ORTHOGONAL CONFIRMATION OF THE SENSITIVITY OF THE PERKINELMER SARS-COV-2 NEW CORONA VIRUS RT-PCR RESULTS

Orthogonal Confirmation of the Sensitivity of the PerkinElmer SARS-CoV-2 New Corona Virus Rt-PCR Results

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Abstract

The PerkinElmer® New Coronavirus Nucleic Acid Detection kit was granted FDA EUA on March 24, 2020. The data submitted to FDA demonstrated 100% specificity and a limit of detection (LOD) of 20 copies/ ml. The kit was identified as having the highest sensitivity of the commercially available kits in a recent study conducted by FDA for all assays that have received EUA (1). By design all FDA EUA COVID assays are semi-quantitative and do not directly correlate with the viral titer. The scientific opinion on the correlation between high cycle-threshold (Ct) values and the presence of virus is still being debated. The purpose of this study is to verify the presence of SARS-CoV-2 genomes in samples with a wide range of RT-PCR cycle threshold Ct values including samples with high Ct (LOD <1000 copies/ ml). Next Generation Sequencing (NGS) was chosen as method of choice (2). The note does not address the clinical significance of low viral copy number in asymptomatic individuals tested early in the course of SARS-CoV2 infection, and in individuals recovered from COVID infection.

Methods

A stored aliquot of extracted RNA from individuals who tested positive on the PerkinElmer® New Coronavirus Nucleic Acid Detection kit was obtained to use as template for NGS. These samples were collected using an anterior nasal swab suspended in molecular transport media (MTM) for transport, and RNA was extracted using the PerkinElmer® chemagic™ Extraction system.

Eight microliters of undiluted extracted RNA was reverse transcribed and amplified using a publicly available SARS-CoV-2 genome-specific ARTIC v3 primer set (3) along with all reagents needed for reverse transcription, amplicon based enrichment and NGS library preparation using Unique Dual Index Barcodes (UDI, PerkinElmer). The resulting amplicon material (50-100 ng) was used to make NGS libraries using the NEXTFLEX® Rapid XP DNA-Seq kit and NEXTFLEX® RNA-Seq 2.0 UDI. Each library was then normalized to 10nM and equal volumes combined to make the final library pool. The final library pool was then prepared and loaded on to an Illumina® NovaSeq® 6000, per manufacturer's instructions, using an Illumina® NovaSeq® 6000 SP Reagent Kit v1.5 (200 cycles) and flow cell and performing a paired-end run (2x75 bp) targeting ~7M reads per library. Data output in the form of FASTQ files were trimmed of adapters via Trimmomatic and aligned to the SARS-CoV-2 Wuhan-hu-1 reference genome (NC_045512.2) via Bowtie2 for bioinformatic analyses. To investigate cross-reactivity with other species alignment comparative analysis was performed by aligning sequences to 29 other bacterial and viral species. Alignment rates were plotted in a heatmap using Python scripting to visualize any potential signaling with species other than SARS-CoV-2.

Results

A total of 149 RT-PCR positive samples with Ct values spanning 10 to 42 cycles were sequenced. This includes 38 samples with a Ct value more than 37. The percentage of sequencing reads that aligned to the SARS-CoV-2 Wuhan-hu-1 reference genome (NC_045512.2) ranged from 38.69% to 99.69%. All samples sequenced showed high sequence specificity to the SARS-CoV-2 virus. No sample had 0% alignment to SARS-CoV-2 and no sample aligned better to any other species other than SARS-CoV-2. The average number of reads across the SARS-CoV-2 genome ranged from 275 to 1198 (average depth) with >90% of the 98 amplicons of the ARTIC v3 primer set represented.

Bioinformatic analysis of NGS data for Ct values ranging 17 to 39 for N gene is shown in Figure 1. Sequence information was obtained for all samples sequenced. Raw clusters per sample ranged from 365,690 up to 2,509,712. Samples with more than 1 million clusters were down sampled for analysis. Low Ct samples showed uniform coverage across the entire 29kb SAR-CoV-2 genome. As sample Ct increased, a gradual decrease in coverage uniformity was observed.

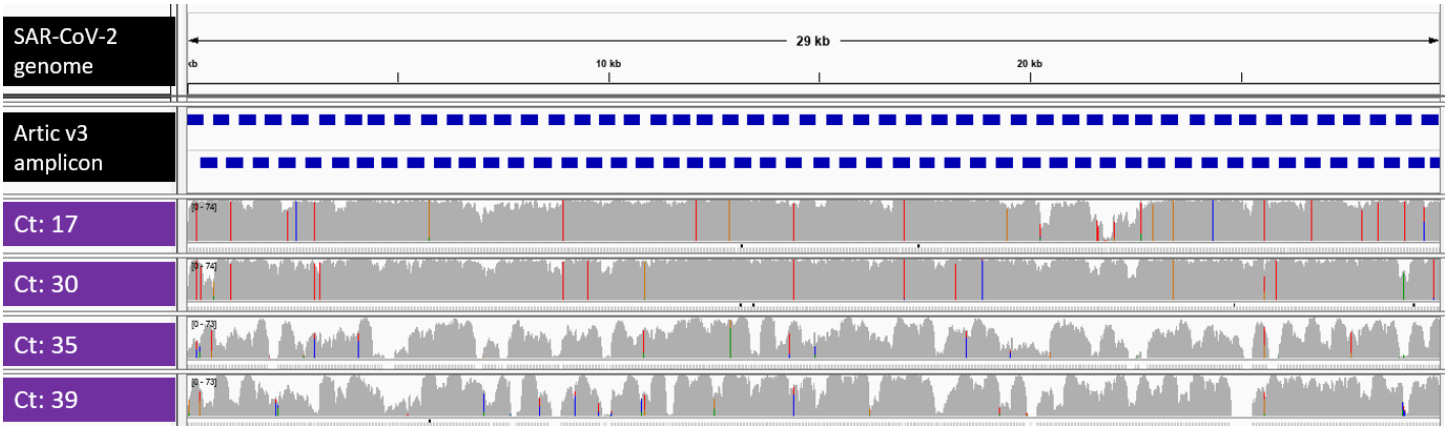

Figure 1: Representative coverage across the 29 kb SARS-CoV-2 genome of samples which tested positive on PerkinElmer® New Coronavirus Nucleic Acid Detection kit. The top panel represents the SARS-CoV-2 WUHAN genomic sequence. The ARTIC V3 amplicons are presented in blue with pool A primers at the top and pool B primers below. The sequence alignment of the Ct values 17, 30, 35, 39 for N gene are shown in the in the purple panels. The Ct value of 17 has the highest viral load (and possible most intact genomic content) which is demonstrated by the high depth of coverage @ ~7SX. A similar coverage was observed for Ct 30. As seen in the two bottom panels though the full genomic sequence coverage for Ct 35 and 39 was not obtained, 10-15% of the SARS-CoV2- genome had unique coverage demonstrating the presence of the viral RNA.

Comparative analysis with other genomes: No sample had 0% alignment to SARS-CoV-2 and no sample aligned better to any other species other than SARS-CoV-2 or hg38. There is a small amount of alignment to the SARS genome, which is due to sequence homology between the SARS-CoV-2 and SARS genomes. This heat map (Figure 2) comparing the Sequencing data to other genomes (29 bacterial and viral genomes) demonstrates that the sequence obtained is unique to SARS-CoV-2.

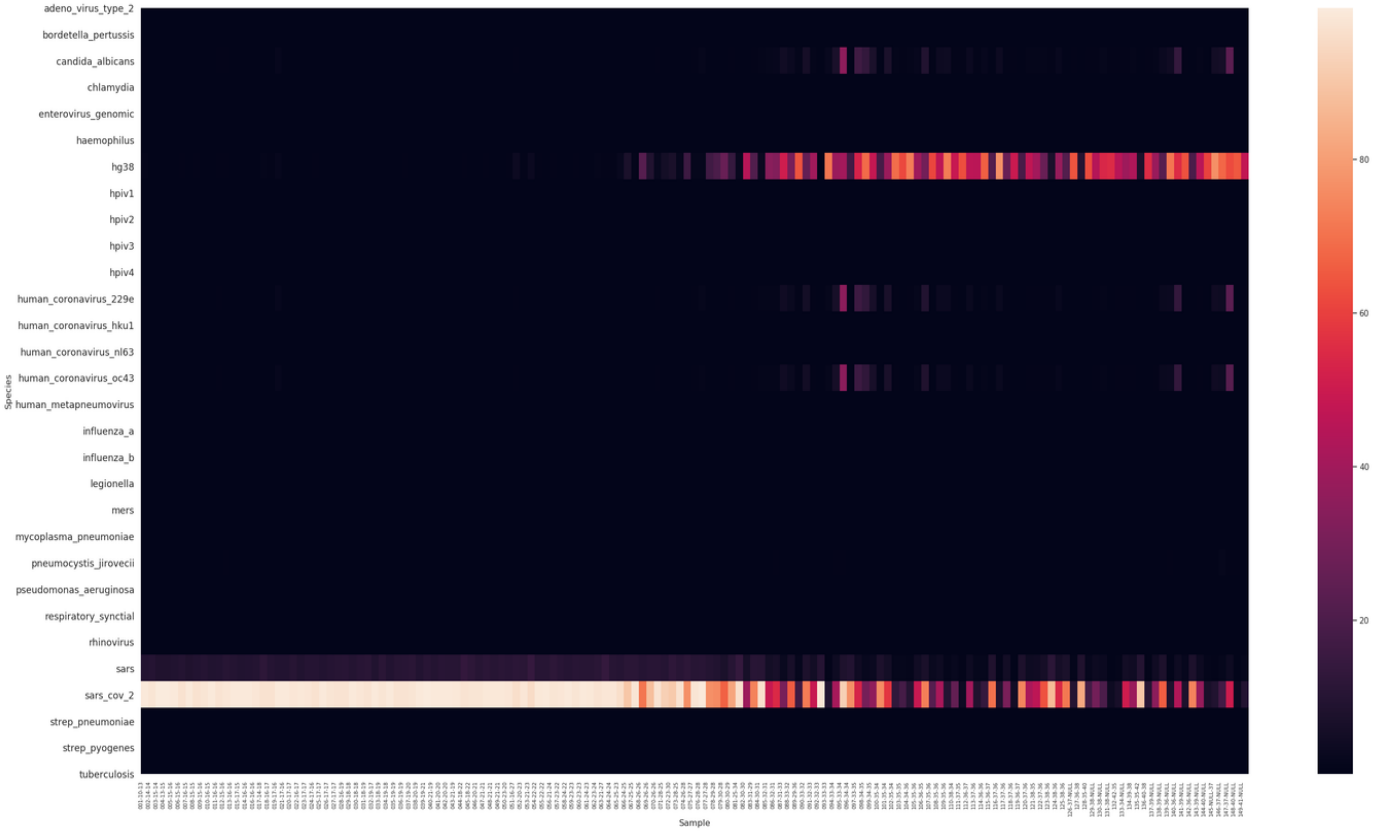

Figure 2: Alignment of reads, against 30 different species, generated from sequencing data of SAR-CoV-2 positive RNA samples demonstrating the unique sequence detection using the PerkinElmer New Coronavirus assay in low to high Ct ranges. Rows represent species, and columns represent samples sorted from low Ct to high Ct (left to right). The heatmap scale is from 0 to 100, representing percent of sample reads aligning to each species. The dark area of the heat map indicates no alignment to the species listed on the left except for the SARS-CoV-2 and human hg38 genomes represented in patterned bars. Residual coverage, presumably artifacts or homologous sequences, were observed in candida albicans and two other human corona virus species hku1 and oc43.

The bioinformatics analysis of NGS data with reference to the Wuhan sequence and the comparative analysis with other genomes confirmed the presence of SARS-CoV-2 in samples associated with a wide range of Ct values including high Ct values generated using the PerkinElmer® New Coronavirus Nucleic Acid Detection kit in the range of Ct >37→<42.

Conclusion

This study was specifically undertaken to demonstrate the analytical sensitivity and specificity of the PerkinElmer® New Coronavirus Nucleic Acid Detection kit. The FDA reference panel study demonstrated sensitivity of 180 NDU/mL. The next closest commercially available kit is at 450 NDU/ml. This data reported on the FDA website shows that there is a 1000-fold difference in Limits of Detection (LOD) between these assays (1). Though RT-PCR as a method is quantitative, the EUA assays are semi-quantitative because they do not determine the viral titer.

Multiple publications have demonstrated the correlation of the severity of COVID disease to low Ct values and high viral loads, the clinical utility of high Ct and of low viral load is still being debated in the literature. Currently it is unknown if these individuals range from being asymptomatic to symptomatic. This study demonstrates that the viral RNA is present in the high Ct value range of Ct <37- <42 and the sequence is unique to SARS-CoV-2. This confirms that the detected Ct values are reflective of the presence of the SARS-CoV-2 virus and they are not an artifact or contamination.

Recent data has shown that at these high Ct values retesting after 12 hours will very likely yield a negative result (4). Similar studies performed on samples with Ct >30 about 3- 25% of the samples retested as negative depending on the kit used (5,6,7). Also, it is important to remember that if retesting is performed on a different assay which has a LOD higher than the PerkinElmer New Coronavirus kit the results are very likely to be negative. Lastly, variation in specimen collection techniques and collection time may also affect results. To date, in spite of extensive precautionary effort it has been difficult to control the increased number of COVID cases. It is time to consider the impact of low viral loads and the impact of these results in the spread of the SARS-CoV-2 virus.

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