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Review

Diagnosis of Viral Infections: A Review of the Current Assays and the Prediction of the Future

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Abstract: Different types of diagnostics methods were introduced to the public due to the recent global pandemic. From the real-time Reverse Transcript Polymerase Chain Reaction (RT-qPCR) to antigen Lateral Flow Assay (LFA), these tests allow relatively accurate and rapid diagnosis of the infection with SARS-CoV-2 virus. Because each assay has separate principles, benefits, and limitations, the diagnostic methods in the future, especially with the rapid development of new techniques, could provide quick, accurate, and accessible Point-Of-Care Testing (POCT). New technologies could be used to identify the virus for future epidemics or other infections that occur in every-day lives, potentially stopping another global pandemic from affecting the community, the economy, and the government.

***Keywords*:** SARS-CoV-2, RT-qPCR, assays, diagnosis, LFA, point-of-care

1. Introduction

Since December of 2019, the COVID-19 pandemic, caused by SARS-CoV-2, has affected many across the globe. According to the World Health Organization (WHO) Coronavirus Dashboard, there have been more than 570 million confirmed cases of the coronavirus and 6 million deaths as of August 1st, 2022. As Chinese scientists released the "initial sequence of the virus" genome of the virus to the public on January 10, 2020 (as informed by Edward Holmes, a virologist and evolutionary biologist at University of Sydney and a member of a consortium led by Yong-Zhen Zhang of Shanghai Public Health Clinical Center & School of Public Health) researchers internationally began to craft vaccines and In Vitro Diagnostics (IVD) methods ranging from molecular assays (MDx) to antigen tests to control and prevent the virus from spreading further [1-2]. Even after the COVID-19 pandemic, the diagnostic industry is expected to grow by 2.4% to USD 85.81 billion by 2030. And undoubtedly, IVD and MDx play a huge role in the industry's market cap [3].

In August of 2022, I had an opportunity to work with the researchers of NanoHelix Co., Ltd., a biotechnology company that provides molecular biological reagents and raw materials for molecular diagnostics internationally. Throughout my time there, I investigated and analyzed the current diagnostic methods for COVID-19, including the Polymerase Chain Reaction (PCR) and the rapid lateral flow antigen tests for SARS-CoV-2. Specifically, I went through the testing procedure of PCR from creating a mixture of the raw materials to running and interpreting the test results from the PCR machine.

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Along with the boom in the viral diagnosis industry, my experience at NanoHelix inspired me to investigate the possible methods that may be integrated for future viral pandemics like COVID-19. This review will discuss the present diagnostics utilized during the pandemic, as well as the possible tests that may be employed for the future detection of viruses.

2. Current diagnostic assays for COVID-19

2.1 *Polymerase Chain Reaction (PCR)*

The Polymerase Chain Reaction (PCR) test is one of the most used methods for COVID-19 diagnostics because of its accuracy and its ability to test large numbers of samples at once [4]. PCR itself is a technique used to amplify the DNA sample exponentially over time to create enough copies in order to facilitate the process of analysis. Consequently, it has been used as a common tool in medical and biological labs to sequence the DNA in the early stages of an infection, help detect the gene of the pathogen in an infection, or generate forensic data from the small DNA samples. It was invented by Kary B. Mullis in 1983, and he was awarded the Nobel Prize in Chemistry for his creation.

2.1.1 *Stages of PCR*

PCR can be broken down into three major stages: denaturing, annealing, and extending. In the denaturing stage, the double helix of the DNA is split into single strands due to a heat of around 94-95 °C, which breaks the hydrogen bonds connecting the two strands. The single strands are used as templates for replication in the following steps.

In the annealing stage, the primers, called forward and reverse primers, attach to the complementary strand of the DNA to initiate the replication process. They are heat-resistant, short pieces of the DNA that are designed to be complementary to the sequence of the target DNA strand [5]. These primers indicate the start and the end of replication and are necessary for the polymerase enzyme to build the complementary DNA in the next stage. Lastly, the annealing stage works best in around 50-68 °C [6]. More specifically, the optimum temperature is around 5 °C lower than the melting temperature of the primers because too low temperatures may cause the primers to bind nonspecifically to the template.

The final step is the extending stage, where Taq polymerase enzyme starts from the primers and uses the deoxynucleotide triphosphates, also known as dNTPs or DNA nucleotides, to build the complementary strand of the single-stranded DNA. Taq polymerase, named after Thermus aquaticus, is a thermostable DNA polymerase that works best at higher temperatures compared to other polymerases [7]. The optimal temperature for the enzymes is 72°, but the extending stage may be between 30 °C to 90 °C for less timing dedicated towards change in temperature from the previous stage. All the three steps, denaturation, annealing, and extension, make up one cycle, and PCR usually runs for 25-35 times for the best output. However, according to Thermo Fisher Scientific, it may be required to run more than 40 times if the DNA copy is fewer than 10 copies but less than 45 cycles due to the appearance of nonspecific products [8].

2.1.2 *Variations*

PCR tests come in several different types. For coronavirus diagnosis, researchers mainly use Reverse-Transcript PCR (RT-PCR), real-time PCR (qPCR or quantitative PCR), or a combination of both. The reverse-transcript PCR has one more step at the beginning than a regular PCR, which is to reverse-transcribe RNA to complementary DNA (cDNA) because PCR cannot amplify RNA. Like many other infectious viruses, SARS-CoV-2 is composed of RNA. To synthesize cDNA from RNA, either the non-sequence specific primers or sequence-specific primers is used [9]. After the reverse-transcription, the cDNA will be used as a template for PCR.

Another variation of PCR is real-time PCR, also known as quantitative PCR or qPCR for short. This variation records the quantitation of the target DNA sequences with fluorescence while the PCR machine amplifies them. These machines include a standard thermocycler platform with an excitation source, a fluorescent-detecting camera, and external computer and software to process the collected fluorescent data [9]. One of the most used fluorescence detection methods is the TaqMan PCR. TaqMan PCR uses a dye-labeled nucleic acid probe, which is labeled with two fluorescent moieties [10]. These include a reporter and a quencher. When the two moieties are close to each other, the quencher

absorbs the fluorescence released by the reporter; however, during the extending stage of PCR, the Taq polymerase cleaves the probe and separates the reporter and the quencher, thereby allowing the reporter to release the fluorescence. The computer software detects and records the increased fluorescence in the data. Overall, the ability to quantitate the amount of targeted DNA sequence makes real-time PCR highly accurate and useful for the coronavirus diagnosis.

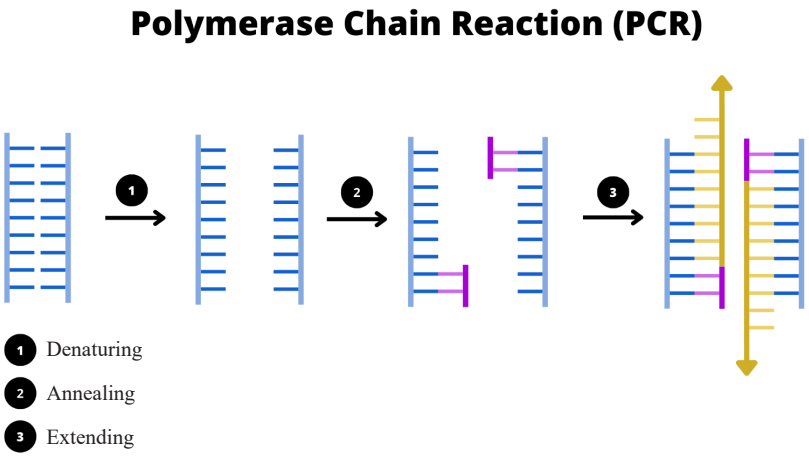


Figure 1. Three stages of PCR. In the diagram, blue represents the original DNA, purple represents primers, and yellow represents the complementary DNA nucleotides

2.1.3 *Example RT-qPCR method and results*

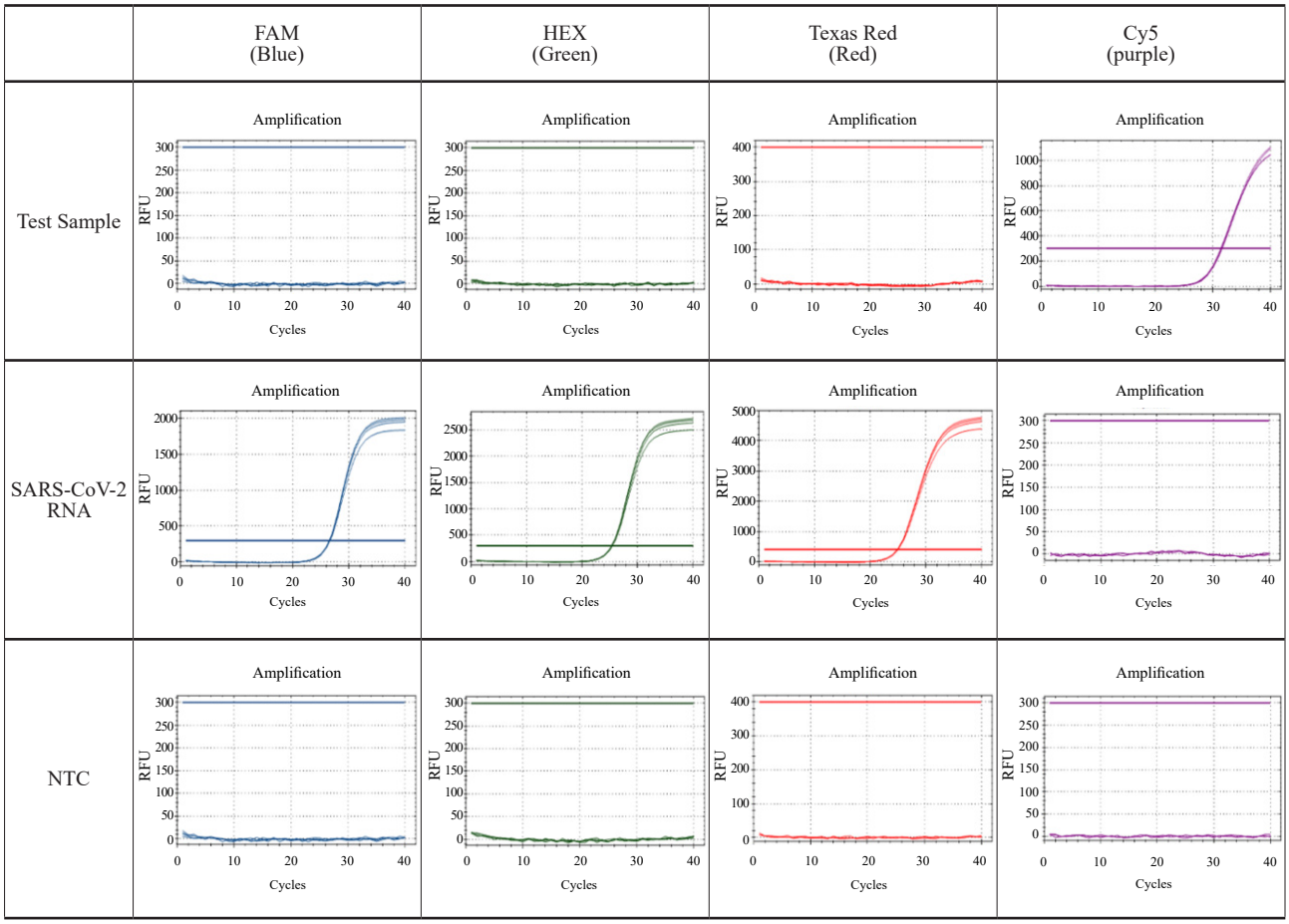
Many of the current molecular assays used to test COVID-19 are a combination of both reverse-transcript PCR and quantitative PCR (often referred to as RT-qPCR). During my time at NanoHelix, I had an opportunity to test out RT-qPCR myself.

This experiment consisted of three separate templates: a nucleic acid sample, RNase-free Water (Negative Control or No Template Control [NTC]), and a sample SARS-CoV-2 (Positive Control or PC). The initial step consisted of preparation of a master mix that consists of 2 μL mixture of enzymes (enzyme mix or EM), 4 μL 5× buffer mix (reaction mix or RM), 4 μL mixture of primers and probes (oligo mix or OM), and 7 μL distilled, sterile, RNase-free water. The measurements were for one round of the PCR test. For the experiment, each template was tested four times for accuracy and precision, meaning that a total of 12 times (3 template × 4 repetition + 3 more times to ensure sufficiency of the components) of each component was used. The vortex and the centrifuge were utilized to mix these components. Then, the master mix was split into 12 separate wells in a 96-well plate, and each of the four wells were mixed with 3 μL of the test sample, NTC, and PC each. After this process, the well plate was placed into a small box before moving to the room with the PCR machine to avoid any possible contamination or exposure to strong lights, as failures in sample collection and storing methodology could create false-positive or false-negative results [11].

In the PCR room, the instrument used to run the test was called the Bio Rad CFX96 Real-time PCR detection. The CFX96 used the following fluorescents to detect the following genes in the sample: FAM to detect S gene, HEX to detect ORF8 gene, Texas Red to detect N gene, and Cy5 to detect IC (RNase P) gene. The PCR system temperature conditions are as shown in Table 1.

The threshold for each of the genes was set at 300 RFU (relative fluorescence units) for S, ORF, and IC (RNase P) genes and 400 RFU for the N gene. For the test to be valid, the Ct (cycle threshold) value of the PC had to be less than 27 for each of the genes, while the Ct value of the NC had to be greater than 37 or N/A for each of the genes. If this case was not true, the test had to be redone due to possible contamination or machine error during the experiment. Each individual Ct value for the target genes of all the samples are illustrated in Figure 2. Based on the Ct results, it is apparent that this test is valid because the values for PC and NC are within the range of the threshold. The values of

the test sample demonstrate that it is negative from the virus because the RFUs for S-gene, ORF8, and N-gene did not exceed the threshold within the determined Ct cycles. Likewise for future tests, the sample can be categorized as either positive, negative, or inconclusive based on the values. The threshold and the determining Ct cycles may differ depending on the device and the manufacturing company.



Template	Florescence and target gene	Ct Value				
		1	2	3	4	Average
Test Sample	FAM (S-gene)	N/A	N/A	N/A	N/A	**N/A**
	HEX (ORF8)	N/A	N/A	N/A	N/A	**N/A**
	Texas Red (N-gene)	N/A	N/A	N/A	N/A	**N/A**
	Cy5 (RNase P)	31.35	31.42	31.50	31.60	**31.47**
SARS-CoV-2 RNA	FAM (S-gene)	26.62	26.48	26.55	26.45	**26.53**
	HEX (ORF8)	25.46	25.36	25.30	25.29	**25.35**
	Texas Red (N-gene)	25.01	24.84	24.83	24.84	**24.88**
	Cy5 (RNase P)	N/A	N/A	N/A	N/A	**N/A**
NTC	FAM (S-gene)	N/A	N/A	N/A	N/A	**N/A**
	HEX (ORF8)	N/A	N/A	N/A	N/A	**N/A**
	Texas Red (N-gene)	N/A	N/A	N/A	N/A	**N/A**
	Cy5 (RNase P)	N/A	N/A	N/A	N/A	**N/A**

Figure 2. The test results for each template. The chart shows the change in the RFU value compared to the number of cycles. It directly reflects the data set, which are the Ct values for each of the template, fluorescence, and target gene, illustrated below

Table 1. The temperature conditions for each step of the RT-qPCR. Although the optimum temperature for the enzymes is 72 °C, the cycle does not change the temperature for simplicity and faster speed in PCR testing

Step	PCR Condition	Cycle(s)
cDNA synthesis	50 °C for 10 min	1
Enzyme activation	95 °C for 3 min	1
PCR amplification	95 °C for 1 sec	2
	60 °C for 20 sec	
	95 °C for 1 sec	40
	60 °C for 5 sec	

2.2 *Antigen Lateral Flow Assays (LFA)*

Antigen Lateral Flow Assays (LFAs), also simply known as Antigen tests, are another most used tests to diagnose COVID-19. It is popular for being quick, accessible, and inexpensive. Consequently, it is widely accepted among users and the regulatory authorities, allowing many individuals to diagnose themselves at home without visiting the doctor's office. Although its accuracy is lower than the PCR test, some health professionals also utilize the LFAs for quick diagnosis. The technical aspect of the LFAs was established by Plotz and Singer in 1956 from their latex agglutination assay [12]. Since then, this test has been used for quite a time for pregnancy and diseases like HIV, Ebola, malaria, Zika virus, flu variants, water pollutions, and other foodborne contaminants [13].

LFA can be categorized into two different types: Lateral Flow Immunoassay (LFIA) and Nucleic Acid Lateral Flow Assay (NALFA) [14]. LFIA exclusively recognizes antibodies, while NALFA recognizes nucleic acids, which are commonly amplified during PCR. This article focuses on the LFIA technique as it is used to detect SARS-CoV-2 during the pandemic.

2.2.1 *Principle and method*

In LFIA, the sample flows through the device and uses the antibodies to display whether the targeted antigen exists in the sample or not. There are several parts to the LFIA device: the sample pad, conjugate release pad, test line, control line, and the absorbent pad. The sample pad is where the test taker inserts their sample. The sample is mixed with buffer salts and surfactants on the pad to optimize it to work best in the device. The mixed liquid flows down to the conjugate release pad, where the label, usually colloidal gold nanoparticles or fluorescence, and conjugate antibodies that are designed to target a specific antigen are located at. If the sample has the targeted antigen, it attaches to the labeled conjugate antibody. Colloidal gold nanoparticles are more commonly used than fluorescence because they change the colors when they aggregate due to the shift to longer wavelength within the surface plasmon band, making it easier to identify the existence of certain antigens without the use of another machine to detect fluorescence [15]. The sample mixed with labels and conjugate antibodies moves to the test line, where there is a fixed antibody that attaches to the targeted antigen. If the targeted antigen exists, the antibody attaches to the antigen, its conjugate antibody, and the label from the conjugate pad in a way that the label faces the exterior. When enough labels are close to each other, they aggregate and change in color. If the targeted antigen does not exist, the antigen does not attach to the fixed antibody and move on. Whether the solution attaches to the test line or not, the remaining moves onto the control line. In the control line, the conjugate antibody from earlier should attach to the fixed antibody regardless of the existence of the targeted antigen, while again having the label face outwards. This should automatically change the color in the control line. If the color does not change, this indicates an error in the device, and the test taker must retake the test. Finally, the remaining solution empties in the absorbent pad.

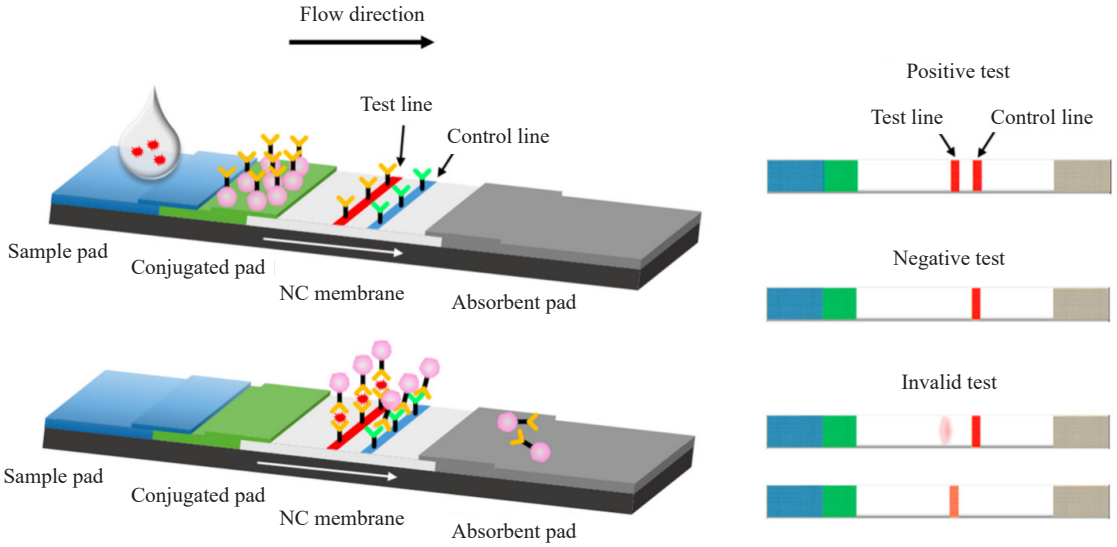


Figure 3. The structure of lateral flow immunoassay and the test result interpretation. The left illustration represents the case when the sample has the targeted virus [16]

2.3 *Benefits and limitations of PCR and LFA*

Both testing methods have several advantages and disadvantages inherent to them. Starting with the benefits, PCR testing allows large replication of genes in a relatively short period of time compared to the previous method of culturing. This facilitates researchers' ability to sequence, detect, and analyze fewer common organisms in the sample. PCR also sets up for other testing methods, such as the nucleic acid lateral flow assay, making itself useful in many circumstances. Lastly, the different variations of PCR allow researchers to analyze different forms of samples in several different ways. For example, RT-PCR allows the analysis of RNA samples instead of DNA samples, while qPCR allows scientists to quantify the targeted gene of the sample.

However, PCR does come with some limitations. Because this molecular assay replicates a large number of genes, even small contamination within the sample can cause a false-positive response. So, only a trained medical professional may conduct accurate PCR tests at a lab. Additionally, designing the correct primers takes a significant amount of time because some combinations of sequences are possible in the genes that are not the targeted gene. Without careful consideration, the test results can display false-positive results. Even after designing the correct primers, they sometimes bind to regions of DNA that are similar to the primer, allowing for another false-positive response. Moreover, the test could take up to a day or two because PCR requires advanced technology, making it necessary to send the sample to a lab for testing. This also makes the test more unaffordable and expensive compared to the Antigen test.

The lateral flow immunoassay also has several benefits. Unlike PCR, it is fast and requires low cost. The test kit can be bought in a local pharmacy for a low price and the test taker can self-diagnose based on the qualitative results within 15-30 minutes, allowing for point-of-care use. Additionally, it has a long life without needing to be refrigerated. This demonstrates its accessibility in many environments, as well as its ability to be shipped to underserved countries for their use. Lastly, the LFAs not only detect antigens, but it is also designed to detect several other types of samples, such as proteins, haptens, nucleic acids, and amplicons.

One of the downsides of LFIA is that it is less sensitive compared to PCR testing, especially with a small number of antigens during the early stages of the infection. Because of its low accuracy, health professionals often recommend test takers to test again with RT-PCR if found positive with the antigen test. This also means that a negative result does not eliminate the possibility of an infection. Finally, the LFAs are mostly qualitative or semi-quantitative, meaning it does not display the amount of targeted antigens unlike the qPCR test.

2.4 *Other tests*

Although PCR and Antigen tests were the most used diagnostic methods, there are other tests that were verified to diagnose SARS-CoV-19. Some of the more popular ones include the antibody test, isothermal nucleic acid amplification, next generation sequencing, and mass spectroscopy, all of which have been used for diagnosing other diseases.

2.4.1 *Antibody test*

Unlike the antigen test, the antibody test looks for the antibodies created by the B-cells in the body to fight the antigens. It is detected through the blood of people who have been previously infected with the coronavirus. However, it is also possible to detect antibodies from vaccines, meaning the test isn't as reliable to diagnose the current infection [17]. The first blood antibody test for HIV was approved by the FDA in March of 1985 [18]. However, since then, a lot about the test has changed, such as testing saliva instead of blood and quicker test results.

2.4.2 *Isothermal nucleic acid amplification*

Unlike PCR testing, isothermal nucleic acid amplification doesn't require any changes in temperature. One of the popular types of this test is reverse transcription loop-mediated isothermal amplification, short for RT-LAMP. It is a one-step molecular assay that can quickly replicate sequences of the targeted RNA, making it useful for diagnosing RNA viruses. This was previously used for MERS-CoV and SARS-CoV, and it has also been shown to be effective in diagnosing the coronavirus. This technology was invented in 2000 and has been used for diagnosing viruses like the hepatitis B virus and the West Nile virus [19]. However, because of its complexity, the primers are more difficult to design.

2.4.3 *Next Generation Sequencing (NGS)*

The next generation sequencing technology, also known as NGS, is a massive sequencing machine that is also used to detect the targeted sequence of the RNA or DNA or understand the entire genome. For the coronavirus pandemic, NGS allowed comprehensive analysis of the SARS-CoV-2 virus, which researchers used to create the vaccines and the diagnosing assays. The first version of this technology was launched in the 2000s, and the company was later bought by Illumina [20]. Although NGS can identify the viral agents without prior knowledge, it also creates a massive amount of data, costs more, and takes a long time to process [4].

2.4.4 *Mass spectrometry*

Mass spectrometry is an analytic tool that is used to measure the mass-to-charge ratio of the present molecules. They detect the type, amount, structure, and chemical composition of the compound [21]. Although mass spectrometry is less sensitive in diagnosing COVID-19 compared to PCR and some of the other methods, it can identify the metabolomic, lipidomic, and proteomic profiles that are related to the diseases' profiles [22]. However, the original purpose of this technology was not to replace DNA or RNA sequencing, but rather to provide an alternative to diagnosing the virus and its mutants with the proteins they produce [23].

3. Prediction for future diagnostic assays

The current diagnostic assays each have different benefits and limitations that allow for better use in some situations than others. For example, Nucleic Acid Amplification Tests (NAATs) are relatively more accurate, but they take longer time for results and are expensive. Antigen tests, on the other hand, are less accurate, but are highly accessible and quick. The best diagnostic assay would be a combination of all the benefits: fast, accurate, and accessible.

3.1 *Point-Of-Care Testing (POCT)*

Point-of-care testing refers to the diagnostic tests that happen either at the patient's home or at a nearby test site. POCT results come out relatively quickly, usually in less than an hour, and may also be excecated by the patients themselves. As more people look for convenient, quick, and accurate diagnostics, monitoring, or screening, POCTs are becoming popular among the public. Some examples of currently existing POCT include blood glucose monitoring and home pregnancy tests.

POCT's quick diagnostics come with a cost. They may be inaccurate, usually performed by non-trained people, sometimes intended to only screen purposes, and unable to detect inferences.

This type of testing is crucial for preventing a pandemic like COVID-19. Convenient methods of diagnosis would allow healthcare professionals to have quick and accurate test results to take actions, such as issuing quarantines to certain people or mandatory masking. There are several issues with the current methods of testing: the results either take several hours to come out, or they are not accurate enough to qualify to be POCT. One way to resolve PCR's inability to produce quick results and the necessity of a laboratory is by allowing a portable microfluidic-based cartridges or chips to amplify and detect the virus - this would allow for easier point-of-care testing outside of a laboratory [24]. To improve the antigen lateral flow assay test, its sensitivity must increase to allow more accurate results.

3.1.1 *Future POCT technology*

To combat these issues, there have been several proposed ideas. The first one is a revolution in the LFA tests by replacing the nanoparticles with magnetic nanoparticles and combining LFA technology with the Isothermal Nucleic Amplification technology [24]. The magnetic nanoparticles could be used to quantitate the measurements if used with an external reader and improve selectivity and sensitivity after being functionalized with an active biomolecule [25]. Another promising technology is the electrochemical biosensor, which has been used to detect viruses, proteins, nucleic acids, and small molecular antibodies. One type of electrochemical sensor, called label-free EIS, is highly popular due to its quick detection of the characteristics of various biological analytes [24]. Two common types of label-free EIS are impedimetric sensors, which measure the change in the charge conductance and capacitance as the targets bind, and the field effect transistor [24, 26]. Last but not least, chip-based nucleic acid detection has emerged to be popular because they can potentially allow automation from preparing the sample to detecting the target nucleic acid in an inexpensive portable device, which follows the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free, and Deliverable) platform guidelines [24]. Some technologies created using the chip-based nucleic acid detection methods include pre-concentration of virus particles, nucleic acid extraction and concentration in microfluidic chips, and nucleic acid detection in microfluidic systems [24].

3.1.2 *Use of artificial intelligence*

Another way for better diagnosis is to introduce an Artificial Intelligence (AI) system that can interpret the diagnostic results and recommend certain drug therapy to the patient based on the results. For example, with the use of qPCR (with possibly more sensitive fluorescence dye detection method for different diagnosis at once), the artificial intelligence can detect the specific virus to understand the infection of the patient. Based on its decision, AI can recommend certain drugs to the patient. When this technology becomes highly reliable, it may be able to prescribe a medicine, too, without requiring a doctor to crosscheck the prescription. With further integration of natural language processing, the computer would be able to communicate with the patient to consider both the patients' symptoms they explain and the test result to provide the correct prescription in a friendly and approachable manner. The use of humanoids would be able to expedite the wait time, as there may be a high number of people waiting with not enough healthcare professionals.

3.2 *Economic growth prediction*

Undoubtedly, point-of-care testing and molecular diagnostics have the potential to grow significantly in the upcoming years, especially after the stimulus from the recent coronavirus pandemic. They are both becoming popular options for clinical, laboratory, and at-home use for detecting different types of diseases, infections, proteins, antigens,

and other targets. According to the PR Newswire press release through Bloomberg, Grand View Research, Inc. published a market analysis stating that the POCT market would be worth USD 68.59 billion by 2030 [27]. This is an increase at a CAGR of 6.8% since 2022. The research also indicated that Asia Pacific is expected to grow significantly because of higher prevalence in cancer, diabetes, cardiovascular diseases, and other infectious diseases.

In addition to the increase in the POCT market, the molecular diagnostics market industry is also expected to show remarkable growth: a massive increase at a CAGR of 11.1% by 2030 [28]. In other words, the global market for the molecular diagnostic industry would rise to USD 37.19 Billion by 2030. Similar to the POCT market, Asia Pacific is estimated to have one of the largest growths in the promotion of health by the government.

4. Conclusion

The global coronavirus pandemic brought attention to the public of the significant harm that a single virus can do to global communities. Some of the effects include undermining the health of the public and the economy, as well as negatively affecting and changing the lifestyle of people. Even though viruses have caused several epidemics and outbreaks for the past hundreds of years, humans did not invest enough in preparing for a pandemic. In the 21st century alone, there have been more than 10 outbreaks that did not spread globally [29]. Especially with the increase in globalization, the capacity for the virus to spread further has increased, and SARS-CoV-2 illustrated the effect clearly. Through the experience of COVID-19, it is imperative to understand the importance of prevention of the virus at an early stage, and diagnostics is one of the best ways for prevention.

Methods like molecular assays and immunoassays, as well as future advancements, are crucial to facilitate the process of avoiding big pandemics. For COVID-19, PCR and Antigen LFA tests were highly utilized as one of the main ways in preventing further infections. However, emphasis in point-of-care tests and artificial intelligence uses could combine the advantages of the current assays to create a more advanced one. With future investments, research and development, and advancements in the diagnostic industry, the globe will be better prepared for the next viral outbreak.

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Conflict of interest

The author has no conflict of interest regarding this content.

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