DirectDetect SARS-CoV-2 Direct Real-Time RT-PCR Study Using Patient Samples

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ABSTRACT: COVID-19 is an infectious disease that caused a global pandemic affecting people worldwide. As disease detection and vaccine rollout continue to progress, there is still a need for efficient diagnostic tools to satisfy continued testing needs. This preliminary study evaluated a novel SARS-CoV-2 diagnostic test called DirectDetect SARS-CoV-2 Direct Real-time reverse transcriptase polymerase chain reaction (RT-PCR) based on a limited sample size of 24 respiratory samples from 14 SARS-CoV-2-positive patients. The test is advantageous compared to others on the market since it does not require viral transport medium or viral RNA extraction prior to nucleic acid amplification and detection. This capability transforms the hours-long sample preparation time into a minutes-long procedure while also eliminating the need for many costly reagents which may be difficult to obtain during the surge in nucleic acid-based testing during the pandemic. The results show a positive agreement of 94.7, 100, and 94.7% between dry sample swabs, treated samples, and untreated samples tested using the DirectDetect SARS-CoV-2 Direct Real-time RT-PCR compared to tests used in a clinical laboratory, respectively. The findings indicate that DirectDetect can be used for multiple different sample types while reducing the number of reagents and time needed for diagnosis. Although this study shows promising results using the DirectDetect results, further validation of this test using a larger sample set is required to assess the true performance of this test.

1. INTRODUCTION

In 2019, a novel strain of coronavirus known as severe acute respiratory syndrome 2 (SARS-CoV-2) caused a global pandemic outbreak of coronavirus disease (COVID-19). Since the first reporting of the virus, it has spread throughout the world to millions of people, causing millions of deaths with increased morbidity and mortality in older patients or those with coexisting health conditions. Moreover, the containment of the virus has been complicated by the existence of asymptomatic carriers, people with no symptoms of the virus. It has been noted that the viral load in asymptomatic patients is similar to that in symptomatic patients, indicating the ability for these people to transmit the virus. While asymptomatic patients may later develop symptoms, this group of people demonstrates the importance of widespread testing for SARS-CoV-2 as part of the public health response. The commonly used procedure for the detection of SARS-CoV-2 is outlined in Figure 1, which begins with a nasopharyngeal (NP), oropharyngeal (OP), or nasal swab to collect a sample from the upper respiratory tract. After collection, the swab is placed directly into a viral (universal) transport medium (VTM) where it can then be transported from the sampling site to the clinical laboratory. Once the sample is in a clinical laboratory of an appropriate safety level, nucleic acid extraction is performed to remove the viral RNA from the rest of the virus material prior to analysis by transferring the sample into lysis buffer. The lysis buffer often contains a guanidinium-based inactivating agent and a non-denaturing detergent to inactivate SARS-CoV-2 if it is present in the sample. Once lysed, the nucleic acids are isolated and purified using either a solid-phase method (i.e., magnetic beads or silica matrices) or a solution-based method (i.e., centrifugation or Chelex). A typical nucleic acid extraction method using solid-phase reversible immobilization (SPRI) bead-based extraction is shown in Figure 1 where the lysed sample and the SPRI beads are mixed, incubated, magnetically separated, alcohol-washed, and eluted. Following the sample preparation, nucleic acid amplification and detection are performed to assess whether the sample contains SARS-CoV-2. Overall, the most commonly used amplification and detection method for SARS-CoV-2 molecule-based testing is real-time RT-PCR and remains the worldwide “gold standard” given its high sensitivity and specificity. Even patients with a low viral load in their upper respiratory tract after recovering from symptoms can still test positive for SARS-CoV-2 using...
real-time RT-PCR. However, the availability of the currently authorized real-time RT-PCR assays for SARS-CoV-2 diagnostic testing is still limited as large-scale application of testing efforts is continuously hindered by worldwide supply chain issues and a shortage of human resources for fast and efficient testing. The recent progress in the diagnostic effort has also been exploring reverse transcriptase loop-mediated isothermal amplification (RT-LAMP) as an attractive alternative to RT-PCR, however, our efforts have been centered around expediting and improving the current real-time RT-PCR testing procedure.

This study presents and analyzes a new COVID-19 diagnostic test called DirectDetect SARS-CoV-2 Direct Real-time RT-PCR (PerkinElmer, Waltham, MA, USA). Currently, this kit is commercially available for the detection of SARS-CoV-2 from environmental surfaces. The aim of this study, however, is to provide a preliminary evaluation regarding the effectiveness of the DirectDetect assay for the successful detection of SARS-CoV-2 in patient sample swabs. As shown in Figure 1, this test removes the need for VTM as well as reagents and equipment needed for viral RNA extraction from samples.

Since the outbreak of COVID-19, there have been several detection tests developed across the world for screening and diagnostic purposes. Of those tests, there are some that also use methods that avoid nucleic acid extraction and only a select number have received Emergency Use Authorization (EUA) from the U.S. Food and Drug Administration (FDA),

Figure 1. Detection of SARS-CoV-2 by the commonly used manual real-time reverse transcriptase polymerase chain reaction (RT-PCR) procedure vs the DirectDetect procedure. The overall assay times for these procedures from sample collection to result differ from up to 4 h for the conventional method to up to 2 h for DirectDetect. Created with BioRender.com.
| company                                      | assay name                          | specimen used                              | gene targets                      | amplification | transportation media needed |
|----------------------------------------------|-------------------------------------|--------------------------------------------|-----------------------------------|---------------|-----------------------------|
| Bioeksen R&D Technologies Ltd.               | bio-speedy direct RT-qPCR            | NP or OP swab                              | ORF1ab gene                       | real-time RT-PCR | yes                         |
|                                              | SARS-CoV-2                          |                                            | IC detects RNase P gene           |               |                             |
|                                              |                                     |                                            | N gene using CDC-validated primer/probesets |               |                             |
|                                              |                                     |                                            | internal human ribonuclease P (RP) control |               |                             |
| Yale School of Public Health, Department of Epidemiology of Microbial Diseases | saliva direct                       | saliva                                     | S gene                           | real-time RT-PCR | no                          |
|                                              |                                     |                                            | ORF1ab gene                       |               |                             |
|                                              |                                     |                                            | IC RNA                            |               |                             |
| DiSorin Molecular LLC                         | Simplexa COVID-19 direct assay       | NP, nasal swab (NS), nasal wash/aspirate (NW), or bronchoalveolar lavage (BAL) | S gene                           | real-time RT-PCR | yes                         |
|                                              |                                     |                                            | ORF1ab gene                       |               |                             |
|                                              |                                     |                                            | IC RNA                            |               |                             |
| Quidel Corporation                            | Lyra direct SARS-CoV-2 assay         | nasal, NP, or OP                          | non-structured polyprotein (pp1ab) | real-time RT-PCR | yes                         |
|                                              |                                     |                                            | process control (PRC)             |               |                             |
| Fluidigm Corporation                          | Advanta Dx SARS-CoV-2 RT-PCR assay   | saliva                                     | N gene, RNase P IC                | real-time RT-PCR | no                          |
| PerkinElmer                                   | DirectDetect (not approved as an EUA test) | NP, OP, or nasal swab                   | N gene                            | real-time RT-PCR | no                          |
|                                              |                                     |                                            | ORF1ab gene                       |               |                             |
|                                              |                                     |                                            | IC (bacteriophage MS2)            |               |                             |
Table 2. LoD Results for DirectDetect Using the AccuPlex SARS-CoV-2 Reference Material Kit, n = 12 for Each Group

| sample input volume (µL) | concentration of viral RNA (cp/µL) | amount of viral RNA per reaction (cp/reaction) | number tested (replicates) | SARS-CoV-2: N gene (FAM) | SARS-CoV-2: ORF1ab gene (ROX) | internal control (HEX) |
|-------------------------|-----------------------------------|-----------------------------------------------|-----------------------------|--------------------------|-------------------------------|--------------------------|
|                         |                                    |                                               |                             | Ct (avg) SD positive     | Ct (avg) SD positive         | Ct (avg) SD positive       |
| 2.5                     | 10                                 | 25                                            | 12                          | 37.68 0.89 (100%)        | 35.48 0.56 (100%)            | 30.52 0.34                |
| 2.5                     | 5                                  | 12.5                                          | 12                          | 37.66 0.94 (83.3%)       | 35.28 0.74 (100%)            | 30.09 0.30                |
| 2.5                     | 2.5                                | 6.25                                          | 12                          | 38.60 0.89 (83.3%)       | 36.12 0.92 (100%)            | 29.99 0.35                |
| 2.5                     | 1.25                               | 3.13                                          | 12                          | 38.89 0.63 (41.7%)       | 36.98 0.65 (100%)            | 29.84 0.30                |
| 2.5                     | 0.625                              | 1.56                                          | 12                          | 38.87 1.35 (16.7%)       | 38.21 0.64 (100%)            | 29.82 0.25                |
| 2.5                     | 0.3125                             | 0.78                                          | 12                          | 0 (0.0%)                 | 38.77 0.06 (25.0%)           | 30.10 0.43                |

2. RESULTS

2.1. DirectDetect Limit of Detection. The limit of detection (LoD) of the DirectDetect assay was tested using two target genes (N and ORF1ab) from positive recombinant virus particles with sequences from SARS-CoV-2 genome. Table 2 presents the results for this study where the LoD at which 100% of samples detected was 12.5 copies/reaction. The percent of positive sample detection decreased as the copies of viral RNA per reaction decreased; however, 2/12 replicates of the N gene and 8/12 replicates of the ORF1ab gene were still identified at 1.56 copies per reaction. Per the requirements of the DirectDetect assay, only one of the N or ORF1ab genes need to be identified to consider SARS-CoV-2 as detected; therefore, at 1.56 copies per reaction, 8/12 samples are confirmed positive, in this case.

2.2. SARS-CoV-2 Detection Using DirectDetect in Patient Samples. Overall, most patient samples were kept at 4 °C and tested within 72 h of being procured unless there was an invalid test caused by the IC being undetected; in this case, the sample was retested as soon as possible. On average, the patient samples for this study were first tested 2.25 days following sample procurement, but the number of days ranged from 0 to 7 due to delays in testing caused by invalid test (Table S1). In each case, no dry swab samples were stored beyond the recommended storage time. Dry swabs were lysed (initial step in the DirectDetect protocol) within the storage time frame, and test repeats were done using the lysed solution. The expected result showed a Ct (threshold cycle) value of ≤40 for all samples including negative and positive controls for the IC (HEX) amplification condition.

This showed that the nucleic acid amplification was successful given that the IC is included in every sample; hence, it will always be amplified. The negative control would show no signal (undetermined or Ct > 42) for both the N gene (FAM) and ORF1ab gene (ROX), where the positive control needed to show amplification for the two genes. Once the controls were analyzed, the patient samples could be interpreted for SARS-CoV-2 detection. An overview of the results for the patient sample testing for different sample types and genes is provided in Figure S1 where the Ct value for each result is plotted. From the total of 24 dry swab samples tested, 22 samples tested positive for SARS-CoV-2 and 2 tested negative for SARS-CoV-2. For patient samples tested with RNA extraction (treated wet swab), 20 samples tested positive...
and 4 tested negative. Last, for patient samples in transport medium tested without RNA extraction (untreated wet swab), 21 samples tested positive and 3 tested negative (Table S2). All of the 24 negative samples show true negative outcomes using DirectDetect (Table S3). The Ct values of the positive and negative controls for the respective samples included in the RT-PCR analysis can be found in Table S5. All controls needed to pass the metrics defined by this protocol for the test results to be considered for analysis.

The DirectDetect assay was tested on a total of 24 patient samples from 14 individuals under three different conditions. While Figure S1 shows the spread and the median of the Ct values in all the samples, Figure 2 shows a relationship and correlation of Ct values from different nasal swabs in dry and wet conditions tested from the same patient. The median Ct values for N gene were 33.99, 34.09, and 32.70 for dry swab, treated wet swab, and untreated wet swab conditions, respectively. Here, the treated wet swabs’ median Ct values for N gene from DirectDetect tests were higher compared to the median Ct values of the initial reference test (Ct\text{median} = 30.10; based on 9 out of the 14 patient samples) used for the confirmation of the presence of SARS-CoV-2 in patients (GeneXpert and Genmark). The median Ct values for ORF1ab gene were 36.65, 34.00, and 34.34 for dry swab, treated wet swab, and untreated wet swab conditions, respectively. Last, the median Ct values for the IC were 35.46, 32.07, and 32.26 for dry swab, treated wet swab, and untreated wet swab conditions, respectively.

When examining the Ct values for different genes from different starting samples, it is clear that there are variations between the conditions tested (Figure 2). However, there were no statistically significant differences in Ct values for the two target genes between dry swab and treated wet swab samples, with the difference in medians of 0.09 and 2.66 Ct values for N gene (FAM) and ORF1ab gene (ROX), respectively (Wilcoxon; N: \( p = 0.49 \), ORF1ab: \( p = 0.32 \)). This also confirms that while there are some variations between dry swab and untreated wet swab, the untreated wet swab is comparable to dry swab with no significant difference in Ct values based on the positive results. There was, however, a statistically significant difference for the IC (HEX) between dry swab and treated wet swab conditions with the difference in medians of 3.39 Ct values (Wilcoxon; \( p = 0.025 \)). Similarly, there was also a statistically significant difference for the IC (HEX) between dry swabs and untreated swabs with the difference in medians of 3.20 Ct values (Wilcoxon; \( p = 0.013 \)). Last, no statistically significant differences were found in Ct values for the three target genes between treated wet swab and untreated wet swab samples, with the difference in medians of 1.39, 0.61, and 0.19 values for N gene (FAM), ORF1ab gene (ROX), and IC (HEX), respectively (Wilcoxon; N: \( p = 0.46 \), ORF1ab: \( p = 0.80 \), and IC: \( p = 0.88 \)), again suggesting that treated wet swabs and untreated wet swabs are comparable and has no significant difference in Ct values based on the positive results. The findings reported here are expanded in Figures S2–S4.

The different starting samples were further compared by evaluating the positive agreement in SARS-CoV-2 detection between the groups (Table S6). The concordance between SARS-CoV-2 detection using dry swabs and treated wet swabs was 95.0%, dry swabs and untreated wet swabs was 95.2%, and treated wet swabs and untreated wet swabs was 95.2%. All of the 24 negative patient samples had no false-positive. Additionally, the positive agreement between each sample condition and the clinical test which confirmed the original COVID-19 diagnosis was also calculated. The reference clinical diagnosis reported 20/24 samples tested positive (Table S4); therefore, the overall concordance between the testing conditions and the clinical test was 94.7% for dry swab samples, 100% for treated samples, and 94.7% for untreated samples for positive samples (Table 3) and 100% for negative samples in all conditions. This is comparable to the negative samples tested with the same reference kits.\textsuperscript{12,13}

3. DISCUSSION

3.1. DirectDetect Can Detect SARS-CoV-2 from Dry Patient Samples without the Need of Nucleic Acid Extraction. Based on the LoD and testing of low number of patient sample, DirectDetect has been shown to be a simple
Table 3. Evaluation of Positive Agreement for Overall SARS-CoV-2 Detection Using DirectDetect and Commercial Real-Time RT-PCR Assay

|                     | commercial real-time RT-PCR assay\(^a\) |              |              |
|---------------------|----------------------------------------|--------------|--------------|
|                     | positive | negative | positive | negative | positive | negative | positive | negative |
| dry swab            |          |          |          |          |          |          |          |          |
| positive            | 18       | 1        |          |          |          |          |          |          |
| negative            | 1        | 3        |          |          |          |          |          |          |
| total               | 19       | 4        |          |          |          |          |          |          |
| positive agreement: | 94.7%    | (18/19)  |          |          |          |          |          |          |
| treated wet swab    |          |          |          |          |          |          |          |          |
| positive            | 18       | 0        |          |          |          |          |          |          |
| negative            | 0        | 4        |          |          |          |          |          |          |
| total               | 18       | 4        |          |          |          |          |          |          |
| positive agreement: | 100%     | (18/18)  |          |          |          |          |          |          |
| untreated wet swab  |          |          |          |          |          |          |          |          |
| positive            | 18       | 1        |          |          |          |          |          |          |
| negative            | 1        | 3        |          |          |          |          |          |          |
| total               | 19       | 4        |          |          |          |          |          |          |
| positive agreement: | 94.7%    | (18/19)  |          |          |          |          |          |          |

\(^a\)GeneXpert, GenMark ePlex.

Real-time RT-PCR technique for detecting SARS-CoV-2. Overall, this work shows the capabilities of DirectDetect as it was further tested using multiple variations of biospecimen starting samples, which include dry nasal swabs, transportation media-suspended nasal swabs, and RNA-purified samples from nasal swabs. Successful viral detection from these different specimens shows the flexibility and adaptability of DirectDetect. The ability to detect SARS-CoV-2 from a dry or non-purified sample can allow for simpler sample preparation and processing protocols compared to the conventional real-time RT-PCR testing by removing the nucleic acid extraction step. This can allow for high-throughput testing for patients suffering from COVID-19 without the need for an expensive nasal swab stabilizing VTM or nucleic acid extraction kit while also reducing the real-time RT-PCR reagents used per sample reaction. Recently, the use of RT-LAMP assay without the need of nucleic acid purification has been shown to be an alternative to RT-PCR; further comparison of DirectDetect and RT-LAMP tests is important for future studies.\(^{14,15}\)

Findings from this study show that the median Ct value for all testing conditions and genes are relatively consistent over the 24 patient samples tested (Figure S2). This value ranged from 32.07 for the treated wet swab IC to 36.65 for the ORF1ab gene dry swab; however, there were no trends identified between a condition or gene and the Ct values. One consistency throughout the data was that there were variations in Ct values between conditions tested (Figure 2). It was possible to have a sample detected for one condition and not the others, but the only statistically significant differences in Ct values identified in this study were within the IC gene tested between the dry swab and treated or untreated wet swabs. This could have been caused by different sample conditions and differences in sample preparation procedures but does not impact the results of the target testing since the amplification is successfully detected within the IC in all cases.

Reference tests used to validate the positive and negative samples showed a high positive agreement (94.7% for dry swab samples, 100% for treated samples, and 94.7% for untreated samples). However, given that the reference tests target alternative genes and profiling, the Ct values of the DirectDetect test and the reference test show different performances (Tables S2–S4). Also, the differences in the sample procurement times (initial sample collected, reference test validation, and procurement again after validation) may account for the difference in Ct values.

3.2. Nasal Swabs with or without Viral Transportation Media Can Be Used for COVID-19 Diagnostics. The positive agreement between testing conditions was also analyzed in this study. The percent of samples that tested positive between both sample swab preparation types show that although there was variation in the Ct values, for the true COVID-19 detection among different conditions, there was still a positive agreement of 95% or more (Table S6). In addition to evaluate the positive agreement between the samples tested with DirectDetect, it was also critical to understand the positive agreement with the original clinical diagnosis. When performing this analysis, it was found that the positive agreement for the dry sample swab was the highest, indicating that it worked the best out of the three conditions tested. One reason for wet swabs performing worse than dry swabs may be due to the sample input being diluted during virus resuspension and incubation in the VTM. Sample dilution can also be further observed in the resuspension buffer during the nucleic acid extraction step. Additionally, the extraction step creates room for error, given the additional number of steps in the protocol; this decreases possible positive agreement results. The normalization of the lysed solution and the VTM is different; therefore, the input viral load into real-time RT-PCR of the dry swab and untreated/treated swabs can be different.

While the results suggest that DirectDetect can identify SARS-CoV-2 in nasal swabs, there was still less than 100% positive agreement between DirectDetect, and the commercially available real-time RT-PCR assay was used for the initial diagnosis. It is hypothesized that one reason for this discrepancy is due to the delay between sample procurement and sample testing where SARS-CoV-2 viral RNA could have degraded. In addition to delays between procurement and testing, many of the samples were collected days after the original positive test using the commercial assay, up to 13 days (Table S1). This delay could lead to a decrease in the viral load within the patient as they recovered from COVID-19 and therefore affect the positive agreement between DirectDetect and the original clinical assay as well. In a typical clinical patient nasal sample swab, the average RNA load is $6.76 \times 10^5$ copies per whole swab until 5 days after the symptom onset.\(^{16}\)

In this study, this hypothesis was tested as samples 12, 13, and 14 were cases of the same patient retested again every day for 3 days after the initial testing to see if there was a change in viral load. The Ct value for a patient changed from one visit to the next, indicating a change in the viral load of the patient by the third test. A larger Ct value indicates a smaller viral load as it will take more PCR cycles for the fluorescence to reach the threshold and a Ct value of 0 means that SARS-CoV-2 was no longer detected in that sample.

Additionally, beyond comparing DirectDetect to the commercial assay original diagnosis, one more hypothesis for the difference in positive agreement between testing conditions (dry, treated, and untreated) in this study is that two different samples were collected and used for each sample, a dry nasal
swab and a transport medium suspended nasal swab. It is likely that virus attachment to the cotton swabs contact area may have been bypassed, potentially leading to false-negative results in cases.17

3.3. LoD in DirectDetect Is Comparable to That of Other Commercial Diagnostic Kits. As SARS-CoV-2 spread rapidly and the global pandemic worsened, a surge in the development and authorization of molecular diagnostic tests presented a challenge in comparing the testing performances.18 The exuberant number of methods being developed by independent researchers was causing many unstandardized comparisons. However, as the emergency use of diagnostics for SARS-CoV-2 was deemed essential, the FDA granted emergency use authorizations for tests and specified the requirement for reference materials to establish an absolute LoD for each method to evaluate the performance and directly compare the assays.19 A similar LoD method was used in this study for evaluating the sensitivity of DirectDetect by testing a known quantity of inactivated virus to assess viral detection in samples. However, the GeneXpert and Genmark Eplex assays used to confirm positive patient samples were not tested for LoD, but the claimed sensitivity of the reference kits is 131 copies/mL20 and 1 × 10⁶ copies/mL21 respectively. The observed test LoD for DirectDetect was 1667 copies/mL (25 copies/reaction) for both the N and ORF1ab target genes to be detected in 100% of samples or 832 copies/mL (12.5 copies/reaction) for 100% of samples to test positive for SARS-CoV-2 using DirectDetect according to the manufacturer’s instructions, testing positive for at least one of the target genes. These metrics are comparable to or better than most of the other FDA EUA-authorized SARS-CoV-2 PCR test kits with nucleic acid extraction methods (180–6 000 000 units/mL).21 While the PerkinElmer New Coronavirus Nucleic Acid Detection Kit (PerkinElmer, Inc., Waltham, MA, USA) and the Viracor SARS-CoV-2 assay (Viracor Eurofins Clinical Diagnostics, Lee’s Summit, MO, USA) have an LoD of 180 units/mL, these kits use swabs in transport medium, while DirectDetect does not require that reagent but has still shown that it is capable of detection within that range. Overall, DirectDetect is one of the few SARS-CoV-2 PCR tests capable of diagnosis without the need for transport medium of which there are currently only four EUA kits which use dry NP or nasal swabs. The LoD for these kits range from 60 000 to 540 000 units/mL, showing DirectDetect is more sensitive than these other assays. Compared to other diagnostic kits using no nucleic acid extraction, mentioned in Table 1, DirectDetect had a LoD that is as sensitive as four of the five tests and is approximately 300 times more sensitive than the Lyra Direct SARS-CoV-2 assay (540 000 units/mL).21,22 DirectDetect, like many other PCR tests, based on the LoD study, has performed better than any of the EUA-authorized SARS-CoV-2 molecular diagnostic tests.21

Furthermore, these results show that the DirectDetect kit can contribute significantly to the global COVID-19 screening efforts and simplify the process of diagnosing patients. Reducing the number of procedural steps and reagents used for proper detection of SARS-CoV-2 among patients is an area of improvement many testing kits have focused on and DirectDetect was able to achieve. Additionally, DirectDetect has the potential to be further implemented in decentralized diagnostic efforts by providing patients the option to self-collect the testing specimen for rapid and reliable monitoring since only a cotton swab for nasal specimen retrieval is required with no need for monitoring reagent contamination. Last, the DirectDetect assay can be further modified to target other gene sites and monitor other nucleic acid-based pathogens or variants of SARS-CoV-2.

3.4. Limitation of the Study. A distinct limitation of this study has been the low number of accessible patient samples. Therefore, the presented findings here show only the preliminary results of effectively detecting SARS-CoV-2 using DirectDetect. Out of the 24 samples, 7 dry swab samples (without VTM), 6 treated (with VTM and RNA extraction), and 6 untreated (with VTM and no RNA extraction) wet swab samples were tested again due to invalid results or to reconfirm the results (Table S2). The validity of these tests is determined by the success rate of IC amplification; without the amplification in the IC, all other amplifications of the N or ORF1ab target sites are automatically invalidated. One cause for this can be the degradation of the reagents. While rerunning samples increases the overall turnaround time to result, even when repeating the real-time RT-PCR step for the DirectDetect assay two times, the timing is still comparable to one conventional real-time RT-PCR assay run time as described in Figure 1. However, rerunning tests is still not ideal and a consideration where refinement of this assay may need to be employed.

An additional limitation to note is that the Ct values of the DirectDetect real-time RT-PCR test for the treated wet swabs overall exhibited a higher number compared to the Ct values of the initial reference test. In cases this can indicate the possibility of false-positive results, however the negative control, non-patient samples confirm the distinct amplification of our target genes in every test. In the future, including negative patient samples could further validate the test results. Moreover, the “gold-standard” RT-PCR method Ct values differ by patient to patient, but overall Ct values of <31 depicted the best representation of the viral load of the patient in the initial 7 days of infection.23 Thus, comparable to “gold-standard” RT-PCR, DirectDetect performed with higher number of Ct values. Although the cross-reactivity and specificity were not investigated in this study, they were previously validated by the PerkinElmer New Coronavirus Nucleic Acid Detection Kit.24,25

4. CONCLUSIONS
Overall, the goal of this work was to evaluate the DirectDetect SARS-CoV-2 Direct Real-time RT-PCR test. LoD testing for DirectDetect indicated that the test was comparable to or better than other COVID-19 detection assays approved for EUA by the FDA, especially other tests using dry swabs. Additionally, this study indicates that DirectDetect can be used for multiple different sample types. Although the goal of the assay is to use dry sample swabs for detection of SARS-CoV-2 without the need for RNA extraction materials and reagents, this work showed that DirectDetect can be adapted and successful using a sample of the transport medium directly to the RT-PCR assay (untreated wet swab) or a sample that undergoes RNA extraction from the transport medium before RT-PCR (treated wet swab) as well. Thus, this procedure can successfully identify SARS-CoV-2 using minutes-long or hours-long sample preparation techniques or further implemented for decentralized diagnostic efforts. In the future, it is possible that this sample preparation can be adapted for other infectious diseases or variants of SARS-CoV-2 and be an easy-to-implement test due to the reduced amounts of reagents,
materials, and time needed between sample procurement and analysis.

5. MATERIALS AND METHODS

5.1. Patient Selection and Sampling. Approval for patient selection and sampling was granted by The Miriam Hospital IRB (protocol 208820). Patients were enrolled in a separate study that collected multiple longitudinal specimens (NP, OP, saliva, and nasal specimens) from patients admitted to the hospital with COVID-19, all of whom had a positive test for SARS-CoV-2 performed in the hospital clinical laboratory using a commercial kit with the nucleic acid purification step for positive samples. As a reference kit, GeneXpert System test, Xpert Xpress, for SARS-CoV-2, Flu A/B, and RSV (Cepheid, Sunnyvale, CA, USA) was used for samples 1–4 and 6, and Genmark Eplex Respiratory Panel 2 (GenMark Diagnostics, Carlsbad, CA, USA) was used for samples 5 and 7–24. These tests have a similar workflow as the Figure 1 manual RT-PCR procedure but with an automated process. Both assays use nasal swabs in VTM that is used for downstream processes and target genes N1 and N2.19,20 The RNA extraction steps in these assays both use magnetic bead-based solid-phase extraction methods. For this study, two nasal samples were collected from each patient: one swab was collected in a dry tube and the other in VTM. The nasal swab sample collected in the VTM was used for both DirectDetect and reference kit; however, these were two distinct samples from the same patient. Once the patient tested positive in the reference test initially, the same patient was asked to come again to provide more samples to be tested using the DirectDetect protocol. There were a total of 14 patients enrolled in this study and 24 samples retrieved for positive samples and 24 negative samples. Additional metrics regarding when the patient samples were initially, the same patient was asked to come again to provide more samples to be tested using the DirectDetect protocol.

5.2. DirectDetect Sample Preparation for SARS-CoV-2. Although DirectDetect SARS-CoV-2 Real-time RT-PCR for Environmental Surfaces (PerkinElmer, Waltham, MA, USA) was designed to be used with dry swab samples collected from environmental surfaces that were not placed in the transport medium, to test the robustness of this assay, both dry patient sample swabs and patient samples collected in transport medium were tested. The samples placed in the transport medium were tested under two different conditions. In the first condition, a sample of the transport medium was added directly to the RT-PCR mix (Figure 3, step 3), which is referred to as an “untreated wet swab”, this is dissimilar to the dry swab as it bypasses the process buffer step. In the second condition, the sample underwent RNA extraction from the transport medium before the sample was ready for RT-PCR, referred to as a “treated wet swab”. This resulted in three different sample preparations for analysis using DirectDetect: dry swab, treated wet swab, untreated wet swab.

Following nasal swab specimen collection, the swab was placed in a clean, dry collection tube according to the manufacturers’ instructions (Figure 1) or transport medium. The dry samples are stable at room temperature for up to 24 h between collection and testing or 72 h when stored between 2 and 8 °C. If the specimen was not tested within this time frame, it was frozen at −70 °C or colder until testing could be resumed. The dry patient sample swabs were also prepared for RT-PCR using the manufacturer’s instructions for environmental surfaces starting. The process buffer, after the adding the dry sample swab, was directly prepared for downstream real-time RT-PCR. The process buffer was stored at −20 °C for additional testing if required. For the treated sample swabs undergoing RNA extraction before RT-PCR, the chemagic Viral DNA/RNA 300 Kit (PerkinElmer, Waltham, MA, USA), a SPRI bead-based extraction kit, was used for the extraction of SARS-CoV-2 viral RNA. Standard operating procedures provided by the manufacturer were followed, and the purified RNA was extracted into a purified suspension and placed into a clean tube for further processing with DirectDetect.

5.3. LoD and Real-Time Reverse Transcriptase Polymerase Chain Reaction. The DirectDetect SARS-CoV-2 Direct real-time RT-PCR assay was first tested using the AccuPlex SARS-CoV-2 Reference Material Kit (Seracare Life Sciences Inc, Milford, MA, USA) to analyze the LoD for this assay. This material is a non-infectious recombinant Alphavirus which has a real viral protein coat and includes target regions in the ORF1a, RdRp, E, and N regions. The concentration of the reference material used is 10,000 copies/mL; thus, the LoD was tested with 25, 12.5, 6.25, 3.13, 1.56, or 0.78 total copies in the RT-PCR assay of a 15 μL reaction, 2.5
μL of the input volume, and 12.5 μL of master mix (Table 4). Each of the conditions was tested with 12 replicates.

Table 4. DirectDetect Kit Components and Reagent Volumes per Sample*

| component name           | volume (384-well) | main ingredients                                      |
|--------------------------|-------------------|-------------------------------------------------------|
| nCoV reagent A           | 3.75 μL           | buffers, dNTPs, Mg²⁺                                   |
| nCoV reagent B           | 0.75 μL           | TE buffer, primers, and probes                         |
| nCoV enzyme mix          | 0.5 μL            | Taq DNA polymerase, reverse transcriptase, RNase inhibitor, and UNG |
| nCoV reagent D           | 0.5 μL            | PCR enhancer for DirectDetection                       |
| nCoV IC                  | 5 μL              | SARS-CoV-2 synthetic RNA oligos encapsulated in recombinant virus |
| nuclease-free water      | 2 μL              | TE buffer, bacteriophage MS2                           |
| patient sample           | 2.5 μL            | SARS-CoV-2 Negative Control                            |
| nCoV direct positive control | 2.5 μL           | SARS-CoV-2 synthetic RNA oligos encapsulated in recombinant virus |
| nCoV negative control    | 2.5 μL            | TE buffer                                              |

*From DirectDetect SARS-CoV-2 Direct Real-time RT-PCR kit info.

The real-time RT-PCR used in DirectDetect targeted the N and ORF1ab genes of SARS-CoV-2. TaqMan probes were used to generate target-specific signals to identify the two genetic regions where the N gene was labeled with FAM and the ORF1ab gene was labeled with ROX fluorescent dyes. To evaluate the assay in real time, an RNA IC (bacteriophage MS2) was also used in every sample and was labeled fluorescently with the HEX fluorescent dye. Last, deoxyuridine triphosphate and uracil-DNA glycosylase (UNG) were also incorporated into this RT-PCR assay as a method to reduce carryover and false-positive results caused by contamination of reagents or samples.

Table 4 outlines the reagents and volumes used to prepare the samples for RT-PCR. To begin, nCoV Reagent A, nCoV Reagent B, nCoV Enzyme Mix, nCoV Reagent D, nCoV IC, and nuclease-free water were all combined, vortexed, and spun down in a 200 μL tube (USA Scientific, Ocala, FL, USA). Next, 2.5 or 5 μL, depending on the RT-PCR machine being used, of patient sample, nCoV Direct Positive Control, or nCoV Negative Control was added to the previously combined reagents. A 5-fold dilution of the positive control was performed using TE buffer before it was added to the reagent mix. All tubes were vortexed and centrifuged for 5 min at 350g, as suggested by the manufacturer; however, a quick spin was also found to be sufficient for this step. Following this step, the samples were pipetted from the reagent tube to a Bio-Rad Hard-Shell 96-Well or a 384-Well PCR Plate (Hercules, CA, USA). The Bio-Rad CFX96 Touch Real-Time PCR Detection System or the Bio-Rad CFX384 Touch Real-Time PCR Detection System (Bio-Rad Laboratories, Hercules, CA, USA) was used for real-time RT-PCR in this study.

5.4. Testing and Statistical Analysis. Following real-time RT-PCR, the IC signals were first analyzed. Table 5 outlines how the samples were evaluated following successful negative and positive control evaluation. All patient samples were run using DirectDetect at least three times, and the Ct value presented are the average of these three trials. The LoD for DirectDetect was also tested using the AccuPlex SARS-CoV-2 Reference Material Kit (Seracare Life Sciences Inc, Milford, MA, USA) before patient sample testing. Twelve trials were run for six different amounts of the input RNA reference material (25, 12.5, 6.25, 3.13, 1.56, and 0.78 copies per reaction) for this assay. Considering that the results have a continuous response (dependent variable) and are described in terms of multiple levels or categories (dry swab, treated wet swab, and untreated wet swab), a t-test (parametric) or Wilcoxon test (non-parametric) was performed for statistical analysis of the real-time RT-PCR data using JMP Pro 15 (SAS Institute Inc, Cary, NC, USA).

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.1c05595.

Additional information collected regarding patient samples and raw Ct data and analysis of DirectDetect patient results using dry swab, treated wet swab, and untreated wet swab samples (PDF)

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Table 5. Patient Sample Evaluation for SARS-CoV-2 Using DirectDetect Real-Time RT-PCR Based on the Ct Value of Each Fluorophore Corresponding with a Different Gene during RT-PCR

| IC (HEX fluorophore) | N gene (FAM fluorophore), ORF1ab gene (ROX fluorophore) | result interpretation |
|----------------------|--------------------------------------------------------|-----------------------|
| Ct ≤ 40              | both targets undetected or Ct > 42                      | SARS-CoV-2 not detected |
| Ct value detected    | both targets Ct ≤ 42                                   | SARS-CoV-2 detected    |
| Ct value detected    | one target Ct ≤ 42                                     | SARS-CoV-2 detected    |
| Ct > 40 or undetected| both targets undetected or Ct > 42                      | invalid test and retest sample |
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Notes
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