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SARS-CoV-2 Virus Real-Time PCR

The Alaska State Public Health Laboratories in Anchorage and Fairbanks use real-time reverse-transcriptase polymerase chain reaction (rtRT-PCR) to accurately identify the presence or absence of SARS-CoV-2 viral genetic material (ribonucleic acid, or RNA) in clinical specimens collected from the respiratory tract of patients. The upper respiratory specimens most often tested by our laboratories are nasopharyngeal swabs, nasal swabs, and oropharyngeal swabs. Real-time RT-PCR remains the gold standard for detecting SARS-CoV-2 worldwide, not just in the United States, because of its superior sensitivity and specificity when compared to other known diagnostic tests for this infection.

How does rtRT-PCR work?

After your specimen is collected, it is transported to our laboratory on ice packs to help preserve the integrity of the specimen and potential viral particles. Real-time RT-PCR happens in 2 steps: Extraction and Amplification.

1. **Extraction (sometimes called "Isolation of RNA"):** Total nucleic acid is isolated from the specimen by chemically removing all other components of the cellular material (i.e. proteins and lipids). This is generally done using high concentrations of chaotropic salts in combination with molecular-grade ethanol. Once the nucleic acids are isolated from the specimen, they are subjected to a PCR test designed for SARS-CoV-2.
2. **Amplification (this is PCR, or replication):** Real-time RT-PCR involves 3 key components: primers, probe, and *Taq* polymerase enzyme. It also involves an initial step of turning the SARS-CoV-2 RNA into complementary DNA with a different specific reverse-transcription enzyme. Once that step is completed, DNA copies (or amplification) can then be made millions of times as long as the 3 components are available. The primers are short chains of oligonucleotides (built from A, C, T, and G bases) that are made to attach to specific target regions of the complementary virus DNA. These primers then act as start and end points for the *Taq* polymerase to replicate (or synthesize) that region. As the *Taq* adds the complementary bases to build a new strand of the target area, it finds a fluorescent probe which sits between the primers. The enzyme will cause the probe to be released and detected by fluorescent spectroscopy at the end of the synthesis. This process describes one PCR cycle. The synthesis of new target strands can be done again and again, exponentially creating more and more new strands and released probes to allow for optimum detectability. Using a calibrated thermal cycler, a full PCR test of 40 cycles takes about 1.5 hours. Between the two public health laboratories in Alaska, we are currently using 9 thermal cyclers to keep up with testing nearly 3,000 specimens daily. See Figures 1 and 2 for further explanations on the testing process and resulting PCR plots.

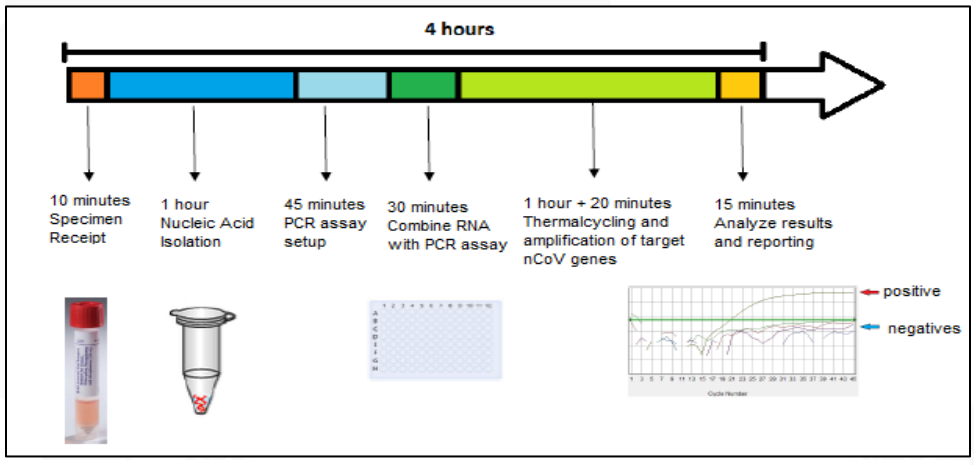

Figure 1. Total time required to test one specimen by real-time PCR. The Alaska Public Health Laboratories must batch specimens (93 specimens per plate) to get the required throughput and keep up with the current demand for testing.

What does a Ct value mean?

Ct stands for "Cycle Threshold." The Ct number refers to the number of cycles needed to see undeniable exponential growth of reporter dye fluorescence. Most diagnostic PCR assays run multiple cycles in order to allow for the various concentrations of viral RNA present in clinical specimens to have a chance to be detected. For instance, if someone is sampled when they are shedding a lot of virus, the sample will have a low cycle threshold meaning detection of the virus was possible during early cycles (i.e. 20 cycles, Ct=20). If the specimen contains low levels of viral RNA, it may take more cycles in order for amplification to begin. These specimens will have late cycle threshold numbers (i.e. Ct=33). Assays are critically evaluated to find end-points (sometimes called cut-offs) describing the number of cycles to run before positive specimens are not reproducible. This really describes the assay's limit of detection. All assays are designed a little differently and various endpoints are established by manufacturers and evaluated by the FDA. Most PCR assays for infectious diseases have Ct cutoffs in the range of 35-40 with any detection below the cutoff considered positive. At the Alaska State Public Health Laboratories, our main PCR assay for SARS-CoV-2 has an end point at 37 cycles, and our backup assay to maintain capacity has an end point of 40 cycles.

Some other important details about Ct values:

- Ct cutoffs are established by test manufacturers through evaluation of known positive and negative samples and are approved as a part of the FDA's Emergency Use Authorization process of the test. Clinical laboratories, including the Alaska State Public Health Laboratories, are federally regulated and always perform rigorous in-house evaluation and validation of each new assay before using it to test patient samples. This involves testing known positive and negative samples to ensure the assay is working properly and is not producing false results.
- SARS-CoV-2 diagnostic rtRT-PCR assays are qualitative (yes or no) tests. They are not designed to determine the amount of virus present in the specimen (viral load) because many external factors can influence these results. Ct values can differ immensely from a poorly collected specimen to a well-collected specimen. A common reason for false negatives is poor specimen collection. Other factors that can impact Ct values include proper (or improper) specimen transport, specimen storage temperatures, how many times the specimen has been frozen, and the instrument on which testing is performed.
- The amount of virus present in a person can vary during the course of their illness. A specimen may have a higher Ct value (low viral load) if the patient is early in their infection and the virus is still increasing in their body, and also later in infection when the viral load is decreasing. In both cases the high Ct value still represents a true positive with SARS-CoV-2 nucleic acid detected.
- For SARS-CoV-2, it is still unknown how much virus is needed to transmit virus from person to person and cause new infections. This is one of the many areas of ongoing research.

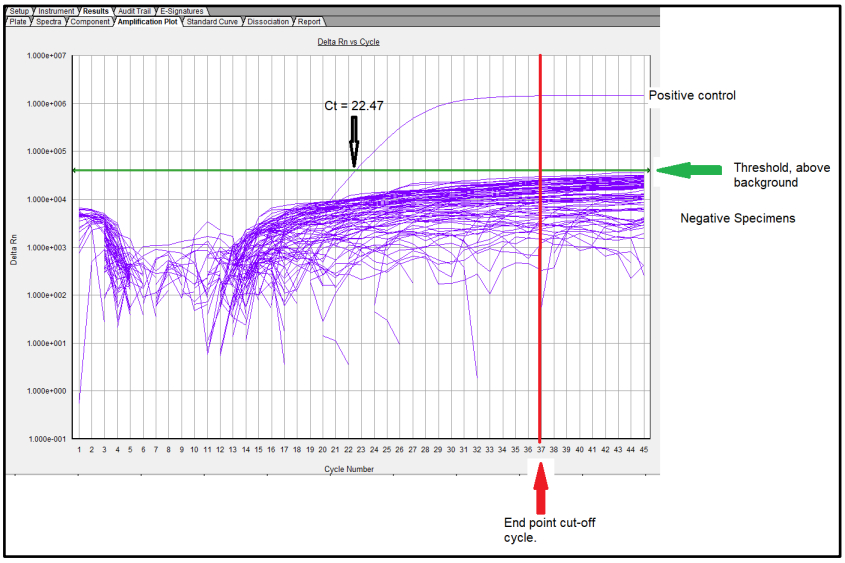

Figure 2. Real-time PCR N1 reaction for SARS-CoV-2 showing threshold, end point, positive control at Ct 22.47, and negative specimen plots

For additional information, please visit the Association of Public Health Laboratories website at:

https://www.aphl.org/programs/preparedness/Crisis-Management/Documents/APHL-COVID19-Ct-Values.pdf