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Review

Roadmap to the Bioanalytical Testing of COVID-19: From Sample Collection to Disease Surveillance

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ABSTRACT: The disease caused by SARS-CoV-2, coronavirus disease 2019 (COVID-19), has led to a global pandemic with tremendous mortality, morbidity, and economic loss. The current lack of effective vaccines and treatments places tremendous value on widespread screening, early detection, and contact tracing of COVID-19 for controlling its spread and minimizing the resultant health and societal impact. Bioanalytical diagnostic technologies have played a critical role in the mitigation of the COVID-19 pandemic and will continue to be foundational in the prevention of the subsequent waves of this pandemic along with future infectious disease outbreaks. In this Review, we aim at presenting a roadmap to the bioanalytical testing of COVID-19, with a focus on the performance metrics as well as the limitations of various techniques. The state-of-the-art technologies, mostly limited to centralized laboratories, set the clinical metrics against which the emerging technologies are measured. Technologies for point-of-care and do-it-yourself testing are rapidly emerging, which open the route for testing in the community, at home, and at points-of-entry to widely screen and monitor individuals for enabling normal life despite of an infectious disease pandemic. The combination of different classes of diagnostic technologies (centralized and point-of-care and relying on multiple biomarkers) are needed for effective diagnosis, treatment selection, prognosis, patient monitoring, and epidemiological surveillance in the event of major pandemics such as COVID-19.

KEYWORDS: *COVID-19, SARS-CoV-2, COVID-19 testing, COVID-19 diagnostics, point-of-care diagnostics, nucleic acid testing, PCR, isothermal amplification, CRISPR, antigen/antibody testing*

The global transmission of the coronavirus disease 2019 (COVID-19), the disease caused by the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), has caused significant mortality and morbidity and has imposed numerous healthcare, economic, and resource challenges. As of September 15, 2020, there were more than 29 million confirmed cases attributed to COVID-19 globally.[1] A unique challenge of this particular disease lies in the contagiousness of undetected asymptomatic, presymptomatic, and mild cases that further its spread.[2,3] Furthermore, COVID-19 symptoms are broad and comparable to other viral respiratory diseases such as the common cold or influenza, making its clinical diagnosis challenging.[4] Given the unavailability of vaccines or highly effective treatments to date, screening, early diagnosis, continuous surveillance, and epidemiological contact tracing are the critically needed strategies for containing the spread of COVID-19 and mitigating its profound impact on global health and economy. Effective diagnostic technologies are foundational to the above-mentioned COVID-19 pandemic mitigation strategies and will be the focus of this Review.

We will initiate this Review by highlighting the importance of the type and time-point of specimen collection, as this greatly affects the clinical sensitivity and specificity of testing. We will review the current state-of-the-art diagnostics technologies and identify the emerging devices, concepts, and approaches that can improve COVID-19 diagnostics. The current state-of-the-art COVID-19 diagnostic technologies form a benchmark against which the emerging technologies are evaluated. For this purpose, we summarize and analyze their performance metrics such as limit-of-detection (LOD), clinical sensitivity, clinical specificity, and analysis time.

We categorize the COVID-19 tests based on the type of analyte being investigated, viral RNA, viral antigens, antibodies, and other biomarkers that are indirectly affected in the human body in the presence of the virus. The advantages and disadvantages of each of these test categories are discussed, and it is identified that the combination of data obtained from multiple tests is needed for advancing capabilities in disease diagnosis, prognosis, and treatment selection, as well as vaccine and treatment development.

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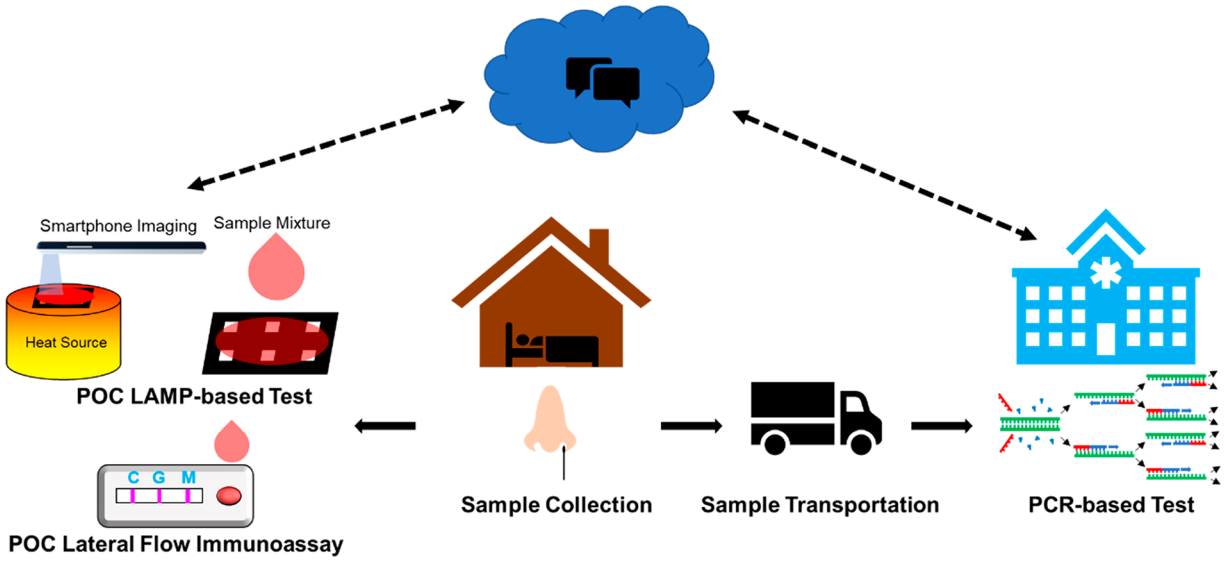

Figure 1. Conceptual schematic of conventional and POC diagnostic test workflow. In the conventional method, the collected specimen is transported to a centralized laboratory for the nucleic acid test, typically performed using polymerase chain reaction (PCR). In the POC methods, the molecular and serological tests are conducted on-site. In case of the colorimetric nucleic acid tests, the assays can be read using a smartphone application and communicated wirelessly to a healthcare professional. Lateral flow immunoassay results can be read visually by patients.

Following the review of the state-of-the-art, we critically review the emerging point-of-care (POC) diagnostic platforms in each category that can be operated at the place and time of need, without reliance on laboratory technicians, and at a low cost. These devices offer great potential for complementing the currently available tests that are based on specimen collection, transport, and centralized testing and allow for rapid screening, home testing, and self-monitoring (Figure 1). If effectively used and integrated within the healthcare system, these POC tests are expected to expedite sample-to-result times, enable widespread decentralized testing that is accessible to the public at home and people in remote and resource-poor settings, increase the overall test rates, and allow for the effective mitigation of the viral spread and the uninterrupted opening of the economy.[5] We finally review the emerging role of artificial intelligence (AI) in analyzing the large influx of data generated from various diagnostics technologies to improve COVID-19 management.

SAMPLE COLLECTION AND PREPARATION

SARS-CoV-2 is detected in a variety of samples such as feces (viral titer: 1×10^7 copies/mL),[6] urine (viral titer: 1×10^2 copies/mL),[7,8] saliva (viral titer: 5×10^4 copies/mL),[9] upper respiratory tract (viral titer: 10^3–10^5 copies/mL) samples (pharyngeal swabs, nasal swabs, nasal discharges), and lower respiratory tract (viral titer: 10^4–10^7 copies/mL) samples (sputum, airway secretions, bronchoalveolar lavage fluid).[10] The antibodies generated in response to active infection are found in blood (IgG: 0.43–187.82 and IgM: 0.26–24.02 (chemiluminescence values divided by the cut-off))[11] and analyzed in serological testing.

For *in vitro* diagnostics of COVID-19, the commonly used specimens are upper respiratory nasopharyngeal (viral titer: 1.69×10^5 copies/mL)[12] and oropharyngeal (viral titer: 7.99×10^4 copies/mL)[12] specimens, nasal midturbinate (viral titer: 1×10^6 copies/mL)[13] samples (collected using a flocked tapered swab), anterior nares (viral titer: 10^3 copies/mL)[14] samples (using a flocked or spun polyester swab), and nasal wash/aspirates (viral titer: 10^4 copies/mL).[12,13] Nasopharyngeal specimens are most widely used due to the ease of collection, high viral load, and sample stability during transportation and storage.[15] Among these, only anterior nares swabs may be currently attained via home self-collection according to the Centers for Disease Control and Prevention (CDC).[12]

Saliva, feces, and urine are non-invasive samples, which are ideally suited for use in the emerging POC COVID-19 tests that require self-collection.[16] Saliva has been successfully used for the detection of respiratory viruses including COVID-19,[16−18] and recent results demonstrate that SARS-CoV-2 can be detected in saliva at high titers.[9] Although faecal samples contain a high concentration of viral nucleic acids, the presence of interfering species (inhibiting enzymes and proteins for nucleic acid amplification) and the difficulty of RNA extraction, make the use of this specimen challenging for the diagnosis of COVID-19.[7] In spite of its noninvasive nature, urine contains a low viral load and at this point, it cannot be used reliably for detecting SARS-CoV-2.[7]

In addition to the sample type, the time-point at which the sample is collected influences the clinical sensitivity of COVID-19 testing. In mild cases, the patients exhibit higher viral loads in the first week of infection, which gradually decreases with the onset of symptoms; however, patients with serious conditions have higher viral titers and longer virus shedding, which lasts for more than 3 weeks.[10,19] Analyzing SARS-CoV-2 in saliva using nucleic acid amplification assays (*e.g.*, real-time reverse transcription-polymerase chain reaction (rRT-PCR)), at the onset of the illness, can produce false-negative results, necessitating follow-up testing using respiratory samples. Respiratory viral shedding peaks at 3–5 days (in mild to medium cases) following the disease onset, indicating the importance of follow-up testing after an initial negative result demonstrated in a suspected patient.[4]

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VIRAL NUCLEIC ACID TESTS

Given the lack of symptoms that specifically distinguish COVID-19 from other respiratory infections, clinicians currently primarily rely on nucleic testing and computed tomography (CT) for evaluating and diagnosing this disease.[4,20] This Review is focused on the bioanalytical technologies, systems that analyze specific biomarkers in patient samples, for COVID-19 testing. The CT-based COVID-19 tests have been reviewed elsewhere.[21,22]

PCR. Among nucleic acid tests, RT-PCR continues to be the gold standard for diagnosing COVID-19.[23] In this method, viral RNA is converted to complementary DNA (cDNA) using reverse transcription, with distinct regions of the cDNA subsequently amplified using PCR.[24,25] Corman *et al.* reported the first validated RT-PCR protocol for detecting COVID-19, where a number of SARS-related viral genome sequences were examined.[26] Of these sequences, two sites comprising of conserved sequences were chosen for the performance evaluation of the protocol: the RNA-dependent RNA polymerase (*RdRP*) gene and the envelope protein (*E*) gene. In this study, *in vitro* transcribed RNA standards that precisely matched the sequence of SARS-CoV-2 were created to assess the limit of detection (LOD); the *RdRP* and *E* genes assays presented a LOD of 3.6 and 3.9 copies/reaction, respectively. To evaluate the clinical specificity, 297 clinical specimens from patients with pre-existing respiratory diseases were examined that contained a wide range of viruses (such as HCoV-HKU1, MERS-CoV, Influenza A and B, *etc.*) at various concentrations (10^5−10^{10} RNA copies/mL). No cross-talk with other respiratory viruses and no false positives were reported (100% clinical specificity);[26,27] however, the clinical sensitivity was not addressed in this work. Corman *et al.* suggested a three-step workflow (first line screening, affirmation of results, and the use of biased tests) for the optimized diagnosis of SARS-CoV-2. First line screening is implemented to identify all SARS-related viruses by targeting several regions of the *E* gene. Following positive testing, the *RdRP* gene is detected using two different primers and Taqman probes, and subsequent biased tests are performed utilizing one of the two probes sequences (*RdRP* 1: FAM(6-carboxyfluorescein)-CCAGGTGGWACR-TCATCMGGTGATGC-BBQ (blackberry quencher) or *RdRP* 2: FAM-CAGGTGGAACCTCATCAGGAGATGC-BBQ).[26] A number of commercially available COVID-19 RT-PCR test kits have been approved by Emergency Use Authorization (EUA). These are summarized in Table S1.

Conventionally, RT-PCR is performed using lab-scale instrumentation at centralized laboratories. Such centralized tests result in long turnaround times (~24−72 h)[28]—associated with sample transport, analysis, and reporting—rely on highly skilled technicians, are not accessible to remote and resource-poor areas due to the high cost of instrumentation and operation, and are not suitable for frequent testing at the POC.[26,29,30] In response, the advancements made over the past few decades in developing miniaturized PCR technologies[31,32] have been rapidly applied to COVID-19 testing. Mesa Biotech has recently developed Accula SARS-CoV-2, a U.S. Food and Drug Administration (FDA)-approved handheld nucleic acid test for detecting COVID-19 (Table S1). This system qualitatively detects viral RNA in 30 min by combining RT-PCR with a lateral flow assay. To conduct the test, nasal or throat samples are diluted in the test buffer and dispensed into a test cassette. The lysis of the virus, reverse transcription of viral RNA to cDNA, amplification, and detection steps all occur within the cassette. The test cassette is then inserted into the Accula Dock, an automated control panel, which regulates reaction temperatures, timing, and fluid flow. After 30 min, the test results are visualized as blue bands on the detection strip in the cassette. The LOD of the Accula SARS-CoV-2 test was determined to be 200 copies/reaction in human clinical matrices (reaction volume: 60 μL). For clinical evaluation of the Accula SARS-CoV-2 test, first, 30 confirmed negative clinical specimens were tested, which resulted in 100% clinical specificity. Next, the negative samples were spiked with SARS-CoV-2 RNA at the concentrations of 400 copies/reaction (2 × LOD), 1000 copies/reaction (5 × LOD), 2000 copies/reaction (10 × LOD), and 10 000 copies/reaction (50 × LOD). The specimens were then randomized for the Accula SARS-CoV-2 test. The test results revealed 100% agreement with the expected outcomes (100% clinical sensitivity).[33,34] In addition, the cross-reactivity of the Accula SARS-CoV-2 test was evaluated by examining 32 potentially cross-reacting organisms (such as Adenovirus, HCoV-HKU1, MERS-CoV, SARS-CoV, Influenza A and B, *Escherichia coli*, *Klebsiella pneumoniae*, *etc.*) in negative throat and nasal swabs. None of the 32 organisms cross-reacted with the test, and no false positives were generated.[34] There are some limitations associated with the Accula SARS-CoV-2 test. As with all PCR tests, this system is prone to false negatives due to the presence of PCR inhibitors or contamination as well as due to issues with specimen collection, storage, or transportation. Therefore, negative test results do not completely rule out the viral infection and should be interpreted in conjugation with other diagnostic tests or clinical assessment. In addition, this is a qualitative test and does not provide any information on the viral loads.[34]

Cepheid (Sunnyvale, CA) has introduced a recent FDA-approved EUA rapid POC test, Xpert Xpress-SARS-CoV-2 (Table S1) that utilizes an automated real time RT-PCR system to amplify and qualitatively detect the *N*-genes and *E*-genes of the virus in the upper respiratory samples. In this device, sample preparation, rRT-PCR amplification, and RNA detection are performed using a single benchtop system (width: 11.5″, height: 18.25″, depth: 17″). This system allows multiple specimens (up to four) to be analyzed simultaneously, yielding a turnaround time of 45 min.[35] Based on manufacturing data submitted to EUA, Xpert Xpress SARS-CoV-2 exhibited an LOD of 75 copies/reaction (250 copies/mL). The clinical sensitivity and specificity of the Xpert Xpress SARS-CoV-2 test were determined using spiked clinical nasopharyngeal swab samples obtained from individuals with symptoms of respiratory tract infection. The negative nasopharyngeal swabs were identified prior to spiking and verified with the Xpert Xpress SARS-CoV-2 test (clinical specificity of 100%). The negative samples were then spiked with SARS-CoV-2 virus at 150 copies/reaction (2 × LOD), 225 copies/reaction (3 × LOD), and 375 copies/reaction (5 × LOD) concentrations. The test results revealed a clinical sensitivity of 100%.[35] In addition, Smithgall *et al.* performed a clinical evaluation of Xpert Xpress-SARS-CoV-2 in comparison with the RT-PCR-based cobas assay (6800 platform, Roche Diagnostics, Indianapolis, IN). A total of 113 nasopharyngeal swabs from patient samples were tested, including 88 positive samples, representing the full range (14−38 cycles) of cycle threshold (C_t) values observed on the cobas assay. High and medium viral concentrations were defined as C_t < 30, whereas

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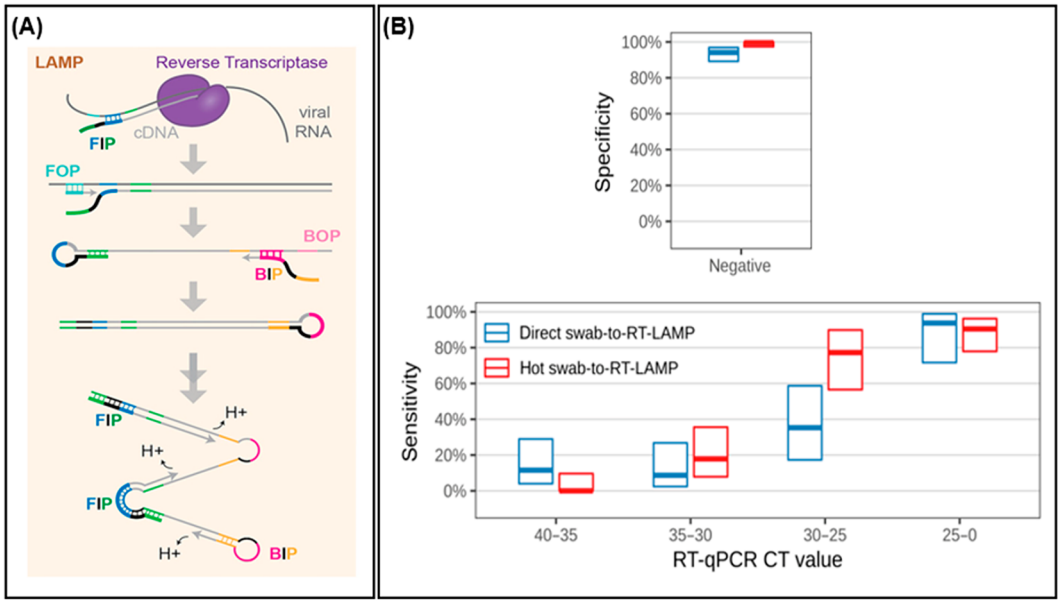

Figure 2. LAMP-based detection systems. (A) General mechanism of LAMP (reprinted with permission from ref 46 under the Creative Commons License (Attribution 4.0 International, http://creativecommons.org/licenses/by/4.0/), Copyright 2020 RNA Society). (B) Specificity and sensitivity comparison between Direct swab-to-RT-LAMP and Hot swab-to-RT-LAMP (Reprinted with permission from ref 55. Copyright 2020 American Association for the Advancement of Science).

C_t > 30 represented low viral loads. After testing all of the 113 patient samples, the overall clinical sensitivity and specificity of Xpert Xpress COVID-19 were determined as 98.9% and 92%, respectively. For C_t < 30, Xpert Xpress COVID-19 was able to accurately detect the viral RNA in every sample (clinical sensitivity of 100%), while for C_t > 30, the clinical sensitivity was reduced to 97.1%.[36] Despite all the advantages offered by Xpert Xpress COVID-19 test in terms of speed, portability and accuracy, improper sample collection and handling can still lead to false negative results, requiring trained healthcare professionals for performing the full assay from specimen collection to analysis.

Although RT-PCR is the gold standard for diagnosing COVID-19, it has several limitations. Like all other RNA-based strategies, this method is susceptible to false negatives stemming from errors in sample collection (collection site and time of sample acquisition), poor specimen handling during viral RNA extraction, existence of PCR inhibitors in poorly treated specimens, diversity in viral load among patients, and varying operating procedures or LODs between different RT-PCR kits. Therefore, negative test results do not fully dismiss the possibility of the viral infection and need to be interpreted in combination with the individual's medical record, clinical symptoms, and other diagnostic test results such as CT scan of the chest.[37] Moreover, the majority of the RT-PCR tests require RNA extraction and purification before reverse transcription and PCR amplification. Although these sample preparation steps are often automated, they add to the instrument complexity and the number of required reagents. The development of testing platforms capable of direct specimen analysis with minimized and simplified sample processing is critically needed for use at the POC.[37] During the COVID-19 pandemic, the shortage of RT-PCR reagents including RNA extraction kits (QIAGEN QIAamp Viral Mini Kit, QIAGEN EZ1 Virus Mini-Kit, Roche MagNA Pure nucleic acid kit) and synthetic oligonucleotides has also been a critical concern.[38−40] In the United States, faulty reagent manufacturing combined with a bottleneck distribution process through CDC's International Reagent Resource (IRR) and increased consumption of reagents following the implementation of a dual specimen testing requirement have led to these shortages.[41] The next section discusses the COVID-19 diagnostic techniques that have been implemented to diversify testing methods and address the shortcomings of RT-PCR.

Isothermal Amplification. Isothermal amplification methods have been developed to replace the thermal cycling steps needed in PCR to simplify, lower the cost of, and reduce the footprint of PCR platforms.[42] Isothermal amplification techniques including loop-mediated isothermal amplification (LAMP),[43] nucleic acid sequence-based amplification (NASBA),[42] transcription-mediated amplification (TMA),[44] rolling circle amplification (RCA),[45] and recombinase polymerase amplification (RPA)[46] have been used for developing COVID-19 diagnostic tests (Table S2).

A prominent example of an isothermal amplification-based POC COVID-19 detection is the ID NOW COVID-19 assay (FDA-EUA designated) developed by Abbott (Table S1), which detects the presence of the *RdRp* gene in nasopharyngeal swab specimens. The ID NOW COVID-19 test begins with the insertion of a sample receiver and a base tube into the ID NOW instrument. The sample is introduced to the receiver that contains a lysis/elusion buffer and is then transported to the base tube via a transfer cartilage to initiate target amplification. Fluorescently labeled molecular beacons are then used to identify the amplified RNA targets. This system exhibited a rapid turnaround time of 5 min for positive results and 13 min for negative results. Based on the manufacturing data, ID NOW COVID-19 possesses an LOD of 125 copies/mL or 25 copies/reaction (calculated from the recommended reaction volume for the ID NOW instrument; the actual reaction volume was not reported). The clinical performance of the device was determined using 30 contrived nasopharyngeal swabs collected from individuals with respiratory symptoms. The test samples were prepared by spiking the

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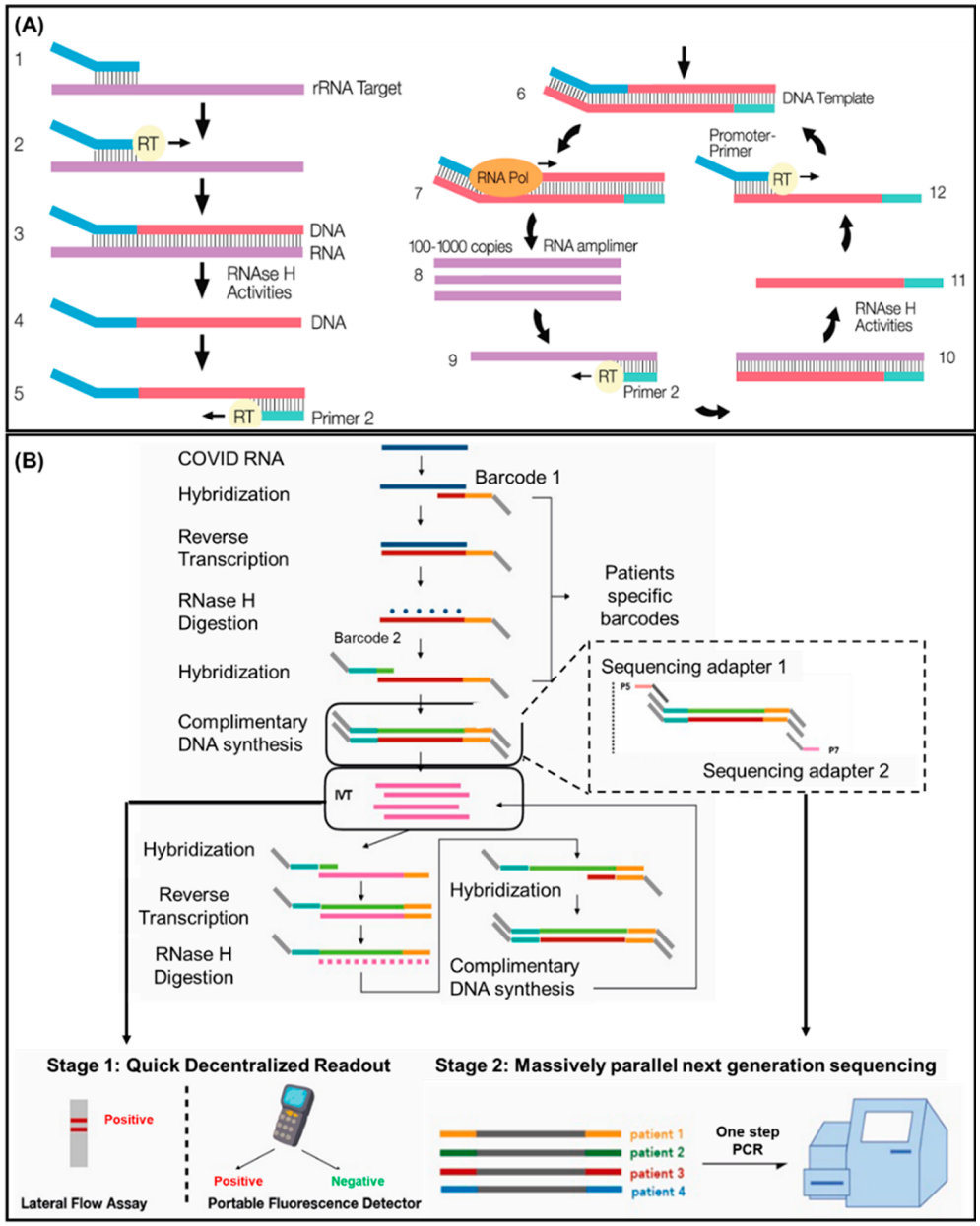

Figure 3. TMA and NASBA. (A) TMA and NASBA share the same mechanism; NASBA employs RNase H to degrade the initial RNA. TMA utilizes RT-DNA polymerase that has intrinsic RNase H activity in TMA (Reprinted with permission from ref 58. Copyright 2004 Elsevier). (B) Schematic of the two-stage INSIGHT workflow (Reprinted with permission from ref 61. Copyright 2020 The Authors).

nasopharyngeal swabs matrices with extracted viral RNA containing target sequences from the SARS-CoV-2 genome. At target concentrations of 50 copies/reaction (2 × LOD) and 125 copies/reaction (5 × LOD), the device showed a clinical sensitivity of 100%. The clinical specificity of the test was evaluated using negative nasopharyngeal samples, which resulted in a 100% negative agreement value.[47,48] The manufacturer did not evaluate the performance of the device using real patient specimens; however, Smithgall *et al.* evaluated the clinical performance of ID NOW in comparison with the RT-PCR-based cobas assay using 113 patient samples (nasopharyngeal swabs). The overall clinical sensitivity and specificity of ID NOW were determined to be 73.9% and 100%, respectively. For C_t < 30, ID NOW was able to accurately detect the viral RNA in all the samples (clinical sensitivity of 100%), while for C_t > 30 it was unable to detect the RNA in most of the specimens (clinical sensitivity of 34.3%).[36] The high false-negative rate of ID NOW COVID-19 at low viral concentrations was also reported in other studies.[49,50] Despite a rapid turnaround time, this platform offers low throughput as it only analyzes a single sample at a time.

LAMP. LAMP, the most commonly used one-step isothermal amplification method, employs four to six primers to identify six to eight distinct regions of target DNA for a highly specific amplification reaction. In this process, *Bst* DNA polymerase mediated strand displacement elongates target nucleotides into stem loop structures containing up to 10^9 copies of the target sequence, in under 1 h (Figure 2A).[51−53] This particular technique is often combined with a reverse transcription step (RT-LAMP) to detect RNA targets.[54]

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Yu *et al.* reported an RT-LAMP-based diagnostic platform for COVID-19, referred to as iLACO, that colorimetrically detects SARS-CoV-2 with an LOD of 2,000 copies/reaction in 20 min. The clinical sensitivity of the LAMP assay was determined to be 89.9% using 248 clinical samples; however, the authors did not evaluate the clinical specificity of the assay. Although this method uses one step isothermal amplification, it still requires an extra sample preparation step for viral RNA extraction.[56] Thi *et al.* developed another colorimetric LAMP assay for detecting SARS-CoV-2 (swab-to-RT-LAMP) that did not necessarily use RNA extraction, demonstrating an LOD of Ct < 30 that corresponds to 1000 copies/reaction (80×10^3 copies/mL). The RT-LAMP assay was evaluated using 768 pharyngeal swabs from positive pretested clinical samples. The clinical performance of the RT-LAMP with processed samples (RNA extraction) yielded a clinical sensitivity of 97.5% and a specificity of 99.7%. The group then evaluated the RT-LAMP assay without sample processing (direct-swab-to-RT-LAMP using 235 aliquots from 131 clinical samples) and using a 5 min heating step at 95 °C prior to amplification (hot-swab-to-RT-LAMP using 343 aliquots from 209 clinical samples), which indicated a loss in performance when eliminating RNA extraction (Figure 2B).[55] The limitation of the Swab-to-RT-LAMP test is in its low sensitivity at high C_t values when unpurified samples are used. However, Swab-to-RT-LAMP holds the potential for POC diagnostics due to its simple operation.

A well-documented drawback in the use of colorimetric and pH indicators in the detection of RT-LAMP amplicons is the occurrence of nonspecific amplification and primer−primer interactions that can generate a detectable signal in the absence of the target; leading to false positives. The detection of specific barcoded sequences (*e.g.*, by combining LAMP with CRISPER) is reported to overcome this shortcoming.[37]

NASBA and TMA. NASBA and TMA are two mechanistically similar isothermal amplification methods (Figure 3A) that first transcribe the target RNA into a double-stranded RNA:DNA hybrid using reverse transcription. Following the degradation of the RNA strand from the hybrid, cDNA strands are generated, which are used to create antisense copies of the original RNA target using T7 RNA polymerase.[57,58] NASBA uses RNase H to degrade the initial RNA from the RNA-DNA hybrid; however, TMA uses the reverse transcriptase for this purpose.[59] Gel electrophoresis, fluorescent probes, and colorimetric assays are used to subsequently detect the products of NASBA and TMA.[60]

Unlike PCR, NASBA yields single stranded RNA, which is detected using probe hybridization without any denaturation steps.[62] NASBA offers a higher amplification efficiency compared to PCR, which in turn reduces the overall error frequency stemming from the lower number of amplification cycles.[42] Leveraging these advantages, Wu *et al.* designed an Isothermal NASBA-Sequencing based High Throughput Test (INSIGHT) for detecting SARS-CoV-2 RNA (Figure 3B). In this method, complementary molecular beacons are added to a part of the amplified sequence for the visualization of the amplicons on a POC lateral flow assay. A proof-of-concept lateral flow assay was demonstrated; however, the assay was only validated using fluorescent readout in 12 saliva samples spiked with synthetic RNA, through which an LOD of 10−100 copies/reaction (500−5000 copies/mL) was achieved in 2 h. To assess the applicability of INSIGHT in clinical diagnostics, it has to be validated, in its lateral flow configuration, using a large number of clinical samples.[61]

TMA has also been used in the developing COVID-19 diagnostic tests. Hologic's "Panther Fusion" system (Table S1) is able to simultaneously screen for COVID-19 and other respiratory viruses using the same patient sample and collection vial. The developed TMA-based Hologic Aptima SARS-CoV-2 assay for the Panther fusion system is capable of performing 1000 tests within 24 h, obtaining the first results in 3.5 h.[48] Gorzalski *et al.* evaluated the Panther Fusion platform in RT-PCR and TMA modes for COVID-19 detection using 116 previously evaluated clinical nasopharyngeal swabs. In these modes, a higher clinical sensitivity (98.1% (52/53) versus 96.2% (51/53) for RT-PCR) and lower LOD (5.5×10^3 copies/mL (1.98×10^3 copies/reaction) versus 5.5×10^4 copies/mL (1.1×10^4 copies/reaction) for RT-PCR) were obtained for TMA compared to RT-PCR.[44]

RCA. Rolling circle amplification (RCA) utilizes circular DNA templates to hybridize with specific target sequences and achieve amplification of 1000 fold (linear RCA) for a single binding event (Figure 4). The DNA or RNA polymerase

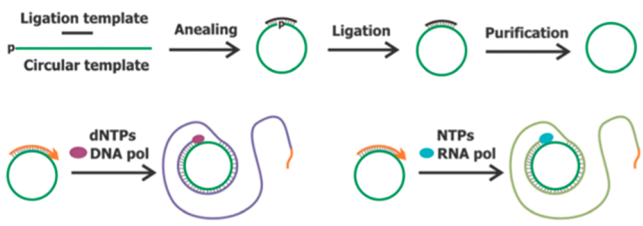

Figure 4. Schematic of the RCA mechanism (reprinted with permission from ref 45. Copyright 2014 Royal Society of Chemistry).

facilitates this amplification by adding dNTPs to a primer annealed to the circular DNA template (formed by ligating the padlock probe) producing repetitive sequences containing long single stranded DNA or RNA sequences, which can be cleaved using enzymes to produce several copies of the target DNA/RNA fragments.[45] These fragments act as a feeder sequence to bind to dye-labeled sequences for colorimetric detection on lateral flow strips (LFS).[63,64] Huang *et al.* developed an assay for the colorimetric detection of SARS-CoV-2 using padlock probe RCA, which detected the RCA product by analyzing hydrogen ions released during the dNTPs addition in the synthesis of DNA strands using a pH indicator. The LOD of the assay was determined to be 2.5 pM (6×10^7 copies/reaction or 1.5 x10^9 copies/mL) using the synthetic glycoprotein gene for SARS-CoV-2 suspended in buffer with analysis time of 30 min at room temperature.[64] RCA is less prone to errors and contamination due to the multiplication of the original DNA target multiple times, as opposed to using newly synthesized DNA as templates in PCR.[48,65] The RCA assay developed here requires evaluation using clinical specimens to further determine its clinical performance.

RPA. RPA differs from all the aforementioned isothermal techniques as it employs recombinase and polymerase to amplify target nucleic acids (Figure 5).[46,66] RPA has the added advantage of faster reactions (20 min) at lower temperatures (37−42 °C) compared to other isothermal techniques such as LAMP (60−65 °C), which makes it applicable to rapid POC COVID-19 diagnostics.[67] While currently not used for COVID-19 testing in a standalone fashion, it has been used

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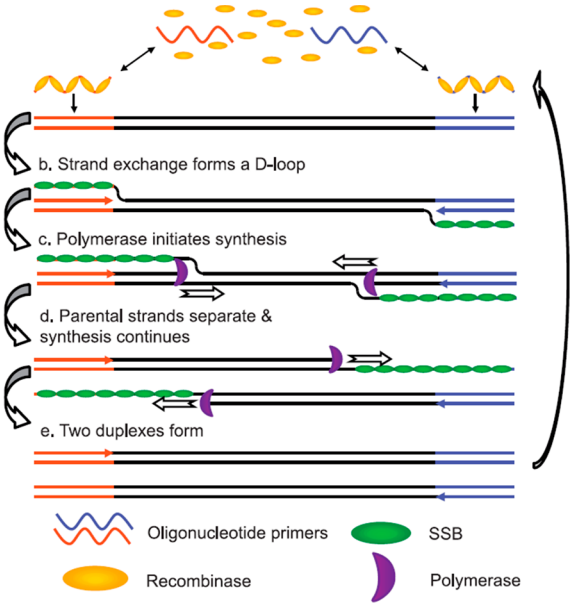

Figure 5. Schematic of the RPA mechanism. The three core proteins, recombinase, single-strand DNA binding protein (SSB), and polymerase, enable DNA amplification at a low constant temperature (37 °C) (Reprinted with permission from ref 66. Copyright 2014 PLOS).

in combination with CRISPR (CRISPR-FDS), which will be described in the following section.[68]

CRISPR. Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) is a genome editing tool developed in the 1980s, which is capable of cleaving all types of nucleic acid targets (i.e., double or single stranded DNA and RNA) at programmable sites.[69,70] CRISPR, segments of genetic material (commonly found in prokaryotes) consisting of repeated short sequences of nucleotides interspersed at regular intervals, and CRISPR-associated proteins (Cas) are used in biosensing to generate cleaved reporter sequences ensuing from site-specific cleavage by the CRISPR/Cas complex.[70] CRISPR/Cas systems detect viral nucleic acids using a guided RNA strand composed of two parts: crispr RNA (crRNA), a sequence complementary to the target nucleic acid, and a tracer RNA, a binding scaffold for the Cas nuclease. Following the guided recognition of viral RNA, Cas can cause cleavage in two ways: (1) sequence indiscriminate cleavage of ssDNA and (2) site-specific double strand break (DSB) on target and nontarget nucleic acid molecules in the vicinity of the Cas enzyme, producing single stranded reporter nucleic acids (Figure 6A).[71,72] The cleavage activity is subsequently leveraged to build reporter systems for colorimetric/fluorescent readout in CRISPR diagnostics.[73] Some of these Cas nucleases include Cas12[74,75] and Cas13,[76] which have been employed for nucleic acid detection. Figure 6B demonstrates an overview of CRISPR-based nucleic detection using Cas12 and Cas 13, followed by lateral dipstick readout. A number of commercialized and emerging CRISPR-based platforms for rapid detection of SARS-CoV-2 are listed in Tables S1 and S2.

Specific High-sensitivity Enzymatic Reporter unLOCKing (SHERLOCK)[73] based on Cas 13 (RNA specific) and HOLMES[79] (one-HOur Low-cost Multipurpose highly Efficient System)/ DETECTR[80] (DNA Endonuclease-Targeted CRISPR Trans Reporter) based on Cas12 (DNA specific) have been developed for detecting viral nucleic

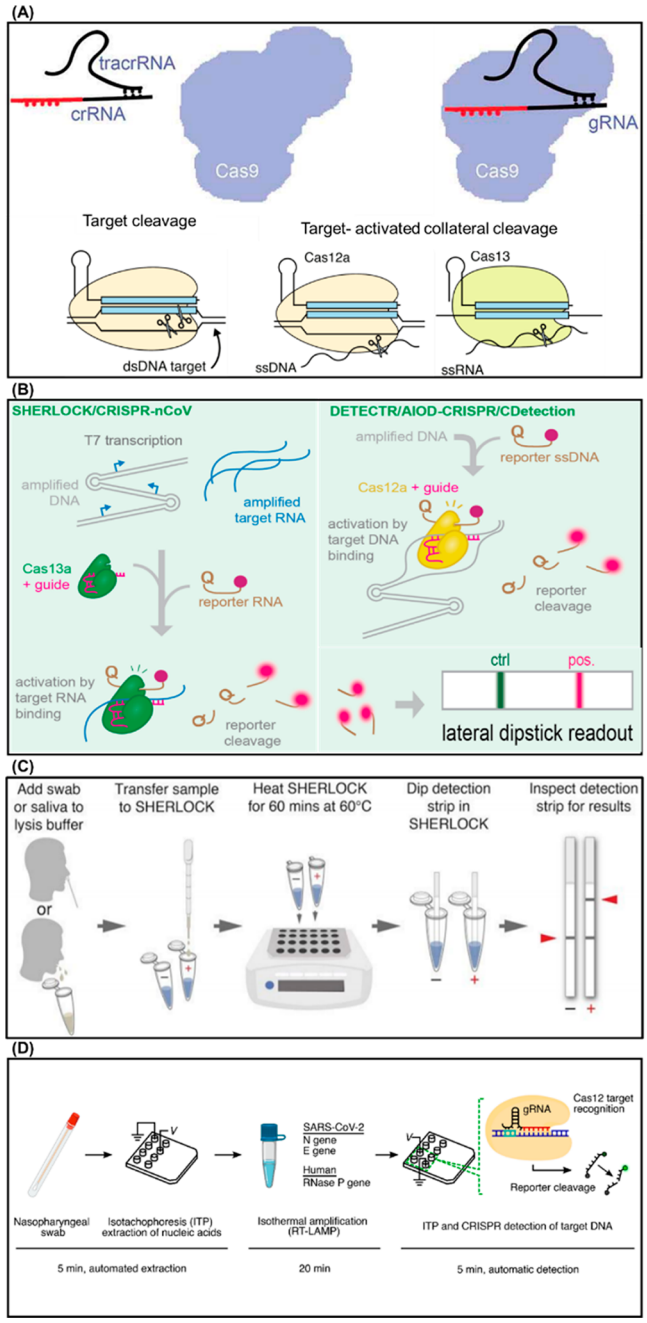

Figure 6. CRISPR-based techniques. (A) Guide RNA (gRNA) components: crRNA, tracrRNA, and Cas endonuclease (Reprinted with permission from ref 71. Copyright 2018 Taylor and Francis). Site specific double strand break (DSB) on target and indiscriminate single strand sequence collateral cleavage (Reprinted with permission from ref 72. Copyright 2018 Springer Nature). (B) Overview of CRISPR-based nucleic acid detection. After the guided recognition of specific target sequences in amplified RNA or DNA, activated Cas cleaves reporter molecules which can be sensed using a lateral flow assay (Reprinted with permission from ref 46. Copyright 2020 RNA Society). (C) STOP-COVID test based on SHERLOCK-LFS platform (Reprinted with permission from ref 77. Copyright 2020 The Authors). (D) Isotachophoresis based extraction of nucleic acid, RT-LAMP and CRISPR detection platform (Reprinted with permission from ref 78. Copyright 2020 The Authors).

acids. These systems have been recently employed for the POC diagnosis of COVID-19 due to their strong collateral

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cleavage activities and ease of integration with lateral flow devices and fluorescent signal reporters.[76,81]

Huang *et al.* developed a CRISPR-based fluorescent system (CRISPR-FDS) that detects the SARS-CoV-2 RNA extracted from clinical nasal swabs and amplified using RT-PCR (or RT-RPA). This assay was developed to enhance the LOD of qRT-PCR by using CRISPR to release a fluorescent reporter that could be read using a fluorimeter. Their analysis demonstrated an LOD of 50 copies/mL (1.5 copies/reaction) with CRISPR-FDS versus 1000 copies/mL obtained with qRT-PCR. Using 29 clinical nasal swab samples, they demonstrated a clinical sensitivity of 100% and clinical specificity of 72%. Despite a low LOD and a high clinical sensitivity, this system demonstrated a low specificity for clinical implementation. Additionally, the system needs to be validated using a larger number of clinical samples for assessing its true clinical applicability. In its current configuration, performed using a 96-well plate and a bulky fluorescent detector, this system is difficult to use in POC settings. Broughton *et al.* validated a multistep CRISPR-Cas12 assay, the SARS-CoV-2 DETECTR, for the detection of viral RNA extracted from clinical nasopharyngeal and oropharyngeal swab samples in 30−40 min on LFS.[82] Using this device, the viral RNA was subjected to reverse transcription and isothermal amplification using RT-LAMP, followed by Cas12 detection of predefined coronavirus sequences. The resultant cleavage of a reporter molecule by Cas 12 confirmed the presence of the virus with an LOD of 1000 copies/reaction (10^4 copies/mL) for the *in vitro* transcribed synthetic viral RNA; however, the LOD deteriorated to 300 000 copies/reaction (3×10^6 copies/mL) in the presence of universal transport media (UTM). Testing of extracted RNA from 23 nasopharyngeal and oropharyngeal swabs (11 COVID-19 positive and 12 containing other respiratory viruses) demonstrated a clinical sensitivity of 95% and specificity of 100% against PCR.

In SHERLOCK, a CRISPR technique popularly employed in COVID-19 detection, the target sequence is amplified with RPA or RT-RPA, thus eliminating the need for a thermal cycler. For SARS-CoV-2 determination, two specific sgRNAs, targeting *Orf1ab* gene and *S* gene are typically used. To develop a POC COVID-19 diagnostic test, a multistep CRISPR-Cas 13a assay was implemented,[83] in which RNA extraction was followed by isothermal application using RT-RPA, RNA transcription by the T7 RNA polymerase, and cas13a-induced detection. The Cas13a−crRNA complex binding to the amplified RNA target causes the cleavage of the RNA reporters that are subsequently captured on a colorimetric lateral-flow strip or visualized by fluorescence. With this method, a LOD of 46 copies/reaction (2300 copies/mL), a clinical specificity of 100%, and a clinical sensitivity of 100% were obtained using fluorescence, whereas the sensitivity drops to 97% with the lateral-flow readout. In spite of outstanding results, obtained using 534 clinical samples used in a head-to-head comparison with PCR, the authors indicated that the SHERLOCK protocol requires physical separation between sample preparation, amplification, and detection steps to minimize RNase contamination causing false positives in complex samples; indicating that the system needs to be better integrated for use outside a molecular diagnostic laboratory. Joung *et al.* developed SHERLOCK Testing in One Pot (STOP), combining isothermal amplification and CRISPR-mediated detection (Figure 6C).[77] This test was performed at a single temperature with one fluid handling step and a simple visual LFS to detect 100 copies/reaction (2000 copies/mL) in contrived saliva and nasopharyngeal swab samples. A study performed with a small cohort of clinically obtained nasopharyngeal swab samples (17 samples) demonstrated a clinical sensitivity and specificity of 100%; which needs to be further validated using a larger sample size. It should be noted that this method still relies on conventional RNA extraction to use with the one-pot amplification/CRISPR detection, making it difficult to implement at the POC. Microfluidic chips powered using isotachophoresis (ITP) were employed to combine the SARS-CoV-2 RNA extraction and detection steps (Figure 6D). ITP was implemented for the automated purification of target RNA from nasopharyngeal swab samples. This was followed by RT-LAMP and on-chip ITP-CRISPR fluorescent detection of SARS-CoV-2 *E* and *N* genes. An LOD of 200 copies/reaction (10 000 copies/mL) of RNA extracted using ITP and an assay time of 30 min were obtained using contrived as well as eight clinically obtained nasopharyngeal swab samples.[78] In its current state, the usage of a bulky electrical power supply and fluidics requiring repeated washing and drying steps using vacuum make this method cumbersome, indicating the requirement for further miniaturization and integration for use in POC applications.

The integration of CRISPR with biobarcodes, isothermal amplification, and LFS formats significantly simplify its operation and reduce its reliance on expensive equipment, making it an excellent candidate for developing POC diagnostic technologies. In spite of this, predesigned reaction kits are currently not commercially available for performing CRISPR, making the development, optimization, and commercialization of new assays a lengthy process. Additionally, due to its reliance on multistep nucleic acid amplification, precise target quantification remains challenging using CRISPR based methods.[68]

Sequencing. Techniques based on nucleic acid sequencing have been implemented for the detection of COVID-19. These techniques provide base-pair level information essential to mutation tracing and COVID-19 strain recognition.[46] While traditional DNA sequencing is expensive and time-consuming, portable and rapid sequencing approaches based on nanopore sequencers are suitable for POC COVID-19 detection.[46] Nanopore sequencing, offered commercially by the Oxford MiniION sequencer,[84] relies on the use of electrophoretic force to translocate DNA, RNA, or protein molecules through an orifice (Figure 7).[85]

Wang *et al.* combined nucleic acid amplification with real time sequencing, using the MinION sequencer, to detect 11 of the virulent gene fragments of the SARS-CoV-2 genome with an LOD of 10 copies/mL in 1 h.[87] Harcourt *et al.* isolated and

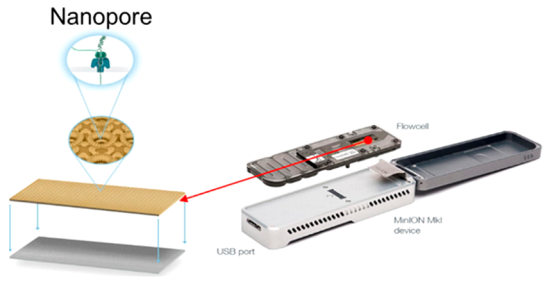

Figure 7. Commercial Oxford MiniION sequencer (Reprinted with permission from ref 85. Copyright 2016 Elsevier).

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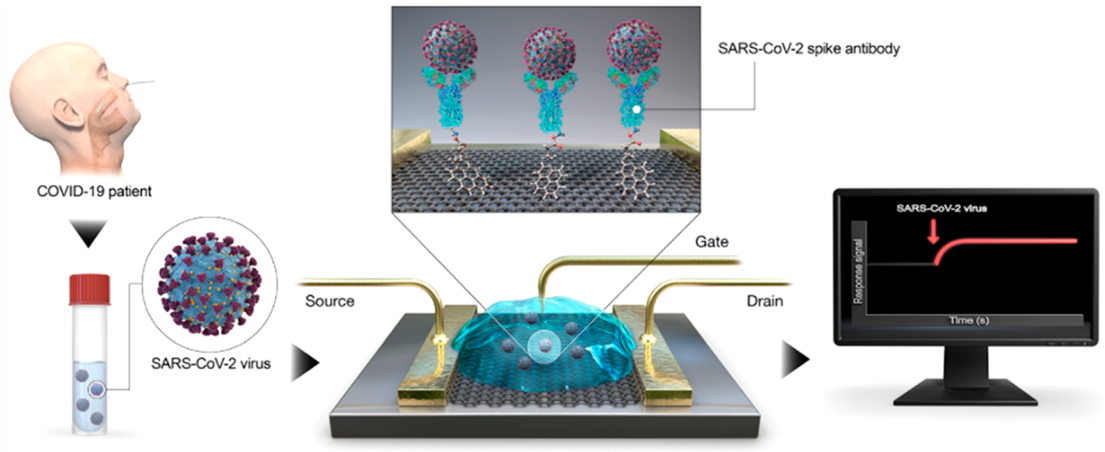

Figure 8. Graphene FET-based detection of SARS-CoV-2 spike protein (Reprinted with permission from ref 99. Copyright 2020 American Chemical Society).

sequenced the entire viral RNA genome for the first COVID-19 infected patient in the US, to be used as the US strain reference, using this method.[88] Li *et al.* demonstrated a combined LAMP-Nanopore Flongle real-time sequencing workflow, wherein COVID-19 RNA was amplified using LAMP for 30 min, prior to being transferred to the sequencing element. The combined approach has an LOD of 21.2×10^3 copies/mL (212 copies/reaction), which can be performed in under 2 h.[86]

These examples demonstrate the potential of utilizing portable sequencing methods in POC diagnostics; however, these need to be combined with nucleic acid amplification for reaching the sensitivity needed for clinical diagnostics and are often faced with challenges related to clogging when interfaced with raw biological samples.[89]

VIRAL ANTIGEN AND ANTIBODY TESTS

The SARS-CoV-2 structural proteins, immune-response antibodies, and inflammatory and proinflammatory response biomarkers can also be utilized for screening and monitoring COVID-19. The structural proteins include the spike glycoprotein (S), envelope protein (E), matrix protein (M), and nucleocapsid protein (N).[90] The receptor-binding spike protein is critical in facilitating viral entry into host cells and redetermining host tropism and as such remains the primary target for antigen-based detection.[91] The other three proteins are essential for the overall functionality of the virus and are involved in assembly, budding, envelope formation, and pathogenesis.

The antibody-based methods have focused on detecting Immunoglobulin M (IgM) and Immunoglobulin G (IgG) against the S proteins.[92] IgM antibody administers the first line of defense against the initial exposure to the virus, while Immunoglobulin G (IgG) antibody confers long-term immunity.[93] Some of the FDA-approved EUA and emerging protein-based detection systems are listed in Table S1 and S2, respectively.

Viral Antigen Testing. Antigen detection is typically faster and less expensive than nucleic acid detection, as it does not rely on target amplification and uses simpler designs.[94] SARS-CoV-2 antigen tests either detect the membrane-bound spike proteins or the nucleocapsid proteins[95] that are typically targeted using specific antibodies produced in animals. In these assays, the lysed sample is deposited on the test slides/strips coated with the capture antibody. Following the addition of the secondary antibody tagged with an enzyme or dye, a colorimetric signal is generated that can be visualized by the naked eye or using a fluorimeter.[96]

In May 2020, the FDA issued an EUA for the first COVID-19 antigen test, Sofia SARS Antigen Fluorescent Immunoassay (FIA) (Table S1).[97] This clinical laboratory improvement amendments (CLIA) certified immunofluorescence test detects viral nucleocapsid proteins in nasopharyngeal samples. Following treatment with a lysis buffer, the specimen is dispensed into the sample well of a lateral flow test cassette. The SARS-CoV-2 antigens, if present, bind to the detection particles on the test strip and are then spatially isolated in a specific region containing antibodies to produce a fluorescent line. This FIA provides automated results in 15 min using the Sofia 2 and Sofia analyzers (toaster size platforms for florescent detection), thus enabling rapid testing at near-patient settings. This assay was tested using 209 nasal and nasopharyngeal swabs spiked with heat-inactivated SARS-CoV-2, and a clinical sensitivity of 80% and clinical specificity of 100% were obtained at a tissue culture infective dose ($TCID_{50}$) of 1.13×10^2/mL (56 pfu/mL). The "COVID-19 Ag Respi-Strip" from CORIS BioConcept is another antigen test that utilizes a dipstick for the detection of viral nucleocapsid proteins (Table S2) using colloidal gold nanoparticles functionalized with monoclonal antibodies that induce a color change on a test strip in the presence of the virus. This test demonstrated an LOD of 12×10^3 pfu/mL, as well as a clinical sensitivity of 60% and clinical specificity of 100%, obtained using 138 clinical nasopharyngeal swabs.[98]

Seo *et al.* reported a highly sensitive field-effect transistor (FET)-based platform for detecting SARS-CoV-2 antigens in clinical nasopharyngeal swab samples (Figure 8; Table S2). The sensor uses highly conducting graphene sheets functionalized with specific antibodies against the SARS-CoV-2 spike protein. The performance of the sensor was determined using purified antigens, cultured virus, and nasopharyngeal swab specimens from COVID-19 patients. The device was capable of detecting the SARS-CoV-2 spike protein at concentrations as low as 1 fg/mL in phosphate-buffered saline and 100 fg/mL in clinical transport medium. In addition, this FET-based sensor successfully detected SARS-CoV-2 in culture medium (LOD: 16 pfu/mL) and clinical samples (LOD: 242 copies/mL).[99] Specificity testing revealed that the antibody binds to

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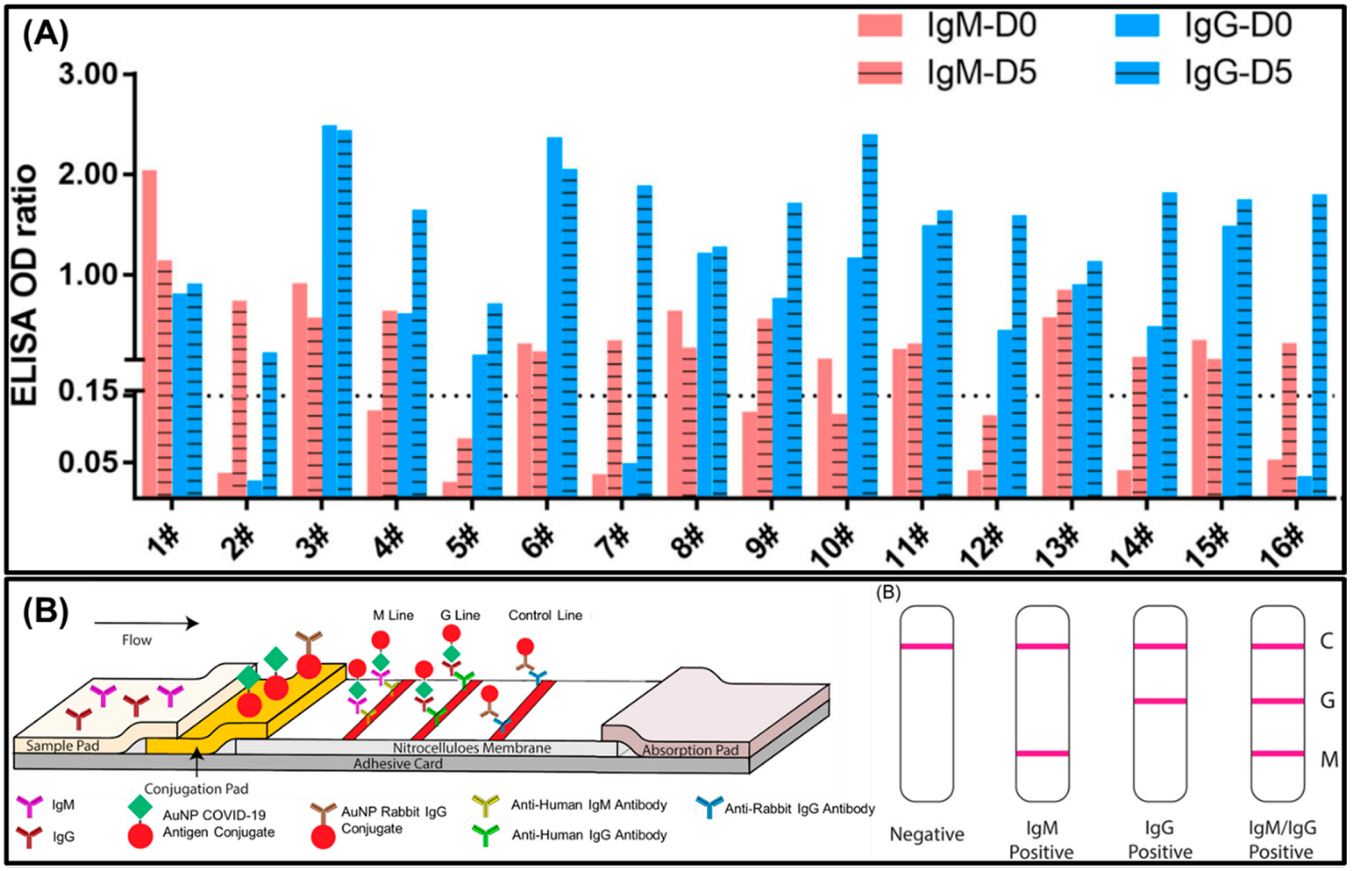

Figure 9. Serological testing. (A) Antibody response on 16 COVID-19 positive samples using an ELISA kit. The dashed line indicates the cut-off, determined based on data from healthy controls (Reprinted with permission from ref 101. Copyright 2020 Taylor and Francis). (B) LFS immunoassay for simultaneous detection of IgM and IgG antibodies against COVID-19 (Reprinted with permission from ref 105. Copyright 2020 John Wiley and Sons).

the SARS-CoV-2 spike protein but not to the MERS-CoV spike protein or bovine serum albumin (BSA). A thorough investigation is needed to demonstrate cross reactivity with other coronaviruses.

Despite being ideally positioned for the POC diagnosis of COVID-19 due to rapid sample-to-result time and compatibility with visual and instrument-free readout, a major drawback of antigen detection platforms is their low clinical sensitivity at low viral loads. This stems from the fact that these tests do not use an amplification step, thus requiring that the viral loads remain adequately high to produce a detectable signal. Another factor affecting the efficacy of viral protein detection is the unavailability of antibodies specific to the targeted proteins. This can be mitigated by utilizing aptamer or peptide chemistry; however, given the high similarity of SARS-CoV-2 proteins with the MERS and SARS-CoV proteins,[95] it is required to carefully select the targeted epitopes on SARS-CoV-2 proteins for probe development to avoid cross reactivity.

Antibody Testing. Detection of the viral RNA and antigens can be challenging due to the variation of viral load over the course of the disease and the possibility of mutations in the viral genome.[9] Particularly in the early stage of the disease, the viral nucleic acid and protein tests of infected individuals may turn out to be negative while it has been shown that the patient's body has built immunity.[100] The antibodies produced in response to SARS-CoV-2 proteins offer a wider window of time for indirect diagnosis of COVID-19 and for monitoring disease progression. These serological tests are also essential for understanding the epidemiology of SARS-CoV-2 and may provide answers pertaining to the scope of infection such as transmissibility, virulence, and mortality rate.[100] Recent studies have shown that IgM and IgG antibodies are detectable up to 22 and 48 days, respectively, following COVID-19 symptom presentation in a patient.[20,101,102] Furthermore, even though the viral load is low during the recovery stage, the levels of IgG and IgM antibodies are reported to be approximately 4-fold higher in this stage compared to the acute phase, making them ideal markers for the surveillance of COVID-19 and identification of convalescent plasma donors.[20,103]

The antibody detection methods include colloidal gold immunochromatography, enzyme-linked immunosorbent assay (ELISA), and chemiluminescence immunoassay.[20] Zhang *et al.* introduced the first antibody response study on SARS-CoV-2 since the identification of the virus.[101] They developed an in-house ELISA kit to detect IgG and IgM antibodies using a cross-reactive N protein from another SARS-related virus, Rp3, which is 92% identical to SARS-CoV-2. After testing 16 COVID-19 positive patient specimens (blood, oral and anal swabs) for IgG and IgM, they discovered that the antibody titers were elevated over the course of 5 days from the onset of symptom presentation (Figure 9A). On the first day of sample collection (D0), 50% and 81% of patients tested positive for IgM and IgG antibodies, respectively with 81% and 100% of patients testing positive for IgM and IgG, respectively, on the

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fifth day (D5).[101] One general limitation of serological testing is that there is a time delay between the start of infection and the generation of detectible antibodies, making these tests more suitable for disease and epidemiological monitoring than diagnostics.[104]

Autobio Diagnostics has recently introduced an FDA EUA designated (April 2020) anti-SARS-CoV-2 LFS immunoassay (Table S1) for rapid detection of IgG and IgM antibodies in plasma and serum within 15−20 min. The device consists of a cassette with two test strips for each antibody. The test strips are selectively precoated with anti-human monoclonal antibodies (anti-IgG and anti-IgM). SARS-CoV-2 recombinant spike protein antigens are conjugated with colloidal gold nanoparticles and then deposited to the test reservoirs, where conjugation between the antibodies and gold-labeled antigens is initiated. In the presence of IgG and IgM antibodies in the samples, the labeled gold colorimetric reagents generate a visible red/pink-colored band in IgM and IgG designated strips. Validation studies were performed on 717 clinical specimens and the outcomes were compared to the SARS-CoV-2 PCR test results. Respiratory tract specimens were collected for PCR testing between 1 to 7 days after the onset of symptoms. Serum and plasma samples were collected for the antibody tests between 1 and >30 days after specimen collection for the PCR tests. This serological LFS immunoassay reveals overall positive and negative agreements of 88.2% and 99.0%, respectively.[106] This device only works with serum or plasma rather than whole blood, which limits its use to laboratories. As of August 6, 2020, the FDA EUA designation of this anti-SARS-CoV-2 rapid test has been revoked. Li *et al.* developed a paper-based POC LFS immunoassay (Table S2) for the simultaneous detection of IgM and IgG antibodies against SARS-CoV-2 in blood.[105] The test kit includes a sample dilution buffer and a cartridge enclosing a test strip containing a sample well, a conjugation zone, and three detection lines (Figure 9B): M line (containing anti-human IgM antibodies), G line (containing anti-human IgG antibodies), and C line (control band, containing anti-rabbit IgG antibody). The conjugation pad contains colloidal gold nanoparticles (AuNP) labeled with recombinant antigen from SARS-CoV-2 and AuNP-rabbit IgG. IgG and IgM antibodies are captured by the AuNP-SARS-CoV-2-antigen conjugates. As the AuNP-conjugated IgM and IgG antibodies pass through the strip, they bind with antibodies immobilized on the M and G lines, respectively, changing the color of the strips to purplish-red. In a validation study including 525 patient blood samples, these tests demonstrated a clinical sensitivity of 88.7% when considering either biomarker and 64.5% when simultaneously detecting both IgM and IgG antibodies, as well as a clinical specificity of 90.6%. The simplicity of this rapid IgM/IgG test and its compatibility with no or rudimentary readout equipment make it ideally suited for POC applications. The downside to this LFS test is that negative results do not conclusively rule out the possibility of a viral infection, and follow-up nucleic acid tests are necessary. Furthermore, positive results may also stem from a current or previous infection with other coronaviruses.[105]

Antibody profiling against various SARS-CoV-2 proteins can guide the discovery of biomarkers that are useful for the control and treatment of COVID-19. An immuno-proteomic microarray for SARS-CoV-2 has been developed by Jiang *et al.* to analyze IgG and IgM antibody responses in the sera of 29 recuperating COVID-19 patients. As expected, high IgG and IgM antibody responses were observed against SARS-CoV-2 proteins, particularly N and S1 proteins (a subunit of spike protein).[107,108] It was shown that IgG response against the S1 protein is directly correlated with the concentration of lactate dehydrogenase (LDH), while it is inversely correlated with lymphocyte percentage. Other SARS-CoV-2 proteins such as ORF9b (accessory protein 9b) and NSP5 (non-structural protein 5) also demonstrate significant antibody responses. This SARS-CoV-2 proteome microarray provides antibody profiling capabilities that support new diagnostic, treatment, and vaccination research efforts;[107] however, it includes 18/28 of the proteins encoded in the genome of SARS-CoV-2,[90] none of which were prepared using mammalian cells, potentially affecting the antibody−antigen interactions. Moreover, only 29 clinical samples were tested and, as such, increasing the number of samples and diversifying the time point of specimen collection can further reveal the dynamics of antibody profiling. The company PEPperPRINT has developed a peptide-based proteomic microarray, PEPperCHIP, for serological testing of COVID-19.[109] They translated the entire SARS-CoV-2 viral proteome into overlapping peptides that are printed onto glass slides. Upon incubation of the glass slides with patient samples, the target antibodies (IgG and IgM antibodies) bind to epitopes recognized within individual peptides. The PEPperCHIP device can also facilitate the comparison of the resulting response profile across different samples to monitor B-cell responses over time, which can be used to study the correlation of autoimmune diseases with B-cell responses and COVID-19.[110]

POC antibody tests using the LFS design do not provide quantitative analysis important for assessing the immunity of patients to future infections. Additionally, these tests commonly suffer from low specificity[105,111,112] caused by the cross-reactivity of employed antigens with other coronavirus antibodies, Epstein−Barr virus, rheumatoid factor, and heterophile antibodies, making antigen selection the key to developing specific antibody tests.[37,112]

PATIENT RESPONSE BIOMARKER TESTING

Some COVID-19 patients rapidly develop acute respiratory distress syndrome (ARDS) along with other severe complications, leading to multiorgan failure.[113] Interestingly, a majority of these severely ill patients do not exhibit acute clinical symptoms in the early phase of the disease, making early diagnosis and treatment of severe COVID-19 paramount to successful patient outcomes.[114] There are several proteins and cellular markers that can be tested for follow-up monitoring, determination of disease severity, and formulation of treatment plans. Some of these biomarkers include C-reactive protein (CRP), ferritin, D-dimer, lymphocytes, LDH, cytokines (e.g., interleukin-6 (IL-6)), glucose, and angiotensin-converting enzyme 2 (ACE2).[20,115,116] Chen *et al.* conducted a study on 99 confirmed COVID-19 patients in Wuhan Jinyintan Hospital from January 1−20, 2020. Findings reveal that COVID-19 positive patients exhibited a decrease in lymphocyte count ($0.9 \pm 0.5 \times 10^9$ /mL), from the physiologically normal range ($1.1-3.2 \times 10^6$), with elevated levels for lactate dehydrogenase (LDH) (260.0−447.0 U/L; healthy range of 120.0−250.0 U/L) and glucose (7.4 ± 3.4 mmol/L; healthy range of 3.9−6.1 mmol/L). A marked increase in the concentration of infection-related biomarkers such as IL-6 (6.1−10.6 pg/mL), ferritin (808.7 ± 490.7 ng/mL), and CRP (51.4 ± 41.8 mg/L) from the normal range (0.0−7.0 pg/mL, 21.0−274.7 ng/mL, and

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0.0−5.0 mg/L respectively) was also seen in positive patient samples, indicating the potential applicability of these biomarkers in predicting COVID-19 outcomes.[113] Another hematological study performed by Gao *et al.* on 43 COVID-19 adult patients was used to compare the changes in glucose, CRP, IL6, and D-dimer in severe versus mild cases of the disease. This study indicated that the concentrations of glucose (median: 7.7 mmol/L; 5.3−9.9 mmol/L versus median: 6.0 mmol/L; 5.5−7.1 mmol/L), CRP (39.4 ± 27.7 mg/L versus18.8 ± 22.2 mg/L), IL-6 (median: 36.1 pg/mL; 23.0−59.2 pg/mL versus median: 10.6 pg/mL; 5.1−24.2 pg/mL), and D-dimers (median:490.0 ng/L; 290.0−910 ng/L versus median: 210 ng/L; 190−270 ng/L) were higher in the severe versus the mild groups.[117]

Uncontrolled immune responses triggered by systemic cytokine storms, which unleash an excessive level of cytokines such as IL-1, IL-1β, IL-6, IL-8, TNF-α, and granulocyte-macrophage colony-stimulating factor (GM-CSF), are a leading cause of ARDs.[114,115,118] IL-6 is released by immune cells, upon activation by viruses or bacteria, to stimulate other immune cells. Since IL-6 is released during the initial stages of an infection, it can be utilized as a biomarker to assist healthcare professionals in early identification of critically ill COVID-19 patients. Typically, the detection of IL-6 is performed using standard ELISA;[119] however, the FDA has issued an EUA for the Elecsys IL-6 immunoassay (June 2020), developed by Roche (Basel, Switzerland), for the quantitative detection of IL-6 in serum or plasma collected from COVID-19 patients (Table S1). In this assay, patient serum or plasma is incubated with a biotinylated monoclonal IL-6-specific antibody (Ab1), followed by incubation with a monoclonal IL-6-specific antibody tagged with a ruthenium complex (Ab2) and streptavidin-coated magnetic microparticles (MPs) to form a sandwich conjugate (MP-Ab1-Ag-Ab2), which is then placed at an electrode using an external magnet. The ruthenium complex mediates the detection of IL-6 using electrochemiluminescence.[120] The LOD of the assay is estimated as 1.5 pg/mL with a test time of 18 min, and a throughput of up to 300 tests/h. No substantial cross-reactivity was reported in samples spiked with 50 000 pg/mL of other cytokines such as IL-1α, IL-1β, IL-2, IL-3, IL-4, IL-8, IL-γ, and TNF-α.[120] The clinical performance of Elecsys IL-6 was evaluated using a data set of 49 PCR-confirmed symptomatic COVID-19 patients, where 19 of them were in critical conditions and required mechanical ventilation. Using a cut-off of 35 pg/mL, the assay was able to identify 16 of the 19 patients that required respiratory support ([IL-6] > 35 pg/mL), leading to a clinical sensitivity of 84.2%. Of the 30 patients that did not require mechanical ventilation, 19 of them were recognized by the Elecsys IL-6 test ([IL-6] ≤ 35 pg/mL), which resulted in clinical specificity of 63.3% (Figure 10). This clinical specificity suggests that Elecsys IL-6 test should be employed in conjugation with other biomarker tests (such as CRP tests) in order to identify severe cases that require mechanical ventilations.[121] Elecsys IL-6 requires large benchtop equipment and sample preparation steps for plasma/serum separation from blood, thus limiting its usage to hospitals and centralized laboratories.[119] Alba-Patiño *et al.* recently introduced a nanoparticle-based mobile biosensor for rapid detection of IL-6 in whole blood. This biosensor employs a paper immunoassay and gold nanoparticles for colorimetric detection of IL-6, which can be read using a smart phone. This assay

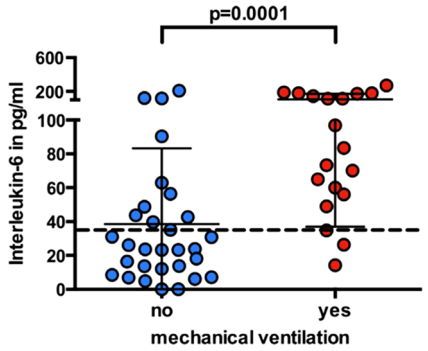

Figure 10. Clinical performance of Elecsys IL-6. Data are represented as means (SDs) (Reprinted with permission from ref 121. Copyright 2020 Elsevier).

demonstrated an LOD of 12.5 pg/mL using IL-6 spiked blood with an assay duration of 18 min.[119]

ACE2 is highly expressed by epithelial cells of the lung, intestine, kidney, blood vessels, and mucosa of the oral cavity.[122,123] The entry of SARS-CoV-2 into the host cells is facilitated by the binding of spike proteins to ACE2 receptors on the surface of host cells.[124,125] Upon this binding event, ACE2 can undergo ADAM17 (a disintegrin and metalloproteinase 17)-mediated ectodomain shedding form the cells resulting in circulating ACE2 with catalytic and bioactive capability.[126] Similar to neutralizing antibodies, circulating ACE2 can potentially inhibit spike proteins and prevent the virus from further spreading to target cells.[127] Recent research suggests that COVID-19 mortality rate is higher in >60 year old men with existing chronic diseases (such as hypertension, cardiovascular diseases, diabetes, *etc.*) and secondary ARDS,[128] which might be related to the declined level of ACE2 activity in these patients.[129] Therefore, monitoring the level of circulating ACE2 may assist with the prognosis of COVID-19.[127] It is also hypothesized that ACE2 spike protein-based vaccine and recombinant human ACE2 may be used for COVID-19 treatment.[129] In this context, rapid, sensitive, and accurate tests are needed to measure the level of circulating ACE2 in accessible physiological fluids such as blood, saliva, and urine.

The difficulty in using the biomarkers discussed in this section for COVID-19 management is their clinically variable range and their lack of specificity to COVID-19. Since deviation from the clinical range for these biomarkers can be related to other diseases and infections, these tests should be used as a complementary tool with molecular and viral antigen tests to predict patient outcomes. The integration of test data from SARS-CoV-2 RNA, antigens, antibodies, and other biomarkers is critically needed to generate diagnostic, prognostic, and predictive information and guide the physicians in effective data-driven treatment decision making.

EMERGENCE OF AI FOR THE DIAGNOSIS AND PROGNOSIS OF COVID-19

In principle, AI has the potential to learn from a constant influx of data related to COVID-19 to recognize patterns (diagnosis), explain behaviors, and predict future outcomes.[130] Lately, there has been a large surge of research focusing on training AI models to diagnose COVID-19 via X-ray and CT chest radiography images. A recent review by Bullock *et al.* argues

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that AI can be as accurate as humans in COVID-19 diagnosis.[131]

Rohaim *et al.* developed a hand-held colorimetric AI-assisted RT-LAMP device for rapid detection of SARS-CoV-2 RNA.[132] An automated image acquisition system and AI-based image processing models were used to reduce the analysis time of the RT-LAMP assay (30 min) and avoid any subjectivity related to operator interpretation of the colorimetric RT-LAMP results. Two separate AI-assisted image processing algorithms were evaluated in this study, Sum of Absolute Difference (SAD) and deep learning Convolutional Neural Network (CNN). The SAD algorithm was able to identify SARS-CoV-2 infected samples with 81.25% accuracy. A limitation of the SAD algorithm was observed when the model was not able to achieve a common threshold value for some of the image sets due to the existence of bubbles and alterations in lighting in the test tubes. In contrast, deep learning CNN models can automatically recognize concealed patterns from given data sets, with no need for any domain knowledge. In this paper, a data set with 4821 cropped images were used for AI training. In addition, a software application was implemented to automatically read the data in clusters from the data set and feed it to the algorithm. This feature enhanced the memory efficiency and real-time data augmentation. The deep learning CNN algorithm was tested using 891 test tube images that were not introduced to the model before. The model was able to detect the tubes containing infected samples with an accuracy of 98%. Once the sample is identified as positive, the process will stop, and the results are returned. This AI-assisted colorimetric detection was able to sense a clear color change as early as 20 min depending on the viral loads; however, clinical studies are still needed to validate the performance of this platform in real clinical situations.

The use of AI in predicting the severity of COVID-19 can assist healthcare professionals in classifying critically ill COVID-19 patients from asymptomatic cases, thus allocating resources more efficiently.[130] Jiang *et al.* used an AI model (predictive analytics) to learn past medical history data acquired from 53 COVID-19 positive patients from two hospitals in china to predict patients at risk of developing ARDS with 80% accuracy.[133] An obvious drawback of this study is the size of the data set which limits the clinical spectrum of COVID-19 severity. This AI model requires further refinement and validation with an expanded clinical data set.

Despite the promise of AI for use in COVID-19 diagnosis and prognosis, only a few models have the operational maturity to perform effectively given the lack of historical COVID-19 data. In most of these studies, CT scans, biomarker profiles, and genome sequence data sets are limited to certain hospitals.[134] To apply AI in a clinical setting, the current regulatory and quality frameworks must be considered to enable AI-based decision making while respecting privacy laws.[131]

CONCLUSION AND FUTURE OUTLOOK

COVID-19 diagnostic technologies have emerged as means for containing the pandemic, preventing its potential future waves and the safe and measured reopening of the economy. In the ever-evolving race toward widespread and accurate testing, conventional nucleic acid detection techniques such as RT-PCR are the well-established front runners. However, time-consuming sample preparation, need for complex laboratory infrastructure and highly trained technical personnel, reagent shortages, and false-negative outcomes stemming from low viral loads or erroneous sample collection methods have fueled the adaptation of other RNA-based methods. Methods based on isothermal amplification and gene editing (CRISPR/Cas) have demonstrated great potential for developing POC tests, mostly based on lateral flow strips, that operate with simple instrumentation and process flow, opening the route toward do-it-yourself and home-based testing. Despite the current progress in developing RNA-based POC diagnostic devices for COVID-19, it is critically needed to validate the performance and reliability of these technologies with real-life clinical samples to assess their true clinical applicability and obtain regulatory approval, overcome limitations related to separate sample preparation steps, minimize user exposure to the virus, and solve issues related to reagent and device manufacturing scale-up.[37]

In addition, assays for analyzing viral antigens, human antibodies, and other immunological biomarkers (*e.g.*, cytokines) have been developed for diagnosing COVID-19, performing epidemiological assessment of the recovered patients, and monitoring the immune response of the patients over the course of the disease and during vaccine clinical trials, respectively. These tests analyze protein biomarkers, enabling them to operate without nucleic acid amplification. These assays have the benefit of facile integration into POC assays (e.g., LFS); however, they have the drawback of reduced sensitivity and specificity, especially when testing with crude clinical samples particularly blood.

Although each of the above-mentioned classes of diagnostic technology offer advantages and disadvantages, data combined from multiple technologies are critically needed for early diagnosis, treatment selection, disease monitoring, epidemiological surveillance, and vaccine and treatment development. Platform and data integration in conjunction with AI are expected to combine COVID-19 diagnosis with predictive analysis and prognosis to enable more effective treatment decision making and disease management. However, due to the limited COVID-19-specific data sets, AI is far from implementation for immediate COVID-19 analysis. In the meantime, it is imperative to support ongoing and widespread collection of COVID-19 diagnostic data to train AI for better diagnosis and prognosis of the disease in the future.

Finally, the scientific research in the area of molecular diagnostics has been intensified over the past few months in the fight against COVID-19; however, it builds on decades of innovation in this area. Similarly, the new knowledge and technologies developed in the context of COVID-19 will help advance the diagnostic field for the immediate use, but more importantly toward building preparedness for the future potential infectious disease outbreaks.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssensors.0c01377.

Summary of the existing and emerging COVID-19 diagnostic tests (PDF)

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Notes

The authors declare no competing financial interest.

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VOCABULARY

Clinical Sensitivity, the frequency of positive test results in patients with the disease; Clinical specificity, the frequency of negative results in patients without the disease; Limit of Detection (LOD), the quantity or concentration of a given analyte that can be detected with reasonable certainty for a given analytical method; Point-of-Care Tests, diagnostic tests that can be performed at the vicinity of the patient at the time and place of patient care; Artificial intelligence, the ability of a computer to perform tasks, such as visual perception and decision-making, that generally require human intelligence

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