RNA reference materials are useful for standardizing COVID-19 tests, study shows

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NIST researcher Megan Cleveland uses a PCR machine to amplify DNA sequences by copying it numerous times through a series of chemical reactions. Credit: M. Cleveland/NIST

Scientists track and monitor the circulation of SARS-CoV-2, the virus that causes COVID-19, using methods based on a laboratory technique

called polymerase chain reaction (PCR). Also used as the "gold standard" test to diagnose COVID-19 in individuals, PCR amplifies pieces of DNA by copying them numerous times through a series of chemical reactions. The number of cycles it takes to amplify DNA sequences of interest so that they are detectable by the PCR machine, known as the cycle threshold (Ct), is what researchers and medical professionals look at to detect the virus.

However, not all labs get the same Ct values (sometimes also called "Cq" values). In efforts to make the results more comparable between labs, the National Institute of Standards and Technology (NIST) contributed to a multiorganizational study that looked at anchoring these Ct values to a reference sample with known amounts of the virus.

Researchers published their findings in the journal *PLOS One*.

SARS-CoV-2 is an RNA virus: Its genetic material is single-stranded instead of double-stranded like DNA and contains some different molecular building blocks, namely uracil in place of thymine. But the PCR test only works with DNA, and labs first must convert the RNA to DNA to screen for COVID-19. For the test, RNA is isolated from a patient's sample and combined with other ingredients, including short DNA sequences known as primers, to transform the RNA into DNA.

When running a PCR test, labs often use the Ct value to make a positive or negative diagnosis of COVID-19. Labs often set a "cutoff" Ct value above which they interpret and can declare a patient "negative" if the virus is not detected after a certain number of cycles. But even though different labs can accurately detect the virus using their PCR tests, they use their own test methods and instruments, which could lead to different Ct values.

"You can kind of think of it like baking cookies—in the same oven,

from the same recipe, cookies that you bake for 10 minutes will probably be softer than cookies baking for 15 minutes, but if something in the recipe was different, if the oven didn't have the same temperature, if you used a different type of baking sheet, for example, then it might not be as comparable," said NIST researcher Megan Cleveland, a co-author of the study.

So, for this study, a multiorganizational research team set out to explore how much Ct values could vary among different labs when they ran PCR tests on the same reference samples containing known amounts of the SARS-CoV-2 virus. Two reference materials with carefully measured concentrations of the SARS-CoV-2 RNA were developed by a group of organizations and institutions led by INSTAND, an interdisciplinary scientific society in Germany that promotes quality assurance in medical laboratories. RM 1 had an estimated viral load of 10 million (10^7) copies per milliliter, and RM 2 had an estimated viral load of one million (10^6) copies per milliliter.

To ensure these values were accurate, three national metrology institutes—the UK National Measurement Laboratory for Chemical and Bio-Measurement (LGC), Physikalisch-Technische Bundesanstalt (PTB, in Germany) and NIST—measured and validated the reference materials using digital PCR. Digital PCR follows the same steps as traditional PCR but is a more advanced version. In digital PCR, the sample is partitioned into thousands of tiny droplets. A compound is also added so when the targeted DNA is detected it gives off a glow, allowing researchers to confirm if the sample is positive for the coronavirus with the presence of the fluorescent molecules.

The reference materials were sent out to a total of 305 laboratories in Germany, which yielded 1,109 data sets to be analyzed. The Ct values differed between labs depending on the test system or PCR equipment used and the targeted DNA sequences of the virus. For example, PCR

assays aiming to detect a key gene (the "N" gene) in the COVID-19 virus had a range of Ct values between 17.6 and 26.9 for RM 1, while for RM 2 the range was between 20.7 and 30.1. The ranges between the two RMs overlap (20.7 to 26.9) even though they have different viral load concentrations, which shows it's not possible to tell the precise concentration from Ct values alone, said Cleveland.

The differences in Ct values among labs showed the usefulness of reference materials as a tool to help labs compare and standardize their results.

Some have previously viewed the Ct value as a way to measure the viral load, the amount of virus in a person's body. But the variation in Ct values across labs underscores that the two variables are merely correlated with one another. For example, if an individual has a higher viral load, then their Ct value would be low because it would take fewer cycles to amplify the virus's genetic material.

"A lower Ct value means more DNA with the coronavirus starting out in the sample. It correlates to the viral load. But Ct can vary depending on the extraction material or the method used. All of these things can affect at which point the DNA is detected," said Cleveland.

However, by using the appropriate reference materials, Ct values can potentially be converted to an actual virus concentration value in copies per microliter, which would be one way to quantify the viral load, though this wasn't a focus of the study, said NIST researcher and co-author Peter Vallone.

Some have also suggested using Ct values to determine how infectious a patient is, another practice that Cleveland cautions against. "There are those in the field who try to say, for example, a Ct value over 30 means a person has COVID but it's not enough to spread the infection to another

person. But you don't know truly which side you are on, infectious or not, with that value," said Cleveland.

Even though Ct values alone don't determine how sick or infected an individual is, understanding these values could help inform decisions on setting criteria for monitoring people affected by the coronavirus. And RNA reference materials can help make these results more reliable and comparable.

"People should use well-characterized reference materials along with their test methods instead of just relying on the Ct values. On its own, the Ct values are not easily comparable between testing laboratories, but researchers having access to reference materials and understanding their importance is beneficial," said Cleveland.

More information: Laura Vierbaum et al, RNA reference materials with defined viral RNA loads of SARS-CoV-2—A useful tool towards a better PCR assay harmonization, *PLOS ONE* (2022). DOI: 10.1371/journal.pone.0262656

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