNA genome assembly; RT-qPCR; SPAdes

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## 1. Introduction

SARS-CoV-2 virus appeared at the end of 2019, when it was found to cause a disease, today known as COVID-19 [1]. To date, a number of different lineages have emerged. Some of the variants that have emerged so far have become widely spread or even dominant for a time. The Alpha variant (B.1.1.7) from September 2020 carries a mutation in the S protein (N501Y) that affects the conformation of the receptor-binding domain and 13 other B.1.1.7 lineage-defining mutations [2]. The Beta variant (B.1.351), which appeared in May 2020, was characterized by seven mutations in the spike protein. Later, the Gamma variant (P.1) appeared with 17 unique mutations, followed by the Delta variant (B.1.617.2) with 29 mutations in multiple proteins, and Omicron (B.1.1.529), which carried more than 50 new point mutations (30 associated with residue changes in the spike protein). Occasionally, coinfection can occur, leading to recombination and the appearance of recombinants among other variants that have actually been documented [3]. Scientists around the world have been collecting genomic or transcriptomic data on SARS-CoV-2 and infected patients, which has made it possible to study the infection and how the host responds to it at the molecular

level. To date, 16,011,577 SARS-CoV-2 genome data have been submitted into the Gisaid system [4].

Assembling the SARS-CoV-2 genome became standard procedure, since it is necessary for NGS data analysis. When it comes to the routinely used methods utilized in standard clinical sample processing during this pandemic, mostly targeted enrichment strategies were used, based on either amplicon or hybridization-probe approaches. There are different designs or protocols that became routinely used, based on standardized academic research or commercially available solutions, e.g., the ARTIC nCoV2019 protocol, which is available in a standardized set of oligonucleotides from IDT as a result of the ARTIC network initiative focused on SARS-CoV-2 [5] or Respiratory Virus Oligo Panel kit from Illumina. Such targeted solutions are perfect when it comes to the need for high throughput, short turnaround times for sample analysis, as well as good economical effectiveness in cases where focused analysis—on detection of SARS-CoV-2 or panel of clinically relevant respiratory viruses—is the issue. However, from a scientific perspective, this brings only a limited amount of disease-related information as the response of the patient to the viral infection is complex and should be studied in association with the currently known relevant biological context. For studying the biological context at the least local (or global) microbiome and local host transcriptome should be involved. Therefore, if we would like to go over this limitation (of focused viral detection analysis), we should use metatranscriptomic analyses as the method of choice [6].

In contrast with procedures focused on viral genome assembly, in our study, the virus genome was assembled by using short-read RNA-seq metatranscriptomic data originating from clinical samples represented by nasopharyngeal swabs. As a part of the project with a wider focus, this paper is focused on describing how RT-qPCR Ct value can predict the feasibility of further analysis of viral RNA in studies with such an experimental design.

## 2. Materials and Methods

### 2.1. Study Approval

Sample collection was performed as part of the clinical study approved by the Ethical Committee of Bratislava Self-Governing District under the identifier 03228/2021/HF from 12 January 2021. All patients have filled out the questionnaires with relevant information regarding their health status in relation to COVID-19 and signed informed consent.

### 2.2. Samples

Nasopharyngeal swabs from patients showing COVID-19 symptoms (79 patients). were obtained in two primary regimens Patients hospitalized with severe symptoms of the disease at the collaborating hospitals were enrolled in the study (30 patients). Patients with mild or any symptoms of the disease were recruited in mobile testing facilities for SARS-CoV-2 by a company providing routine laboratory diagnostics from the population during the COVID-19 pandemic (25 patients). Voluntary donors with no symptoms were recruited and then tested with RT-qPCR and used as a negative control (24 samples). A total of 24 donors in this study were asymptomatic, but positive for COVID-19. In total, 103 individuals were part of this study.

### 2.3. Nucleic Acid Extraction

Nasopharyngeal swab specimens were collected from COVID-19 patients and controls and stored in a viRNAtrap collection medium (GeneSpector Innovations, Prague, Czech Republic) at 4 °C. Total nucleic acid was extracted using a Sera-Xtracta Virus/Pathogen Kit (Cytiva® Sera-XtractaTM virus/Pathogen Kit (Global Life Sciences Solutions Operations, Little Chalfont, UK)) according to manufacturer instructions. An amount of 400 µL of the nasopharyngeal swab medium was used for nucleic acid extraction with final elution to 50 µL of nuclease-free water. RNA was quantified with the Qubit™ RNA High Sensitivity Assay Kit (Invitrogen, Eugene, OR, USA). RNA isolates were stored at −80 °C.

### 2.4. RT-qPCR

The presence of SARS-CoV-2 was determined by RT-qPCR using the COVID-19 Real Time Multiplex RT-QPCR Kit (Labsystems Diagnostics, Vantaa, Finland) and RT-qPCR platform ABI QuantStudio 6 Real-Time PCR System (ThermoFisher, Waltham, MA, USA) utilizing the original manufacturers' protocols. The amplification cycle threshold of Ct values < 40 was needed to evaluate the sample as positive.

### 2.5. RNA Library Preparation and Sequencing

The metatranscriptomic libraries were prepared using a KAPA RNA HyperPrep Kit with RiboErase (HMR) (Kapa Biosystems, Salt River Cape Town, South Africa) according to the original protocol of the manufacturer. For quantity and quality control of prepared libraries, a Qubit 1X dsDNA High Sensitivity Assay Kit on Qubit 3.0 (Invitrogen) and an Agilent High Sensitivity DNA Kit on an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) were used. Sequencing of pooled libraries was performed on NextSeq 500 and NextSeq 2000 (Illumina, San Diego, CA, USA) platforms using 2 × 75 or 2 × 100 paired-end sequencing setups, respectively.

### 2.6. Data Quality Control and Preparation for Analysis

Quality control was performed using FastQC v0.11.9 [7]. Reads were processed with Trimmomatic v0.39 (CROP:96 HEADCROP:10 LEADING:22 TRAILING:22 SLIDINGWINDOW:4:22 MINLEN:25, and our own set of adapter sequences was used in the ILLUMINACLIP step) [8]. Parameters were chosen according to FastQC results. All mentioned tools were used as Linux command line instances with Conda.

### 2.7. Genome Assembly

Trimmed reads were mapped to the SARS-CoV-2 genome using the BWA-MEM algorithm. Subsequently, those reads were extracted using the samtools view from samtools v1.6 [9] and Picard SamToFastq from Picard v2.27.4 [10]. Reads were mapped as a paired set; otherwise, the parameters of mapping were set to default. The assembly of SARS-CoV-2 was performed using the coronaspades.py tool from Spades [11]. All mentioned tools were used as Linux command line instances with Conda.

### 2.8. SARS-CoV-2 Variants Identification

SARS-CoV-2 variant identification was performed independently of genome assembly using the Galaxy pipeline "Mutation calling, viral genome reconstruction and lineage/clade assignment from SARS-CoV-2 sequencing data" [12]. As an input, we used reads mapping on the SARS-CoV-2 genome. The first step of the pipeline is variation analysis with the following key components: BWA-MEM for mapping, Lofreq for variant calling, and SnpEff for variants' annotation [13]. Then, the next step is variant reporting. The third step is to generate consensus sequences and then identify SARS-CoV-2 clades/lineages using Pangolin and Nextclade [14,15].

### 2.9. Statistics of Ct and Assembly

For the purpose of assembly quality estimation, the LG50 value of the assembly was computed using the Linux command line instance of Quast software (QUAST v5.2.0) [16]. To prove the relationship between the assembly and RT-qPCR, a correlation coefficient was computed between RT-qPCR Ct values (E gene) and LG50 values. The correlation coefficient was computed by dividing the covariance by the product of the two variables' standard deviations:

$$\mathrm{Corr}(X,Y) = \mathrm{Cov}[X,Y]/(\mathrm{StdDev}(X) \times \mathrm{StdDev}(Y))$$

The same formula was used to obtain the correlation coefficient between Ct values and the success of the SARS-CoV-2 clade assignment (scored 0/1 for unassigned/assigned).

Undetermined values of Ct value were ignored, although for the purpose of visualization data on a graph, a value of 40 atop of the maximal value was assigned.

## 3. Results

This work is part of a bigger project studying the transcriptome and metatranscriptome of COVID-19 patients. We assembled genomes of SARS-CoV-2 from these samples and noticed the correlation between the success rate of assemblies and measured Ct values from RT-qPCR.

### *3.1. Assembly Performance*

For assembly of the genome, we used the SPAdes assembler (coronaspades mode), and it was performed from SARS-CoV-2 mapped reads. Out of 79 samples, 36 genomes were assembled as one scaffold of at least 28,000 nt long, while in another 6 samples, assembly was fragmented in 2 or more scaffolds, but its alignment covered almost the whole reference genome.

### *3.2. Presence of the Virus and Its Strain*

Here, we report results on 79 samples from patients positive for COVID-19, whose positivity was proved by RT-qPCR. We assigned viral reads to specific WHO variants and clades. Results were correlated with assembly success. A total of 24 samples were assigned as Alpha variant (clade 20I), 12 samples as Delta (clade 21J, 21I in 1 sample), 6 samples as Omicron (clade 21L, and 1 sample with 22B), 1 sample was assigned to 20C clade, and 1 was reported as recombinant.

### *3.3. RT-QPCR and Assembly*

We examined the Ct values from RT-qPCR and whether these values could predict assembly success (statistics of Ct values according to COVID-19 severity are shown in Table 1). Samples with lower Ct values (E gene) were mostly assembled. The median Ct value for samples with a completely assembled genome was 24.24, while in the case of fragmented assembly, it was 28.76. The median of samples with failed genome assembly was 32.95. The correlation coefficient between Ct value and NG50 value of assembly was −0.81. In total, eight samples had undetermined Ct values and were left out from correlation statistics (seven with failed assembly and one with fragmented assembly). The relationship between RT-qPCR Ct value and NG50 assembly quality metrics is shown in Figure 1, where three Ct value zones of successful, unsuccessful, and unsure assembly can be seen. The grey zone of unsure assembly is between Ct values of 25 and 30, while everything that is lower was always successfully assembled and everything that is higher always failed. This visualization shows how the quality of metrics changes in relation to RT-qPCR Ct value. None of the negative samples were assembled into a complete genome, nor into the genome fragmented into more scaffolds.

**Table 1.** RT-qPRR Ct value statistics for symptoms-derived group of patients and all patients.

| Symptoms | Min Ct Value | Max Ct Value | Mean | St. Deviation | Interquartile Range |
|---|---|---|---|---|---|
| **Severe** | 14 | 37.3 | 27.9 | 6.7 | |
| **Mild** | 15.4 | 33.6 | 24.8 | 5.3 | |
| **Asymptomatic** | 19.2 | 38.8 | 29 | 4.7 | |
| **All** | 14 | 38.8 | 28.42 | 7 | 10.93 |

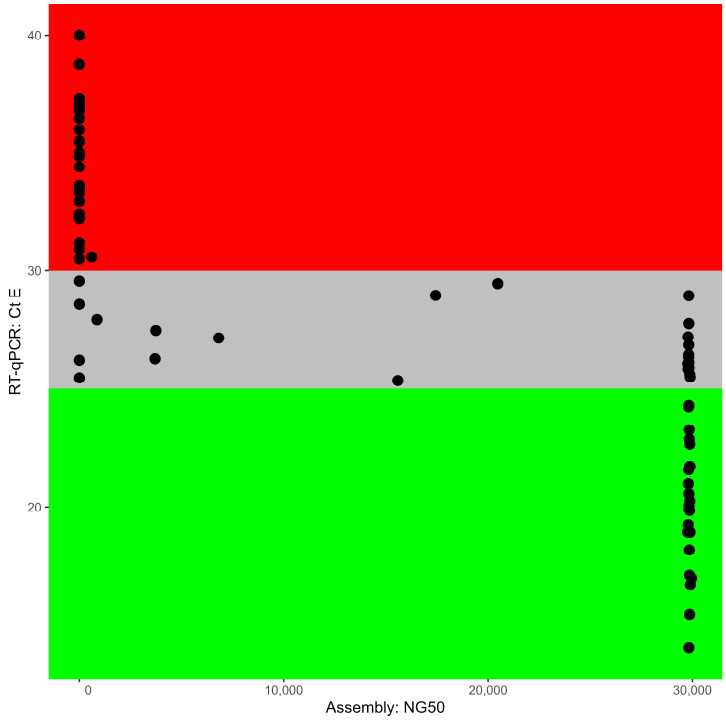



**Figure 1.** Dot plot graph showing relationship between SARS-CoV-2 assembly success (NG50 value) and RT-qPCR Ct value. Here, we can see 3 highlighted zones—the green zone under Ct value < 25, where all samples were assembled into a complete genome, and the grey zone between Ct values of 25 and 30 where genomes were assembled completely, fragmented, or assembly failed. None of the samples over Ct value 30 were assembled into the SARS-CoV-2 genome.

### *3.4. RT-QPCR and Lineage/Clade Assignment*

For samples with lower Ct values (E gene), the SARS-CoV-2 clade was mostly assigned (correlation coefficient with successful clade assignment = −0.78, while 8 Ct values were undetermined, left out of correlation statistics, and all unassigned). For samples where the assembly was not successful enough to assign a clade to the virus, the median Ct value was 33.54. The minimum value was 25.46. The Ct value was not determined for eight samples. The median of the determined samples was 24.24 with a maximum value of 30.57. Samples with a Ct value < 25 appear to be viable for both virus clade assignment and assembly.

## 4. Discussion

Although we originally performed our study with a focus on the identification of potential novel biomarkers resulting from human transcriptome and metatranscriptome analysis of samples from COVID-19 patients with different severity of the disease, here, we reported dependency of SARS-CoV-2 genome assembly and lineage assignment on Ct value from diagnostic RT-qPCR. This work is a collateral output of the complex research but is an independent and specifically focused part of it and a brief report of this finding. Exploring the relationship between RT-qPCR Ct value and further analysis of data on the virus would be logical (through higher viral load leading to more viral reads in the dataset), and it certainly was questioned before; however, here we confirmed and described this relationship in a specific experimental design for a metatranscriptomic study. This means that RNA sequencing of human tissue from COVID-19 patients containing viral reads between those of humans and bacteria was analyzed instead of using the standard procedure for genome assembly with viral RNA isolation.

We performed multiple steps of data analysis to achieve the observation. First, assembly of the genome was necessary. There are various assemblers fit for this job. The benchmarking study by Gupta and Kumar (2022) recommends SPAdes, IDBA, and ABySS out of the eight assemblers they tested [17]. We tested the performance of SPAdes our-

selves, trying various strategies as well as Trinity, which was not compared by Gupta and Kumar (2022). These results are about to be published as "Possibilities of assembling whole genome of clinically relevant pathogens directly from clinical samples sequenced as metatranscriptomic data" (Hadzega et al., 2023). For presenting results here, we chose the best-performing assembly strategy.

It was observed that samples with a Ct value under 25 were always assembled, samples between Ct values of 25 and 30 were in a kind of grey zone—where assembly might be successful, but not always—and samples over a Ct value of 30 were never successfully assembled.

Previously published studies have shown that the use of different approaches for SARS-CoV-2 genome sequencing is capable, although targeted resequencing (either amplicon or hybridization-based) with subsequent full genome assembly involved were working horses when it comes to massively applied diagnostic and epidemiologic surveillance studies. Additionally, short- as well as long-read sequencing solutions were used with an acceptable ratio when it comes to turn-around times, throughput, and cost-effectiveness of such testing [6–18]. However, when it comes to the research potential with the focus not only on diagnostics but also complex study of viral infection and local and potentially global host reaction, the most relevant strategy is the metatranscriptomic analysis starting directly from clinical samples. With this approach, the host transcriptome, microbiome, as well as viral genome could be analyzed in parallel if there is enough of the viral genome; in addition, a full viral genome could be assembled. In a comprehensive study by Xiao et al. of complete genomes, inter-individual and intra-individual variations of SARS-CoV-2 from serial dilutions of a cultured isolate as well as clinical samples covering a range of sample types and viral loads were analyzed and, in the case of metatranscriptomic analyses, similar findings as in our study were reported. Their analysis was performed on only eight clinical samples with a focus on the correlation between Ct values and successful SARS-CoV-2 genome assembly, and further study showed the limiting Ct value for the completeness of assembly was a Ct value $\leq$ 24.5 (corresponding to conc. $\geq 1 \times 10^5$ viral genome copies per milliliter) [6].

In our study, we used Quast's NG50 value as the quality metric of assembly. In comparison with more standard N50 values, it compares assembled contigs to the reference genome, not to the assembly itself. With this value, it was possible to evaluate assembly success in relation to Ct value. In computing the correlation coefficient, we ignored undetermined Ct values from RT-qPCR. By this simplification, we meant to eliminate the risk of incorrect methodology if the undetermined values were replaced by a number, although it probably causes a slight underestimation of the resulting number for the correlation coefficient. Our study strength was in the number of samples, which we believe is enough for the presented statistics.

To sum up our main point, in this study, it was proven that with certain sample parameters (here represented by Ct value), it is possible to obtain a variety of information from whole transcriptome studies, such as a complete SARS-CoV-2 genome from a mix of human and bacterial reads. Specifically, if the RT-qPCR Ct value is under the surprisingly firm threshold, assembly of the viral genome is possible.

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**Institutional Review Board Statement:** The study was conducted in accordance with the Declaration of Helsinki, and approved by the Ethics Committee of Bratislava's self-governing region (12 January 2021).

**Informed Consent Statement:** Informed consent was obtained from all subjects involved in the study.

**Data Availability Statement:** Raw sequencing data used in this study are available on European Nucleotide Archive (ENA database), under study submission PRJEB62682 (www.ebi.ac.uk/ena).

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