



Comparative Assessment of SARS-CoV-2 Diagnostics in Nigeria

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Comparative Assessment of SARS-CoV-2 Diagnostics in Nigeria

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A Thesis in the Field of Biology
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Abstract

Viral pandemics are a major threat to public health, as they have the potential to overwhelm health systems and cause a significant loss of lives and economic meltdown. The COVID-19 pandemic highlighted not only the impact of pandemics but also the critical role of diagnostics in curbing their spread. Early and accurate diagnosis of Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) was very important in the effective management, control, and prevention of the pandemic. Several diagnostics tools were deployed during the COVID-19 pandemic, such as the gold standard, quantitative reverse transcription polymerase chain reaction (RT-qPCR), point-of-care (POC) Rapid Diagnostic Tests (RDTs), and enzyme-linked immunosorbent assay (ELISA). None of these diagnostics tools deployed in Africa were manufactured locally. Nigeria and other African countries relied on diagnostics donated from developed countries. Therefore, many commercially available diagnostic kits were utilized during the pandemic. This thesis evaluated the performance of the different commercial RT-qPCR diagnostic test kits (all one-step reverse transcriptase real-time PCR assays), CRISPR-based detection (mCARMEN) and viral RNA extraction methods (Qiagen and AviPure extraction kits, which are used for manual and automated extraction, respectively) that were deployed in a genomic reference lab in Nigeria. Nasopharyngeal (NP) swab samples from known positive SARS-CoV-2 cases with precise Ct values (divided into ranges Ct <25, 25-35 and 35-40) were used for the evaluation. The result showed that although both automated (AviPure) and manual (Qiagen) extractions were efficient for nucleic acid extraction for

the RT-qPCR assays, Qiagen, a manual extraction kit, had better RNA concentration yield. All the RT-qPCR assays were specific to SARS-CoV-2. However, the best-performing RT-qPCR commercial test was the DaAn Gene kit, while the Allplex had the lowest performance rate. mCARMEN was also very sensitive (100%) and specific for SARS-CoV-2. For sequencing assay, only samples with low and moderate Ct had complete genomic sequence using Illumina short read sequencing, while with the Oxford Nanopore (ONT), the complete sequence was seen at low, 73.3% at mid and 6.7% at high Ct. Reviewing the performance of different diagnostics deployed during the pandemic will enable us to select the best-performing diagnostics for future pandemics.

KEYWORDS: Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), RNA extraction methods, SARS-CoV-2 quantitative reverse transcription polymerase chain reaction (RT-qPCR), Sensitivity, Specificity, mCARMEN, Cycle threshold (Ct), Sequencing.

Dedication

This research work is dedicated first to God Almighty, then to the best gift God gave me, my husband Jonah, my amazing kids Jayden, Jaymin, and Jedidah, and to the world's best mentor ever, Prof. Pardis Sabeti and my amazing co-thesis director Dr Chinedu A Ugwu. Their unwavering love, support, and belief in my abilities have been the driving force behind my pursuit of knowledge and academic excellence. Their encouragement, guidance and understanding during the ups and downs of this research journey have been a constant source of strength. I am eternally grateful for their presence in my life and for being my guiding light. This accomplishment is a testament to their belief in me, and I dedicate this work to them with profound gratitude and love.

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Chapter I.

Introduction

The COVID-19 pandemic has become one of the most devastating global disease outbreaks. Over 770 million people have been infected, and about 6 million people died from the outbreak as of September 2023 (WHO 2023). The disease is caused by a novel coronavirus (2019-nCoV), also known as the Severe Acute Respiratory Syndrome Coronavirus 2 (SARS-CoV-2), which emerged in China in 2019 and then spread throughout the world. (Zhu et al.,2020). SARS-CoV-2 belongs to the β -coronavirus family and shares extensive genomic identity with bat coronaviruses, suggesting that bats may be the natural host (Kowalik et al., 2020). It is a zoonotic virus and the seventh identified coronavirus (CoV) to infect humans. It causes a respiratory disease with symptoms similar to those of the common cold (Bassetti et al., 2020).

Because SARS-CoV-2 is a novel CoV, diagnostic tests were not available in the early days of the pandemic. Many of the molecular diagnostic tools that were deployed for the detection of the disease underwent expedited certification and approval (Moshkovits et al., 2022). For resource-limited countries like Nigeria, most of the diagnostic tests that were deployed during the pandemic were from external sources, such as through donations or research grants, and none were manufactured locally or in-country. This resulted in the use of different diagnostic test kits from different companies with little or no time for validation of the test kits' performance.

The tests that were deployed during the pandemic include Quantitative Reverse Transcription PCR (RT-qPCR) tests for SARS-CoV-2, point-of-care rapid diagnostic tests

(RDT), and antigen and antibody-based ELISA tests. The RT-qPCR became the most common test for clinical detection of SARS-CoV-2 and will be the major focus of my thesis (Oliveira et al., 2020; Kubina et al., 2020; Loeffelholz et al., 2020; Venter et al., 2020).

RT-qPCR tests for SARS-CoV-2 were designed to detect highly conserved sequences of the spike (S), nucleocapsid (N), envelope (E), RNA-dependent RNA polymerase (RdRP), and Open Reading Frame 1ab (ORF 1ab) of SARS-CoV-2 (Oliveira et al., 2020; Kubina et al., 2020; Loeffelholz et al., 2020; Venter et al., 2020). Sometimes there is discordance in the results obtained from the RT-qPCR kits from two different companies (Wang et al., 2021). With the emergence of numerous variants of SARS-CoV-2 capable of evading detection, the performance of many diagnostic tests was questioned, especially for the tests that targeted the S protein of the SARS-CoV-2. This is because the S protein receptor is under intense immune pressure due to interaction with the immune system and is thus likely to mutate (López-Cortés et al., 2022).

Now that the pandemic is waning, this project aims to evaluate the performance of the numerous molecular diagnostics tests that were deployed during the COVID-19 pandemic in a genomic reference lab (ACEGID) in Nigeria. During the pandemic, ACEGID was one of the major reference labs involved in testing and confirmatory diagnosis of COVID-19 in Nigeria. At ACEGID, we used different RT-qPCR kits that we received through donations for the diagnosis of COVID-19. Sometimes, we had discordance in the result from different RT-qPCR kits, and we relied on sequencing for confirmation. Therefore, evaluating the performance of the various test kits that were deployed during the pandemic will provide vital information for epidemic preparedness, as it will help responders, as well as test developers and manufacturers, better understand

the quality of their products and strengthen public health response.

Objectives and Research Aim

In this study, my aim is to carry out a comparative assessment of the common diagnostic kits deployed during the COVID-19 pandemic in a national and continental regional genomic reference center in Nigeria (ACEGID). This comparison will include tests based on RT-q-PCR, sequencing, and CRISPR-based detection, as well as the impact of molecular extraction of nucleic acid on the diagnostic test result.

The objectives of this study are to:

Review the performance of some commercial COVID-19 RT-qPCR kits deployed during the pandemic.

Test for efficacy, sensitivity and specificity of the test kits deployed during the pandemic.

Identify the best method for extraction of the SARS-CoV-2 virus nucleic acid for optimal diagnostic test performance.

We will consider three RT-qPCR COVID-19 diagnostic kits: (1) AllPlex SARS-CoV-2 Master Assay, (2) AviMol Dri SARS-CoV-2 and (3) DaAn Gene Detection Kit for 2019 Novel Coronavirus (2019-nCoV). We will assess these kits in comparison with each other and a CRISPR-based test. Also, this project explores the process or mechanism involved in the generation of nucleic acid material for RT-qPCR testing. For this reason, we will also assess two different RNA extraction methods, Qiagen Viral RNA Isolation

Kit and AviPure RX Nucleic Acid extraction kit, by comparing their performance in manual versus automated extraction methods. The outcome of this study will highlight the most sensitive, specific diagnostic kits amongst the listed, used during the COVID-19 pandemic and provide necessary information that will guide future deployment of diagnostics during disease outbreaks.

Definition of Terms

cDNA (Complementary DNA) is a DNA molecule that is produced from the reverse transcription of messenger RNA templates.

COVID-19 (Coronavirus disease 2019) is an infectious disease caused by SARS-CoV-2 that emerged in 2019 and led to a global pandemic.

CRISPR-Dx (Clustered Regularly Interspaced Short Palindromic Repeats Repetitive DNA sequences- diagnostics) are diagnostics that use a defense mechanism of bacteria against viruses to detect genomic sequences in a sample.

Ct (Cycle Threshold) is the intersection between an amplification curve after each cycle of a PCR reaction and a threshold line. It is a relative measure of the concentration of the target in the PCR reaction. (Higher Ct = less virus, lower Ct = more virus.)

E gene (Envelope Gene) is a viral gene that encodes the protein forming the viral envelope. The expression of this gene enables retroviruses to target and attach to specific cell types and to infiltrate the target cell membrane. They are also known to be fusion machines.

Hypoxemia is a condition of below normal level of oxygen in the blood with the potential to result in shortness of breath.

ORF 1ab (Open Reading Frame 1ab) encodes three SARS-CoV-2 proteins that are broadly recognized as drug targets and are key components for infection and disease progression.

IC (Internal Control) functions as a Quality Control (QC) testing used to monitor and assure the dependability of test findings produced by the laboratory.

mCARMEN (microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic Acids) is a CRISPR-based diagnostic using microfluidics to allow for multiplexed testing.

MERS-CoV (Middle East Respiratory Syndrome Coronavirus) is a zoonotic coronavirus transmitted between animals and people.

N gene (Nucleocapsid Gene) is a type of structural/multifunctional protein of SARS-CoV-2, which is a potential target for the detection of viruses.

NP (Nasopharyngeal) are samples taken from deep inside the nose, reaching the back of the throat.

OP (Oropharyngeal) are samples taken from the middle part of the throat (pharynx) just beyond the mouth.

RdRP (RNA-dependent RNA Polymerase or RNA replicase) is a SARS-CoV-2 enzyme that catalyzes the replication of RNA from an RNA template. Specifically, it catalyzes the synthesis of the RNA strand complementary to a given RNA template.

RNA (Ribonucleic Acid) is a polymeric molecule essential in various biological roles in coding, decoding, regulation, and expression of genes; present in all living cells.

RT-qPCR (Quantitative Reverse Transcription Polymerase Chain Reaction) is used for the quantification, detection and measurement of products generated during each cycle

of PCR. The technique is used to find out how much of a specific section of DNA there is in a sample and uses either non-specific fluorescent dyes or sequence-specific DNA probes containing fluorescent reporters.

SARS-CoV (Severe Acute Respiratory Syndrome Coronavirus, also known as SARS-CoV-1) is a coronavirus infection that causes respiratory disease in humans. It is the cause of the Severe Acute Respiratory Syndrome (SARS) outbreak in 2002-2003.

SARS-CoV-2 (Severe Acute Respiratory Syndrome Coronavirus-2) is a coronavirus that causes respiratory disease in humans. It is the cause of COVID-19.

S gene (Spike Gene) codes for the SARS-CoV-2 spike protein that binds with the human receptor to allow the virus to enter cells.

The History and Epidemiology of SARS-CoV-2

Coronavirus disease was first documented in 1931. Its name is derived from the Latin word "corona" (crown). The name alludes to the virus's distinctive appearance under an electron microscope as spherical particles with a rim of projections like the solar corona (Hamre & Procknow, 1966). CoVs belong to the family Coronaviridae, which is known to typically produce mild respiratory diseases in humans. Human coronaviruses (HCoVs) were first reported in the mid-1960s when two species were isolated from persons with the common cold: HCoV-229E (Hamre & Procknow, 1966) and HCoV-OC43 (McIntosh *et al.*, 1967). Since then, seven different types of CoVs have been detected in humans. Three of seven CoVs are highly pathogenic and are suggested to have originated from bats: severe acute respiratory syndrome coronavirus (SARS-CoV or SARS-CoV-1), the Middle East Respiratory Syndrome Coronavirus (MERS-CoV), and SARS-CoV-2 (Zhu *et al.*, 2020).

The first Coronavirus pandemic, caused by SARS-CoV, was identified in Foshan, Guangdong, China, in 2002. (Yang *et al.*, 2020). The origin of the outbreak was thought to be due to zoonotic disease, but the intermediate host was unknown (Yang *et al.*, 2003). Another similar respiratory syndrome outbreak emerged in June 2012 in Saudi Arabia and was named MERS and found to be caused by MERS-CoV (Wang *et al.*, 2013). In late 2019, another novel Coronavirus emerged in Wuhan, China and was later named SARS-CoV-2 by the World Health Organization (WHO) (Huang *et al.*, 2020).

On 11 March 2020, the WHO officially declared COVID-19 a global pandemic. As of 16th October 2023, over 771 million cases have been reported globally, with over 6 million deaths (WHO, 2023). The United States (US) has the highest number of reported infections (109,023,428) and deaths (1,180,183) of any country in the world. The continent of Africa has recorded 12,843,125 cases and 258,840 deaths, and Nigeria has recorded 267,146 infections and 3,155 deaths (Worldometer, 2023). The disease has spread to about 231 countries, with new incidence cases reported daily (Worldometer, 2023). Currently, the cases are going down with the introduction of COVID-19 vaccines and an increase in the number of individuals with natural immunity from previous infections.

Structure and genome of SARS-CoV-2

Microscopic imaging has shown that SARS-CoV-2 has a crown-like surface projection (Prasad *et al.*, 2020). It maintains key structural components such as Spike (S) Protein, Nucleocapsid (N) Protein, Envelope Proteins (E), and Membrane Proteins (M), as shown in Figure 1. These proteins are required for viral genome synthesis, replication,

virion-receptor attachment, virion, and viroporin creation, all of which help in virus entry, growth and spread (Bertram et al., 2011).

The S protein plays a major role in cell attachment, antigenic recognition, viral fusion, and antibody neutralization (Belouzard et al., 2012). Wan et al. (2020) reported that SARS-CoV-2 is optimized to bind to angiotensin-converting enzyme II (ACE2) human receptors. The receptor-binding domain (RBD) of the S protein is the most variable part, and it differs for each type of CoV. The S protein cleavage takes place at two sites, causing the formation of a six-helix bundle that allows the fusion of the virus-cell membrane and host cell membrane, resulting in the release of the viral genome into the cytoplasm (Bosch et al., 2003). The genome of CoV consists of positive-sense single-stranded RNA, which is used as a template to directly translate pp1a and pp1b, which are processed further to proteins essential for the formation of the replication transcription complex (RTC) present in double-membrane vesicles (Snijder et al., 2006).

The SARS-CoV-2 RTC synthesizes a set of sub-genomic RNAs (sgRNAs) in a discontinuous manner (Hussain et al., 2005). The positive sgRNA serves as an mRNA for all structural and accessory genes, whereas the negative-sense strand of sgRNA serves as a template for the production of sub-genomic and genomic positive sense mRNAs (Sawicki et al., 2007). Following the replication and synthesis of mRNAs, structural proteins get transcribed (Snijder et al., 2003). These structural proteins are inserted into the endoplasmic reticulum and transferred to endoplasmic reticulum-Golgi intermediate compartments (Ke et al., 2020).

The M protein is the most abundant protein in the envelope. It plays a key role in the assembly of the virion and interacts with other structural proteins (N, S, and E). This protein is exposed inside the viral envelope.

The N protein is a highly basic phosphoprotein that combines with RNA to form the helical nucleocapsid. This protein is involved in the processes linked to the viral genome. Moreover, it is also involved in the virus replication cycle and the host's cellular response to viral infection (Srinivasan et al., 2020).

The E protein is the smallest among the structural proteins. It is the least abundant and the most difficult to understand among the four structural proteins. (Schoeman & Fielding, 2019). Although it lacks a cleavable signal peptide that classifies it among the type II transmembrane proteins, it is very important in the production and maturation of the virus.

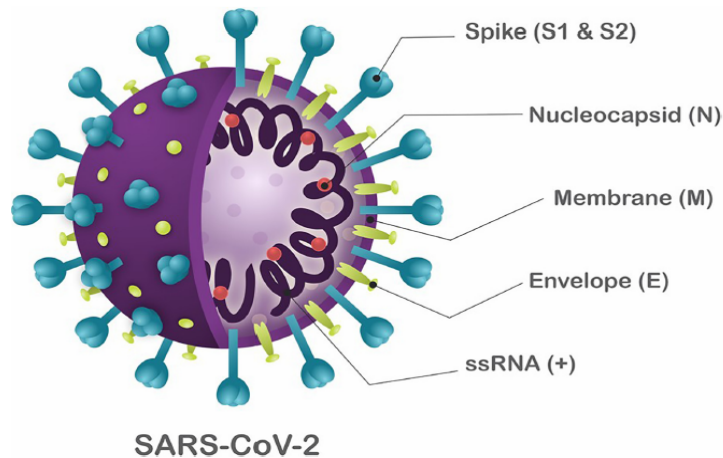


Figure 1: Structure of SARS-CoV-2 (Santos, 2020).

This genome is present inside circular nucleocapsid (N) proteins and further encapsulated by an envelope (E) protein (Li, 2016). Other structural proteins include the spike (S) and membrane (M) proteins (Han Y et al., 2019).

Pathogenesis and Clinical Manifestation of SARS-COV-2

SARS-CoV-2 Infects ciliated bronchial epithelial cells and type II pneumocytes predominantly, where it binds to the surface receptor, ACE2, through the S glycoprotein found on its surface (Hoffmann et al., 2020). The affinity between the virus's surface proteins and its receptors is a critical step for viral entry. The affinity of S glycoprotein of SARS-CoV-2 for ACE2 binding efficiency is 10–20 times more than that of SARS-CoV-1, which might explain SARS-CoV-2's strong infectious capacity (Letko et al., 2020). Common symptoms associated with suspected cases of SARS-CoV-2 includes, fever, dry cough, diarrhoea, vomiting, muscle ache, shortness of breath. Other less common symptoms include chest pain, headache, sore throat etc (Wang et al., 2020, Chen et al., 2020)

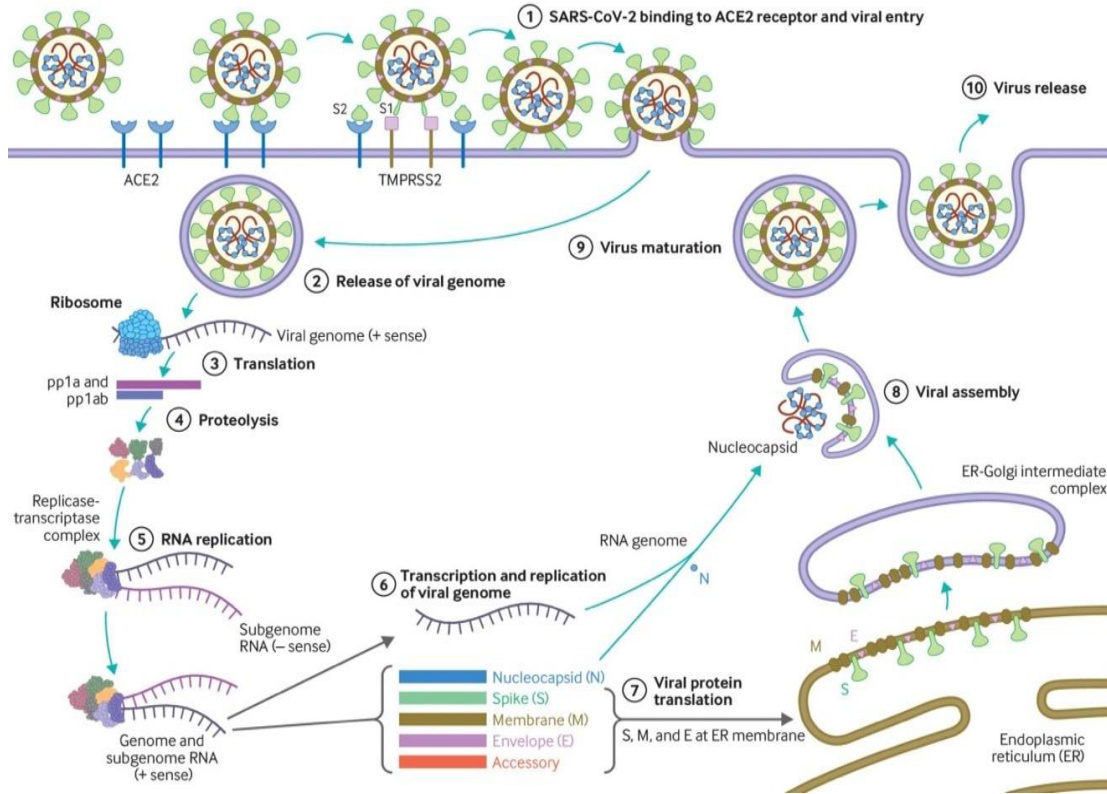


Figure 2: Pathogenesis of SARS-COV-2 (Muge Cevik BMJ, 2020)

The virus binds to the host's transmembrane serine protease 2 (cell surface protein), which is predominantly expressed in airway epithelial cells and vascular endothelial cells, as the host target cell receptor.

The Need for Early and Robust SARS-CoV-2 Diagnostics

Diagnostics are an essential part of clinical care, effective surveillance and outbreak mitigation strategies. These tests allow for the confirmation of the specific infectious disease in a patient, thereby allowing for more directed and effective treatments by the physician. An accurate diagnosis also makes it possible to better anticipate the course of the disease and take appropriate protective measures, such as quarantining or following up with recent contacts who may have contracted the disease.

Thus, our first line of defense in combating SARS-CoV-2 outbreaks is to quickly identify infected individuals for epidemiological tracing and containment to stop the virus from spreading further. These diagnostics platforms should have the following characteristics to be maximally useful during the emerging outbreak: high sensitivity, high specificity, low cost, ease of use, point-of-care deployment-capable and adaptable to new viruses and variants (Nelson et al., 2020).

This high sensitivity would allow for accurate detection of the SARS-CoV-2 viral particles down to very small copy numbers in patients, reducing the chance of returning false negative results and retaining accuracy even in patients that are asymptomatic or have low viral loads but may still be infectious. High specificity means that the test will only return a positive result on the target of interest and will not detect other closely related viral species, subtypes and anything else that it was not designed to detect, reducing the chance of false positive results that will mischaracterize the infection.

SARS-CoV-2 diagnostic platforms must be cost-effective and readily available in order for the tests to be accessible and widely distributed, as high costs can pose a great challenge to under-resourced communities and underfunded health systems.

During the COVID-19 pandemic, several diagnostic tests were deployed globally. These include molecular tests such as RT-qPCR, immunological tests such as ELISA and bead-based Luminex and genomics tests such as next-generation sequencing

RT-qPCR, the Gold Standard

The gold standard for detecting SARS-CoV-2 is reverse-transcriptase polymerase chain reaction (RT-qPCR), a form of real-time PCR. RT-qPCR works by using RT to convert viral RNA into cDNA and then using qPCR to amplify the cDNA. Fluorescence

probes complementary to the amplicon are added, and exonuclease activity during the amplification phase cleaves the probes to emit fluorescence signals that can be detected if viral RNA is present in the sample (Kralik and Ricchi, 2017). The RT-qPCR platform became the standard method for viral detection, such as for SARS-CoV-2, due to its high sensitivity, specificity and reliability (Watzinger et al., 2004). However, shortcomings exist in the RT-qPCR platform that make it unsuitable as an effective viral point-of-care (POC) diagnostic. RT-qPCR requires an elaborate protocol of RNA extraction, thermal cycling and data processing (Chan et al., 2020). The platform suffers from high per-test costs in the range of \$10 per test and requires specialized equipment and personnel that only large, centralized laboratories can afford. As such, samples are rarely processed and tested at the POC collection site and must be transported to reference laboratories for testing, resulting in long turnaround times (Brendish et al., 2020). Developing countries without the necessary healthcare infrastructure for large-scale RT-qPCR testing struggle more to keep viral pandemics under control, exacerbating existing global health inequities (Giri and Rana, 2020).

The scalability issues of RT-qPCR have grave effects on the detection of viruses with pandemic potential, like SARS-CoV-2 and influenza viruses. The widespread transmission of SARS-CoV-2 in the US and the rest of the world has been attributed to the slow deployment of high-sensitivity tests early in the outbreak, as it was difficult to ramp up and streamline the demanding protocol of RT-qPCR (Lau et al., 2021). The lack of rapid, reliable diagnostic tools led to a breakdown of information as infected individuals did not know that they carried the virus and needed to be isolated from the wider community, leading to excess cases and mortality.

Droplet Digital PCR

The droplet digital PCR (ddPCR) can measure the quantity of the amplifiable region thereby able to pick alterations in the target copy number. This important feature makes it more sensitive than RT-PCR because it is able to quantify the amplicons from samples with low viral load. (Yu et al., 2020). Yu et al. reported that both RT-PCR and ddPCR showed reliable accuracy in samples with high viral load and negative examples, but digital droplet PCR (ddPCR) was better at detection in samples that possessed low viral load (Yu et al., 2020).

Serology

Serological techniques of diagnosis, compared with other methods, are the most widely used for the diagnosis of infections (Zhang *et al.*, 2020). The number of different blood cells, including leukocytes, lymphocytes, neutrophils, platelets, and haemoglobin, undergo changes that can be evidence for the type and severity of the disease. COVID-19 is diagnosed using antigen and antibody of viral proteins that respond to SARS-CoV-2 infection in serology. Serology tests can be used to track illness progression, previous infections, and the development of immunity. Immunoglobulin M (IgM) indicates the early stages of infection, whereas immunoglobulin G (IgG) indicates past infection and post-infection defense (Carter et al., 2020). The test is also critical in epidemiology studies and vaccine development (Carter et al., 2020). According to a study, both IgM and IgG antibodies were identified in all 39 SARS-CoV-2 infected individuals tested after 5 days of infection (Loeffelholz and Tang, 2020). Antibodies generated in reaction to viral proteins allow time for the indirect detection of SARS-CoV-2 (To et al., 2020). This test, which uses antibodies as indicators of SARS-CoV-2 infection, may be useful in diagnosing

COVID-19, but the likelihood of cross-reactivity of SARS-CoV-2 antibodies with antibodies generated against other coronaviruses is a drawback of this approach (Udugama et al., 2020).

Rapid Antigen Test

This is an immunoassay that utilizes the lateral flow technology to detect proteins from pathogens. The principle involves coating antibody on paper strip tagged with a colour signal such that when sample containing the antigen is dropped on the paper a colour signal will be seen in the form of line. Double line is positive while single line is negative. The good thing of rapid test is that they are quick, cheap and requiring no complex technical capacity.(Loeffelholz and Tang ,2020) (Harpaz et al., 2020). Thus, rural communities without laboratories can conduct a diagnosis of suspected patients using rapid antigen tests (Udugama et al., 2020). However, rapid antigen tests are limited by their reduced sensitivity.

CRISPR-Based Detection Assays

Clustered, regularly interspaced short palindromic repeats are abbreviated as CRISPR. It is the defining feature of acquired immunity in bacteria, consisting of a nucleotide sequence of repeats obtained from a bacteriophage or plasmid (Marraffini and Sontheimer, 2010).

The CRISPR system permits viral DNA to be integrated into the CRISPR locus. CRISPR sequences are transcribed into RNA, which is then combined with proteins (supplied by Cas genes) to form interference complexes that use information on RNA molecules to match base pairs in viral DNA (Garneau et al., 2010).

CRISPR has also been used to produce programmable transcription factors, allowing scientists to target, activate, or silence certain genes (Larson et al., 2013). The recently developed CRISPR-based assays are an attractive alternative to traditional RT-qPCR assays for viral diagnosis. They meet the above criteria by being highly sensitive, specific and programmable, relying on complementary base pairing between the target RNA or cDNA and the CRISPR-RNA (crRNA) sequence. Both CRISPR-Cas13 and Cas12-based assays have been developed for viral detection using extracted nucleic acids as input, and there have been promising developments to simplify the assays for POC deployment.

Microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic Acids (mCARMEN)

Microfluidic Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic Acids (mCARMEN) is a CRISPR- and microfluidics-based technology that enables the detection of the nucleic acids of multiple pathogens simultaneously in a single sample and run. The technology is scalable and has a high throughput for samples, able to process as many as 192 samples in a single run (Welch et al., 2020). The technology is applicable to both the DNA and RNA of pathogens.

The ability of mCARMEN to detect multiple targets is enabled by multiplex PCR amplification of target nucleic acids and the microfluidics enabled on Fluidigm instruments. The former allows for simultaneous amplification of targets to a detectable level, while the latter allows for spatial detection of amplified nucleic acid of targeted pathogens of interest.

Currently, mCARMEN has been developed for the detection of ten respiratory viruses. This panel is referred to as the Respiratory Virus Panel (RVP). Viruses detected in this panel are as follows; SARS-CoV-2, Influenza A (FLUAV, human-associated subtypes), Influenza B (FLUBV), human respiratory syncytial virus (HRSV), human metapneumovirus (HMPV), human parainfluenza virus type 3 (HPIV-3), human beta coronavirus strain HKU1 (HCoV-HKU1), human alphacoronavirus strain NL63 (HCoV-NL63), and human beta coronavirus strain OC43 (HCoV-OC43). However, other panels can be developed for the detection of various pathogens of interest or of epidemiological importance (Welch et al., 2020).

Next Generation Sequencing (NGS) Technology

The advent of genomic tools for pathogen detection has enabled us to answer many unknown questions about the pathogen's biology and immune response. One such genomic tool is Next Generation Sequencing (NGS). NGS reveals the entire genome of the pathogen, and this can be correlated with the functional phenotype. This has enabled the precise identification of viral, bacterial, or parasitic pathogens with a high degree of sensitivity (Bhat and Rao, 2020). The method is based on the detection and subsequent analysis of double-stranded DNA that has been extracted from an infected organism, either in the form of total RNA that is reverse-transcribed into cDNA (in cases of an RNA virus) or DNA (in the case of a bacterium, parasite, or DNA virus) (Bhat and Rao 2020). A sequencing library is then prepared in order to break the DNA template into smaller and more manageable fragments, and adapters are added to serve as primers for downstream amplification or sequencing. This sequencing reaction involves repeated chemical reactions that are carried out in cycles and detected automatically using a flow cell. Results

from NGS can be verified by designing primers based on the identified sequences running qPCR or RT-qPCR using total DNA or RNA. (Bhat and Rao, 2020). Ultimately, this verification allows other assays to be developed for quicker and more reliable detection of the pathogen, which is why NGS is so important as a first step in controlling epidemics (Quer et al., 2022).

With NGS becoming a mainstream tool in infectious disease detection and management, it has become very useful and helpful, considering it requires no prior knowledge of the host or the pathogen (Massart et al. 2017). NGS is able to precisely identify non-human sequences for the purpose of characterizing the strains or even detecting resistance genes (Quan et al. 2008). It has ultra-high throughput capabilities and is largely scalable (Hert, Fredlake, and Barron, 2008). Despite these benefits, NGS still has its drawbacks, which become particularly pertinent in resource-constrained environments. NGS technologies are complicated, platforms are expensive, and sample preparation requires a significant level of expertise or training (Bhat and Rao, 2020). This makes NGS largely impractical for conducting routine diagnostic analyses in places where funding and resources are insufficient.

Nucleic Acid Extraction

An important procedure in molecular biology is the extraction of biomolecules, DNA, RNA, and protein (Wink, 2006). DNA, RNA, and protein can be extracted from any biological material, including live or conserved tissues, cells, virus particles, or other materials (Wink, 2006). In general, there are three steps to effective nucleic acid purification: Denaturation of nucleoprotein complexes, effective disruption of cells or tissue, and nuclease inhibition (for example, RNase for RNA extraction and DNase for

DNA extraction) (Doyle, 1996). Contaminants such as protein, carbohydrates, lipids, or other nucleic acids should be avoided in the target nucleic acid, for example, DNA free of RNA or RNA free of DNA (Buckingham and Flaws, 2007). The quality and purity of the isolated nucleic acid will have a direct impact on the outcomes of all subsequent scientific research (Cseke *et al.*, 2003).

RNA Extraction

RNA plays an essential role in many biological functions, such as protein control and biosynthesis. Once isolated from the cell or tissues, RNA is an unstable molecule with a short half-life (Brooks, 1998). rRNA (ribosomal RNA) (80%-90%), messenger RNA (2.5–5%), and transfer RNA (tRNA) are all examples of naturally occurring RNA (Buckingham and Flaws, 2007). Because RNA is sensitive to deterioration, special attention and safeguards are necessary while isolating it (Buckingham and Flaws, 2007; Kojima and Ozawa, 2002). The presence of RNases, which are enzymes found in RNA, makes it especially unstable in the blood, all tissues, and most bacteria and fungi in the environment (Brooks, 1998). Strong denaturants have always been used in intact RNA isolation to inhibit endogenous RNases (Doyle, 1996). RNA extraction relies on good laboratory techniques and RNase-free techniques. RNases are heat-stable and refold following heat denaturation. Because they do not require cofactors, they are difficult to deactivate (Doyle 1996). The extraction procedure should be as fast, accurate, and reliable as possible while minimizing the danger of cross-contamination (Loeffler, 2004).

Types of RNA Extraction

Conventional Method: (Guanidinium Thiocyanate-Phenol-Chloroform Extraction)

This involves the use of liquid solvents that are either polar or nonpolar in nature. This could be done by reflux, percolation or maceration.

In nucleic acid samples, salt is a frequent contaminant. It has always been necessary to eliminate it from nucleic acid samples prior to any downstream processing or analysis. To desalt the sample containing the nucleic acid, one or more separation and/or purification procedures are required (Smarason, 2003). After desalting, the primary steps of nucleic acid purification include cell lysis, which breaks the cellular structure to produce a lysate, deactivation of cellular nucleases such as DNase and RNase, and separation of required nucleic acid from cell debris (Doyle, 1996). Ulrich et al. were the first to mention the use of Guanidinium isothiocyanate for lysing cells in RNA extraction (1977). The procedure was time-consuming. As a result, Chomczynski and Sacchi (1987) used a single-step method known as Guanidinium thiocyanate-phenol-chloroform extraction to replace it (Sambrook and Russel, 2001), wherein phenol/chloroform is used to extract the homogenate at a pH lower than normal. Guanidinium thiocyanate is a chaotropic substance that aids in the breakdown of proteins. After extraction with an acidic solution, including guanidinium thiocyanate, sodium acetate, phenol, and chloroform, RNA is separated from DNA in this single-step method (Chomczynski and Sacchi, 2006). In acidic conditions, total RNA will remain in the upper aqueous phase of the entire mixture, while DNA and proteins will remain in the interphase or lower organic phase. The entire RNA is subsequently recovered using isopropanol precipitation (Sambrook and Russel, 2001).

Solid-Phase Nucleic Acid Extraction (Magnetic Bead Based Nucleic Acid Purification.)

The majority of commercial nucleic acid extraction kits include solid-phase nucleic acid purification. Compared to traditional techniques, it provides for faster and more effective filtration (Esser et al., 2005). Many of the issues that come with liquid-liquid extraction may be avoided, such as inadequate phase separation. A spin column, which is operated under centrifugal force, is commonly used for solid-phase purification (Gjerse et al., 2009). In comparison to traditional techniques, this technology can purify nucleic acids quickly. Four critical steps in solid-phase extraction are cell lysis, nucleic acid adsorption, washing, and elution (Kojima and Ozawa, 2002).

Magnetic separation is a simple and effective method for purifying nucleic acids in today's world. Magnetic or paramagnetic particles are used in this innovation contained in polymers such as magnetizable cellulose. (Nargessi, 2005). In the presence of specific concentrations of salt and polyalkylene-glycol, magnetizable cellulose can bind to nucleic acids. A commercially accessible extraction kit based on the idea of magnetic bead-based nucleic acid purification is available (Bio-Nobile, 2003). The reagents included in this kit are designed to be used with magnetic instruments, making them unique. If you're dealing with micro-tubes, this magnetic tool is a must-have. It's a practical gadget that uses magnetic particle technology to produce separations. There are no organic solvents required, and there is no need for repetitive centrifugation, vacuum filtering, or column separation with this kit. A modified alkaline lysis method is followed by nucleic acid binding to magnetic particles in this protocol. Magnetic particles are captured with the bound nucleic acid using the magnetic instrument, and impurities are removed using the

wash buffer supplied. With the elution buffer, the nucleic acid is eluted from the magnetic particles (Bio-Nobile, 2003).

Automated Extraction System

This is a large, expensive, and complex instrumentation designed for high-throughput sample processing that has helped to simplify the isolation of nucleic acids (Promega, 2008). This system was created for medium-sized to large laboratories (Loeffler, 2004). The automated nucleic acid extraction method has the ability to cut working time, lower labor expenses, improve worker safety, boost laboratory efficiency, and, in the process, improve the reproducibility and quality of findings (Boyd, 2002). Purification of high-quality RNA from a range of starting materials will be employed in downstream testing applications in clinical laboratories. Obtaining purified samples of appropriate quality and purity is critical (Promega, 2008). Automated extractions should, therefore, be more consistent and repeatable.

Chapter II.

Methods

The purpose of this study was to evaluate the performance of RT-qPCR test kits deployed in a national reference lab in Nigeria during the COVID-19 pandemic. The performance criteria were based on sensitivity, specificity, time, and impact of extraction methods on the RT-qPCR performance and defining a sequencing Ct cut-off.

Study Site

The African Center of Excellence for Genomics of Infectious Diseases (ACEGID), located in the Redeemer's University, Ede, Osun state, is a World Bank funded research laboratory and a national and regional reference laboratory for infectious diseases. ACEGID receives multiple samples from different parts of Nigeria and across the continent for molecular diagnosis and surveillance. ACEGID was chosen as the study site because it is a national and regional reference molecular lab and was supporting both the country and the continent with routine diagnosis and genomic sequencing during the COVID-19 pandemic. Also, the nature of operations of the lab (accessibility to COVID-19 samples, patients' confidentiality, accuracy and integrity of testing outcomes for infectious diseases over the past decades) makes it suitable for this study.

Ethical Statement

COVID-19 patients were recruited using approved protocol by human subjects' committees at ACEGID (HREC) NHREC Protocol Number NHREC/01/01/2007-08/08/2020 NHREC Approval Number NHREC/01/01/2007-30/11/2021B and IRB22-0894 by the Institutional Review Board (IRB) Harvard University Committee on the Use of Human Subjects

All methods were carried out in line with the relevant guidelines and regulations of the approved protocol.

Sample Collection and Selection Criteria

De-identified SARS-CoV-2 Clinical samples were provided by the African Center of Excellence for Genomics of Infectious Diseases. The samples were archived nasopharyngeal swabs stored in viral transport media (VTM), also known as virus preservation solution, from patients with confirmed COVID-19 diagnosis collected during the first and second waves of the COVID-19 pandemic from 2020-2022 stored in -80°C. The samples were randomly selected based on varying Ct's (Threshold Cycle) values and grouped into samples with high, mid and low Ct values. 15 samples each were selected for each group as follows: COVID Negatives, (High) >35 low viral loads, (Mid) 25-35 mid viral load and (Low) < 25 high viral loads as previously confirmed by RT-qPCR from the national and reference lab in Nigeria (ACEGID). Furthermore, 15 Lassa fever positive archived samples (collected in 2018, prior to the COVID-19 pandemic) were selected as negative control samples.

RNA Extraction Protocol

Archived nasopharyngeal (NP) swabs stored in (VTM) of patients with confirmed COVID-19 disease were extracted to obtain the viral RNA using two RNA extraction methods:

Manual method using (QIAamp Viral RNA Isolation Kit) and Automation using (Avipure RX Nucleic Acid Extraction kit).

RNA Extraction of Nasopharyngeal Swabs using QiAmp Viral RNA Isolation Kit

The QIAamp Viral RNA Isolation Kit was used to extract viral RNA from the samples according to manufacturer's instructions (Lot no:56902240).

16 μL of Linearized acrylamide (LA) was added to a fresh 2 mL container of AVE Buffer. Next, buffer AW1 and AW2 was diluted with 25 ml and 30 ml ethanol respectively. 5 μL Beta-Mercaptoethanol (BME) was added to the sample during AVL inactivation, mixed with a vortex mixer for 10 seconds followed by an incubation for 15 minutes at 56 °C. Next, 560 μL of ethanol was added to the sample and mixed with a vortex mixer. and incubated for 5 minutes at room temperature. Next, 650 μL of lysate was added onto QiaAmp Mini Spin columns, without wetting the rim, in two fractions. Washes of the column were done with the respective buffers as well as ethanol. The RNA was eluted from the column in 60 μL of AVE + LA buffer in a clean 1.5 μL tube for viral qRT-PCR (QIAamp Viral RNA Mini Handbook, 2020).

RNA Extraction of Nasopharyngeal Swabs using Avipure RX Nucleic Acid Extraction Kit

AviPure RX nucleic acid extraction kit was used to extract viral RNA from the samples. According to the manufacturer's instructions (Ref no: AVP10196), The pre-made reagents plates includes:

Wash Solution 1 Concentrate, Wash Solution 2 Concentrate, Wash Solution 3 Concentrate lysis buffer magnetic bead, and elution buffer.

The sample plate was prepared by adding 200 μ L of samples into the lysis buffer plate, mixed by vortexing for 1 min. Finally, all plates were loaded into the AviPure Magnetic Particle Processor following the instructions on the LCD. The pre-loaded script was used for the isolation. (Instruction Manual for AviPure Nucleic Acid Extraction Kit, 2020).

Measuring RNA Concentration using Qubit flex fluorometer

The Invitrogen Qubit™ Flex Fluorometer was used to measure the RNA concentration in the isolates (extracts) using the RNA BR (broad range). The software application for the Qubit machine was opened, and a new report was created for this experiment. The standard and working solutions were prepared based on the manufacturer's instructions. The set of standards (1 and 2) was established by pipetting 10 μ l of each standard into 190 μ l of the Qubit working solution, while 2 μ l of samples was added into 198 μ l the qubit working solution. Measurement was taken for 2 standards, and the data was captured. This procedure was done for the rest of the samples, data was saved, and the software application was closed (Qubit™ Flex Fluorometer quick reference, 2020).

Quantitative Polymerase Chain Reaction (qPCR)

The viral RNA extracts obtained from the manual extraction methods was subjected to PCR amplification of the SARS-CoV-2 genes using 3 RT-qPCR kits:

RdRp/ORF1ab gene, N gene and E gene using Allplex SARS-CoV-2 Master assay,

RdRp/ ORF1ab, and N gene using AviMol Dri™ SARS-CoV-2 Kit and

ORF1ab gene and N gene using DAan Gene Detection Kit for 2019 Novel Coronavirus (2019-nCoV).

PCR Amplification of SARS-CoV-2 Genes using Allplex (2019-ncov)

The Allplex™ SARS-CoV-2 plus Variant Assay is a multiplex real-time PCR that detects 4 target genes for SARS-CoV-2. The master mix for the Allplex™ SARS-CoV-2 Assay was prepared according to the manufacturer's instructions (Seegene Inc RVA10284X). It was prepared on ice in a 2.0 ml Eppendorf tube by pipetting (5µl) SC2pVmom and probe mixture (5µl) of EMB (Enzymes) and (5µl) RNase free water multiplied by the number of samples including the number of controls. An overage of 2 was added to account for possible pipetting errors. The mixture was then vortexed and spun down briefly with a centrifuge. 15µl of the master mix was then pipetted into each microwell of the 96-well PCR plate according to the plate map. 5µl of each of the templates was then added into the 96-well PCR plate. The plate was then sealed to avoid spillage and contamination. The plate was then spun down briefly to collect the master mix and template to the bottom of the reaction plates. The plate was then put into the thermocycler for amplification.

The cycling conditions for the thermocycler were: reverse transcription for 1 cycle at 50°C for 20 minutes, pre-denaturation for 1 cycle at 95°C for 15 minutes, denaturation

for 45 cycles at 95°C for 10 seconds and annealing for 45 cycles at 60°C for 15 seconds and 72°C for 10 sec (fluorescence is measured at 60°C and 72°C). The fluorescence probes were FAM, HEX, Cal Red 610, Quasar 670 and Quasar 705 (Allplex™ SARS-CoV-2 plus Variant Assay Instruction Manual, 2021).

PCR Amplification of SARS-CoV-2 Genes using AviMol Dri™ SARS-CoV-2 Kit

The AviMol Dri™ SARS-CoV-2 Kit is a real-time reverse transcription-PCR assay for the qualitative detection of SARS-CoV-2 nucleic acid. This kit is used for the in vitro qualitative detection of SARS-CoV-2 ORF1ab gene and N gene.

The lyophilized (freeze-dried) RT-qPCR mix for the AviMol Dri™ SARS-CoV-2 kit was prepared according to the manufacturer's instructions (Avicenna, AVM218048). 23µl of sample template was pipetted into the dried reaction mixture/probe mixture (individual 0.5 µl PCR) multiplied by the number of samples, including the number of controls. An overage of 2 was added in case of pipetting errors. The mixture was then vortexed and spun down briefly with a centrifuge. The Master mix containing samples was then transferred into a 96-well PCR plate and run on the thermocycler for amplification.

The cycling conditions for the thermocycler were: reverse transcription for 1 cycle at 50°C for 10 minutes, pre-denaturation for 1 cycle at 95°C for 3 minutes, denaturation for 45 cycles at 94°C for 10 seconds and annealing for 45 cycles at 60°C for 1 min (fluorescence is measured at 60°C). The fluorescence probes were: FAM- ORF 1ab gene, VIC-N-gene and Cy5- IC (AviMol Dri™ SARS-CoV-2 Detection Kit for Instruction Manual, 2021).

PCR Amplification of SARS-CoV-2 Genes using DaAn Gene Detection Kit for 2019
Novel Coronavirus (2019-ncov).

The DaAn Gene Detection Kit for 2019 Novel Coronavirus (2019-nCoV) is a one-step RT-qPCR technique. This kit is used for the in vitro quantitative detection of novel coronavirus invitro (2019-nCoV) ORF1ab and N genes.

The master mix for the DaAn Gene kit was prepared according to the manufacturer's instructions (DaAn gene, Cat#DA-930). It was prepared in a 1.5ml Eppendorf tube by pipetting the volume of the ORF1ab solution A (17 μ l) and ORF 1ab solution B (3 μ l) multiplied by the number of samples, including the number of controls and samples prepared. An overage of 2 was added in case of pipetting errors. The mixture was then vortexed and spun down briefly with a centrifuge. 20 μ l of the master mix was then pipetted into each microwell of the RT-qPCR plate according to the plate map. 5 μ l of each of the template samples were then added into the RT-qPCR plate. The plate was then sealed or closed to avoid contamination. The plate was then spun down briefly to collect the master mix and template to the bottom of the reaction plates. The plate was then put into the thermocycler for amplification.

The cycling conditions for the thermocycler were: reverse transcription for 1 cycle at 50°C for 15 minutes, pre-denaturation for 1 cycle at 95°C for 15 minutes, denaturation for 45 cycles at 94°C for 15 seconds and annealing for 45 cycles at 55°C for 45 seconds (fluorescence is measured at 55°C). The fluorescence probes were FAM- N gene, VIC- ORF 1ab gene, and Cy5- IC (Da An Gene Detection Kit for 2019 Novel Coronavirus (2019-nCoV) Kit Instruction Manual, 2019).

RT-PCR Amplification, Detection for mCARMEN

The master mix was prepared on ice in a 2.0 ml Eppendorf tube by pipetting (12.5 μ l) of 5x OneStep RT-PCR buffer, (3 μ l) forward primer pool, (3 μ l) Reverse primer pool, (2 μ l) dNTP mix, (2 μ l) Qiagen enzyme mix and (17.5 μ l) nuclease-free water, multiplied by the number of samples, including the number of controls. An overage of 2 was added to account for possible pipetting errors. The mixture was then vortexed and spun down briefly with a centrifuge. 40 μ l of the master mix was then pipetted into each microwell of the 96-well PCR plate according to the plate map. 10 μ l of each of the templates was then added into the 96-well PCR plate. The plate was then sealed to avoid spillage and contamination. The plate was then spun down briefly to collect the master mix and template to the bottom of the reaction plates. The plate was then put into the thermocycler for amplification.

The cycling conditions for the thermocycler were: reverse transcription for 1 cycle at 50°C for 30 minutes, pre-denaturation for 1 cycle at 95°C for 15 minutes, Amplification for 45 cycles at 94°C for 30 seconds, 58°C for 30 seconds, 75°C for 30 seconds and hold at 4°C.

Next, the cas13 detection reaction mix was prepared by adding 10.4 μ L of 2x Assay loading reagent to each assay Master Mix well, vortex and spin down. Next, the assay master mix was prepared in a 1.5ul nuclease-free tube on ice by adding (1.4 μ l) nuclease-free water, (0.9 μ l) Cas 13a, (2.5 μ l) Lucigen T7 pol, and (8.2 μ l) of 2x assay reagent multiplied by the number of samples including the number of controls and gently flick tube containing mix. Using a clean pipette, I added 12.6 μ l of the assay master mix into each well of the detection assay plate, matching the already prepared previous mix. After which,

3.4 μ l of 1 μ M crRNA was added to the corresponding plate containing the assay master mix. Next a sample master mix was prepared in a 5ml Eppendorf tube on ice by adding (8.52 μ l) nuclease-free water, (1.43 μ l) 10x CB, (0.57 μ l) NEB rNTPs, (0.72 μ l) RNase inhibitors, (0.72 μ l) Loading Reagent (20x), (0.45 μ l) Reporter (16 μ M FAM-7-U), (0.03 μ l) ROX dye (50x), and (0.13 μ l) MgCl₂ 1M multiplied by the number of samples including the number of controls. Vortex and add 12.6 μ l of the sample master mix into the appropriate well without adding MgCl₂ to the negative detection control well. 12.6 μ l of 1M MgCl₂ was added to the rest of the plate wells and vortexed. Next, 12.6 μ l of the sample master mix was added to the wells labelled samples and 1.43 μ l of the sample target was added to the vortex, spun down and placed on ice.

Next, the 192X 24 Fluidigm IFC chip was prepared by opening the aluminium bag. The control line fluid that comes prepared in a syringe was added to the top center well on the chip, avoiding bubbles. Next, 150 μ l of the Actuation fluid was added in the well labeled P1 on the chip, avoiding bubbles. Next, 150 μ l of the Pressure fluid was added to the wells labeled P2 and P3, while 20 μ l of the pressure fluid was also added to the wells labeled P4 and P5. Next, 4 μ l of assay mix from the prepared assay plate was added to the wells on the chip as follows in column 1 (wells 1,4,7,10, etc), skipping one well in that order. While 4 μ l of the sample mix containing target samples was added to wells on both sides of the chip. Next, the Biomark X was turned on, and the IFC chip was loaded after carefully peeling off the seal on the bottom of the chip. The appropriate protocol was selected, and the run started, which lasted for 3 hours.

Sequencing of SARS-CoV-2 using the Rapid Barcoding and Midnight RT-PCR
Expansion (SQK-RBK110.96 and EXP-MRT001)

The Oxford Nanopore Midnight protocol for library preparation of SARS-COV-2 positive samples is an amplicon-based sequencing protocol for identifying SARS-COV-2 and its variants. The assay was prepared according to the manufacturer's instructions.

The Reverse transcription was done using 2ul of a supermix, also known as LunaScript RT supermix) combined with 8ul of the input RNA and set up on a thermal cycler under the preassigned cycling condition. (25°C for 2 minutes, 55°C for 10 minutes, 95°C for 1 minute and a final hold at 4°C.

Next was the Polymerase Chain Reaction (Tiling) done using two pools of multiplex primer to generate 1200 bp amplicons that overlap by approximately 20bp using a master mix of 3.7µl Nuclease-free water, Midnight Primer Pool A (MP A) 0.05µl, Midnight Primer Pool B (MP B) 0.05µl, Q5 HS master Mix (Q5) 6.25µl. After the preparation of the two Master Mixes, 10ul of each pool mix was dispensed for the required number of samples in two different wells. 2.5ul of cDNA was then dispensed into each sample well as required. After this, the sample plate was placed on the thermal cycler with the following conditions: Initial denaturation at 98°C for 30sec, 35 cycles of denaturation, Annealing and extension at 98°C for 15 sec and 65°C for 5 min respectively, and a final hold at 4°C.

The next step was the barcoding. 5ul from the pooled products was aliquoted into a new plate (barcode attachment plate) and mixed with 2.5ul of nuclease-free water and 2.5ul of the barcodes to make a final volume of 10ul. The barcode attachment plate was then placed on a thermal cycler and incubated using the following conditions: 30°C for 2

minutes and 80°C for 2 minutes, after which 10ul from each sample were pooled together in a single tube and cleaned up to remove all nucleic acids (DNA/RNA) that are not of interest, using equal volume of SPRI (Solid Phase Reversible Immobilization) beads. 200ul (80%) ethanol was used to wash twice to debris and molecules that do not attach to the beads. The beads were then air-dried to remove residual ethanol and then eluted with 15ul of elution buffer (EB) and allowed to stand on the magnetic stand for 10 minutes.

The eluted DNA was quantified to know its amount in nanograms per microliter (ng/ul). A qubit dsDNA Assay HS kit was used for this quantification. Standards and samples were prepared as follows: for standards, 190ul of working solution from the dsDNA HS kit was mixed with 10ul for each standard (Standard 1 and 2) in a strip tube. Next was the preparation of samples. 198ul of the working solution was mixed with 2 ul of the sample (pooled sample in this case). The mix was vortexed, spun down and placed on the Qubit system. A qubit flex system was used, and the assay protocol for the dsDNA HS kit was followed. Standards were first used to standardize the system, and the samples were quantified. The value of the sample is then noted.

The Qubit value of the pool in ng/ul is used to make up the sample up to 800ng, and the volume made up to 11ul. Following this, 1ul of Rapid Adapter F (RAP F) is added to the barcoded DNA (pool) and incubated at room temperature for 5 minutes (this process is temperature sensitive and could be regulated by placing the mix on a thermal cycler).

While the sample pool is incubating, a flow cell for the Nanopore sequencing platform is brought out and allowed to acclimatize at room temperature for about 30 minutes to an hour. Upon attaining room temperature, a flow cell check was carried out on the flow cell and the number of available pores was recorded. A flow cell for the GridION

ONT system was used, and the samples were loaded using the GridION system experiment. After the system check, the flow cell was primed for loading of the samples. Next, 30ul of storage buffer was drawn out from the priming port. A priming mix is then prepared (by mixing 1.17ml of Flush Buffer [FB] with 30ul of Flush Tether [FLT]) and loaded twice via the priming port using a 1000ul pipette tip. In the first step, 800ul of the priming mix is loaded gently via the priming port and then incubated for 5 minutes. During this incubation, the 12ul pool (library) is brought out and mixed with 37.5ul of sequencing buffer II (SBII) and 25.5ul of loading beads II (LBII), thus bringing the final volume to 75ul. Upon completion of the 5 minutes incubation, 200ul of the priming mix was then loaded gently via the priming port; however, during this, the SpotON port on the flow cell was opened to allow the wetness of its rim. Immediately, the 75ul library mix is loaded dropwise on the SpotON port and allowed to flow through the port. The SpotON port cover was then gently replaced (alongside the priming port), and the sequencing run was ready to be set up on the system. The library prep kit is selected, a MUX scan for 3 hours is selected, and the run started and allowed to run for 72 hours.

Data Analysis

Microsoft Excel (version 16.39) and GraphPad Prism software (version 8.4.2, 2020) were used for data analysis. Continuous variable comparisons and assessments of relationships were conducted through pair t-tests, correlation analyses, and regression analyses. Descriptive statistics employed geometric mean. A significance threshold of $p < 0.05$ at a 95% confidence interval was employed for all statistical evaluations.

Chapter III.

Results

Manual vs Automated System of Nucleic Acid Extraction

During the COVID-19 pandemic, two different methods of nucleic acid extraction were used at ACEGID: manual and automated. Here, I evaluate the performance of the two different methods of nucleic acid extraction.

RNA was manually extracted from selected nasopharyngeal samples using the QIAamp Viral RNA Mini Kit according to the manufacturer's instructions (QIAGEN; Ref 52906; Lot no 26902240). Also, RNA was extracted using the AviPure RX nucleic acid automated extraction kit (Avicenna; Ref AVP10196; Lot no 2021082010). The extracts from each of the extraction methods were tested to measure the amount of RNA present in them using the Invitrogen Qubit™ Flex Fluorometer. Also, the time spent on each method of extraction was recorded with a stopwatch. The results showed that both methods were efficient in nucleic acid extraction (Fig 3a). However, the manual methods yielded more RNA than the automated method (Fig 3b). In terms of time, the automated method took a shorter time (1-2 hours) to complete compared to the manual method (4-5 hours).

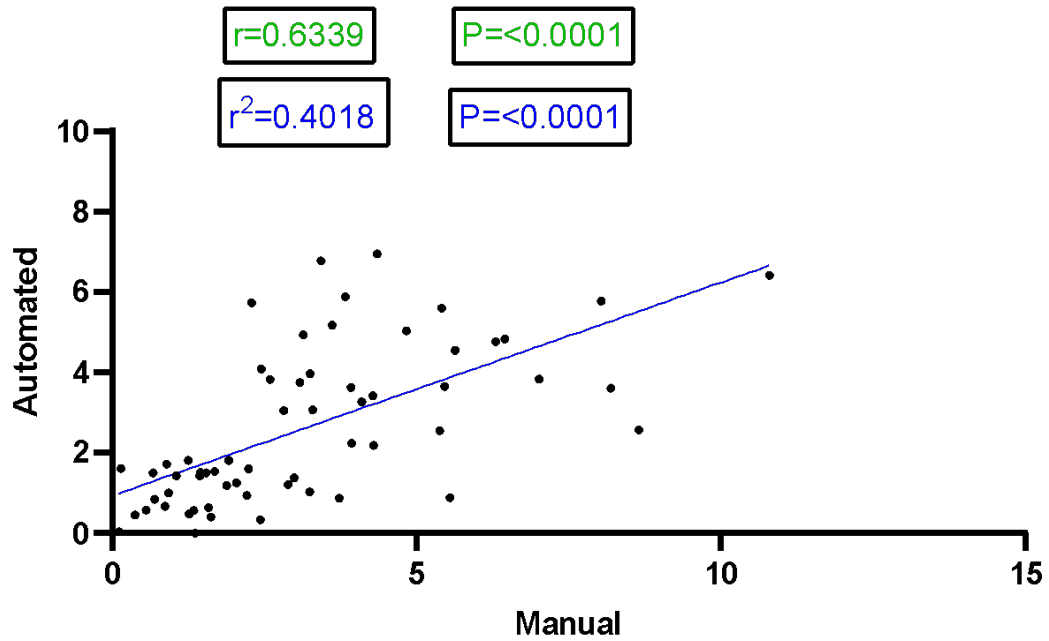


Figure 3a: Correlation between manual and automated methods of nucleic acid extraction

This shows the linear relationship between the concentration of RNA yield (ng/ul) obtained from Qubit between manual and automated extraction. The straight line and r value indicate the direction and strength of the linear relationship. The p -value shows that the relationship between manual and automated extraction is highly significant.

	Manual	Automated
Geometric mean	2.348	1.717

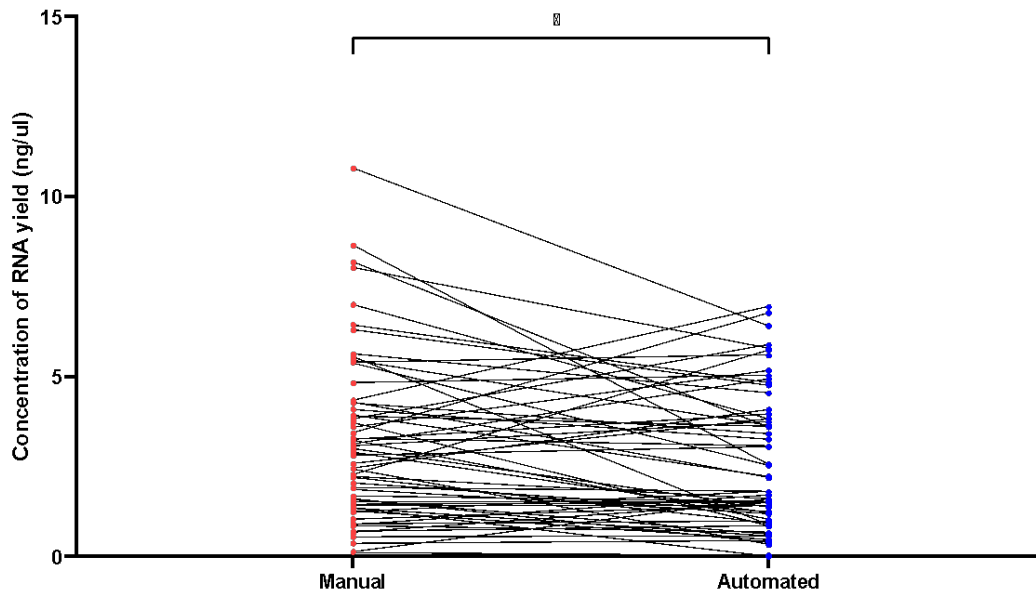


Figure 3b: Nucleic Acid yield

*The concentration of RNA yield (ng/ul) obtained from Qubit between manual and automated extraction. The table depicts the geometric mean at 95% CI. Statistical analysis was conducted using a t-test and p-value denoted by a line with *, implying a significant difference.*

Performance evaluation of qRT-PCR Assays that were deployed during the COVID-19 pandemic in Nigeria

The three qRT-PCR -Allplex CoV-2 Master assay, AviMol Dri SARS-CoV-2 and DaAn Gene detection 2019 novel CoV kits were tested for their sensitivity and reproducibility using known positive SARS-CoV-2 nasopharyngeal samples. The known Ct value of the SARS-CoV-2 N-gene from different samples (grouped into High, Mid, and low Ct values) was compared using the three different kits. The result showed that the three test kits were sensitive to SARS-CoV-2 (Fig 4a). DaAn gene kit was the most sensitive

assay at high and mid Ct values while the Allplex kit was the least sensitive, having Ct values lower than those at the initial low Ct level at low Ct value; the AviMol was the most sensitive while Allplex was the least sensitive kit (Fig 4b). A similar trend was also seen for the RdRp genes across the three different kits (Fig 4c) and (Fig 4d). When the kits were tested for specificity using the Lassa fever virus, which is a different pathogen from SARS-CoV-2, all three kits were specific for SARS-CoV-2, and none detected the Lassa fever virus (Fig4e).

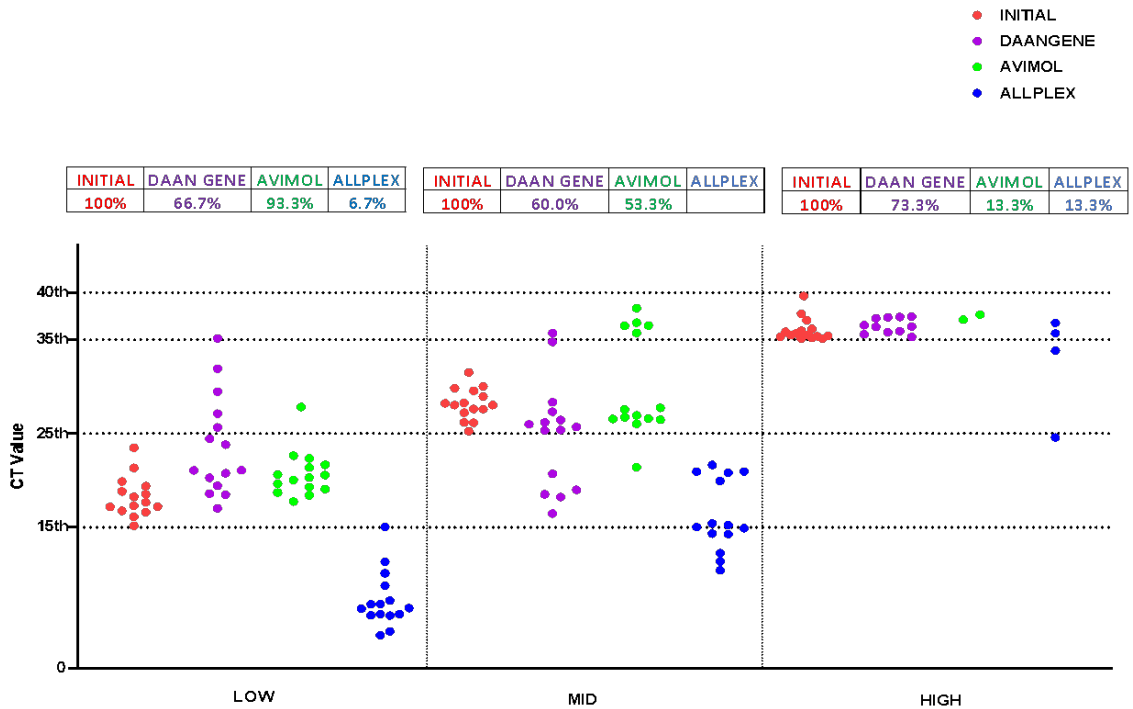


Figure 4a: Performance of the different commercial qPCR kits (N Gene)

The reproducibility of the three qPCR kits specific to SARS-CoV-2 by comparing the initial N-gene outcome with the re-run. The samples were sorted into three groups based on the initial Ct value as High, Mid, and low. The tables show the percentage of the reruns with precise Ct value as the initial.

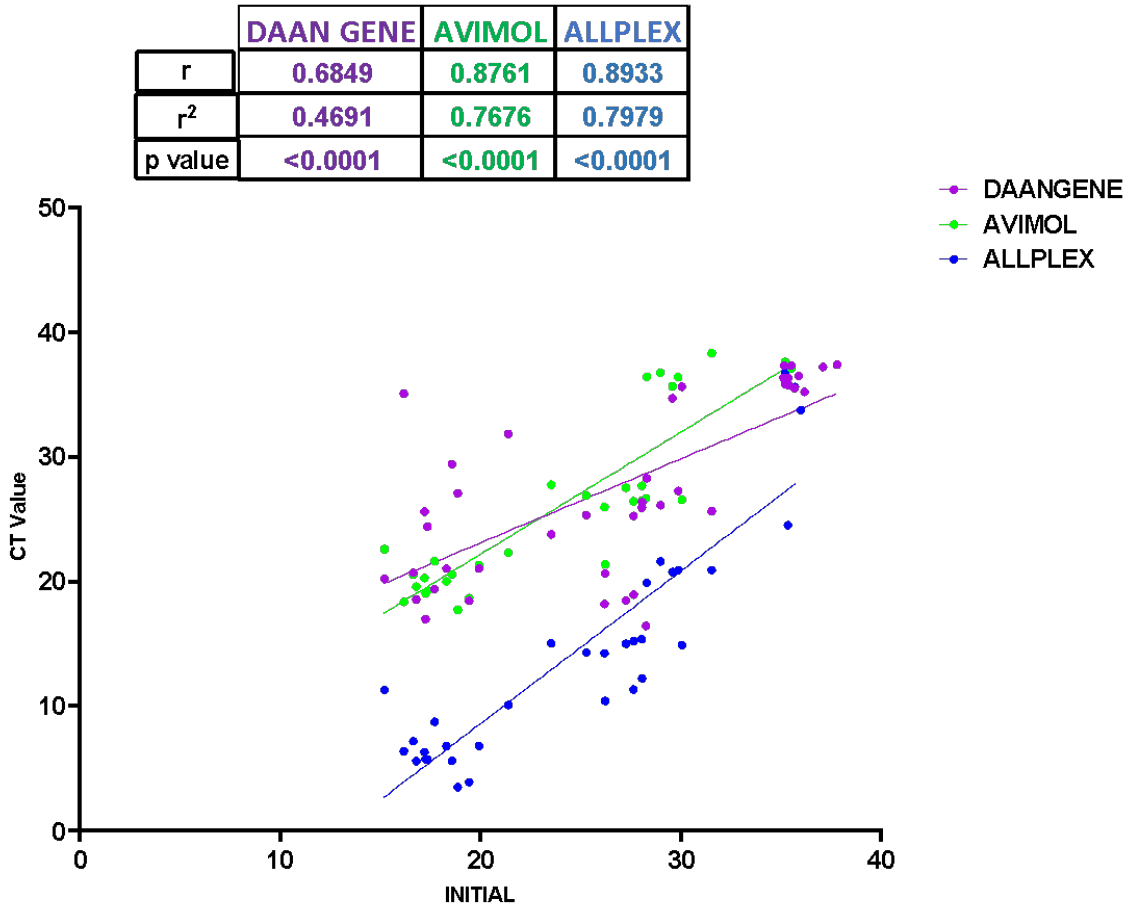


Figure 4b: Correlation analysis of the different commercial qPCR kits (N Gene)

The linear relationship between the initial Ct values and the Ct values of the re-run using the three qPCR kits. The straight line and r value indicate the direction and strength of the linear relationship. The p-value shows that the relationship between the initial Ct values and the Ct values of the re-run using the three qPCR kits is highly significant.

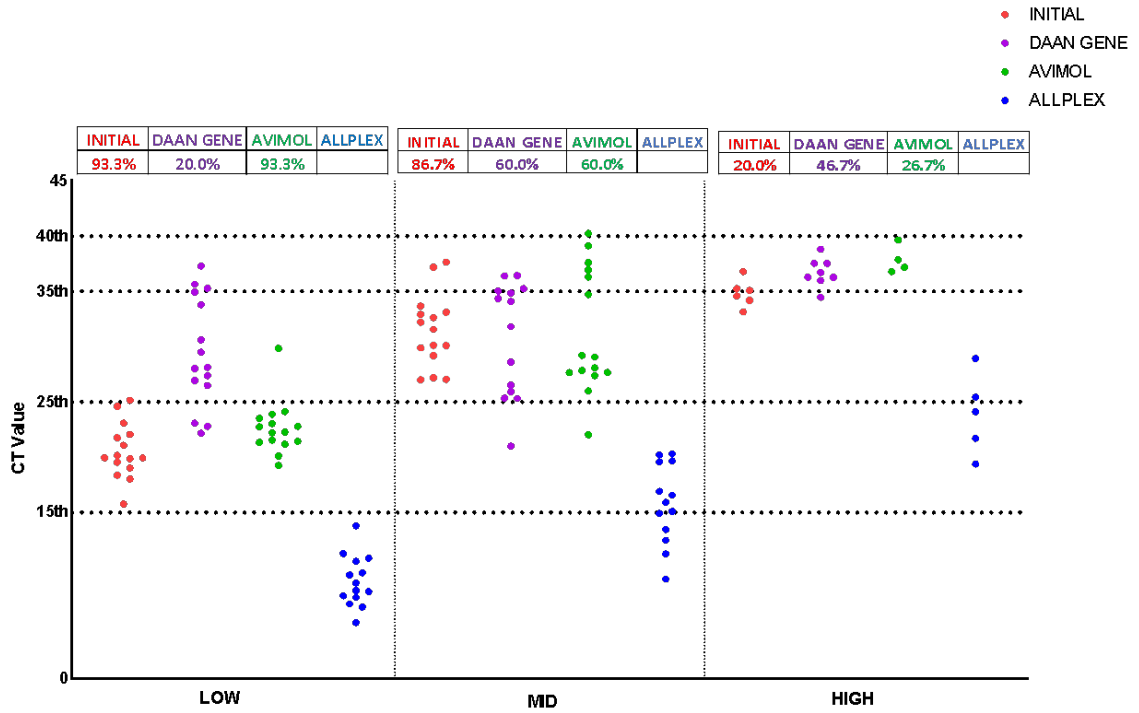


Figure 4c: Performance of the different commercial qPCR kits (RdRp Gene)

This is the initial RdRp gene outcome with the re-run. The samples were sorted into three groups based on the initial Ct value as High, Mid, and low. The tables show the percentage of the re-runs with precise Ct value as the initial.

	DAAN GENE	AVIMOL	ALLPLEX
r	0.08077	0.7308	0.7563
r ²	0.2842	0.8549	0.8697
p value	0.1213	<0.0001	<0.0001

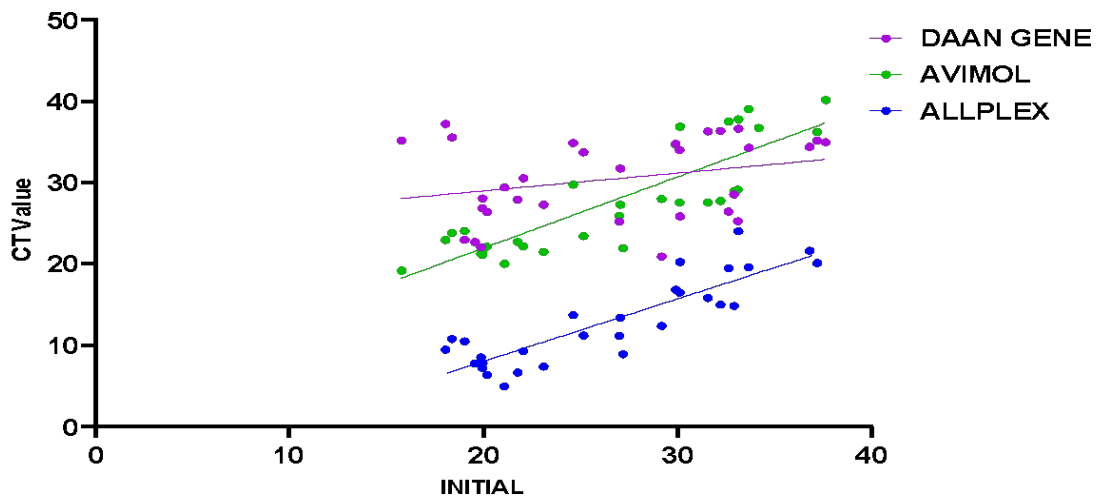


Figure 4d: Correlation analysis of the different commercial qPCR kits (RdRp Gene)

The linear relationship between the initial Ct values and the Ct values of the re-run using the three qPCR kits. The straight line and r value indicate the direction and strength of the linear relationship. The p-value shows that the relationship between the initial Ct values and the Ct values of the re-run using the three qPCR kits is highly significant, except for the DaAn gene.

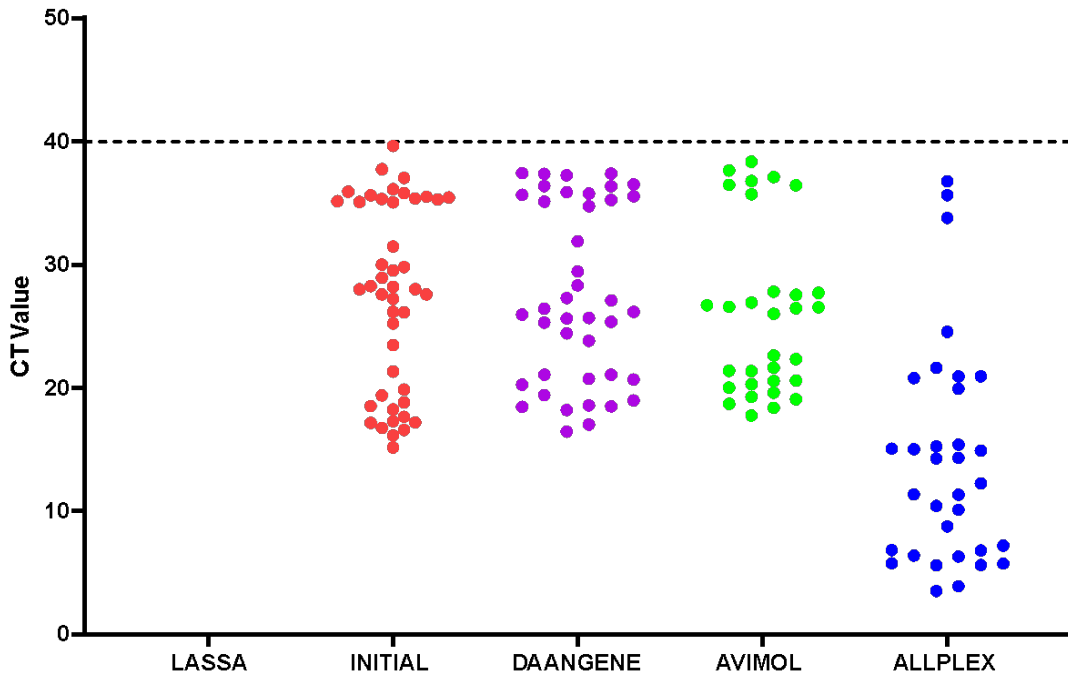


Figure 4e: Performance specificity for SARS-CoV-2

Lassa confirmed samples tested with the three different commercial SARS-CoV-2 kits do not produce any CT values, implying that the kits are specific only to SARS-CoV-2.

Performance evaluation of CRISPR-based SARS-CoV-2 Assay (mCARMEN)

mCARMEN is a CRISPR-based microfluidic chip-based assay that can detect many respiratory viruses within a short time and that is technologically less intensive. Here, I evaluated the performance of mCARMEN to detect known SARS-CoV-2 positive samples at different ranges of Ct values (high, mid, low). Interestingly, mCARMEN detected all positive samples at different Ct values (Fig 5), and as expected, it also detected other respiratory pathogens such as HRSV, FLUAV, FLUBV, HCoV-OC43, HCoV-NL63.

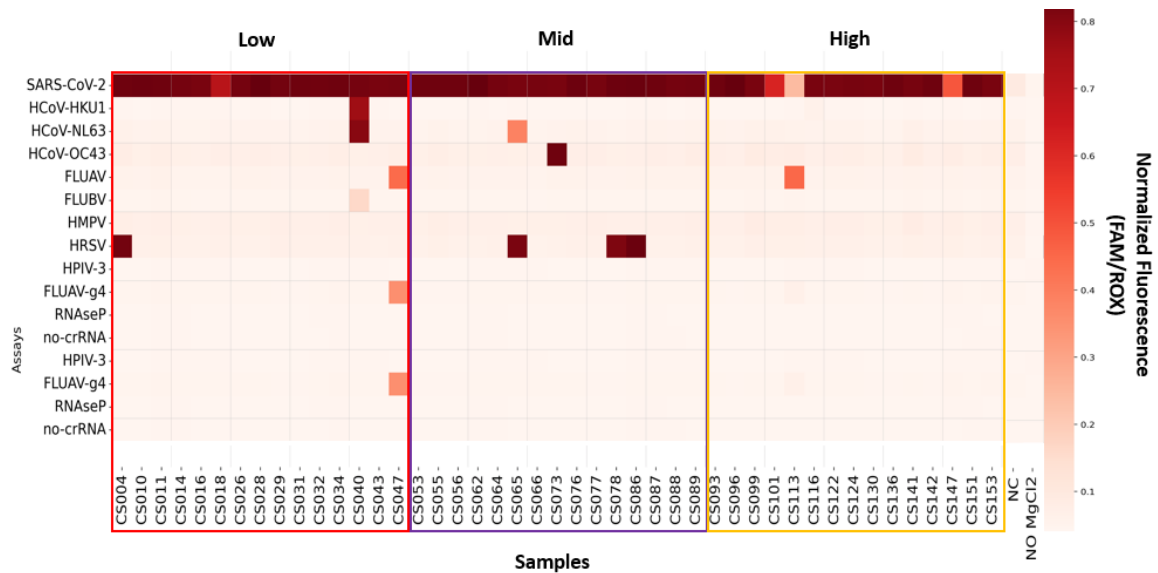


Figure 5: Detection of SARS-CoV-2 and other respiratory pathogens by mCARMEN

Heat map fluorescent data showing SARSCoV-2 at varying CT values and other known respiratory pathogens as co-infections.

Performance evaluation of different genomic sequencing platforms for SARS-CoV-2 detection

Next-generation sequencing was very useful in detecting and tracking the emergence of different variants of SARS-CoV-2 during the COVID-19 pandemic. Here, I compared the performance of two different next-generation sequencing platforms - Illumina and Oxford nanopore platforms. Known SARS-CoV-2 positive samples with different ranges of Ct-value (high, mid, and low) were sequenced with the two sequencing platforms. The result showed that both platforms could only sequence samples with low and mid CT values but not samples with high CT values (fig 6).

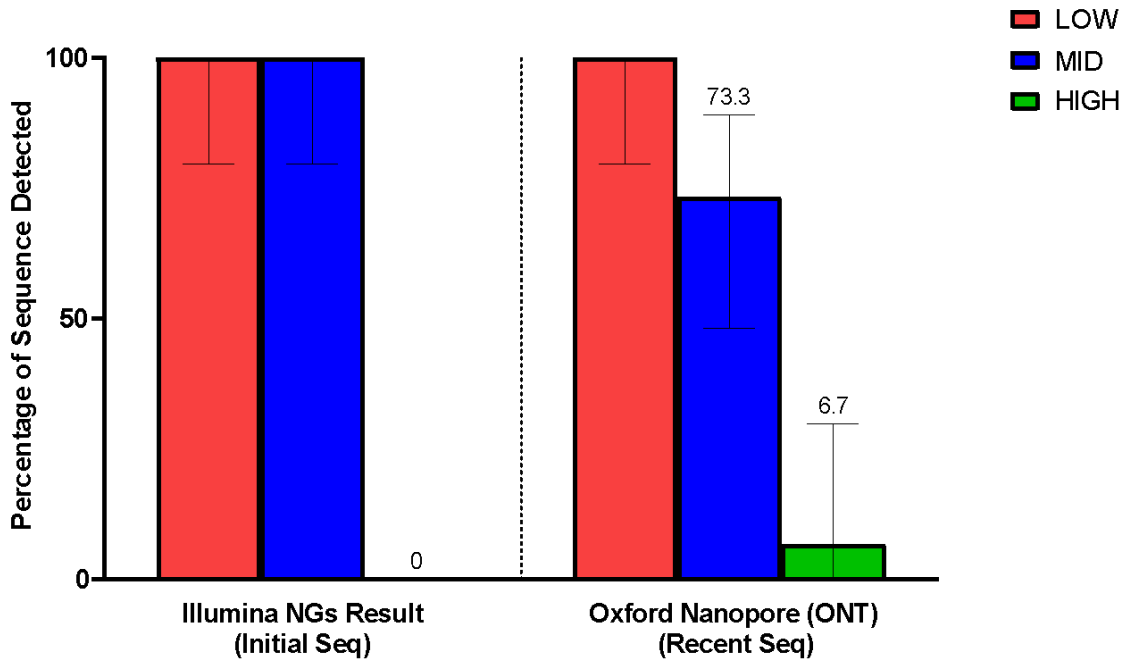


Figure 6: Threshold CT value for SARS-CoV-2 genomic sequencing

The graph shows the proportion of the SARS-CoV-2 sequence at the different Ct values using Illumina NGS and Oxford Nanopore (ONT). Genomes could only be detected in samples with low and mid Ct values but not high Ct values.

Chapter IV.

Discussion

The COVID-19 pandemic resulted in a significant number of deaths and has negatively impacted the global health economy. Like many other infectious diseases, early detection and diagnosis is important in containing an outbreak. For the SARS-CoV-2 virus, diagnostics played a crucial role in containing the pandemic (Kwok *et al.*, 2022). One such role was the identification of the novel SARS-CoV-2 virus as well as the continuous detection of new and emerging variants. Globally, many diagnostics tests were deployed during the pandemic. Molecular tests such as RT-qPCR and NGS were the most effective and commonly used diagnostic methods. During the pandemic, in Nigeria and other resource-limited countries, most of these molecular tests were not locally produced; they thus relied on supply and donations from developed nations (Kamara *et al.*, 2022). For this reason, different molecular tests from different companies were deployed in Nigeria during the pandemic. At ACEGID, a national and regional reference lab, about five different RT-qPCR test kits were used for patients' and travelers' diagnosis of COVID-19. The test kits include DaAn gene kits, BGI, Sansure, Tagpath, Allplex, AviMOI Dri. As a result, sometimes, there was discordance in the results from different tests (Kwok *et al.*, 2022). Now that the pandemic is winding down, it is time to reflect on the lessons learnt from it in order to prepare better for future pandemics.

This study evaluated the performance of different commercial molecular tests as well as nucleic acid extraction kits that were used for the diagnosis of COVID-19 at ACEGID.

Two nucleic acid extraction methods were used at ACEGID, one manual and one automated. The results showed that the manual method of extraction yielded more RNA (ng/ul) compared to the automated method. On the other hand, the automated method was quicker compared to the manual method. Similar or contrasting results have been shown in other labs, especially with the purity and concentration of the RNA (Joseph 2022).

Efforts should be made to improve the automated extraction method, especially for RNA yield, as it will come in handy for high throughput samples and turnaround time during pandemics. For those who cannot afford automated systems, the manual is also a good choice and with more practice and experience, the turnaround time will improve.

Next, I evaluated the performance (sensitivity and reproducibility) of the three commercial qPCR kits (AviMol, DaAn Gene and AllPlex) using an established initial Ct value of the SARS-CoV-2 N and RdRp genes by RT-qPCR at ACEGID as standard. From the results shown, all three kits used were sensitive to SARS-CoV-2, with similar outcomes as (Wang *et al.*, 2021). However, the DaAn gene kit was the most sensitive.

Similarly, a specificity test was done using previously confirmed Lassa Fever positive samples with the three SARS-CoV-2 kits. From the result, there was no amplification (no CT value), implying the kits were specific for SARS-CoV-2. A similar comparison done by (Wang *et al.*, 2021) using different SARS-CoV-2 commercial kits further confirms the specificity of these testing kits across the board. Furthermore, I compared the molecular tests with a CRISPR-based detection assay, mCARMEN RVP

workflow, that can detect about 9 different respiratory pathogens. Interestingly, the result showed similar sensitivity and specificity to the RT PCR. The result agreed with (Welch *et al.*, 2020), as there was no decrease in the assay's ability to detect the SARS-CoV-2 virus at the different CT ranges. In addition, it was able to pick up some of the HCOVS and other respiratory pathogens that were seen (this may be because of the conserved region for the SARS-CoV in the RVP panel) and can be said to be co-infections with other respiratory viruses in addition to SARS-CoV-2, thus validating the assay. (Welch *et al.*, 2020).

Sequencing played a major role during the pandemic by enabling the early sequencing and release of the viral genome, which aided in the synthesis of primers for diagnosis and improved surveillance of the virus significantly (Wang *et al.*, 2020). At ACEGID, we used two sequencing platforms during the pandemic: Illumina Next Generation Sequencing and Oxford Nanopore Technology (ONT). I evaluated the performance of the two platforms. My result revealed that the Illumina platform yielded a full genome from samples with low and mid-Ct values, while Oxford Nanopore didn't perform as well as the Illumina with Mid-Ct. A similar result was obtained from a study by (Tshiabuila *et al.*, 2022). I went further to show that the optimal Ct value to yield a full genome from both platforms was low and mid-Ct (20-30). This agrees with Charre *et al.*, 2020, after a careful evaluation of different next-generation sequencing methods.

Conclusion

The COVID-19 pandemic has put a strain on global public health and economy. During pandemics, usually, there are different available methods for the detection of the

suspected circulating pathogen, most of which are based on the detection of the nucleic acid or protein component of the virus.

This study has provided key information on some of the diagnostic assays which were deployed during the pandemic and how their performance varies. The result showed that the concentration of RNA yield (ng/ul) obtained via manual extraction (Qiagen kit) was significantly higher than via automated extraction (AviPureRX). The performance rate sensitivity test of three different commercial qPCR kits (AviMol Dri, Da AN Gene and AllPlex) showed the DaAn Gene expressed the highest performance rate of 66.7%, and the (specificity) test using a virus different from SARS-CoV-2 showed that the three kits were all specific to SARS-CoV-2. qPCR. On the other hand, the mCARMEN RVP had a 100% performance rate when compared with the qPCR. The Illumina Next Generation Sequencing, in comparison to the Oxford Nanopore Technology (ONT), performed better and was used to determine an appropriate sequencing Ct cut-off.

This study has shown that all the diagnostic tests that were deployed during the pandemic at ACEGID were sensitive and specific; therefore, diagnostic test results were reliable. However, the slight variation in sensitivity emphasizes the need for assay validation prior to deployment during a pandemic to ensure that the best-performing test is used. Also, prior validation before deployment will provide necessary feedback to the manufacturer should there be a need for optimization of the assay kits. Finally, the performance of the CRISPR base test MCARMEN is worthy of mention since it yielded similar results to the RTqPCR test. The future of diagnostic tests during a pandemic is the availability of a cheap, quick, field-deployable diagnostic test, and mCARMEN interestingly fits such a product. In addition, it can also detect co-infection with other

respiratory pathogens. This study has provided field evaluation data for mCARMEN, and the feedback will be useful for future deployment.

Recommendation

The importance of diagnostic roles in disease surveillance to stop the disease from spreading cannot be overstated. It is hereby recommended that:

The Allplex performance can be further evaluated due to its high sensitivity rate at the low Ct level, seeing it had Ct values better than the initial qPCR values. This may be a result of its variant-specific composition.

Further studies should be done to explore other extraction methods to obtain the best quality of the viral genome.

Due to the unstable nature of RNA, an improved storage facility and power supply are required in resource-limited settings to avoid degradation of the viral RNA before it undergoes RT-qPCR amplification, which could limit the integrity of the results derived from the downstream experiments.

Prompt and accurate diagnosis is a key factor in curbing the spread of the SARS-CoV-2 infection. Thus, testing reagents must be delivered in a timely manner to ensure early patient isolation and reduce the spread and transmission of the virus.

The mCARMEN RVP detection assay should be validated further and made a commercial diagnostic assay due to its multiplexed nature and ability to detect several pathogens in a single run.

Appendix 1.

Distribution of samples based on Ct ranges

Table A1: Distribution of samples based on Ct ranges

S/N	Sample ID >35 (High Ct)	Sample ID 25-35 (Mid Ct)	Sample ID <25 (Low Ct)	Lassa Fever positive
1	EPS 10129	EPS 11199	EPS 11225	EPLV 001
2	EPS 10133	EPS 11994	EPS 11950	EPLV 002
3	EPS 10160	EPS 11261	EPS 11947	EPLV 003
4	EPS 10167	EPS 12041	EPS 11931	EPLV 005
5	EPS 10168	EPS 12049	EPS11938	EPLV 009
6	EPS 10174	EPS 12147	EPS 11916	EPLV 016
7	EPS 10177	EPS 12148	EPS 11905	EPLV 028
8	EPS 10210	EPS 12156	EPS 11902	EPLV 030

S/N	Sample ID >35 (High Ct)	Sample ID 25-35 (Mid Ct)	Sample ID <25 (Low Ct)	Lassa Fever positive
9	EPS 10214	EPS 10447	EPS 11868	EPLV 033
10	EPS 10321	EPS 10540	EPS 11861B	EPLV 036
11	EPS 10557	EPS 10563	EPS 11859B	EPLV 054
12	EPS10658	EPS10580	EPS11426	EPLV 056
13	EPS 10784	EPS 10587	EPS 11989	EPLV 057
14	EPS 10789	EPS10659	EPS 11328	EPLV 059
15	EPS 10798	EPS 10736	EPS 12036	EPLV 060

Initial sample selection based on Ct values. Low Ct (high viral load), Mid Ct (average viral load), High Ct (low viral load) and Confirmed Lassa fever positive samples (serves as control)

Appendix 2.

CARMEN-RVP Primers, crRNA, and Reporter sequences

Table A2: CARMEN-RVP Primers, crRNA, and Reporter sequences

Virus	Primer	Oligonucleotide Sequence (5'-3')	Target Gene
1 SARS-CoV-2	Forward primer	GAAATTAATACGACTCACTATAGGGCA ATTAGAGAT GGAACTTACACC	Orf1ab
	Reverse primer	CTTTTtagCTTCTTCCACAATGTC	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACCUAAAACUAUUCACUUCAAUAGU CUGAA	
2 FLUAV	Forward primer	GAAATTAATACGACTCACTATAGGGGC GTGTTGATG AGAACGG	PB1
	Reverse primer	GCCACAAACTTACAATCTTTTACAC	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUGGUGUAUUUCUUUUGUCCAAG AUUCAG	
3 FLUBV	Forward primer	GAAATTAATACGACTCACTATAGGGGG TGGTATGAG CTACTTTTGTGTA	PB1
	Reverse primer	ACTACCTGTGCACATATTCTTGTATA	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUCCAAUGUUUUUGAUGCCUAGU GCUGCU	
4 HRSV	Forward primer	GAAATTAATACGACTCACTATAGGGCT TCACGAAGG CTCCACATA	M
	Reverse primer-1	CCCATATTGTTAGTGATGCAGG	
	Reverse primer-2	GCACCCATATTGTTAGTGATGC	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACGUCUUUUUCUAGGACAUUGUAU	

UGAACA

Virus	Primer	Oligonucleotide Sequence (5'-3')	Target Gene
5 HMPV	Forward primer	GAAATTAATACGACTCACTATAGGGAC CCAAATGA GAAAGACTGTG	F
	Reverse primer	GCAACATTAATTCCTGCTGCT	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACGUCGCAAAGACAUGGUCUCCUC UUGUU	
6 HPIV-3	Forward primer	GAAATTAATACGACTCACTATAGGGAC GAATCAA GATAAATACGGGAG	M
	Reverse primer	ATTGGTAATGATCCAGAGCCA	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUUGUAACUCGGGUCACUGUCAAG AUCAU	
7 HCoV-HKU1	Forward primer	GAAATTAATACGACTCACTATAGGGTT CTGGTAGTG GTCAGGCTA	Orflab
	Reverse primer crRNA	CTTATAGGGTCATTTGTACCTATAGG GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACCGCAUAACAAAUCGUGAAUCACG AACUA	
8 HCoV-NL63	Forward primer	GAAATTAATACGACTCACTATAGGGGT GACTTTGAT ATTGTAGTGGCTT	
	Reverse primer	ATACCACAAATAGTAGCTATAGTCTG C	
	crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUGUGCCACAACAAGAAGAUUAG UAACCA	
9 HCoV-OC43	Forward primer	GAAATTAATACGACTCACTATAGGGTT CTGAGAGTT GGAGTGTTGG	RdRp
	Reverse primer crRNA	ACCAGGAACAACACAAAGTTTC GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUCAGAUCUGUCCUCUUAACAACA AAGAA	

Virus	Primer	Oligonucleotide Sequence (5'-3')	Target Gene
10	RNase P forward primer	GAAATTAATACGACTCACTATAGGGTT	RNase P
RNase P	RNase P reverse primer	GATGAGCTG GAGCCA ATGTGGATGGCTGAGTTGTT	
control	RNase P crRNA	GAUUUAGACUACCCCAAAAACGAAGG GGACUAAA ACUCCGAGUCAGUGGCUCCCGUGUG UCGGU	

Primers are sourced from Eton, crRNA and Reporter is sourced from ID

Appendix 3.

Pictorial representation of a 192.24 chip showing positions for priming (Ps), loading of Assays and Samples

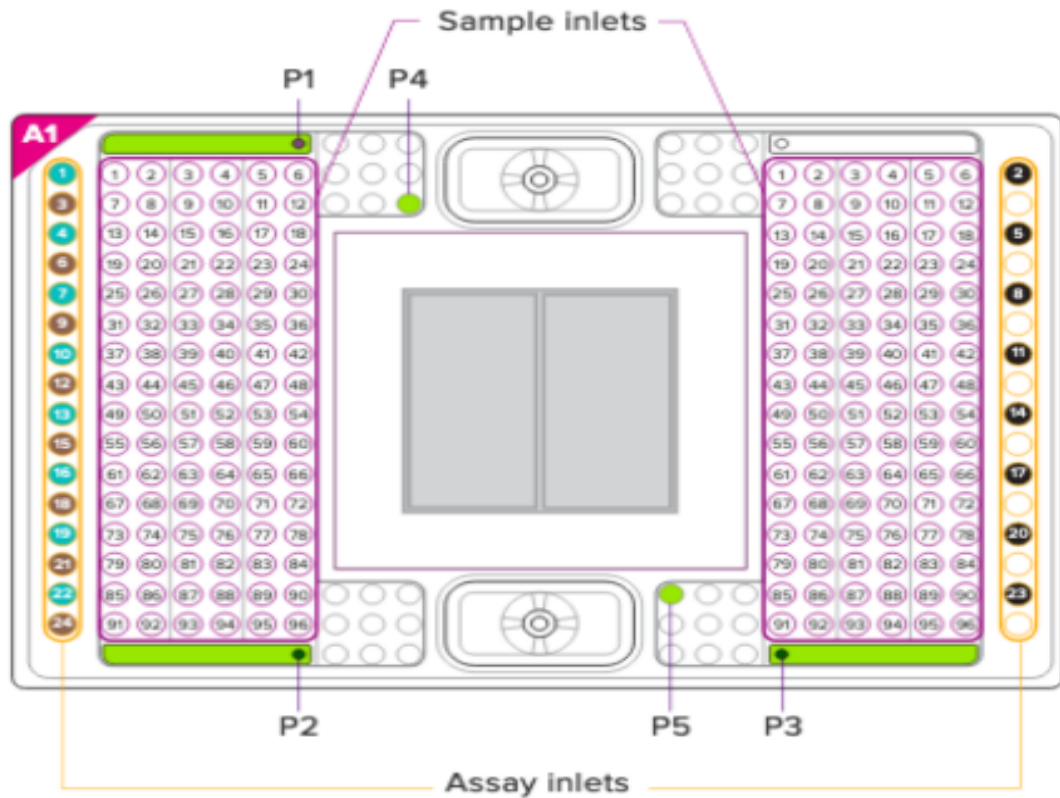


Figure A1: IFC priming and loading

The barcode is always on the left

Assay Detection reactions go into the leftmost and rightmost columns (Assay inlets)

Sample Master Mix Plate 1 goes on the left side. Sample inlets

Sample Master Mix Plate 2 goes to the right side. Sample inlets

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