An Overview of RT-PCR in SARS-CoV-2 Detection and Comparison to Other Methods

Tony Qu

Grade 10 Student at Toronto Secondary School, Ontario, Canada
Academic Program in Medical Science SLAP COVID 19 Research Program
Supervisor/mentor: Dr. Allina Ding Wang (Sunlife Academic Program in Medical Science, Faculty of Medicine, University of Toronto) February 9, 2021

Abstract:

Sars-Cov-2 is a novel β-coronavirus that has emerged into our world in the winter of 2019. In order to prevent the spread of this pandemic, countries constantly need rapid testing tools to identify and treat outbreaks. This review essay will discuss the history of PCR technology, examine the CDC-approved qRT-PCR detection method, and demonstrate comparison with two CRISPR based assays based on effectiveness.

Keywords: SARS-CoV-2, SARS-CoV-2 detection, PCR, Cas12, Cas13

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Introduction:

Sars-Cov-2 is a novel β-coronavirus that has emerged into our world in the winter of 2019 and it is now affecting 184 countries around the world(1)(2). As of December 30, 2020, it has infected 82,115,011 people worldwide, with 1,793,368 deaths (1).

This coronavirus has caused a WHO-declared international pandemic (3). Humanity has experienced many pandemics, from the Black Death of the 14th century, to the yellow fever of the 18th century, and finally the Spanish Flu of the 20th century (4). Similar to previous times, the economy is going into recession as a result of mass unemployment and consumer discouragement in this coronavirus pandemic. The virus causes a respiratory infection that leads to symptoms similar to a severe viral pneumonia (cough, fever, shortness of breath). In some patients, Sars-Cov-2 can also cause organ failure as a result of a dysfunctional immune response (2).

Sars-Cov-2 is similar to other coronaviruses (SARS-CoV and MERS-CoV) as their genomes are all in the form of a single-stranded ribonucleic acid (RNA). The base of the RNA sequences is in the 5'→3' orientation that would later correspond to messenger RNA (mRNA). The RNA virus encodes 4 structural proteins, the nucleocapsid protein, the S-glycoprotein, the matrix protein, and the envelope protein. The S-glycoprotein binds to the angiotensin-converting enzyme 2 (ACE2), found in the lower respiratory tract of humans. For this reason it has allowed rapid human-to-human transmission inside our cross-connected world (2). Scientists also suspect Sars-Cov-2 to come from bats because it has shown a 96.2% similarity to a bat coronavirus, RaTG13 (2).

While the world anxiously awaits for a Sars-Cov-2 vaccine, the WHO urges countries to actively involve in testing and detection of virus infections in order to develop better containment plans (3). A common and widely-approved method for detection and diagnosis is reverse transcription polymerase chain reaction (RT-PCR), which stemmed from Kary Mullis' PCR technology, invented in 1985 (5). This review essay aims to comprehensively explore the technology that allows RT-PCR to function and compare this method to CRISPR related detection methods.

What is PCR:

Polymerase chain reaction, also known as PCR, was first invented in the late 20th century, its original application of the technology was not anywhere near virus detection. It was organically used for the simple mass duplication of small DNA samples, which could then be used for analysis with different applications. Even today, the use of PCR is not limited to the detection of Sars-Cov-2. The technology is also used in the diagnosis of gene defects, detection

of AIDS virus, genome editing, DNA recombination, paternity testing and even forensic science(5)(17).

The success of PCR depends on technological breakthroughs such as the utilization of thermostable Taq enzymes (6). Towards the end of the century, the real time monitoring of DNA amplification (qPCR) through monitoring of fluorescence was introduced, allowing users to measure the momentary amount of DNA amplicons in the sample at a specific time (6). Following this breakthrough, there were thermal cycling machines invented to accommodate this technology.

PCR technology requires only a small amount of DNA samples in a test tube. The genetic information will then be duplicated into bigger amounts (7). The goal of a common PCR test is to amplify specific trace amounts of genetic material, identifying specific parts of DNA. This process mimics cell division, where DNA is divided by first separating its two strands of polynucleotides and then creating a new strand based off of the template. A PCR test performs this task faster and produces large quantities of genetic material (5)(7).

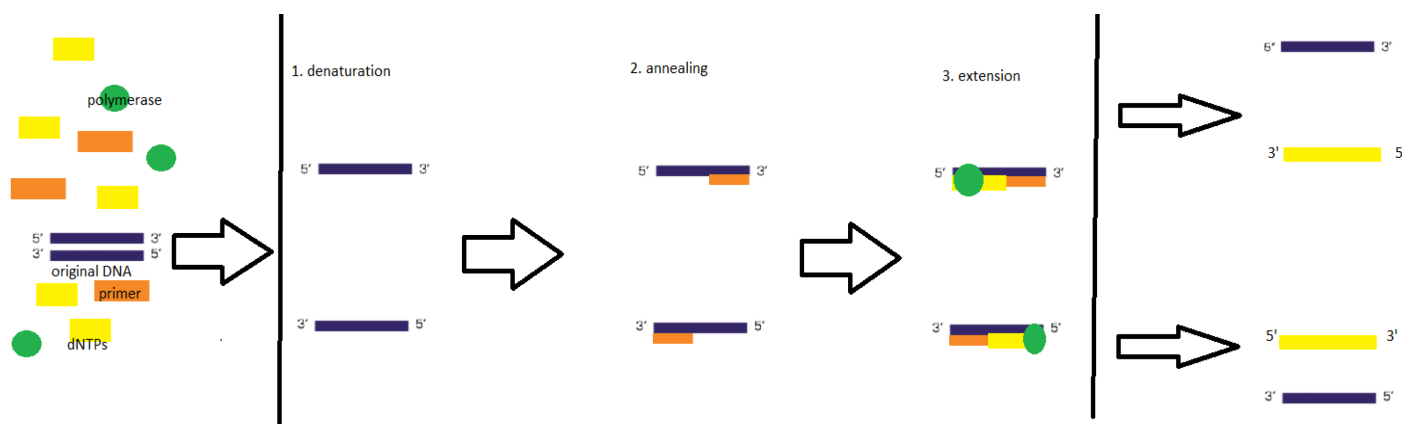


Figure 1: This figure describes the basic process of PCR in quantifying genetic material. Each cycle of PCR involves the 3 processes: denaturation, annealing and extension, and requires primers, dNTPs and polymerase. These cycles can be repeated n times depending on the researchers requirement for quantification.

What is Reverse Transcription PCR:

The first process in a RT-CPR test is that the RNA template gets converted into a complementary DNA (cDNA) using a reverse transcriptase. Through the process of PCR, the cDNA is then duplicated many times as a template. This often involves either real-time quantitative nucleic acid sequence-based amplification (QT-NASBA) or real-time quantitative PCR (qRT-PCR) (8).

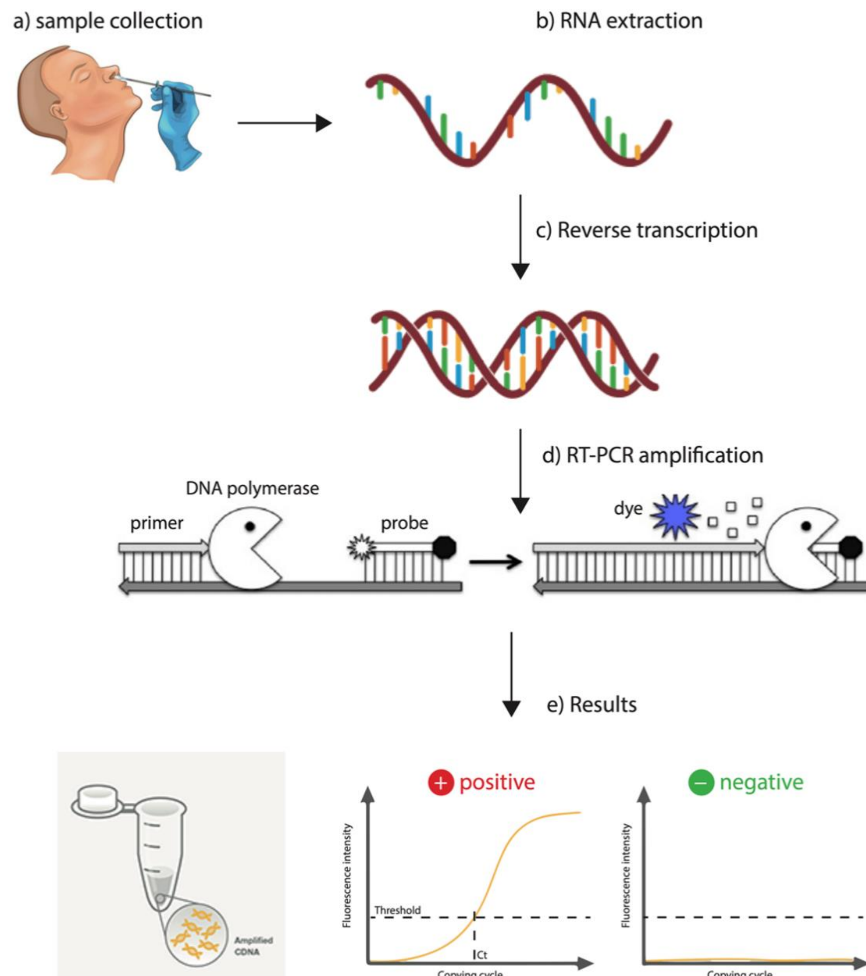


Figure 2: This figure shows the general process of RT-PCR test. *Figure taken from "The Worldwide Test for Covid-19" by Global Biotechnology Insights.*

What is qRT-PCR:

Real-time quantitative PCR, also known as qRT-PCR, is a process that is evolved from RT-PCR and used in many tests. It is used to detect, characterize and quantify nucleic acids for different applications. (9) After the cDNA is created, it will go through 3 processes (the cycle of PCR): denaturation, annealing and elongation. Denaturation is the process that heats up the sample to around 95 celsius to break the cDNA from RNA (10). After creating a single-stranded DNA, the annealing process happens at 58 celsius and allows the primers to bind to the template (10). During elongation, a new strand of DNA will be synthesized by the Taq enzymes at stable temperature (10). Sometimes, fluorescent labeling is used to allow the collection of data as the process is performed. From the 5' end of the newly formed strand, fluorescence can be examined using special tools. The fluorescence signal increases proportionally to the amount of replicated DNA (6). As data is collected during each cycle, the DNA is quantified, thus calling it "real-time".

One-step RT-PCR and Two-step RT-PCR

A RT-PCR test can be done using two methods, the first method is a one-step RT-PCR. This process allows the entire RT-PCR test to be done in one single test tube. The second method is a two-step RT-PCR, which requires the test to be partially done in the first test tube and completed using a second test tube (11). The two-step RT-CPR is considered to be more vulnerable to contamination because the genetic material could be contaminated when it is transported from the first test tube to the second. However, some still prefer two-step RT-PCR because the one-step RT-PCR is prone to degradation of the RNA template and inaccuracy in quantification (11).

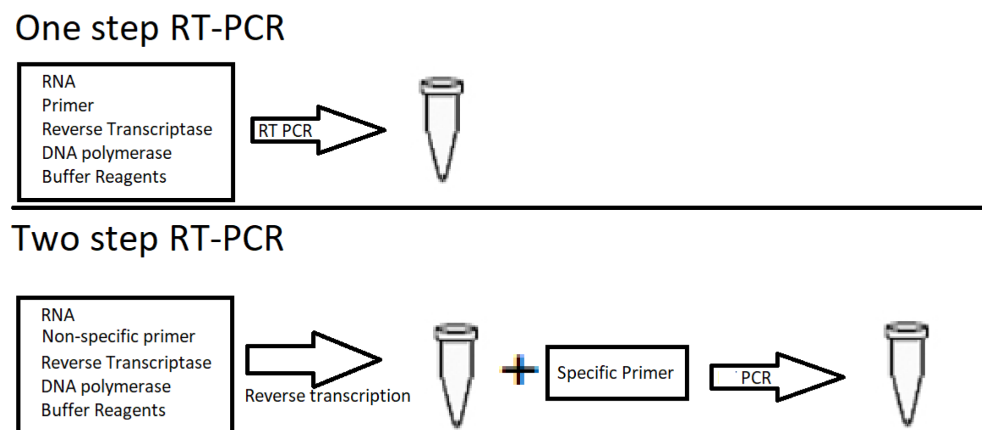


Figure 3: In a one-step RT-PCR, involves one test tube that includes RNA, primers, reverse transcriptase, DNA polymerase, and buffer reagents. The test tube undergoes RT-PCR directly. Differently, in a two-step RT-PCR, the test involves two test tubes. In the first test tube, RNA, non-specific primers, reverse transcriptase, DNA polymerase, and buffer reagents. After the first tube undergoes reverse transcription, the material is added with a specific primer before being transported into the second tube for PCR (denaturation, annealing and elongation).

The Role/Function RT-PCR in Sars-COV-2 Detection

PCR technology only works on DNA (double-stranded) while Sars-COV-2 coronavirus is a RNA (single-stranded) virus, so in order to detect the viral infection RNA needs to be transcripted back to DNA using the reverse transcription technology. The test begins with a specimen from a nasopharyngeal swab. Then, the sample RNA will be separated and collected. Using a one-step RT-PCR process, the isolated RNA is mixed with enzymes (DNA polymerase and reverse transcriptase), DNA building blocks, cofactors, probes and primers that are appropriate for Sars-COV-2 testing (12).

The primers for the SARS-CoV-2 RT-PCR detection involve a novel primer targeting SARS-CoV-2 Nucleocapsid N gene sequence, and two primer pairs each probing to another non-overlapping region of SARS-CoV-2 Nucleocapsid N gene and ORF1ab gene (13). SYBR-Green I dye that binds to DNA is also used for the real-time monitoring of the detection test (as shown in figure 3) (13).

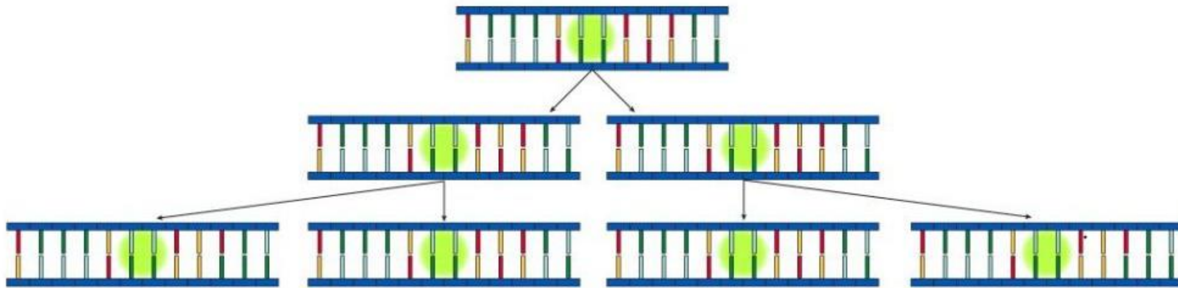


Figure 4: This figure demonstrates the duplication of DNA with SYBR-Green I dye. Researchers observe the fluorescence from the dye to determine the quantification of RT-PCR. *Figure taken from the journal "The Science Behind the Test for the COVID-19 Virus", Discovery's Edge Mayo Clinic.*

This one-step one tube easy-to-develop testing method makes qRT-PCR so prevalent in SARS-CoV-2 testing. Although it could take up to days before a result can be shown for a sample, the test demonstrates adequate accuracy (around 90%) (14). The only downside of this test is that it cannot detect antigens or past infection.

CRISPR Detection Methods

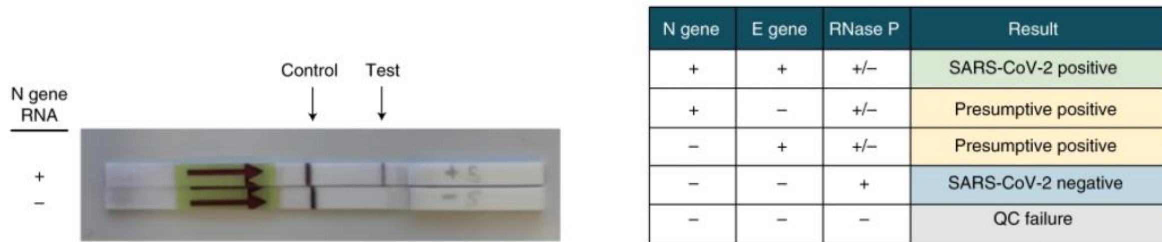
There are many other less popular methods used to detect SARS-CoV-2 infection. Clusters of regularly interspaced short palindromic repeats (CRISPR) are commonly known for its special use of Cas-protein in gene editing. Emmanuelle Charpentier received a nobel prize for her efforts in developing the CRISPR-Cas9 used in editing genomes (15). However, CRISPR-Cas12 and CRISPR-Cas13 have been discovered to be useful for detecting single-stranded RNA viruses. The two CRISPR tests will be the examples discussed and compared to RT-PCR.

CRISPR-Cas12 in SARS-CoV-2 Detection

A Cas12-based test, called SARS-CoV-2 DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR), was reported by Broughton et al. The test uses loop-mediated

amplification (LAMP) from a nasopharyngeal or oropharyngeal sample to undergo reverse transcription and isothermal amplification before being confirmed by a reporter molecule in a Cas12-related process (16).

The primers used in this test targets the envelope (E gene region) and the nucleoprotein (N2 region) genes because they match with the Cas12 guide RNAs (gRNAs). The Cas12 gRNA uses multiple amplicons with probes that are specific to SARS-CoV-2. The SARS-CoV-2 DETECTR assay focuses on the E gene, N gene and human RNase P gene. The DETECTR assay can be run in approximately 40 minutes with 20-30 minutes and 10 minutes in LAMP reaction and Cas12 detection reaction respectively (16).



N gene	E gene	RNase P	Result
+	+	+/−	SARS-CoV-2 positive
+	−	+/−	Presumptive positive
−	+	+/−	Presumptive positive
−	−	+	SARS-CoV-2 negative
−	−	−	QC failure

Figure 5: A result showing detection in both E and N genes is considered to be positive. A result showing at least one detection in either E or N genes is considered presumptive positive. A result showing detection in neither is negative, and it is considered failed if it also shows no detection in RNase P. *Figure taken from journal "CRISPR–Cas12-based detection of SARS-CoV-2" by James P. Broughton et al., Nature Biotechnology*

The DETECTR assay for SARS-CoV-2 is considerably accurate and extremely rapid compared to the qRT-PCR method. Ignoring travel and setup time, this Cas12-based test can be done in less than 40 minutes with a 95% accuracy relative to qRT-PCR (16).

CRISPR-Cas13 in SARS-CoV-2 Detection

Cas13 is a RNA-guided RNase that produces multiple cleavage sites in a single-stranded RNA target with base preferences (18). In a two-step Cas13-based Specific High Sensitivity Enzymatic Reporter UnLOCKing (SHERLOCK) system, Cas-13 is used in

combination with isothermal amplification methods such as reverse transcription recombinase polymerase amplification (RT-RPA) (19). The RT-RPA step, with specially designed primers, occurs before the Cas-13 detection. Cas-mediated nucleic acid probe cleavage can be observed by fluorescence detection or using the lateral-flow strip method, which is an easily portable and readable method (19).

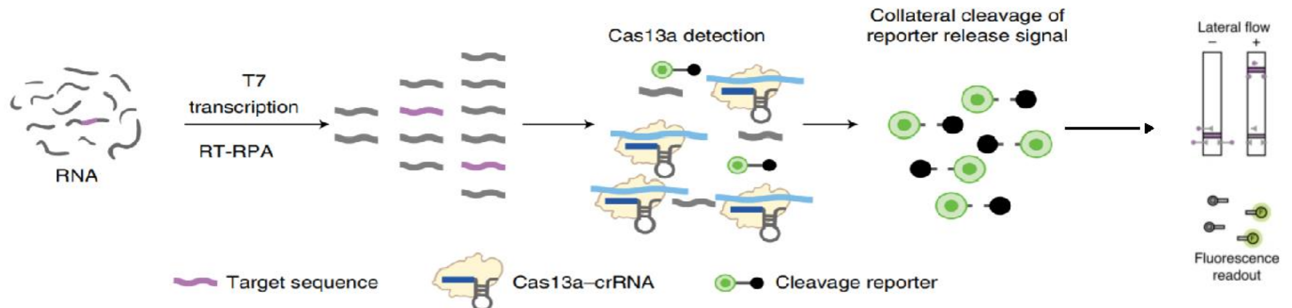


Figure 6: This figure demonstrates the process of a two-step SHERLOCK Cas13-based detection method for Sars-Cov-2. *Figure modified and adapted from "SHERLOCK: nucleic acid detection with CRISPR nucleases" by Max J. Kellner et al. and "Clinical validation of a Cas13-based assay for the detection of SARS-CoV-2 RNA" by Maturada Patchsung et al.*

This Cas13-based test employs high levels of portability, sensitivity, and accuracy. This CRISPR model can be designed into a point-of-care detection method which everyone can use at home (18). Its sensitivity can be summarized by being capable of single-molecule detection in a RNA sample of 1 μL (19). In a clinical sample of 154, the Cas13 assay was able to identify 73 out of 73 negative COVID-19 samples and 78 out of 81 positive COVID-19 samples (18).

Conclusion:

Ths review paper examined three methods that take the genetic approach to detect Sars-Cov-2: qRT-PCR, Cas12 DETECTR, and Cas13 SHERLOCK. As the cases rise in western nations, the need for point-of-care detection methods becomes more salient. While POC CRISPR tests are available in research settings and trials, qRT-PCR, which takes up to days for a patient to receive results, remains to be the most used method by healthcare systems. In the distant future, increased usage of POC tests could help in detecting more remote or immobile communities (rural areas or senior care homes) that don't have access to research labs or hospitals. In the future, for DETECTR and SHERLOCK assays to become more prevalent, they need to meet two requirements. First is to increase accuracy to the point where they are equally or more accurate than qRT-PCR. Second is to be involved in mass production in order to replace the rapid demand of qRT-PCR. There is room for improvements for all three methods. qRT-PCR, DETECTR and SHERLOCK are all either not developed to perform antigen tests or not used for antigen tests. In the future when more vaccines are made, we would need to begin doing antigen tests to ensure the quality of vaccines as well as assess population immunity. Moreover, for the CRISPR-based assays to become more successful, they would need to have lower false negative rates in relation to qRT-PCR. This may be done by using more accurate amplification methods that replace RT-LAMP and RT-RPA. Overall, all three Sars-Cov-2 detection tests demonstrate adequate accuracy, high accessibility, and rapid results.

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