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The Validation of The SalivaDirect Test Performed at Boise State University for the Quantification of SARS-CoV-2 Testing with a Comparison to TaqPath Covid-19 Combo Kit

by

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Committee Approval

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The Validation of The SalivaDirect Test Performed at Boise State University for the Quantification of SARS-CoV-2 Testing with a Comparison to TaqPath Covid-19 Combo Kit

Thesis Abstract—Idaho State University (2021)

Covid-19 caused by SARS-CoV-2 is a global wide pandemic with illness and death reaching into the millions. With hundreds of different testing methods implemented in order to diagnose this disease, Boise State University implemented two: the TaqPath COVID-19 Combo Kit and SalivaDirect. The TaqPath COVID-19 Combo kit used nasopharyngeal swabs with healthcare professionals having to collect them from the university's community. A validation for a more cost effective and sensitive method of testing took place in November 2020 that changed the sample type from nasopharyngeal to saliva and had henceforth broadened the university's testing range. The SalivaDirect testing method was performed on known positive and negative saliva samples on November 27th 2020, and December 11th, 2020 on the university's QuantStudio 5 PCR instruments. Testing found that SalivaDirect was more accurate and precise than the previously used TaqPath, thus lowering the cost and providing quality results to those tested.

Key Words: SalivaDirect, TaqPath COVID-19 Combo Kit, Covid-19, SARS-CoV-2, PCR, Validation

CHAPTER I: INTRODUCTION

In light of the Covid-19 pandemic, multiple health care facilities and universities have opted to find a testing method that would enable them to help diagnose its surrounding community. For Boise State University, the original method was TaqPath COVID-19 Combo Kit. This was able to test 94 patients with every plate with each run, and taking about two hours. This method however proved to be very costly, and with other testing procedures being approved by the Food and Drug Administration, the University looked towards a more sensitive method that would also lower the cost of testing. Given their current instrumentation and equipment their next best option was SalivaDirect. This testing method would test almost the same amount of patient samples, with a higher sensitivity and less cost. In November 2020, Boise State University started taking the authorization steps from Yale University to implement SalivaDirect on their two ThermoFisher Scientific Applied Biosystems QuantStudio 5 Real-Time PCR Systems. Starting with a small validation sent to Yale, and ending with the completed validation given to Boise State University, each step was carefully considered in order to improve the testing method at the university.

HISTORY

The origin of the novel coronavirus is thought to come from animals traded in a live animal market found in Wuhan, China in December of 2019 (Astuti & Ysrafil, 2020). During this time, the Wuhan Municipal Health Commissions in China reported a cluster of pneumonia cases to which the virus was identified as the causative agent. In January 2020, the World Health Organization reported no deaths from this cluster of pneumonia patients in Wuhan, China. They later issued a comprehensive package of technical guidance with the advice given to all countries

on how to detect and manage potential cases based on what was known about Coronavirus at the time (World Health Organization, 2020).

It was on January 11th, 2020, that the Chinese media reported the first known death caused by the novel Coronavirus of which had now infected dozens of people. Following in January, confirmed cases occurred in Japan, South Korea, Thailand, and the United States. These became the first cases to occur outside of mainland China (Taylor, 2020). Human-to-human transmission evidence became strongly supported on January 22, 2020 after a visit conducted by a WHO delegation to the city of Wuhan (Dos Santos, 2020). By late January, planes and trains leaving the city of Wuhan were cancelled by Chinese authorities. They also suspended buses, subways, and ferries as 17 people had died and now more than 570 had been infected by Coronavirus. The first death outside of China was a 44-year-old man in the Philippines during early February. Days later on February 11th, the World Health Organization proposed an official name for the disease caused by the virus. Now known as Covid-19 as an acronym that stands for Coronavirus disease 2019 and was named as such to avoid the stigma of referencing it to any people, places, or animals associated with the virus. Three days later, France announced the first Coronavirus death in Europe; this makes it the first death outside of Asia and the fourth death outside of mainland China where, at this point 1,500 people had died. The United States reported its first death on February 29th. However, two people who had died earlier may have been the first victims of the Coronavirus in the US, but their Covid-19 diagnoses were not discovered until months later (Taylor, 2020).

The World Health Organization declared Covid-19 as a pandemic on March 11th, 2020. This declaration was made due to the speed and scale of the disease's transmission. It wasn't until later that the virus genome was sequenced and found to be genetically related to the

Coronavirus responsible for the SARS outbreak of 2003. The International Committee for Taxonomy of Virus then named the virus Severe Acute Respiratory Syndrome Coronavirus-2 (SARS-CoV-2) (Dos Santos, 2020).

By late March 2020, the United States officially became the country hit hardest by the pandemic with at least 81,321 confirmed infections and more than 1,000 deaths. By early April, the pandemic had infected more than one million people in 171 different countries and had caused at least 51,000 deaths. These numbers nearly tripled by the end of April reaching 2.8 million people who have been infected with over 200,000 deaths; this actual number is suspected to be higher by an unknown degree and would remain so for some time. In late May, nearly four months after the government confirmed the first known case of Coronavirus, more than 100,000 patients passed away in the United States alone. By September global deaths had reached 1 million since the pandemic began in Wuhan, China. By the month of November, the United States then reached 10 million cases with a death toll of 250,000 alone (Taylor, 2020).

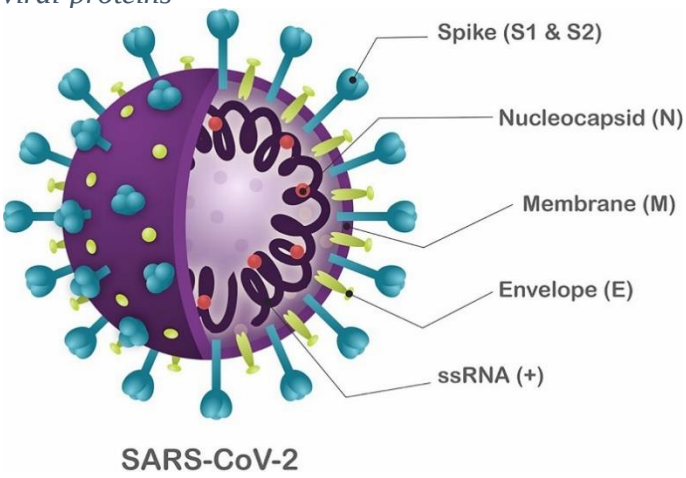
During this pandemic, a worldwide collaboration had begun in creating a vaccine as quickly as possible as numbers continued to rise. In December of 2020, the United Kingdom began vaccinating with the Pfizer vaccine with Margaret Keenan becoming the first person to receive the Coronavirus vaccination. The Food and Drug Administration authorized the Pfizer vaccine for emergency use in the United States, and within the same month they authorized the vaccine made by Moderna for emergency use as well (Taylor, 2020). By late December, the United States began to vaccinate front-line healthcare workers.

SARS-CoV-2 IDENTIFICATION

Severe acute respiratory syndrome Coronavirus 2 also known as SARS-CoV-2 was identified in Wuhan, China as Beta-Coronavirus (nCoV) before being genetically sequenced (Astuti & Ysrafil, 2020). It was previously assigned to group 2b CoV but it is now a member of the lineage B of the genus Betacoronavirus in the family of Coronaviridae and subfamily Coronavirinae. It shares a similar genome organization with other coronaviruses but exhibits a unique genomic structure that includes several specific accessory genes such as ORF3a, 3b, ORF6, ORF7a, 7b, ORF8a, 8b, and 9b (Yadav & Saxena, 2020). SARS-CoV-2 is an enveloped, non-segmented, positive-sense RNA virus; included in the sarbecovirus, ortho coronavirinae subfamily that is found in both humans and other mammals. Bats have been suspected as the reservoir of this virus by finding as much as 96.2% in identical genome sequencing of SARS-CoV-2 with Bat-CoV-RaTG13 (Astuti & Ysrafil, 2020).

SARS-CoV-2 has four main structural proteins along with several accessory proteins; these are the spike glycoprotein, small envelop glycoprotein, membrane glycoprotein, and nucleocapsid protein. The spike protein forms homotrimers protruding on the viral surface and will facilitate binding to the host's cells by attraction with angiotensin-converting enzyme 2 (ACE2) that is found to be expressed in the cells of the lower respiratory tract. This glycoprotein is cleaved by the host cell's furin-like protease into two subunits named S1 and S2. S1 is found to be responsible for the determination of the host-virus range and cellular tropism while S2 mediates virus fusion in transmitting host cells. The nucleocapsid protein is the structural component of the coronavirus localizing in the endoplasmic-reticulum Golgi region that is structurally bound to the nucleic acid material of the virus. This protein is bound to RNA, involved in the processes related to the viral genome, the viral replication cycle, and the cellular

Figure 1: Representation of SARS-CoV-2 with main viral proteins



response of the host cell to the viral infections. The nucleocapsid protein is heavily phosphorylated and suggested to lead to structural changes enhancing the affinity for viral RNA. The membrane glycoprotein/M protein is a structured protein that plays a role in determining the shape of the virus envelope. The M protein can bind to all other structural proteins and helps stabilize the nucleocapsid protein, and promotes the completion of viral assembly by stabilizing the N protein-RNA complex (Astuti & Ysrafil, 2020). Lastly, the envelope protein is the smallest of the viral structure and plays a role in the production and overall maturation of the virus. A visual representation of the virus can be seen in Figure 1.

SARS-CoV-2 attacks the cells of the lungs because of the conserved receptors, namely the ACE2. ACE2 is expressed in the lower respiratory tract such as the type II alveolar cells of the lungs, upper esophagus, stratified epithelial cells, and other absorptive enterocytes such as the ileum and colon, cholangiocytes, myocardial cells, kidney proximal tubule cells, and bladder urothelial cells. Patients infected with SARS-CoV-2 not only experience respiratory problems such as pneumonia that can lead to acute respiratory distress syndrome but may also experience disorders of the heart, kidneys, and digestive tract. Once SARS-CoV-2 enters the body, the spike glycoprotein attaches to the receptor of ACE2 on host cells; this is then followed by the fusion of the viral membrane and host cell. After fusion, the type II transmembrane serine protease (TMPRSS) present on the surface of host cells, clears ACE2 and activates the receptor-attached

spike-like S protein. This then leads to a conformational change and allows the virus to enter the cell. Once inside, mRNA is released by the virus and is ready to be translated into a protein. These subgenomic proteins are translated into structural and accessory proteins such as the M, S, N, and E proteins. These proteins are then enclosed into the endoplasmic reticulum and moved to the endoplasmic reticulum-golgi intermediate compartment where they are assembled into new viruses and later released from the cell (Astuti & Ysrafil, 2020).

SARS-CoV-2 INFECTION, DISEASE, AND TREATMENT

Pathogenic Coronavirus was first reported in 2002 in the Guangdong province of China and is known as SARS-CoV. The second time this virus was seen was in December 2019 in Wuhan and is now known as SARS-CoV-2 (Yadav & Saxena, 2020). The presence of SARS-CoV-2 found in host cells initiates various protective responses as the body's immune system is mediated by cytokines. A case report in Wuhan from 99 Covid-19 patients showed that there is a total increase in the number of neutrophils, interleukin-6 serum, and c-reactive proteins, with a total decrease of lymphocytes. Other research found an increased expression of proinflammatory cytokines and chemokines IP-10, MCP-1, MIP-1A, and tumor necrosis factor-alpha (TNFα). The virus is first encountered by the innate immune system via antigen-presenting cells such as dendritic cells and macrophages. These antigen-presenting cells have pattern recognition receptors (PRR) including Toll-like receptors, NOD-like receptors, RIG-I-like receptors, and other small free molecules that can be found on the host cell. Each of these PRR can induce a different biological response to subsequent protein activation. An antigen-presenting cell will present an antigen of SARS-CoV-2 to CD4+T helper cells by MHC class 1, this leads to releasing IL-12 as a co-stimulatory molecule to further stimulate the Th1 cell activation. This Th1 stimulation then releases IL-12 and IFN-α which increases in MHC class I expression and

natural killer cell activation. Production of proinflammatory cytokines via the NF-κB signaling pathway is then initiated. When SARS-CoV-2 infection occurs interleukin 17 increases. With this increase of IL-17 T cells and neutrophils are able to activate and mobilizes to the areas of infection. Other cytokines released that recruit neutrophils and monocytes to the site of infection activate IL-1, IL-6, IL-8, IL-21, TNF-ß, and MCP-1 (Astuti & Ysrafil, 2020).

Respiratory viruses are transmitted through three modes: airborne, contact, and droplet. Airborne transmission is when smaller droplets containing the virus remain in suspension in the air over a long period and great distances. Contact transmission is when an infection is spread through direct or indirect contact. This can be done with either contact with an infected patient or contact with a fomite, an article or surface that has been contaminated. Lastly, droplet transmission is infection through the exposure of the virus done by contaminated respiratory droplets from an infected person in close contact. Available data suggest that SARS-CoV-2 spreads like most other common respiratory viruses, primarily through respiratory droplet transmission within a short-range. These respiratory droplets are produced during exhalation such as breathing, coughing, singing, sneezing, and speaking. About 40-45% of SARS-CoV-2 infections occur without symptoms and can be spread by people showing no symptoms (CDC, 2019). Studies find that transmission of SARS-CoV-2 may also occur by droplets landing on surfaces to which the virus can remain viable for several hours to days. This then is transmissible from a fomite to a person's mucosa such as their eyes, nose, and mouth (CDC, 2019).

Symptoms of Covid-19 can range from mild flu-like symptoms to severe illness. These symptoms typically appear two to fourteen days after exposure to the virus. Common symptoms include fever or chills, cough, shortness of breath or difficulty breathing, fatigue, muscle or body aches, headache, a new loss of taste or smell, sore throat, congestion or runny nose, nausea or

vomiting, and diarrhea. These are not all the possible symptoms and patients can either be asymptomatic or have some of these symptoms (CDC, 2019). Researchers analyzed data of more than 70,000 Covid-19 cases to find what the most frequently occurring complications are. Around 80% of patients who develop Covid-19 are generally able to recover without the need for special treatment in about two weeks. These patients typically develop flu-like symptoms and would get little to no complications (Kandola, 2021). 53.4% of these patients were admitted to a hospital, with 4.7% being admitted to the intensive care unit, and the remaining 46.6% were outpatients. The most common complications stem from the respiratory, circulatory, central nervous, gastrointestinal, and renal systems, along with hematologic disorders and long-time disturbances of smell and taste (Vince, 2020). Patients can have a severe impact to the lungs leading to difficulty breathing, low levels of oxygen in the blood, lung injuries, and pneumonia (Kandola, 2021). Cardiac symptoms can include acute myocarditis and cardiac arrest. Patients may also experience hematologic disorders such as disseminated intravascular coagulation, and could also have acute kidney failure. The study found that 27.6% of all patients with 81% in the intensive care unit experience pneumonia, 22.6% of all and 75.3% of ICU experienced respiratory failure, 11.8% of all and 50.7 of ICU patients experienced acute kidney failure, and 10.4% of all with 54.1% of ICU patients experienced some form of sepsis (Vince, 2020). Other patients had reported health problems that last for weeks to months after their initial infection including fatigue, shortness of breath, cough, joint pain, chest pain, headaches, muscle pain, and a recurring fever (Kandola, 2021). As of December 2020, there were approximately 128 authorized Covid-19 tests that providers could utilize for testing (FDA, 2020). For patients with suspected Covid-19, a physician can order one of two types of diagnostic. The first is a molecular test which can take different sample types and is typically done by PCR. The samples

can be nasopharyngeal, nasal, throat swabs, or saliva. The second test type is an antigen test which can only use nasal or nasopharyngeal swabs. If a patient suspects that they have already been infected with Covid-19, there is a third test type which is an antibody test. This test utilizes blood from a fingerstick in order to find antibodies to the virus (CDC, 2019).

During this pandemic, the Center of Disease Control has come up with ways to prevent the spread of infection. These include staying at least six feet from others; to cover one's cough or sneeze with a tissue; to wear a mask that covers the nose and mouth in public; refrain from touching one's eyes, nose, and mouth; clean and disinfect frequently touched objects; to stay home when sick; and to wash one's hands with soap and water for at least 20 seconds (CDC, 2019). As of now, when patients exhibit mild symptoms, they are advised to stay home and rest only to leave their home when seeking medical care. For patients whose symptoms are more severe and would need to be admitted to the hospital generally to receive relief of their symptoms, an antiviral drug known as remdesivir, or convalescent plasma can be distributed. Remdesivir, sold under the name Veklury, is an FDA emergency use authorization antiviral drug used for some pediatric patients and adults who have either been hospitalized or are in a health care setting (Center for Drug Evaluation and Research, 2021). Remdesivir is an intravenous nucleotide prodrug of an adenosine analog; it binds to RNA polymerase and inhibits viral replication through the premature termination of RNA transcription (NIH, 2021). The drugs known as chloroquine and hydroxychloroquine have been shown to increase endosomal pH and inhibit the fusion of SARS-CoV-2 and host cell membranes (NIH, 2021). However, the National Institutes of Health evaluated both the safety and effectiveness of these drugs and formally concluded that they provide no clinical benefit to hospitalized patients (NIH, 2021). A final method of treatment is the use of convalescent plasma. Convalescent plasma is plasma collected

from recovered Covid-19 patients and has been found to contain antibodies to SARS-CoV-2. Before the administration of vaccinations, the Food and Drug Administration issued a EUA to use covid convalescent plasma in August 2020 (Harvard Health, 2021).

As of March 2021, there are three emergency use authorization vaccines used to protect against Covid-19. These include Pfizer-BioNTech, Moderna, and Janssen/Johnson & Johnson. Both Pfizer and Moderna use messenger RNA as their mode of vaccination. The vaccine gives cells instructions to make a harmless piece of spike protein, after which it is made by the host's cells. The immune system will recognize that it does not belong and build an immunity to it. Janssen/Johnson & Johnson vaccine's mode is a vector. For this type of vaccine, genetic material from the virus is inserted into a different and weakened live virus such as adenovirus. This virus will then get into the host's cells and gives the cell instructions to make the same spike protein as the mRNA vaccines. The Pfizer is 95% effective in preventing Covid-19, to be given to individuals 16 years and older, and requires that two injections be given 21 days apart with the second dose that can be given up to six weeks after the first dose if necessary. The Moderna vaccine is 94% effective, given to patients 18 years and older, and requires two injections to be given 28 days apart with (like Pfizer) the second dose can be given up to six weeks after the first dose. Lastly, the Jenssen/Johnson & Johnson is 66% effective at 14 days in preventing Covid-19, and 85% effective at least 28 after vaccination. The Johnson & Johnson only requires one injection and is given to patients 18 years and older (Mayoclinic, 2021).

CHAPTER II: VALIDATION

POLYMERASE CHAIN REACTION

In terms of testing, the primary Covid-19 testing method is polymerase chain reaction through the use of reverse transcription (Fraley, 2020). PCR is based on using the ability of DNA polymerase to synthesize a new strand of complementary DNA based on the offered template strand. DNA polymerase adds nucleotides to the preexisting 3' OH group and requires a primer. Primers are short pieces of single-stranded DNA that are complementary to the target strand. SARS-CoV-2 genetic material is RNA, for PCR to happen it would need to go through reverse transcriptase PCR. This form of PCR is preceded by the conversion of the sample RNA and turning it into complementary DNA by a reverse transcriptase enzyme (NCBI, 2017). Reverse transcriptase PCR can be done in either one or two steps. One step combines reverse transcriptase and PCR in a single tube and buffer. This uses reverse transcriptase along with DNA polymerase and only utilizes sequence-specific primers. The two-step method is when the reverse transcriptase and PCR steps are performed in separate tubes with different optimized buffers, reaction conditions, and priming strategies (Thermofisher, 2021). There are particular limitations when using PCR as a testing method in the laboratory. PCR generates copies of the target sequence exponentially and during this phase is it possible to extrapolate back to determine the starting quantity of the target sequence contained in the sample. Units for PCR are known as Ct or cycle threshold. This is the cycle number in which the target gene begins to amplify enough to be detected. Some inhibitors of PCR are usually found within the sample, reagent limitation, the accumulation of pyrophosphate molecules, and self-annealing of the accumulating product. Mainly due to reagent limitation, PCR ceases to amplify the target

sequence ending in a "plateau effect" and thus makes the endpoint of quantification of the end product unreliable (NCBI, 2017).

CLINICAL LABORATORY IMPROVEMENT AMENDMENTS

The Boise State Clinical Laboratory is a 660 sq ft space found in the Science Building on campus. The laboratory utilizes two QuantStudio 5 Real-Time PCR systems for the testing of SARS-CoV-2. The lab also has a KingFisher Flex used for DNA or RNA extraction and a Thermo Scientific Multiskan FC microplate photometer. The lab also performs SARS-CoV-2 antibody testing using an ELISA based kit from ZEUS which detects SARS-CoV-2 antibodies under the FDA EUA authorization. The Clinical Laboratory provides free COVID-19 testing to Boise State University employees and students. They also receive samples from Lewis and Clark College, the College of Idaho, Idaho Legislature, as well as several employers and child care centers found in the valley.

Boise State University's Clinical Laboratory received their CLIA license on September 25, 2020. The site needed an individual with at least one year of high complexity testing experience to be a supervisor and a medical director as a pathologist. To be fully authorized the lab needed a site inspection from CLIA in which inspectors looked through policies and procedures that the laboratory performs, inspected employee files to show that they are trained in SARS-CoV-2 PCR, and pulled random samples in which laboratory personnel would need to perform a proficiency test and explain the result given and how it is put into the laboratory information system properly. The laboratory was then certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C. 263a and meets the requirements to perform high complexity tests (CLIA ID #13D2193886). The lab had its initial site survey on 2/11/21 and passed with no deficiencies.

SALIVADIRECT

SalivaDirect uses real-time reverse transcription-polymerase chain reaction for the qualitative detection of nucleic acid from SARS-CoV-2 found in a patient's saliva. The sample can be collected in a sterile container without preservatives. This testing is only for use under FDA EUA and limited to laboratories that have been designated by the Yale School of Public Health, Department of Epidemiology of Microbial Disease, that includes the Clinical Molecular Diagnostics Laboratory, Department of Pathology, Yale School of Medicine, located at 310 Cedar St., New Haven, CT 06510 that are also certified under the Clinical Laboratory Improvement Amendments of 1988 (CLIA), 42 U.S.C §263a and meet the requirements to perform high complexity tests. SalivaDirect is intended for use by qualified and trained clinical laboratory personnel who have been instructed and trained in the techniques of RT-qPCR and in vitro diagnostic procedures (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

The authorizing agency for the use of SalivaDirect is Yale University. To use their protocol, they needed proof of CLIA licensure as well as a small validation to be sent to them which included at least five positives and five negatives by using the SalivaDirect protocol, a description of the type of PCR equipment used, evidence of experience with SARS-CoV-2 testing with PCR, and the number of PCR samples per week analyzed in the past and anticipated in the future when using SalivaDirect.

The small validation was done on November 24, 2020 in which, MagMAX viral/pathogen proteinase K, NEB Luna Universal Probe One-Step Rt-qPCR kit, and IDT primers and probes were used. A total of 20 samples were tested and results were put into a table to show Cycle threshold values of each sample along with a graph to show amplification curves.

Table 2: Validation sent to Yale University, November 24, 2020

	Date	11/24/2020	
	Proteinase K	MagMAX Viral/Pathogen Proteinase K #A42363	
	RT-qPCR kit	NEB Luna Universal Probe One-Step RT-qPCR Kit	
	Primers-probes	IDT	
	RT-qPCR instrument	QuantStudio 5	
Sample #	Positive or negative?	N1	RP
NEC	Negative / Positive	0	0
NTC	Negative / Negative	0	0
POS	Positive / Negative	31.092	0
1	Negative		24.89
2	Negative		23.958
3	Negative		25.8
4	Negative		24.307
5	Negative		24.214
6	Negative		24.201
7	Negative		21.69
8	Negative		22.993
9	Negative		24.514
10	Negative		26.318
11	Positive	29.441	23.712
12	Positive	32.788	24.055
13	Positive	33.515	25.36
14	Positive	21.064	25.276
15	Positive	24.474	25.64
16	Positive	28.311	24.927
17	Positive	21.892	25.85
18	Positive	24.84	23.103
19	Positive	32.564	24.449
20	Positive	34.083	26.8

Figure 2: Amplification curve graph from November 24 2020 Run sent to Yale University

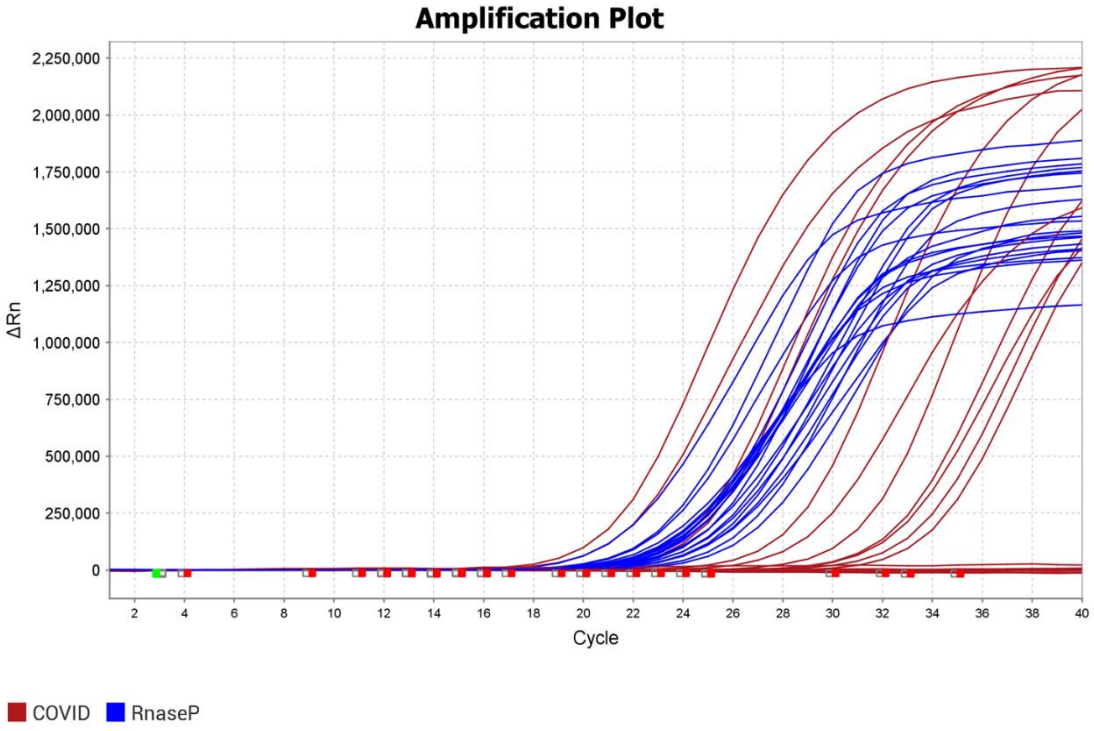


Table 1 is the requested validation results sent to Yale University that shows all required data. N1 is the SARS-CoV-2 target gene and the RP is the RNase P target. Both negative controls show no amplification for either targets, and the positive control has a value of 31.092 as its cycle threshold. Figure 2 shows linear data of the November 24, 2020 validation run, as a visual to when targets begin to amplify and the point in which they begin to plateau signifying the end of reagents available to amplify more copies.

SARS-CoV-2 RNA is generally detectable in saliva during the acute phase of infection. Clinical correlation with patient history with other diagnostic information is necessary to determine the patient's infection status. Based on the emergency use authorization form, positive results are indicative of the presence of RNA from SARS-CoV-2; however, these results do not rule out any co-infection with other viruses or bacteria. Negative results also do not disqualify

SARS-CoV-2 infection and should not be used as the sole basis for patient management decisions (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

SalivaDirect can be broadly implemented as it does not require saliva collection tubes to contain preservatives, it does not require specialized equipment for nucleic acid extraction, and is validated for use with products from multiple vendors. Due to its simplicity and flexibility, it does not get as affected by supply chain bottlenecks as some other assays. SalivaDirect is a nucleic acid extraction-free method. This enables testing of low volume and minimally processed saliva in a dualplex RT-qPCR tested using a validated primer and probe set. SalivaDirect uses 2019-nCoV_N1 and Ribonuclease P primer and probe sets as developed by the US CDC. The human Ribonuclease P probe was modified with a different fluorophore so that the primer and probe set could be combined in a dualplex assay, thus reducing the number of tests to one assay with two sets (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

Based on the emergency use authorization, four controls are run with SalivaDirect. A negative extraction control by using nuclease-free water is used to monitor for contamination during the saliva processing. The negative template control uses nuclease-free water to monitor for contamination of PCR reagents. Twist Synthetic SARS-CoV-2 RNA positive control that has been diluted to 100/µL is used to monitor functioning RT-qPCR reagents. Lastly, a primer and probe set detecting RNaseP is used to ensure that saliva of sufficient quantity and quality is tested and should be found in every sample (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

During a clinical evaluation in the EUA document of SalivaDirect; the performance of SalivaDirect was compared to the authorized ThermoFisher Scientific Taqpath RT-PCR Covid-19 combo kit by testing paired nasopharyngeal and saliva samples. A total of 67 nasopharyngeal and saliva pairs were tested for this study with 37 being positive and 30 being negative. From the 34 nasopharyngeal specimens that was tested positive by the TaqPath COVID-19 Combo Kit, 32 saliva specimens tested positive by SalivaDirect yielding a positive percent agreement of 94.1%. From the 33 negative nasopharyngeal swabs by the TaqPath assay, only 30 saliva specimens tested negative by SalivaDirect making a negative agreement of 90.9%. (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

CHAPTER III: METHODOLOGY

TAQPATH COVID-19 COMBO KIT MATERIALS AND EQUIPMENT

Table 3: TaqPath COVID-19 Combo Kit Materials and Equipment

Item	Vendor	Catalog Number
ABI QuantStudio 5 Real-Time PCR System	ThermoFisher Scientific	A28573
TaqPath COVID-19 Combo Kit	ThermoFisher Scientific	A47814
KingFisher™ Flex Purification System, KingFisher with 96 Deep-well Head	ThermoFisher Scientific	5400630
KingFisher™ Flex 96 Deep-Well Heating Block	ThermoFisher Scientific	24075430
KingFisher 96 deep-well plate, v-bottom, polypropylene	ThermoFisher Scientific	95040450 A48305
KingFisher™ Plastics for 96 standard and PCR formats	ThermoFisher Scientific	97002540
KingFisher 96 tip comb for deep-well magnets	ThermoFisher Scientific	97002534
MagMAX™ Viral/Pathogen Nucleic Acid Isolation Kit	ThermoFisher Scientific	A48310
MagMAX™ Viral/Pathogen Binding Solution	ThermoFisher Scientific	A42359
MagMAX™ Viral/Pathogen Wash Solution	ThermoFisher Scientific	A42360
MagMAX™ Viral/Pathogen Binding Beads	ThermoFisher Scientific	A42362
MagMAX™ Viral/Pathogen Proteinase K	ThermoFisher Scientific	A42363
MagMAX™ Viral/Pathogen Elution Buffer	ThermoFisher Scientific	A42364
TaqPath™ 1-Step Multiplex Master Mix (No ROX)	ThermoFisher Scientific	A28521, A28522, A28523
MicroAmp™ Clear Adhesive Film	ThermoFisher Scientific	4306311
MicroAmp™ Optical Adhesive Film	ThermoFisher Scientific	4311971 and 4360954

ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	ThermoFisher Scientific	A24734
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	ThermoFisher Scientific	A24735
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	ThermoFisher Scientific	4346906, 4366932
MicroAmp™ Adhesive Film Applicator	ThermoFisher Scientific	4333183
Nonstick, RNase-free Microfuge Tubes, 1.5 mL and 2.0 mL	ThermoFisher Scientific	AM12450 and AM12475
Sterilized aerosol barrier (filtered) pipette tips	ThermoFisher Scientific	Thermofisher.com/pipettetips

TAQPATH COVID-19 COMBO KIT

The TaqPath COVID-19 Combo Kit was validated for use at Boise State University in October 2020. TaqPath COVID-19 Combo kit is a two-step process to detect the presence of SARS-CoV-2. The first step is the RNA isolation/extraction to which the nucleic acid is isolated and purified from specimens using the MagMAX Viral/Pathogen Nucleic Acid Isolation Kit and automated process using the KingFisher Flex Purification System. It's designed to recover RNA and DNA from viruses in samples such as the Universal Transport Media or 1x Phosphate Buffered Saline. The second step is the RT-PCR to which this purified nucleic acid is reverse transcribed into cDNA and amplified using TaqPath RT-PCR COVID-19 Kit and Applied Biosystems QuantStudio 5 Real-Time PCR system. The probes anneal to three specific SARS-CoV-2 target sequences located between three unique forward and reverse primers. The target genes are; ORF1ab, N protein, and S protein (Boise State University Clinical Laboratory, 2020).

Nasopharyngeal swabs are the preferred specimen type for the TaqPath COVID-19 Combo kit. Other acceptable samples include oropharyngeal, mid-turbinate, and nasal swabs. The specimen is to be placed in an approved media with a minimum of 1mL of Viral Transport Media, Universal Transport Media, or Sterile 0.9% Saline. Specimen rejection criteria include incorrect swab type, transport media, labeled specimen, and storage temperature to which the patient would need to be contacted and swabbed again for testing (Boise State University Clinical Laboratory, 2020).

Racked samples correlate to a plate map that is scanned prior to any testing to correlate specimen to the correct patient. Lab personnel are required to double check accession numbers on both the rack and the template to ensure that the ending result correlates with the patient. Each TaqPath run is done with two controls, a negative control only containing MS2 and a positive control containing the positive Taqpath Covid-19 Control (Boise State University Clinical Laboratory, 2020).

Reagent preparation include a wash buffer solution, elution buffer, 80% ethanol, binding bead buffer, and binding beads. A total of four deepwell Kingfisher plates are prepared for TaqPath testing. Wash 1 plate contains 500µL of wash solution, Wash 2 contains 1000µL of 80% ethanol, Elution plate contain 50µL of the elution solution, and the final plate will contain proteinase K, the patient's sample, and the binding bead solution. Binding beads must be vortexed prior to prepare the binding bead solution with 265µL of the binding solution to 10µL of nucleic acid magnetic beads to be added into each well. This amount is calculated and added to a 50mL conical tube in which a total of 30 mL of the binding bead solution is made for a full 96-well plate run (Boise State University Clinical Laboratory, 2020).

5µL of Proteinase K is then added to each sample well including the positive and negative control. The patient sample rack and the 96-deepwell kingfisher plate are then taken under a fume hood for processing. Each sample is vortexed for five seconds and 200µL is taken out of the collection tube and added to the corresponding well. 200µL of nuclease free water is added to the negative control well. Binding bead solution should be inverted five times gently and 275µL is added into each well containing a patient's sample. Lastly 5µL of MS2 phage control is added to each well except to the positive control well (Boise State University Clinical Laboratory, 2020).

Maintenance on the KingFisher should be run before each extraction. After which, the program to run the extraction is found under the RNA selection and is called MVP 2Wash 200 Flex. Once the run is started the KingFisher will prompt the user to place each prepared plate into position and run the program automatically for 22 minutes. Once complete the elution plate is removed, covered with an adhesive film and kept on ice. The plate should be gently vortex and centrifuged at 1000 RPM for one minute before preparing for RT-PCR (Boise State University Clinical Laboratory, 2020).

Prepared RT-PCR plates was made daily in a clean room for the number of tests to be performed. Each 96-well reaction plate includes 6.25µL of TaqPath 1-Step Multiplex Master Mix, 1.25µL COVID-19 Real Time PCR Assay Multiplex, 7.50µL Nuclease-free Water, into each well of a 96-well PCR plate including both the controls. From the elution plate, 10µL of the sample is added to each well corresponding to the plate map, and 10µL is taken from the negative control well and added into the negative control well on the PCR plate. The positive control contained 2µL of positive control (diluted TaqPath COVID-19 control) and 8µL of nuclease-free water. The PCR plate is then placed into the QuantStudio 5 Real Time PCR

instrument and proceeded with the preprogramed RT-PCR cycle (Boise State University Clinical Laboratory, 2020).

SALIVADIRECT MATERIALS AND EQUIPMENT

Table 4: SalivaDirect Materials and Equipment

Item	Vendor	Catalog Number
ABI QuantStudio 5 Real-Time PCR System	ThermoFisher Scientific	A28573
Proteinase K Molecular Biology Grade	New England Biolabs	P8107S
Luna Universal Probe One-Step RT-qPCR (2x) Kit	New England Biolabs	E3006E
nCOV_N1 Forward Primer Aliquot	Integrated DNA Technologies	10006830
nCOV_N1 Reverse Primer Aliquot	Integrated DNA Technologies	10006831
nCOV_N1 Probe Aliquot	Integrated DNA Technologies	10006832
RNase P Forward Primer Aliquot	Integrated DNA Technologies	10006836
RNase P Reverse Primer Aliquot	Integrated DNA Technologies	10006837
RNase P Probe	Integrated DNA Technologies	10007062 (ATTO647)
Synthetic SARS-CoV-2 RNA Control 2	Twist Bioscience	102024
MicroAmp™ Clear Adhesive Film	ThermoFisher Scientific	4306311
MicroAmp™ Optical Adhesive Film	ThermoFisher Scientific	4311971 and 4360954
ABY™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	ThermoFisher Scientific	A24734
JUN™ Dye Spectral Calibration Plate for Multiplex qPCR, Fast 96-well	ThermoFisher Scientific	A24735
MicroAmp™ Fast Optical 96-Well Reaction Plate with Barcode, 0.1 mL	ThermoFisher Scientific	4346906, 4366932
MicroAmp™ Adhesive Film Applicator	ThermoFisher Scientific	4333183

Nonstick, RNase-free Microfuge Tubes, 1.5 mL and 2.0 mL	ThermoFisher Scientific	AM12450 and AM12475
Sterilize aerosol barrier (Filtered) pipette tips	ThermoFisher Scientific	Thermofisher.com/pipettetips
MagMAX™ Viral/Pathogen Proteinase K	ThermoFisher Scientific	A42363

SALIVADIRECT

SalivaDirect was validated with two ThermoFisher Scientific Applied Biosystems QuantStudio 5 Real-Time PCR Systems at Boise State University. Equipment used includes: two Applied BiosystemTM QuantStudio 5 Real-Time PCR instruments, single and multichannel adjustable pipettors, cold blocks and ice, centrifuge with a rotor that accommodates standard and deepwell microplates, laboratory vortexes, and laboratory freezers (-30°C to -10°C and ≤ -70°C). Software included the QuantStudioTM Design and analysis software along with the QuantStudio TM design and analysis software to observe sample graphs and Ct values in Microsoft excel. Patients are asked to refrain from eating or drinking 30 minutes before sample collection. Specimens are kept in non-preservative collection tubes and are stored in a specimen fridge before testing (Boise State University Clinical Laboratory, 2020).

Validation testing took place on November 27th 2020 and on December 11th 2020. Known positive and negative samples were taken out of the -70°C freezer and set to thaw for a short amount of time. During this process, 2.5µL of proteinase K was added to each well including the negative extraction control well. A basic plate map was made to identify which known sample was placed into each well. Table 4 is the plate map used during both runs on both QuantStudio instruments. A1 to A10 are all known negatives while B1 to B10 are known positives. PC signifies the positive control, NTC is the negative template control, and NEC is the negative extraction control. A11 and C1 to H12 were intentionally left blank as they contained

no samples and no PCR reagents (Boise State University Clinical Laboratory, 2020). The Table below is the plate map used in both November 27th, 2020 and December 11th, 2020 runs on both instruments.

Table 5: SalivaDirect plate map for validation at Boise State University Clinical Laboratory

	1	2	3	4	5	6	7	8	9	10	11	12
A	**N1**	**N2**	**N3**	**N4**	**N5**	**N6**	**N7**	**N8**	**N9**	**N10**		**PC**
B	**P1**	**P2**	**P3**	**P4**	**P5**	**P6**	**P7**	**P8**	**P9**	**P10**	**NTC**	**NEC**
C												
D												
E												
F												
G												
H												

Samples were then vortexed for homogenization and 50µL was taken from the initial collection tube, and place into the 96-PCR plate using new pipette tips with each sample to ensure sterility. 50µL of Nuclease-free water was added to the negative extraction control well. During the extraction phase the negative template control and the positive control are not put into the plates as they are not needed during this phase. The 96-well PCR plate was then sealed with MicroAMP clear adhesive film and vortexed for one minute. It was then centrifuged and placed in the QuantStudio 5 instrument for five minutes at 95°C. During this time, a PCR mix plate was prepared. Reagents needed for the SalivaDirect PCR mix include 10µL of the master mix, 1µL of Enzyme, and 4µL of the Primer and Probe mix. This end results uses 15µL in PCR mix for every well to be tested and should be multiplied for the amount needed to run a 96-well plate (Boise State University Clinical Laboratory, 2020).

After this extraction phase, both PCR plates are taken into a clean room and 5µL of each sample is then transferred to the PCR reaction mix plate. 5µL of the negative extraction is also

transferred into the new negative extraction control well. 5µL of nuclease-free water is then added to a free well as the negative template control as descripted on the plate map. Lastly, 5µL of Twist Bioscience Synthetic SARS-CoV-2 RNA control 2 that has been diluted to 100/µL is added to a final well as a positive control. This makes the final volume of each well 20µL. The PCR plate is then sealed with a new MicroAMP optical adhesive film and place into the QuantStudio 5 instrument for amplification (Boise State University Clinical Laboratory, 2020).

CHAPTER IV: RESULTS

Covid-19 results are based on the cycle threshold amount in which the target genes in a sample begin to amplify. Based on the FDA emergency use authorization detection of RNase P, the cycle threshold should be less than 35 to indicate saliva of sufficient quantity and quality to be detected. This detection of RNase P is required to report a negative SARS-CoV-2 result. A positive sample must have a cycle threshold value of less than 40 with N1 while RNase P should be at any Ct value. A negative result will have a Ct value of greater than or equal to 40 with N1 and RNase P Ct value is less than 35. Any invalid sample will have a Ct value of greater than or equal to 40 for N1 and RNase P Ct value at greater than or equal to 35. These invalid results should be repeated by retesting the primary specimen by starting the testing over with the extraction phase (Yale School of Public Health, Department of Epidemiology of Microbial Disease, 2021).

Figures 3, 4, 5, and 6 show five positive PCR amplification curves taken from the QuantStudio Design and Analysis software from both runs. All four of these figures show both amplifications of RNase P and the Covid target gene of N1. Figures 3 and 4 were taken from both QuantStudio 5 PCR instruments on the November 27th, 2020 run. Figures 5 and 6 were taken from the same two instruments on the December 11th, 2020 run. While Figures 7, 8, 9, and 10 show five negative PCR amplification cures taken from the QuantStudio Design and analysis software from both runs. All four figures show only RNase P amplification and the data would

show no cycle threshold values for the COVID gene N1. Figures 7 and 8 are from the November 27th, 2020 run from both instruments, while Figures 9 and 10 are from December 11th, 2020 run.

Figure 3: Quant 1 Five Positive Curves November 27, 2020

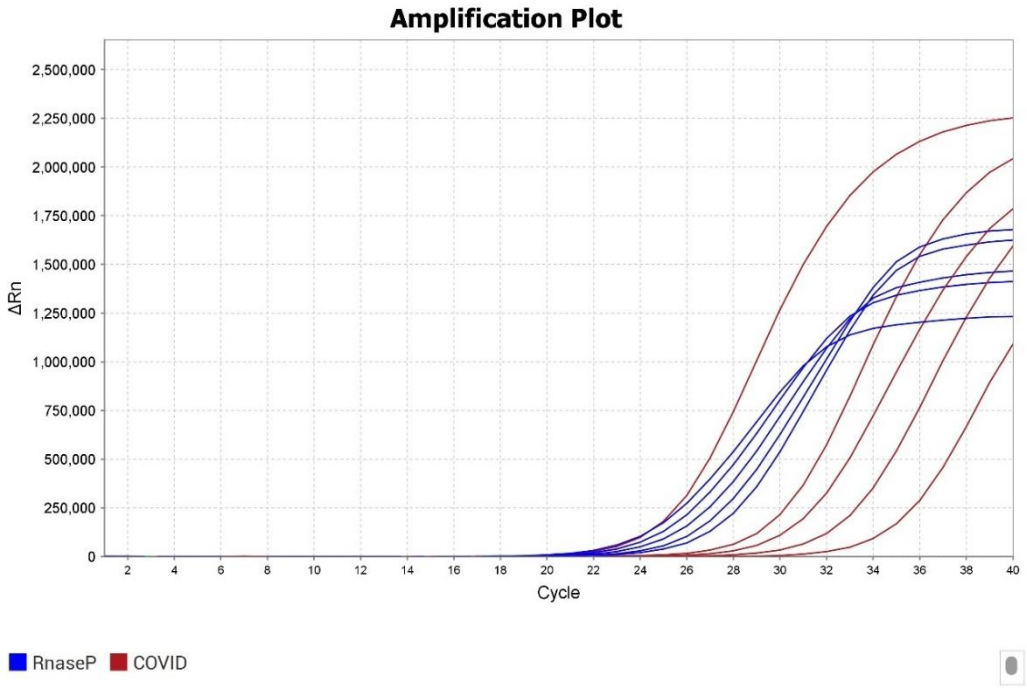


Figure 4: Quant 2 Five Positive Curves November 27, 2020

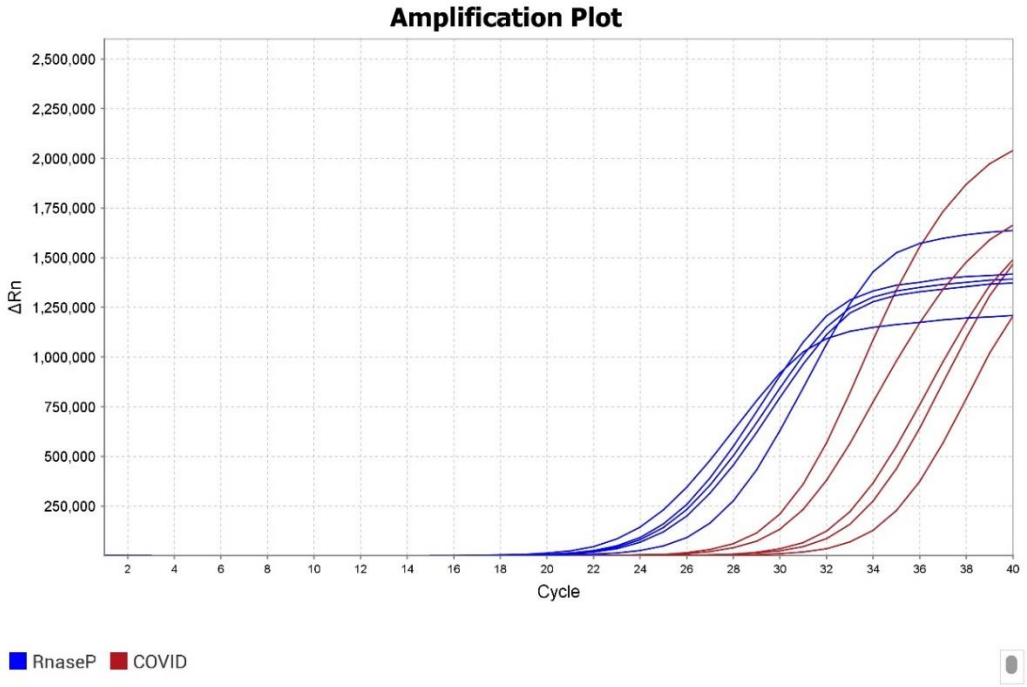


Figure 5: Quant 1 Five Positive Curves December 11, 2020

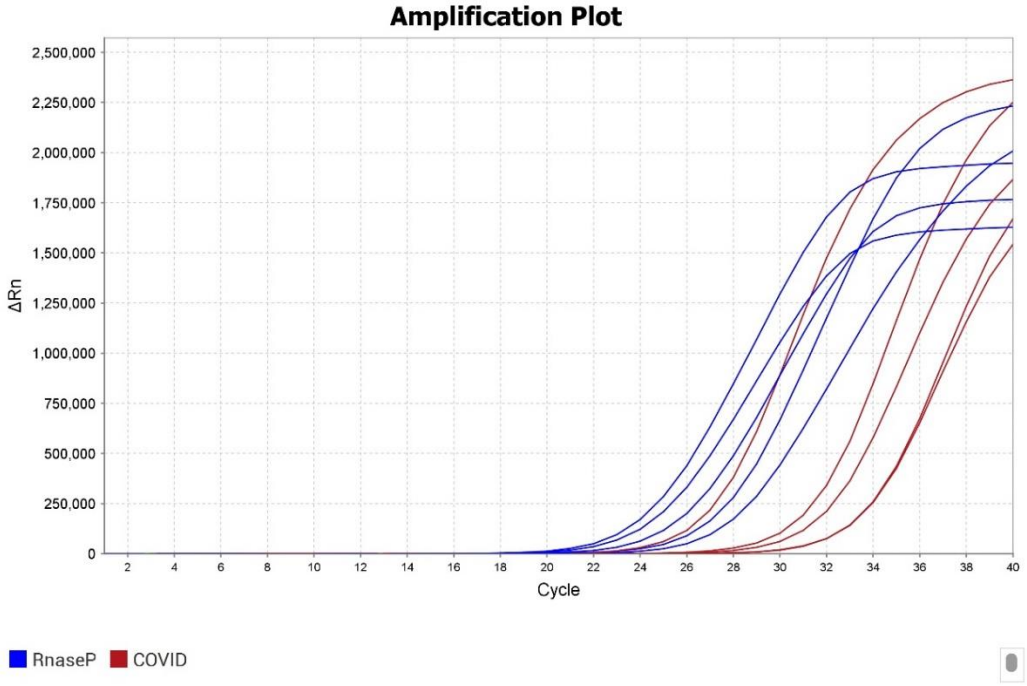


Figure 6: Quant 2 Five Positive Curves December 11, 2020

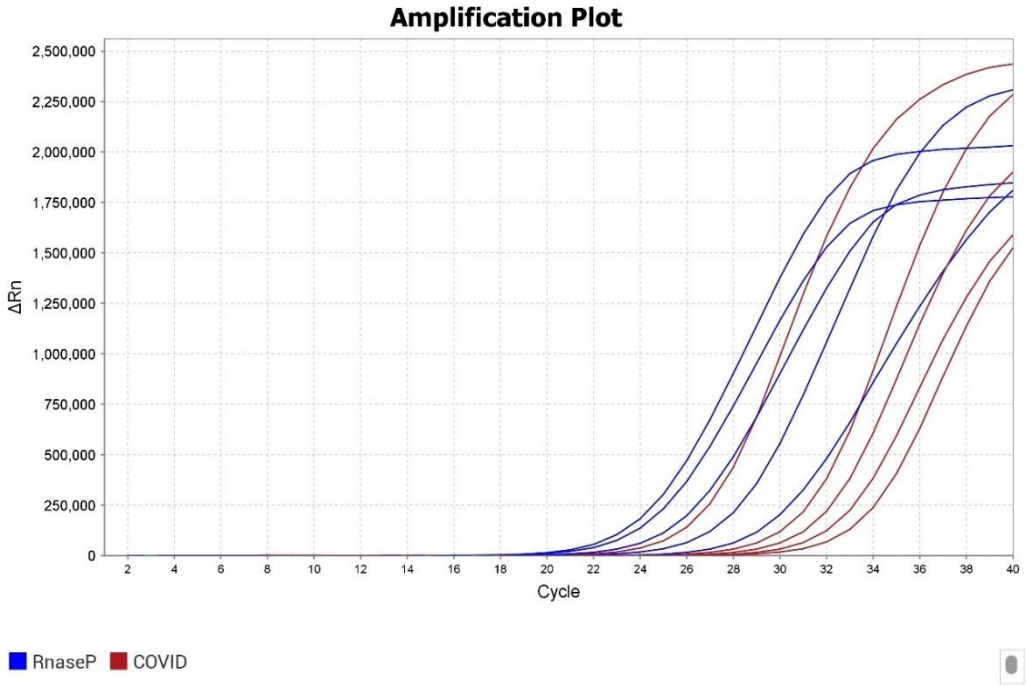


Figure 7: Quant 1 Five Negative Curves November 27, 2020

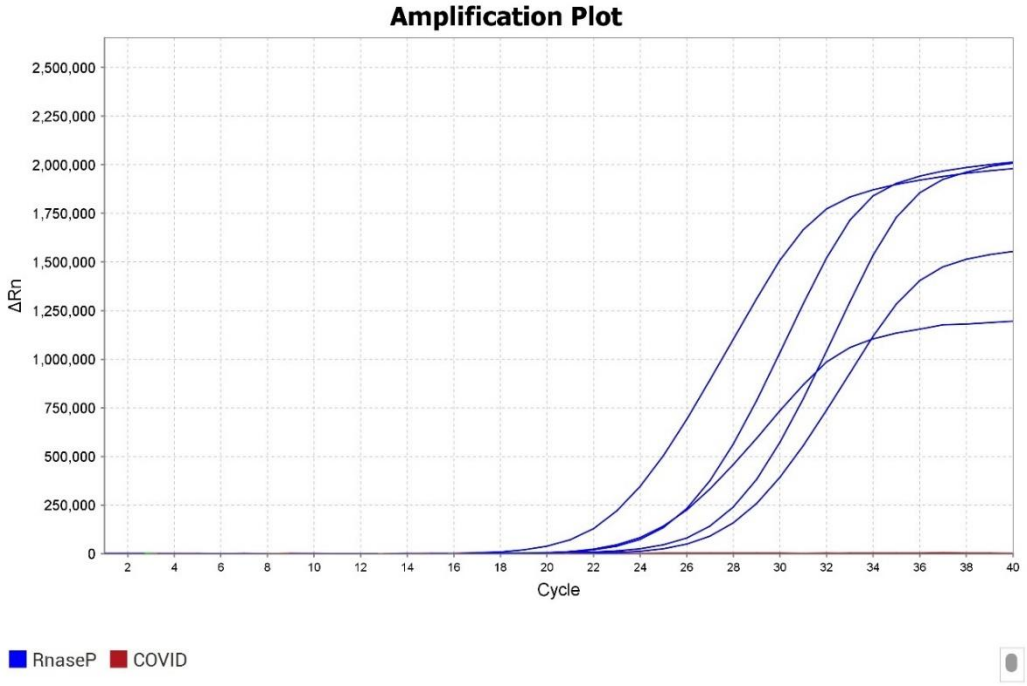


Figure 8: Quant 2 Five Negative Curves November 27, 2020

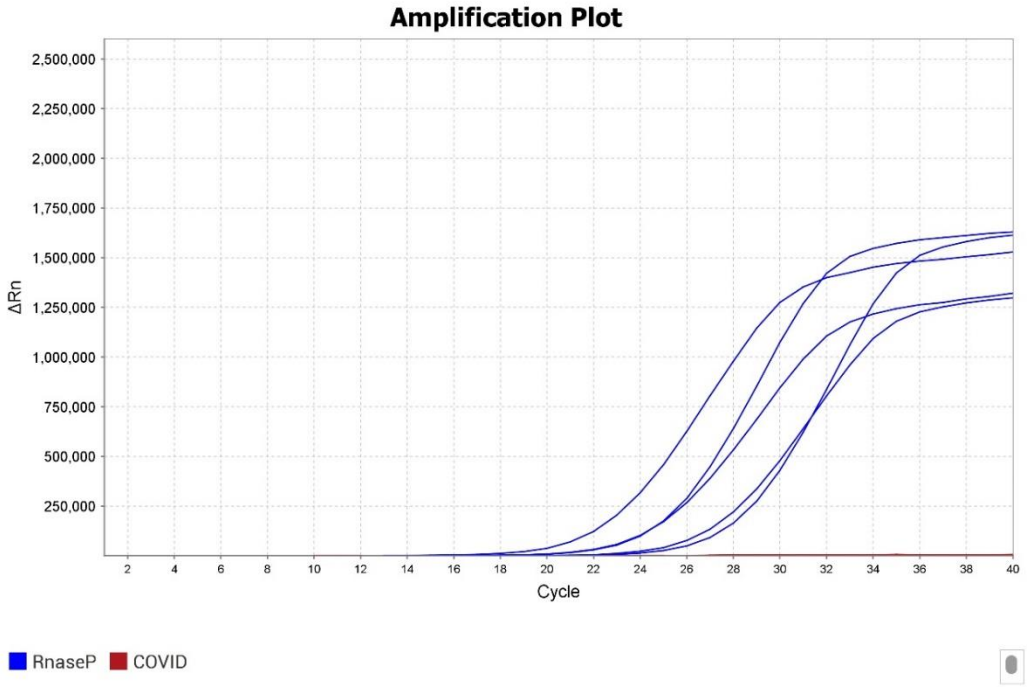


Figure 9: Quant 1 Five Negative Curves December 11, 2020

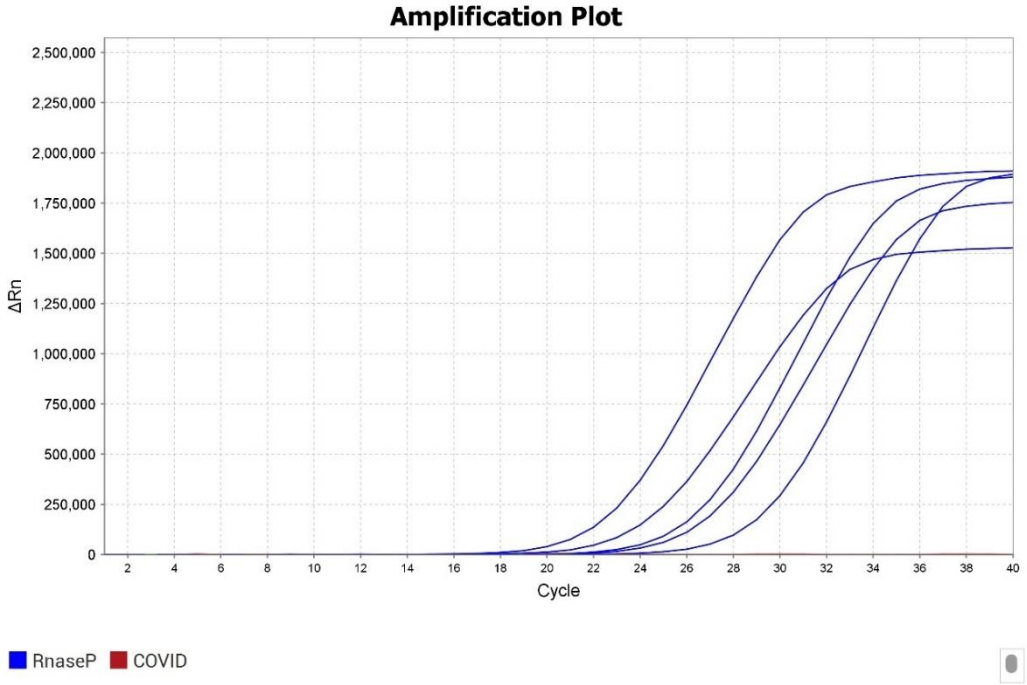
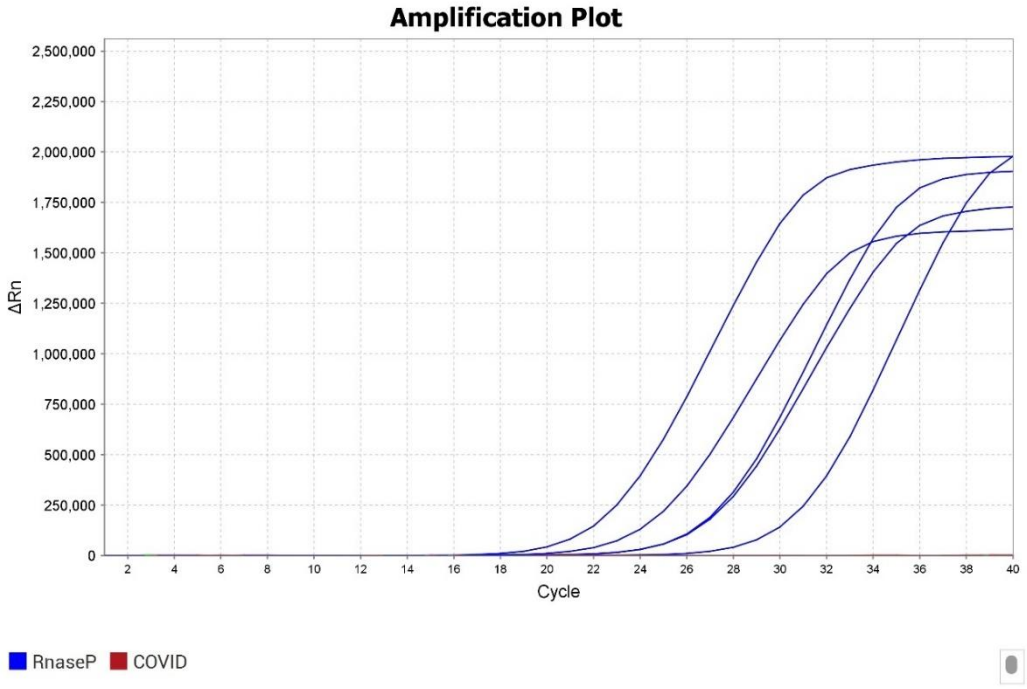


Figure 10: Quant 2 Five Negative Curves December 11, 2020



All eight figures are to show what positive and negative samples look like to show consistency between both instruments and both runs. After about the 30th cycle both Rnase P and the target Covid gene plateau show no change. The QuantStudio Design and analysis software also allows a function which allows the Ct values to be found so interpretation is not from the graph data alone. A third party who programs Boise State University laboratory information system then took the Ct value limits as provided from the emergency use authorization document to designate how positive and negative samples would be uploaded from the QuantStudio 5 instruments.

ACCURACY

Table 6: Samples ran on QuantStudio 5 instrument 1 given results with expected results with whether or not they are in agreement. December 11, 2020

QuantStudio Instrument 1				
Sample Source: Saliva compared to previous nasal swab by Applied Biosystems TaqPath Assay				
	Assigned Sample ID	**QuantStudio Instrument 1 Results**	**Expected QuantStudio Instrument 1 Results**	**Agreement* Yes/no**
1	**N1**	**Negative**	**Negative**	**Yes**
2	**N2**	**Negative**	**Negative**	**Yes**
3	**N3**	**Negative**	**Negative**	**Yes**
4	**N4**	**Negative**	**Negative**	**Yes**
5	**N5**	**Negative**	**Negative**	**Yes**
6	**N6**	**Negative**	**Negative**	**Yes**
7	**N7**	**Negative**	**Negative**	**Yes**
8	**N8**	**Negative**	**Negative**	**Yes**
9	**N9**	**Negative**	**Negative**	**Yes**
10	**N10**	**Negative**	**Negative**	**Yes**
11	**P1**	**Positive**	**Positive**	**Yes**
12	**P2**	**Positive**	**Positive**	**Yes**
13	**P3**	**Positive**	**Positive**	**Yes**
14	**P4**	**Positive**	**Positive**	**Yes**
15	**P5**	**Positive**	**Positive**	**Yes**
16	**P6**	**Positive**	**Positive**	**Yes**
17	**P7**	**Positive**	**Positive**	**Yes**
18	**P8**	**Positive**	**Positive**	**Yes**
19	**P9**	**Positive**	**Positive**	**Yes**
20	**P10**	**Positive**	**Positive**	**Yes**

Table 7: Samples ran on QuantStudio 5 instrument 2 given results with expected results with whether or not they are in agreement. December 11, 2020

QuantStudio Instrument 2				
Sample Source: Saliva compared to previous nasal swab by Applied Biosystems TaqPath Assay				
	Assigned Sample ID	**QuantStudio Instrument 2 Results**	**Expected QuantStudio Instrument 2 Results**	**Agreement* Yes/no**
1	**N1**	**Negative**	**Negative**	**Yes**
2	**N2**	**Negative**	**Negative**	**Yes**
3	**N3**	**Negative**	**Negative**	**Yes**
4	**N4**	**Negative**	**Negative**	**Yes**
5	**N5**	**Negative**	**Negative**	**Yes**
6	**N6**	**Negative**	**Negative**	**Yes**
7	**N7**	**Negative**	**Negative**	**Yes**
8	**N8**	**Negative**	**Negative**	**Yes**
9	**N9**	**Negative**	**Negative**	**Yes**
10	**N10**	**Negative**	**Negative**	**Yes**
11	**P1**	**Positive**	**Positive**	**Yes**
12	**P2**	**Positive**	**Positive**	**Yes**
13	**P3**	**Positive**	**Positive**	**Yes**
14	**P4**	**Positive**	**Positive**	**Yes**
15	**P5**	**Positive**	**Positive**	**Yes**
16	**P6**	**Positive**	**Positive**	**Yes**
17	**P7**	**Positive**	**Positive**	**Yes**
18	**P8**	**Positive**	**Positive**	**Yes**
19	**P9**	**Positive**	**Positive**	**Yes**
20	**P10**	**Positive**	**Positive**	**Yes**

Accuracy is described as the closeness of a measured value to the standard or known value. Tables 5 and 6 show the accuracy between both QuantStudio instruments from the December 11th, 2020 performance. They showed the expected results in alignment with what results were given from both instruments. All controls passed, allowing the test to be valid between both instruments. N1 through N10 are all known negative samples while P1 through P10 are known positives. All twenty known samples were expected to result as such, and it was found that the QuantStudio 5 was able to amplify with the given primers and probes.

PRECISION

Table 8: Shows November 27 and December 11, 2020 runs on both instruments given their results and if they are in agreement.

Worklist ID/s:	QuantStudio Instrument 1: 2020-11-27_134714 2020-12-11_105740				
	QuantStudio Instrument 2: 2020-11-27_131749 2020-12-11_125332				
Panel ID & Expected Results	Precision Data				
	Dates:	11/27/2020 and 12/11/2020			
	Operator:	Shelby Morris MLS (ASCP)^{CM}			
	11/27/2020 Instrument 1	11/27/2020 Instrument 2	12/11/2020 Instrument 1	12/11/2020 Instrument 2	Agreement
N1	**Negative**	**Negative**	**Negative**	**Negative**	Yes
N2	**Negative**	**Negative**	**Negative**	**Negative**	Yes
N3	**Negative**	**Negative**	**Negative**	**Negative**	Yes
N4	**Negative**	**Negative**	**Negative**	**Negative**	Yes
N5	**Negative**	**Negative**	**Negative**	**Negative**	Yes
P1	**Positive**	**Positive**	**Positive**	**Positive**	Yes
P2	**Positive**	**Positive**	**Positive**	**Positive**	Yes
P3	**Positive**	**Positive**	**Positive**	**Positive**	Yes
P4	**Positive**	**Positive**	**Positive**	**Positive**	Yes
P5	**Positive**	**Positive**	**Positive**	**Positive**	Yes
Run%	Results in agreement/total * 100				**100%**
Acceptability Criteria					
Total Precision	100%				

Precision is how close measurements of the same item are to each other. This means if one specimen were to receive a positive result, the other instrument would need to obtain the same result for that sample. Table 7 shows the precision between the two instruments on both run dates. The table shows both instrument run times and run dates within the table which would allow easy access in finding the run for evaluation at a later date. Five samples were chosen to compare results in order to test precision between the instruments and runs with a total agreement of 100%.

Table 9: Shows analytical data from December 11 2020 run on instrument 1

Experiment Name	2020-12-11_105740																	
Experiment Run End Time	2020-12-11 12:36:36 PM MST																	
Experiment Type	Standard Curve																	
Instrument Name	Instrument_1																	
Instrument Serial Number	272512093																	
Instrument Type	QuantStudio™ 5 System																	
Quantification Cycle Method	Ct																	
Signal Smoothing On	true																	
Stage/ Cycle where Ct Analysis is performed	Stage2, Step2																	
User Name																		
Well	Well Position	Omit	Target Name	Task	Reporter	Quencher	CT	Ct Mean	Ct SD	Automatic	Ct Threshold	Automatic	Baseline Start	Baseline End	Amp Status	Cq Conf	CQCONF	HIGHSD
1	A1	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	25	No Amp	0.000	Y	N
1	A1	FALSE	RnaseP	UNKNOWN	CY5	None	23.986	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.990	N	Y
2	A2	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	15	No Amp	0.000	Y	N
2	A2	FALSE	RnaseP	UNKNOWN	CY5	None	26.490	24.931	2.342	TRUE	147,212.924	TRUE	3	16	Amp	0.986	N	Y
3	A3	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	9	No Amp	0.000	Y	N
3	A3	FALSE	RnaseP	UNKNOWN	CY5	None	25.814	24.931	2.342	TRUE	147,212.924	TRUE	3	16	Amp	0.990	N	Y
4	A4	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	19	No Amp	0.000	Y	N
4	A4	FALSE	RnaseP	UNKNOWN	CY5	None	22.119	24.931	2.342	TRUE	147,212.924	TRUE	3	12	Amp	0.982	N	Y
5	A5	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	9	No Amp	0.000	Y	N
5	A5	FALSE	RnaseP	UNKNOWN	CY5	None	28.694	24.931	2.342	TRUE	147,212.924	TRUE	3	19	Amp	0.984	N	Y
6	A6	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	18	No Amp	0.000	Y	N
6	A6	FALSE	RnaseP	UNKNOWN	CY5	None	25.866	24.931	2.342	TRUE	147,212.924	TRUE	3	16	Amp	0.989	N	Y
7	A7	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	8	No Amp	0.000	Y	N
7	A7	FALSE	RnaseP	UNKNOWN	CY5	None	30.610	24.931	2.342	TRUE	147,212.924	TRUE	3	19	Amp	0.988	N	Y
8	A8	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	20	No Amp	0.000	Y	N
8	A8	FALSE	RnaseP	UNKNOWN	CY5	None	23.206	24.931	2.342	TRUE	147,212.924	TRUE	3	14	Amp	0.985	N	Y
9	A9	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	9	No Amp	0.000	Y	N
9	A9	FALSE	RnaseP	UNKNOWN	CY5	None	23.859	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.986	N	Y
10	A10	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	16	No Amp	0.000	Y	N
10	A10	FALSE	RnaseP	UNKNOWN	CY5	None	22.338	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.985	N	Y
12	A12	FALSE	COVID	UNKNOWN	FAM	None	32.758	29.877	4.186	TRUE	170,203.936	TRUE	3	23	Amp	0.993	N	Y
12	A12	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	24.931	2.342	TRUE	147,212.924	TRUE	3	26	No Amp	0.000	Y	N
13	B1	FALSE	COVID	UNKNOWN	FAM	None	31.615	29.877	4.186	TRUE	170,203.936	TRUE	3	23	Amp	0.982	N	Y
13	B1	FALSE	RnaseP	UNKNOWN	CY5	None	24.309	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.984	N	Y
14	B2	FALSE	COVID	UNKNOWN	FAM	None	33.277	29.877	4.186	TRUE	170,203.936	TRUE	3	23	Amp	0.984	N	Y
14	B2	FALSE	RnaseP	UNKNOWN	CY5	None	25.406	24.931	2.342	TRUE	147,212.924	TRUE	3	16	Amp	0.987	N	Y
15	B3	FALSE	COVID	UNKNOWN	FAM	None	33.268	29.877	4.186	TRUE	170,203.936	TRUE	3	24	Amp	0.979	N	Y
15	B3	FALSE	RnaseP	UNKNOWN	CY5	None	23.730	24.931	2.342	TRUE	147,212.924	TRUE	3	14	Amp	0.986	N	Y
16	B4	FALSE	COVID	UNKNOWN	FAM	None	26.581	29.877	4.186	TRUE	170,203.936	TRUE	3	18	Amp	0.985	N	Y
16	B4	FALSE	RnaseP	UNKNOWN	CY5	None	27.709	24.931	2.342	TRUE	147,212.924	TRUE	3	18	Amp	0.988	N	Y
17	B5	FALSE	COVID	UNKNOWN	FAM	None	30.796	29.877	4.186	TRUE	170,203.936	TRUE	3	19	Amp	0.984	N	Y
17	B5	FALSE	RnaseP	UNKNOWN	CY5	None	26.823	24.931	2.342	TRUE	147,212.924	TRUE	3	14	Amp	0.989	N	Y
18	B6	FALSE	COVID	UNKNOWN	FAM	None	32.975	29.877	4.186	TRUE	170,203.936	TRUE	3	24	Amp	0.985	N	Y
18	B6	FALSE	RnaseP	UNKNOWN	CY5	None	23.940	24.931	2.342	TRUE	147,212.924	TRUE	3	15	Amp	0.987	N	Y
19	B7	FALSE	COVID	UNKNOWN	FAM	None	22.034	29.877	4.186	TRUE	170,203.936	TRUE	3	15	Amp	0.973	N	Y
19	B7	FALSE	RnaseP	UNKNOWN	CY5	None	24.972	24.931	2.342	TRUE	147,212.924	TRUE	3	16	Amp	0.979	N	Y
20	B8	FALSE	COVID	UNKNOWN	FAM	None	34.309	29.877	4.186	TRUE	170,203.936	TRUE	3	26	Amp	0.939	N	Y
20	B8	FALSE	RnaseP	UNKNOWN	CY5	None	26.871	24.931	2.342	TRUE	147,212.924	TRUE	3	17	Amp	0.982	N	Y
21	B9	FALSE	COVID	UNKNOWN	FAM	None	25.913	29.877	4.186	TRUE	170,203.936	TRUE	3	17	Amp	0.985	N	Y
21	B9	FALSE	RnaseP	UNKNOWN	CY5	None	22.567	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.985	N	Y
22	B10	FALSE	COVID	UNKNOWN	FAM	None	25.120	29.877	4.186	TRUE	170,203.936	TRUE	3	15	Amp	0.984	N	Y
22	B10	FALSE	RnaseP	UNKNOWN	CY5	None	21.963	24.931	2.342	TRUE	147,212.924	TRUE	3	13	Amp	0.988	N	Y
23	B11	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	19	No Amp	0.000	Y	N
23	B11	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	24.931	2.342	TRUE	147,212.924	TRUE	3	19	No Amp	0.000	Y	N
24	B12	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.877	4.186	TRUE	170,203.936	TRUE	3	10	No Amp	0.000	Y	N
24	B12	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	24.931	2.342	TRUE	147,212.924	TRUE	3	27	No Amp	0.000	Y	N

Table 10: Shows analytical data from December 11 2020 run on instrument 2

Experiment Name	2020-12-11_125332
Experiment Run End Time	2020-12-11 14:29:29 PM MST
Experiment Type	Standard Curve
Instrument Name	Instrument_2
Instrument Serial Number	272512079
Instrument Type	QuantStudio™ 5 System
Quantification Cycle Method	Ct
Signal Smoothing On	true
Stage/ Cycle where Ct Analysis is performed	Stage2, Step2
User Name	

Well	Well Position	Omit	Target Name	Task	Reporter	Quencher	CT	Ct Mean	Ct SD	Automatic Ct Threshold	Ct Threshold	Automatic Baseline	Baseline Start	Baseline End	Amp Status	Cq Conf	CQCONF	EXPFAIL	HIGHSD
1	A1	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	39	No Amp	0.000	Y	Y	N
1	A1	FALSE	RnaseP	UNKNOWN	CY5	None	24.385	25.269	2.627	TRUE	162,206.604	TRUE	3	15	Amp	0.989	N	N	Y
2	A2	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	35	No Amp	0.000	Y	N	N
2	A2	FALSE	RnaseP	UNKNOWN	CY5	None	26.791	25.269	2.627	TRUE	162,206.604	TRUE	3	16	Amp	0.992	N	N	Y
3	A3	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	23	No Amp	0.000	Y	N	N
3	A3	FALSE	RnaseP	UNKNOWN	CY5	None	26.703	25.269	2.627	TRUE	162,206.604	TRUE	3	17	Amp	0.988	N	N	Y
4	A4	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	19	No Amp	0.000	Y	N	N
4	A4	FALSE	RnaseP	UNKNOWN	CY5	None	22.153	25.269	2.627	TRUE	162,206.604	TRUE	3	12	Amp	0.984	N	N	Y
5	A5	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	24	No Amp	0.000	Y	N	N
5	A5	FALSE	RnaseP	UNKNOWN	CY5	None	30.219	25.269	2.627	TRUE	162,206.604	TRUE	3	19	Amp	0.987	N	N	Y
6	A6	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	10	No Amp	0.000	Y	N	N
6	A6	FALSE	RnaseP	UNKNOWN	CY5	None	25.429	25.269	2.627	TRUE	162,206.604	TRUE	3	14	Amp	0.987	N	N	Y
7	A7	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	27	No Amp	0.000	Y	N	N
7	A7	FALSE	RnaseP	UNKNOWN	CY5	None	30.689	25.269	2.627	TRUE	162,206.604	TRUE	3	19	Amp	0.987	N	N	Y
8	A8	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	20	No Amp	0.000	Y	N	N
8	A8	FALSE	RnaseP	UNKNOWN	CY5	None	22.574	25.269	2.627	TRUE	162,206.604	TRUE	3	12	Amp	0.989	N	N	Y
9	A9	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	31	No Amp	0.000	Y	N	N
9	A9	FALSE	RnaseP	UNKNOWN	CY5	None	24.366	25.269	2.627	TRUE	162,206.604	TRUE	3	13	Amp	0.989	N	N	Y
10	A10	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	12	No Amp	0.000	Y	N	N
10	A10	FALSE	RnaseP	UNKNOWN	CY5	None	23.036	25.269	2.627	TRUE	162,206.604	TRUE	3	13	Amp	0.982	N	N	Y
12	A12	FALSE	COVID	UNKNOWN	FAM	None	32.368	29.229	4.158	TRUE	141,411.619	TRUE	3	23	Amp	0.992	N	N	Y
12	A12	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	25.269	2.627	TRUE	162,206.604	TRUE	3	6	No Amp	0.000	Y	N	N
13	B1	FALSE	COVID	UNKNOWN	FAM	None	31.257	29.229	4.158	TRUE	141,411.619	TRUE	3	20	Amp	0.987	N	N	Y
13	B1	FALSE	RnaseP	UNKNOWN	CY5	None	24.296	25.269	2.627	TRUE	162,206.604	TRUE	3	15	Amp	0.986	N	N	Y
14	B2	FALSE	COVID	UNKNOWN	FAM	None	32.198	29.229	4.158	TRUE	141,411.619	TRUE	3	23	Amp	0.988	N	N	Y
14	B2	FALSE	RnaseP	UNKNOWN	CY5	None	25.616	25.269	2.627	TRUE	162,206.604	TRUE	3	15	Amp	0.987	N	N	Y
15	B3	FALSE	COVID	UNKNOWN	FAM	None	33.118	29.229	4.158	TRUE	141,411.619	TRUE	3	24	Amp	0.978	N	N	Y
15	B3	FALSE	RnaseP	UNKNOWN	CY5	None	23.774	25.269	2.627	TRUE	162,206.604	TRUE	3	14	Amp	0.986	N	N	Y
16	B4	FALSE	COVID	UNKNOWN	FAM	None	25.999	29.229	4.158	TRUE	141,411.619	TRUE	3	17	Amp	0.992	N	N	Y
16	B4	FALSE	RnaseP	UNKNOWN	CY5	None	29.566	25.269	2.627	TRUE	162,206.604	TRUE	3	19	Amp	0.986	N	N	Y
17	B5	FALSE	COVID	UNKNOWN	FAM	None	30.296	29.229	4.158	TRUE	141,411.619	TRUE	3	22	Amp	0.987	N	N	Y
17	B5	FALSE	RnaseP	UNKNOWN	CY5	None	27.509	25.269	2.627	TRUE	162,206.604	TRUE	3	18	Amp	0.989	N	N	Y
18	B6	FALSE	COVID	UNKNOWN	FAM	None	32.110	29.229	4.158	TRUE	141,411.619	TRUE	3	23	Amp	0.984	N	N	Y
18	B6	FALSE	RnaseP	UNKNOWN	CY5	None	24.058	25.269	2.627	TRUE	162,206.604	TRUE	3	13	Amp	0.985	N	N	Y
19	B7	FALSE	COVID	UNKNOWN	FAM	None	21.509	29.229	4.158	TRUE	141,411.619	TRUE	3	11	Amp	0.994	N	N	Y
19	B7	FALSE	RnaseP	UNKNOWN	CY5	None	24.927	25.269	2.627	TRUE	162,206.604	TRUE	3	15	Amp	0.994	N	N	Y
20	B8	FALSE	COVID	UNKNOWN	FAM	None	33.155	29.229	4.158	TRUE	141,411.619	TRUE	3	24	Amp	0.968	N	N	Y
20	B8	FALSE	RnaseP	UNKNOWN	CY5	None	27.184	25.269	2.627	TRUE	162,206.604	TRUE	3	18	Amp	0.982	N	N	Y
21	B9	FALSE	COVID	UNKNOWN	FAM	None	24.942	29.229	4.158	TRUE	141,411.619	TRUE	3	15	Amp	0.989	N	N	Y
21	B9	FALSE	RnaseP	UNKNOWN	CY5	None	22.466	25.269	2.627	TRUE	162,206.604	TRUE	3	12	Amp	0.989	N	N	Y
22	B10	FALSE	COVID	UNKNOWN	FAM	None	24.564	29.229	4.158	TRUE	141,411.619	TRUE	3	15	Amp	0.991	N	N	Y
22	B10	FALSE	RnaseP	UNKNOWN	CY5	None	21.985	25.269	2.627	TRUE	162,206.604	TRUE	3	11	Amp	0.990	N	N	Y
23	B11	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	3	No Amp	0.000	Y	N	N
23	B11	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	25.269	2.627	TRUE	162,206.604	TRUE	3	22	No Amp	0.000	Y	N	N
24	B12	FALSE	COVID	UNKNOWN	FAM	None	Undetermined	29.229	4.158	TRUE	141,411.619	TRUE	3	7	No Amp	0.000	Y	N	N
24	B12	FALSE	RnaseP	UNKNOWN	CY5	None	Undetermined	25.269	2.627	TRUE	162,206.604	TRUE	3	23	No Amp	0.000	Y	N	N

Tables 8 and 9 show analytical data taken from the December 11th 2020 run from both QuantStudio 5 instruments. From these tables, one can see the experiment name, end time, the testing instrument, and Ct values from each well. These Ct values are used in the LIS in order to determine a positive or negative result from the sample. It should be noted that A1 to A10 contain known negatives in both plates, while B1 to B10 are known positives. A12 contains the positive control in which only the covid gene should be amplified as there should not be any RNase P. B11 and B12 contain both the negative extraction control and the negative template control to which should not have any amplification from either target genes.

CHAPTER V: DISCUSSION

Prior to December 14th, 2020, Boise State University tested Covid-19 samples through the TaqPath COVID-19 Combo Kit. TaqPath COVID-19 Combo Kit is an FDA EUA for the qualitative detection of nucleic acid from SARS-CoV-2. It uses RT-PCR on upper respiratory specimens such as nasopharyngeal, oropharyngeal, and nasal swabs. When TaqPath was in use at Boise State University specimens were collected by nasopharyngeal swab and place in a tube with 3 mL of phosphate buffer. Health professionals of the testing center swabbed patients and while personal protective equipment was worn by personnel, there was still some small risk of infection from patient to healthcare worker. This also proves hazardous when needing to swab small children, who do not enjoy the procedure. Once SalivaDirect was implemented, this decreased the risk taken by healthcare professionals as patients of all ages are able to collect the sample themselves. Patients are also able to collect a sample at home as the collection container does not need a preservative. Originally SalivaDirect samples were collected in small tubes that came with a collection straw, but the lab personnel found that this added limitations to the testing process. A minimum quantity was not required at the time, and samples would get stuck in the collection straw rather than the tube. To eliminate this, the laboratory has now taken away the collection straw and now requires a minimum of 2mL. This has eliminated the problem with insufficient sample. With TaqPath, there was always at least 3mL of the phosphate buffer in the tube, however if the sample was not collected correctly it would result in a negative when the patient could truly be Covid-19 positive.

In December 2020, the FDA released a SARS-CoV-2 Reference Panel that allows a comparison of the analytical performance of the different molecular in vitro diagnostic assays used to detected SARS-CoV-2. In this panel, one can find a sensitivity mean estimate of EUA

authorized diagnostic tests which states that the limit of detection in NDU/mL for Thermo Fisher TaqPath COVID-19 Combo Kit at 180000 NDU/mL. SalivaDirect as limit of detection of 18000 NDU/mL making it 10 times more sensitive than the TaqPath COVID-19 Combo Kit (FDA, 2020).

The TaqPath method uses MS2 Phage Control as an internal process control and is used for nucleic acid extraction. While this is a good internal process control, it does not provide the knowledge of correct specimen collection. Thus, false negatives from the TaqPath could attribute to improper sample collection. SalivaDirect's internal control is RNase P which is a type of ribonuclease that cleaves RNA that is found in human saliva. This allows lab personnel and the LIS to detect if a sample of efficient quantity and quality has been provided. For TaqPath to run it contains three specific SARS-CoV-2 target sequences which are the ORF1ab, N gene, and S gene. If only one of these genes amplifies the test is inconclusive and the sample would need to be run again. Meanwhile, based on the data above there is only one target sequence of SARS-CoV-2 that SalivaDirect amplifies along with RNase P; for patients, as long as there is a cycle threshold value less than or equal to 35 Ct for RNase P they would get a result.

Before any testing, the start-up cost including all non-consumables and the laboratory information system was a total of $313,417.47. This included: computers, the two QuantStudio PCR instruments, Kingfisher instrument with head, biosafety cabinets, Sorvall clinical centrifuge, pipettes, plate vortex, a 96-well magnet stand, Ultra-low temp freezer, lab benches and chairs, the start-up cost of using StratusDX plus a monthly fee, and the middleware. The only testing equipment that is shared between SalivaDirect and TaqPath are the computers, the two QuantStudio instruments, pipettes, plate vortex, and centrifuge. In terms of cost, it takes approximately $505,522.60 to run the TaqPath COVID-19 Combo kit for seven weeks with a

total number of 24,500 samples that could be tested. This includes the Combo kit, 96-well plates, adhesive covers, 15mL conical tubes, Kingfisher 96 microplate, Kingfisher deep well tip comb, Kingfisher deep-well 96 plates, Taqpath 1-step multiplex master mix, MicroAmp optical 96-well plates, MicroAmp optical adhesive covers, MicroAmp clear adhesive covers, nuclease free water, MagMax viral/pathogen nucleic acid kit II, 100 mL reservoir, Molecular biology ethanol, 50 mL conical tubes, and MicroAmp Plate seals. Meanwhile, for SalivaDirect, it would take approximately $23,248.00 to run the same number of tests in the same timeframe.

Based on Boise State University's performance, the typical turnaround time from sample collection to result was 24 hours at max. Most tests were resulted in less time and were dependent on sample collection quality and whether or not an invalid result was obtained. In terms of processing time, it takes both SalivaDirect and TaqPath COVID-19 Combo kit the same amount of time to process if a valid result is obtained. From start to finish, it takes about two hours to run a full plate for the TaqPath COVID-19 Combo kit, this includes making the kingfisher plates, adding the samples and adding MS2, running the kingfisher for 20 minutes, plating the extracted samples to a PCR plate, then finally amplifying the target sequences on the QuantStudio 5 instrument. SalivaDirect also takes approximately two hours; while it cuts out the need of the kingfisher stage its amplification process takes 30 minutes longer than the TaqPath.

Another major difference between the two is if an invalid result is obtained. With SalivaDirect it is understood that if an invalid result is given, you must start from the beginning with the extraction phase once more. On the other hand, with the TaqPath COVID-19 Combo kit, it would depend on the reasoning why an invalid result was given. If there is enough sample from the 96-deepwell kingfisher plate, one can just have the sample replated into a new PCR plate and amplified again. If MS2 was not added, or if one of the other reagents that take place

during extraction is not added correctly, the operator would have to start from the original sample as well. This runs the risk of another invalid result being obtained if the proper steps were not performed.

CHAPTER VI: CONCLUSION

SARS-CoV-2, the cause of Covid-19 is an ongoing pandemic with new cases finally starting to slow down. Hospitals and universities that provide testing are starting to see the effects of proper testing, informed patients, and the roll out of effective vaccinations. As we continue to understand it molecularly, we continue to learn better diagnostic tools, treatments, and prevention methods to combat the virus in hopes of bringing new cases to a stop. Starting with TaqPath COVID-19 Combo Kit, Boise State University tested their community and gave accurate results to patients. After validation of the SalivaDirect, the university decided to switch methods and use SalivaDirect to test the students, and faculty of its own campus, the College of Idaho, and Lewis and Clark State College. Boise State University has now moved on to testing the Idaho Legislature, high school students, and are now preparing to test students in kindergarten through twelfth grade.

SalivaDirect methodology has improved overall cost, quality of test results with its higher sensitivity, provided better sample collection, and in some small way decreased turnaround time. SalivaDirect uses a simple non-invasive method to collect samples, has stability without the need for preservatives, and an overall accurate way of testing to provide the best results to the community. While the money is given to the university for testing by the United State Government, the university saves money given by using SalivaDirect. This method also allows a non-invasive way of testing patients of all ages as it is much easier for patients to collect their own saliva to be sent to the clinical laboratory then the need of trained health care professionals to collect a quality sample. With SalivaDirect's high sensitivity, Boise State University will continue to broaden its testing range and provide quality results back to the patients until the pandemic comes to a full stop.

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