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Perspective

Molecular Diagnosis of COVID-19: Challenges and Research Needs

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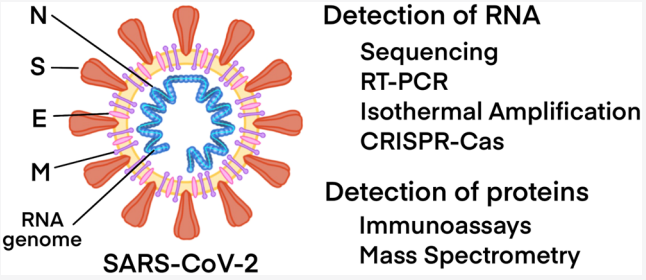
ABSTRACT: Molecular diagnosis of COVID-19 primarily relies on the detection of RNA of the SARS-CoV-2 virus, the causative infectious agent of the pandemic. Reverse transcription polymerase chain reaction (RT-PCR) enables sensitive detection of specific sequences of genes that encode the RNA dependent RNA polymerase (RdRP), nucleocapsid (N), envelope (E), and spike (S) proteins of the virus. Although RT-PCR tests have been widely used and many alternative assays have been developed, the current testing capacity and availability cannot meet the unprecedented global demands for rapid, reliable, and widely accessible molecular diagnosis. Challenges remain throughout the entire analytical process, from the collection and treatment of specimens to the amplification and detection of viral RNA and the validation of clinical sensitivity and specificity. We highlight the main issues surrounding molecular diagnosis of COVID-19, including false negatives from the detection of viral RNA, temporal variations of viral loads, selection and treatment of specimens, and limiting factors in detecting viral proteins. We discuss critical research needs, such as improvements in RT-PCR, development of alternative nucleic acid amplification techniques, incorporating CRISPR technology for point-of-care (POC) applications, validation of POC tests, and sequencing of viral RNA and its mutations. Improved assays are also needed for environmental surveillance or wastewater-based epidemiology, which gauges infection on the community level through analyses of viral components in the community's wastewater. Public health surveillance benefits from large-scale analyses of antibodies in serum, although the current serological tests do not quantify neutralizing antibodies. Further advances in analytical technology and research through multidisciplinary collaboration will contribute to the development of mitigation strategies, therapeutics, and vaccines. Lessons learned from molecular diagnosis of COVID-19 are valuable for better preparedness in response to other infectious diseases.



The coronavirus disease of 2019 (COVID-19) has resulted in nearly 8 million reported cases and more than 430 000 deaths worldwide, as of June 15, 2020. The causative infectious agent of this pandemic is the severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2).[1−4] The newest addition to the *Coronaviridae* family and the *Betacoronavirus* genus, SARS-CoV-2 joins the previously known SARS-CoV and Middle East respiratory syndrome coronavirus (MERS-CoV). These viruses are so named due to their *corona*-like (the Latin word for crown) appearance under electron microscopy and their potential for causing acute respiratory distress syndrome (ARDS). Coronavirus-induced ARDS is characterized by cytokine storm syndrome, which is a positive feedback loop that can cause a surge of pulmonary inflammation and fluid extravasation causing respiratory failure. ARDS, respiratory and/or cardiac failure, and sepsis due to secondary bacterial infection are major causes of mortality due to COVID-19.[4−7]

SARS-CoV-2 is an enveloped positive sense single-stranded RNA (ssRNA) virus, which means that the viral capsid is enclosed within a lipid bilayer and that the viral genome, not its complementary sequence, encodes viral proteins. Coronavirus genomes, ranging from 26 kb to 32 kb in length, are the largest of any RNA virus. An infectious SARS-CoV-2 virion (~100 nm in diameter) contains the 29 903 nucleotide (nt) RNA genome, along with four structural proteins: envelope (E), nucleocapsid (N), membrane (M), and spike (S) proteins (Supporting Information Table S1).[1,2] N proteins bind the RNA genome in a helical symmetry, resembling beads on a string, and this genome structure is surrounded by a lipid bilayer that is embedded with E, M, and S proteins.

Infection of SARS-CoV-2 is mediated by binding of the spike protein to angiotensin-converting enzyme 2 (ACE2) receptors on the surface of host cells.[8,9] In the host cells, the RNA genome of SARS-CoV-2 is replicated and the four

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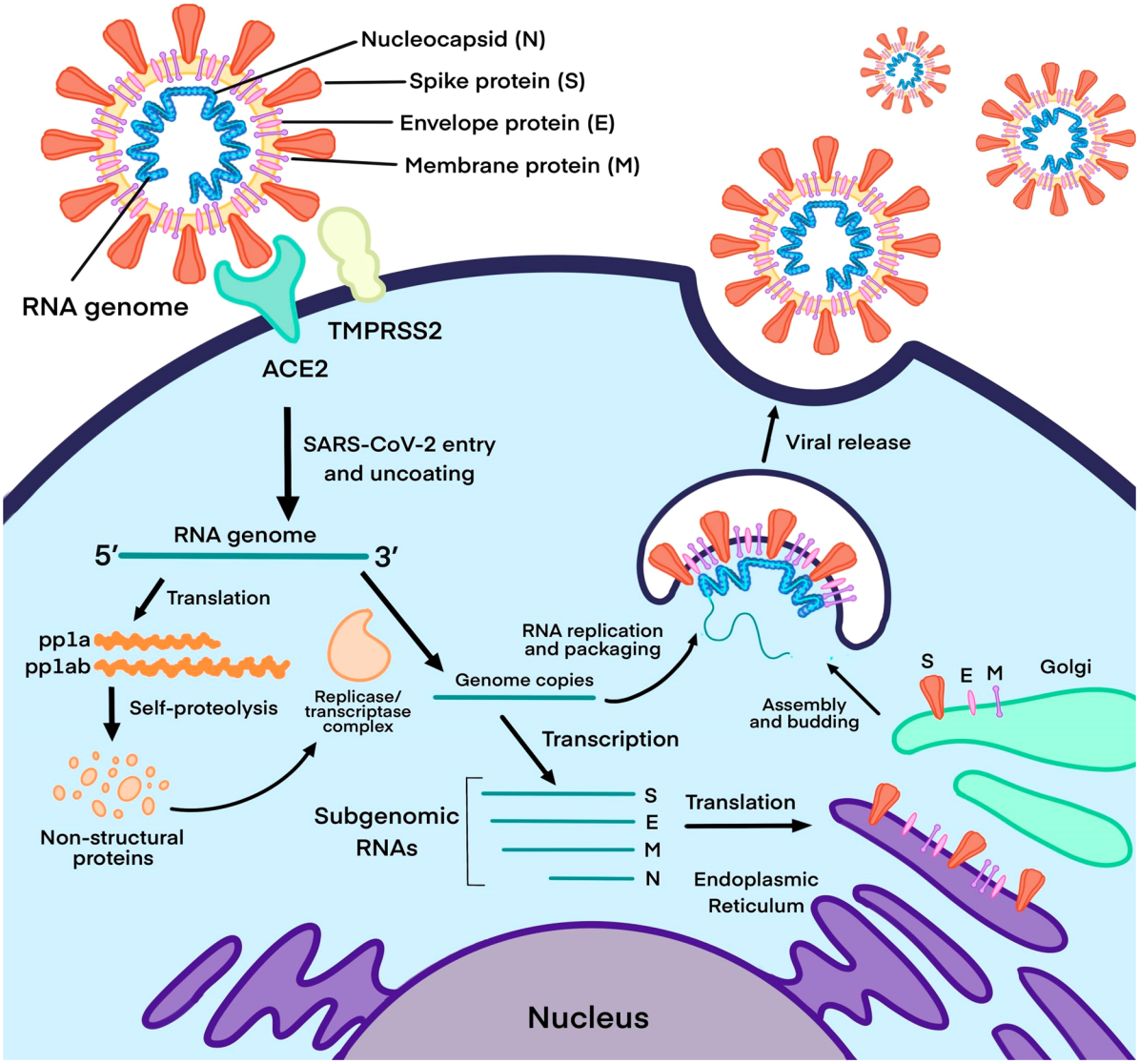

Figure 1. Schematic presentation of SARS-CoV-2 and its life cycle.[9−11] Infection of SARS-CoV-2 is mediated by binding of the receptor binding domain of the S1 region of spike protein to angiotensin-converting enzyme 2 (ACE2) receptors on the surface of host cells.[8,9] The spike protein is subsequently primed by cleavage at the S1/S2 site by the transmembrane protease serine 2 (TMPRSS2),[9] which exposes a fusion peptide that merges viral and cell plasma membranes. This membrane fusion at the cell surface deposits the genome into the cytoplasm, leading to translation of *ORF1a* and *ORF1b* and production of the polyprotein 1a (pp1a) and pp1ab, respectively. Pp1a and pp1ab are self-cleaved into 16 nonstructural proteins (Nsps) by the viral proteases Nsp3 and Nsp5. Nsps 1 to 16 coalesce to form a replicase/transcriptase complex (RTC) containing multiple enzymes, such as the Nsp7-Nsp8 primase, the Nsp12 RNA dependent RNA polymerase (RdRp), the Nsp13 helicase/triphosphatase, the Nsp14 exoribonuclease, the Nsp15 endonuclease, and the Nsp10-Nsp16 N7- and 2′O-methyltransferases.[2,13] Within this RTC, the RdRp polymerizes full length and partial length RNA complementary to the viral genome (negative sense RNA) which serve as templates for nascent synthesis of positive sense RNA genomes as well as subgenomic RNA species. The subgenomic RNAs encode the aforementioned structural proteins (E, M, S, N) as well as putative accessory proteins.[10,11] The E, M, and S proteins enter the endoplasmic reticulum (ER), and the N proteins bind positive sense RNA genomes, and these virion components are subsequently combined in the ER-Golgi apparatus compartment (ERGIC). These newly formed SARS-CoV-2 viruses are then released from cells through vesicle transport (exocytosis).

structural (E, M, S, N) and 25 nonstructural proteins are synthesized.[10,11] Packaging of the genomic RNA with the structural proteins results in the formation of new SARS-CoV-2 viruses (Figure 1).

Coronaviruses replicate RNA genomes and subgenomic RNAs exclusively from RNA templates and do not require a DNA step in their viral life cycle. Unique to coronaviruses, the 3′ to 5′ exonuclease activity of nonstructural protein 14 (Nsp14) confers proofreading, thereby enhancing genomic replication fidelity. Unlike other RNA viruses that undergo error-prone replication, coronaviruses use Nsp14 exonuclease, which is the first identified proofreading enzyme encoded by an RNA virus and is likely an adaptation to accommodate the large RNA genomes of coronaviruses.[12] This proofreading function implies that coronaviruses mutate at a less frequent rate than other RNA viruses.

Molecular diagnosis of COVID-19 primarily relies on the detection of RNA of the SARS-CoV-2 virus.[14−16] Detection of viral proteins is also useful, although it has not yet been applied to the diagnosis of COVID-19. Seroconversion is approximately 13 days (median) for IgM and IgG.[17] Many test kits for the detection of IgM and IgG antibodies in human serum

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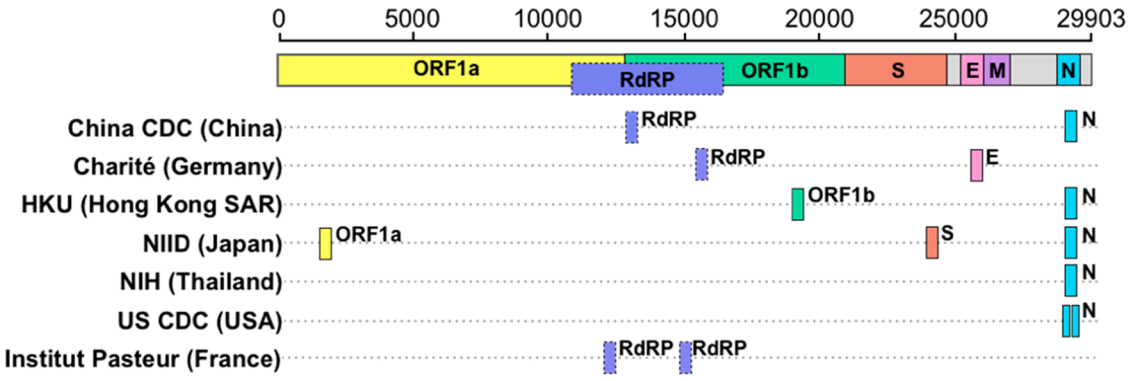

Figure 2. Genome organization of SARS-CoV-2 and the relative positions of gene targets detected using seven reverse transcription polymerase chain reaction (RT-PCR) methods shared by the World Health Organization (WHO) as its in-house assays.[37] ORF, open reading frame; RdRP, RNA-dependent RNA polymerase; S, spike protein; E, envelope protein; and N, nucleocapsid protein.

have been developed. The promises and challenges of antibody testing have captured the world's attention.[18,19] However, molecular diagnosis of COVID-19 is faced with many challenges. For example, the variable and very low viral loads in different types of specimens collected at different times during the course of the infection (Table S2) present a wide range of challenges from sample collection, handling, and treatment to analytical specificity and limit of detection. Additionally, the dynamic humoral response to SARS-CoV-2 exposure causes challenges for serological testing. These analytical challenges directly impact the validity of molecular diagnosis, including concerns over clinical sensitivity (the percentage of sick individuals correctly identified as sick) and clinical specificity (the percentage of healthy individuals correctly identified as healthy). In this Perspective, we discuss strategies for the molecular diagnosis of COVID-19, highlight challenges in detecting SARS-CoV-2, and identify opportunities to assist in finding solutions to these challenges. We focus on molecular detection of the viral RNA and proteins of SARS-CoV-2.

DETECTION OF VIRAL RNA

Metagenomic next generation sequencing (NGS) was used to identify and discover SARS-CoV-2, the causative agent of COVID-19, at the time of the initial outbreak.[1] Total RNA was extracted from a bronchoalveolar lavage fluid sample. RNA was reverse transcribed to DNA and amplified using polymerase chain reaction (PCR). Ribosomal RNA (rRNA) was depleted during this process. The amplified DNA was sequenced using a method of sequencing by synthesis. The four different nucleotides were labeled with different fluorophores, so the sequence was revealed by monitoring the fluorescence generated during polymerase elongation. Reads in short length (150 bp) were assembled and aligned with databases, and human nucleic acids were excluded. The full SARS-CoV-2 genome was obtained by analyzing the longest contigs (overlapping DNA regions) which were also sequences in high abundance. Wu et al.[1] reported the first SARS-CoV-2 genome sequence (GeneBank accession number MN908947) on January 7, 2020. Since then, the number of released SARS-CoV-19 sequences has increased dramatically. As of June 15, 2020, more than 46 000 SARS-CoV-2 genome sequences have been publicly shared under the Global Initiative on Sharing All Influenza Data (GISAID).[20]

The early identification and sequencing of SARS-CoV-2 by Wu et al.[1] and Zhou et al.[2] enabled the rapid development of reverse transcription (RT) PCR techniques for detecting specific sequences of the SARS-CoV-2 genome[21] (Figure 2). At the beginning of the COVID-19 pandemic, presumptive cases were identified using RT-PCR assays and confirmed with genome sequencing. Because genome sequencing is more expensive and time-consuming than RT-PCR, current molecular diagnosis of COVID-19 is primarily based on RT-PCR detection of viral RNA of SARS-CoV-2. Although RT-PCR is a well-established technique, challenges remain in the overall process of detecting the viral RNA, from the initial sample collection and treatment to the subsequent amplification and detection.

Collection of Specimens. Viral loads of SARS-CoV-2 differ in various types of clinical specimens. RT-PCR detection of SARS-CoV-2 in eight types of clinical specimens were compared: bronchoalveolar lavage fluid, fibrobronchoscope brush biopsy, sputum, nasal swabs, pharyngeal swabs, stool, blood, and urine.[22] The highest positive rates (93%) were observed for bronchoalveolar lavage fluid, followed by sputum (72%), nasal swab (63%), fibrobronchoscope brush biopsy (46%), pharyngeal swabs (32%), stool (29%), blood (1%), and urine (0%). Thus, respiratory specimens are generally collected to detect SARS-CoV-2.[23−25] Recent studies[23,26,27] have revealed that viral loads of SARS-CoV-2 in upper respiratory specimens reach peaks soon after symptom onset and decline with time after 1 week. The viral load profile of SARS-CoV-2 is more similar to that of influenza, which is present at high levels at around the time of symptom onset. In contrast, the viral loads of SARS-CoV and MERS-CoV reach peaks at around 10 days and 14 days, respectively.

The World Health Organization (WHO) has recommended nasopharyngeal swabs, oropharyngeal swabs, and nasopharyngeal or endotracheal washes as upper respiratory specimens in ambulatory patients.[14] Nasopharyngeal swab specimens are usually collected because this method of collection is relatively less invasive. Lower respiratory specimens include sputum, endotracheal aspirate, and bronchoalveolar lavage. Sputum production relies on productive coughs from patients. Asymptomatic or presymptomatic patients may be unable to produce sputum. One challenge of collecting lower respiratory specimens is the risk of exposure to SARS-CoV-2 for personnel collecting specimens because of the requirement for close-contact with potential COVID-19 patients and irritation of respiratory airways during sampling. When requesting patients to produce sputum or probing with nasopharyngeal swabs, the

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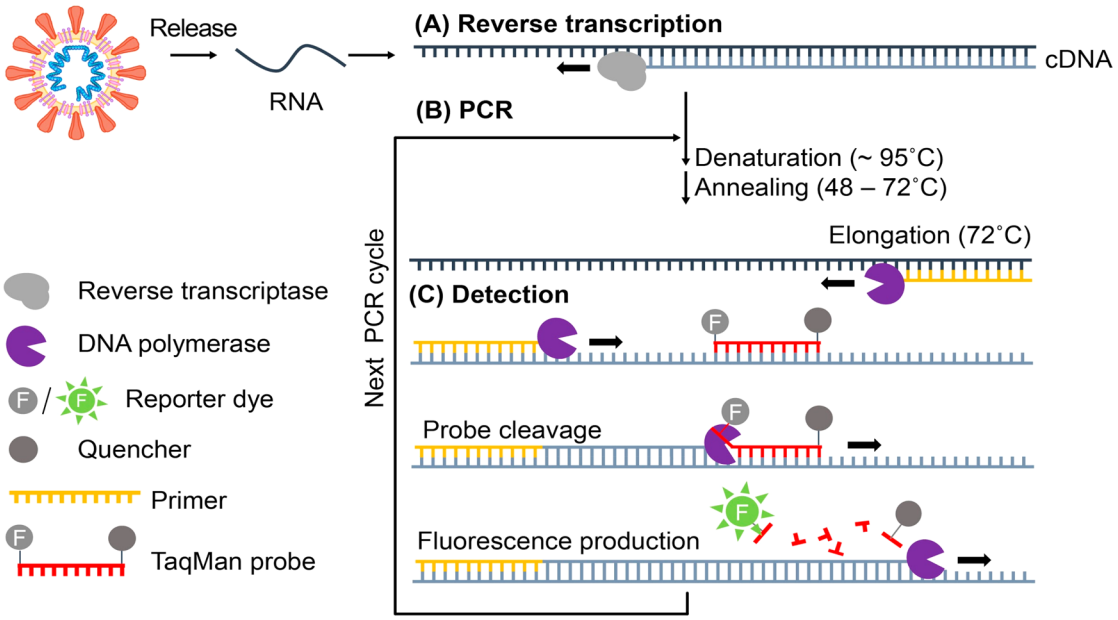

Figure 3. Principle of the RT-PCR assay. (A) Reverse transcription (RT) of the viral RNA produces complementary DNA (cDNA). (B) PCR amplification of the cDNA target starts from denaturation of RNA-cDNA hybrids, followed by annealing of a pair of primers to complementary sequences on the cDNA target and the complement of the cDNA target, and polymerase-dependent elongation of primers. (C) A custom-designed specific oligonucleotide sequence labeled with a reporter dye and a quencher at either end of the sequence serves as a detection probe. The intact probe emits no or negligible fluorescence because the close proximity of the fluorophore to the quencher results in fluorescence energy transfer and fluorescence quenching. When the cDNA is amplified, the amplicons act as templates to which the detection probes (e.g., TaqMan probes) bind. The DNA polymerase uses its 5′-3′ exonuclease activity to break down the TaqMan probe, separating the quencher from the fluorophore, restoring the fluorescence. For each strand of DNA amplified, a quencher is cleaved from the fluorophore. Increases in fluorescence intensity correspond to increased amounts of the amplicons.

sampling process could cause sneezing or coughing and the production of aerosols.

The WHO recommends using Dacron or polyester flocked swabs for collecting nasopharyngeal and oropharyngeal specimens and sterile containers for nasopharyngeal and oropharyngeal washes, sputum, endotracheal aspirates, and bronchoalveolar lavage fluid.[28] After collection, samples must be packaged appropriately and transported to the laboratory as soon as possible. Specimens should be shipped and stored at 2−8 °C until testing. The maximum storage time is 2 days for nasopharyngeal or oropharyngeal washes, sputum, endotracheal aspirate, and bronchoalveolar lavage and 5 days for nasopharyngeal and oropharyngeal swabs, serum, and whole blood.[14] Specimens that must be stored for longer than these recommended times should be kept at −70 °C. It is also important to ensure the adherence of adequate safety procedures during specimen collection, packaging, storage, and transport.

Release of Viral RNA. Because the genomic RNA is encased within the nucleocapsid of virions, the first step in the detection of SARS-CoV-2 in patient specimens is to extract high-quality viral RNA. RNA is extremely sensitive to degradation by ribonucleases (RNases). Improper RNA extraction causing loss or degradation of viral RNA contributes to poor clinical sensitivity.[29] Moreover, the RNA extraction process is time-consuming and requires laboratory equipment and trained personnel.

Table S3 (Supporting Information) lists the commercially available RNA extraction kits, their operational principles, and the length of time required for their use. RNA is conventionally extracted using organic solvents. One type of organic extraction uses phenol-guanidine isothiocyanate (GITC), which is capable of rapidly denaturing nucleases and stabilizing RNA. However, this method of RNA extraction is manually intensive, time-consuming, and requires the caustic GITC reagent. Residual salts and organic solvents also contaminate the extracted RNA. These contaminants, such as divalent cations, can inhibit downstream RT-PCR. RNA purification using magnetic particles and spin columns removes organic solvents and contaminants and is amenable for automation. RNA extraction/purification robotics and kits have been successfully developed with some being recommended by the United States Centers for Disease Control and Prevention (CDC).[21,29−31] However, the increasing demand for RNA extraction kits stresses supply chains and manufacturers worldwide, which hinders the capacity of rapid diagnosis of COVID-19.

Extraction-free techniques, such as those that lyse viral particles to release RNA for subsequent detection, are easier to perform and amenable for point-of-care (POC) testing. However, the development of techniques that do not require RNA extraction and purification must address issues of efficiency and matrix effects, such as the influence of complex specimen matrixes that can inhibit the downstream amplification process. For example, detergents added during sample treatment to deactivate viruses can inhibit PCR reaction.[32] Another challenge is the difficulty in accessing the viral RNA because it is packaged by viral proteins. One solution is to preheat samples to denature the viral proteins and release the RNA. Preheating of specimens followed by RT-PCR amplification and direct detection of SARS-CoV-2 RNA has been successfully applied to nasopharyngeal and oropharyngeal swab samples. However, the sensitivity of SARS-CoV-2 detection using RT-PCR in specimens pretreated with a

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heating step is poorer relative to those treated with RNA extraction kits but better than untreated samples.[33] A recent study by Pan et al.[34] has suggested that heat treatment to release RNA may adversely impact the ability of RT-PCR to detect specimens containing low viral loads, which can contribute to the number of false negatives.

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Assays. Targets for the detection of SARS-CoV-2 include the genes encoding for the N, E, and S proteins, the open reading frame 1ab (Orf1ab), and the RNA dependent RNA polymerase (RdRP) gene, which is located within Orf1ab[31,35] (Figure 2). The choice of targets affects the analytical specificity of RT-PCR assays. The E gene is highly conserved among all beta coronaviruses, and the N gene may cross-react with other coronaviruses.[13] The RdRP gene can be used to differentiate SARS-CoV-2 from the SARS-CoV virus.[2] The S gene is also useful for differentiating SARS-CoV-2 because it is highly divergent from other coronaviruses.[2] At the time of the outbreak, the WHO quickly made available seven RT-PCR assays for diagnosis of COVID-19 developed by scientists from around the world (Figure 2). These protocols provide access to standard technical guidelines for viral RNA detection. The protocols from Germany of the first real-time RT-PCR assays targeting the RdRP, E, and N genes of SARS-CoV-2 were published on January 23, 2020.[21] Their RT-PCR assay targeting the RdRP gene resulted in the highest analytical sensitivity (3.6 RNA copies/reaction at 95% detection probability).[21] In assays developed later, the use of the N gene as the RT-PCR target resulted in SARS-CoV-2 detection that was 10 times more sensitive than when the Orf1b gene was used.[31] Since then, the Foundation for the Innovation of Research Diagnostics (FIND), a WHO collaborating center, has collated commercially available tests to detect SARS-CoV-2.[36] Researchers can choose different targets or multiple targets of RT-PCR assays according to their needs.

Figure 3 depicts typical processes of RT-PCR assays for SARS-CoV-2. The reverse transcription reaction converts the viral RNA to complementary DNA (cDNA). PCR exponentially amplifies the cDNA, and the amplified DNA is detected in real time using fluorescence probes. As a new strand of DNA is synthesized, a hybridized reporter probe (e.g., TaqMan probe) is cleaved by the 5′ exonuclease activity of the polymerase and the quencher is removed from the fluorophore, resulting in the generation of fluorescence. Thus, the fluorescence intensity reflects real-time amplification of the DNA sequences and is used for quantitative detection of the target DNA. Another common strategy to produce fluorescence is to use double-stranded DNA (dsDNA) binding dyes (e.g., SYBR Green). TaqMan probes provide higher specificity than SYBR Green because hybridization of the TaqMan probes with specific target sequences is required to produce the fluorescence signal whereas SYBR Green is intercalated in any sequence of dsDNA.

Progress in RT-PCR assays for the detection of SARS-CoV-2 has been made very quickly, with many versions of RT-PCR assays and kits developed in-house or commercialized. As of early April 2020, 25 of the 28 products (89%) that were issued emergency use authorization (EUA) by the United States Food and Drug Administration (FDA) are RT-PCR test kits (Table S4).[38] However, several limitations and issues require further research. First, the positive detection rate was reported to be as low as only 30−60%, suggesting a high rate of false negatives.[35,39,40] Several factors were speculated to result in this high false negative rate, such as errors in sampling and inappropriate selection of clinical specimens,[41] individual variations in viral load,[42] and the differences in the operation procedure and the limit of detection (LOD) of various RT-PCR kits.[33] False negative results can also arise from PCR inhibitors present in improperly treated samples or extracts. Second, PCR requires controlled temperature cycling, refrigerated reagent storage, and professionally trained operators. These resources may not be widely accessible.

Isothermal Amplification Techniques with Point-of-Care (POC) Potential. Although RT-PCR is the gold standard for the molecular diagnosis of COVID-19,[3,21,43] its requirement of a thermal cycler is not ideal for POC applications. Alternative exponential amplification techniques, such as loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), rolling circle amplification (RCA), exponential amplification reaction (EXPAR), and exponential strand displacement amplification (E-SDA), can each be performed at a single temperature. Without requiring thermal cycling, these exponential amplification techniques have potential for POC applications. LAMP and RPA provide similar sensitivity achievable by PCR for detecting low copy number of nucleic acids, while other isothermal amplification techniques are unable to achieve the same level of sensitivity.[44]

RPA uses a recombinase-primer complex to open dsDNA targets, facilitating the primer to bind to its complementary sequence within the target, thereby circumventing the heat denaturing step typically required in PCR. Polymerase-dependent elongation of the primer uses the target dsDNA as templates to produce multiple copies of the original target. As the new copy is being synthesized, the double-stranded DNA template downstream is separated to single-stranded DNA (ssDNA) through the strand displacement activity of the polymerase. An ssDNA-binding protein (SSB) present in the RPA reagent cocktail temporarily binds to the ssDNA and prevents it from hybridizing back to dsDNA.[45] RPA can be completed in 10 min at a reaction temperature of 37−42 °C and is therefore amenable for the development of POC assays.

Also suitable for POC settings, LAMP typically operates at 60−65 °C to achieve exponential amplification of nucleic acid targets without requiring temperature cycling.[46] LAMP uses three pairs of primers:[47] two inner primers, two outer primers, and two loop primers. The inner and outer primers bind with the target and their extension produces a dumbbell structure composed of two stem-loops on either end. The loop and inner primers hybridize to the loop region of the dumbbell to initiate DNA amplification. The newly formed amplified DNA is double-stranded. The intrinsic strand-displacement activity of the polymerase enables multiple cycles of amplification by separating the dsDNA into ssDNA.[47]

Several RT-LAMP assays have been developed to target different gene regions of SARS-CoV-2,[48,49] with fluorescence[49] or colorimetric[48,50] readouts. One strategy to produce fluorescence is to use Calcein, a fluorescein complex.[51] The fluorescence of Calcein is initially quenched when Calcein is bound to manganese. The pyrophosphates generated from the DNA amplification reactions sequester the manganese and release the Calcein. Free Calcein can bind to magnesium ions, increasing the intensity of its fluorescence emission. Using Calcein, Yan et al.[49] developed an RT-LAMP assay for the visual detection of SARS-CoV-2. They correctly identified 58/58 positive and 72/72 negative patients, confirmed with

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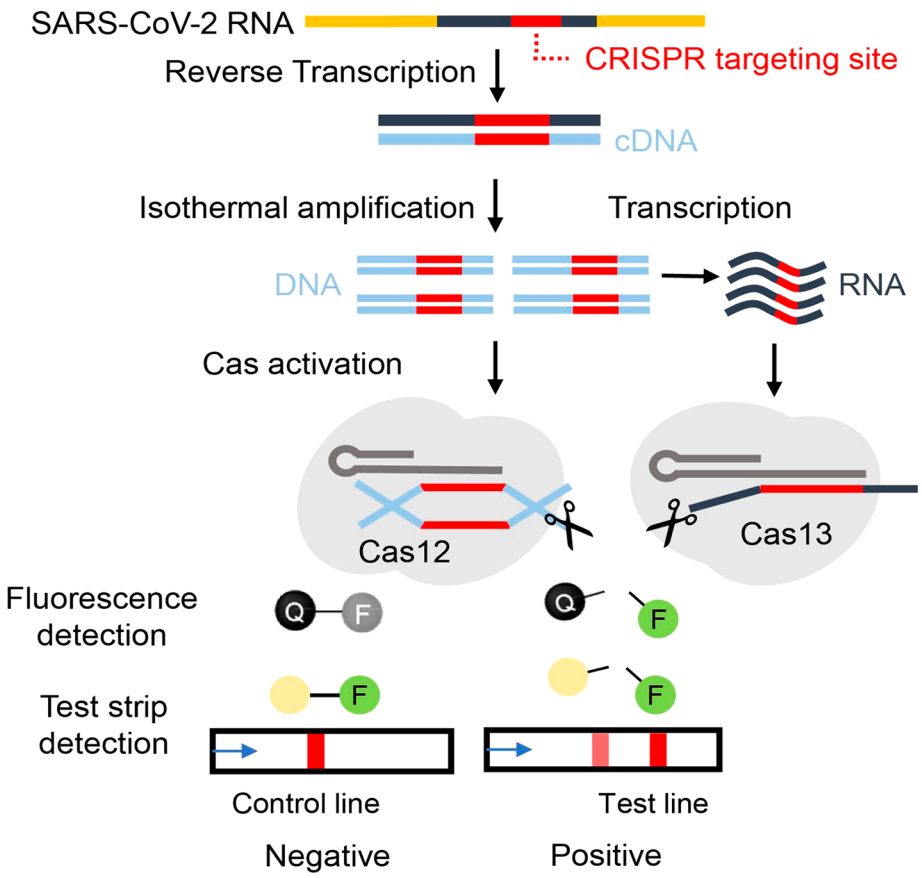

Figure 4. Schematic of isothermal amplification incorporating the CRISPR technology for the detection of SARS-CoV-2.[54,55] SARS-CoV-2 RNA is first extracted from patient specimens. The purified RNA is then reverse transcribed to cDNA and amplified through isothermal techniques, e.g., RT-RPA and RT-LAMP. The cDNA amplicons are either added directly to the CRISPR-Cas12 system or transcribed to ssRNA first and then added to the CRISPR-Cas13 system. Cas12 is activated by dsDNA with a CRISPR targeting sequence (in red) to cleave ssDNA reporters. Cas13 recognizes RNA containing CRISPR targeting sequences and cleaves its RNA reporters. In a fluorescence assay, the cleavage of the reporter generates fluorescence. In a lateral flow assay, the cleavage of the reporter leads to the appearance of a test line.

parallel RT-PCR testing. This RT-LAMP assay of the patients' RNA sample extracts only required a 60 min incubation at 63 °C.

Colorimetric readouts for amplification techniques using enzymatic polymerization can also be achieved using pH indicators. During DNA polymerization, each addition of a dNTP (deoxyribonucleoside triphosphate) releases a hydrogen ion which decreases the pH of the solution. The decrease in pH corresponds to the extent of DNA polymerization. For visual readout of their RT-LAMP, Baek et al.[50] used phenol red, which changes color from pink (pH 8.8) to yellow (pH < 8.0), to indicate the occurrence of amplification. After a 30 min amplification at 65 °C, RT-LAMP achieved detection of 200 copies of SARS-CoV-2 RNA extracted from COVID-19 patient samples.

A drawback of the use of Calcein or pH indicators for detection in RT-LAMP is the risk of false positive results. This issue arises because any nonspecific amplification, such as that caused by nontarget sequences or primer−primer binding (primer dimers), also generates detectable signals. A solution to this problem is to detect the specific sequences of the amplification products. A successful approach is exemplified by the incorporation of LAMP or RT-LAMP with the Clustered Regularly Interspaced Short Palindromic Repeats (CRISPR) technology.

Isothermal Amplification Incorporated with CRISPR Technology. CRISPR-based diagnostics arose from the discovery of microbial CRISPR and CRISPR-associated (Cas) proteins. Two types of Cas proteins, Cas12 and Cas13,[52,53] are particularly useful for analytical applications. The Cas12 or Cas13 protein is guided by a CRISPR RNA (crRNA) to target a specific nucleic acid sequence, while the single-stranded region of the crRNA is complementary to the target (Figure 4). The functions of Cas12 and Cas13 are different. Cas12 targets ssDNA,[53] while Cas13 targets ssRNA.[52] The targeting sequences (~17 nt) of Cas12 are chosen to be next to a protospacer adjacent motif (PAM) (e.g., 5′-TTTV). PAM facilitates the unwinding of dsDNA targets and the binding between crRNA and DNA targets. This binding induces a conformational change of Cas12 and activates its collateral cleavage activity to cleave any ssDNA indiscriminately. However, Cas13 targets ssRNA,[53] and its operation does not need a PAM sequence. Cas13 only has some preference of the first nucleotide next to the 3′ end of the targeting sequence (~28 nt). Upon targeting, Cas13 cleaves ssRNA nonspecifically.

CRISPR techniques, using both Cas12 and Cas13, have been incorporated into isothermal amplification approaches for the detection of SARS-CoV-2[54,55] (Figure 4). The CRISPR targeting sequence must be considered and determined when designing primers for the amplification of SARS-CoV-2 RNA.

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The CRISPR targeting sequence is within the amplicon and in between the primers. The crRNA-Cas complex scans amplicons and specifically binds to those with the complementary target sequence. Upon targeting, Cas proteins are activated to perform their known collateral cleavage activity, the nonspecific cleavage of multiple ssRNA substrates by Cas13 or ssDNA substrates by Cas12.

Signaling reporters, which are short, single-stranded nucleic acids dually labeled with a fluorophore and quencher, can be used as substrates.[55,56] The cleavage of the signaling reporter separates the quencher from the fluorophore and restores fluorescence signals that can be monitored in real time or visualized under LED light. Alternatively, substrates dually labeled with a biotin and a fluorescein amidite (FAM) probe can be used for detection on test strips.[55] Gold nanoparticles (AuNPs) on the lateral flow strip are coated with anti-FAM antibodies to capture FAM. In the absence of the target, the reporters remain intact and are captured by streptavidin on the control line, resulting in an accumulation of AuNPs which generates a red band, indicating a negative test. In the presence of the SARS-CoV-2 RNA target, the amplicons activate the Cas protein to cleave signal reporters which allows the freed FAM and attached AuNPs to travel to the test line, producing another red band indicating a positive test. The signaling reporters are in excess; so, in a positive test, excess intact reporters are captured at the control line as well.

CRISPR-based detection checks the sequences of the products from isothermal amplification and only produces signals when the correct sequences exist, which provides a higher analytical specificity than using nonspecific fluorescent dyes or pH indicators. On the other hand, an assay using Cas12 or Cas13 alone is not sensitive enough to detect the viral RNA in specimens.[56] Isothermal amplification achieves exponential amplification in a short period of time, which overcomes this limitation of CRISPR technology in terms of analytical sensitivity.

One of the pioneer CRISPR diagnostic platforms, DETECTR (DNA endonuclease-targeted CRISPR trans reporter),[53] has been used with LAMP.[55] Broughton et al.[55] posted a protocol using DETECTR for SARS-CoV-2 testing on February 15, 2020. They used RT-LAMP to amplify extracted SARS-CoV-2 RNA, leading to the generation of dsDNA products. The dsDNA products were detected using the collateral cleavage of reporters by Cas12. Lateral flow strips were then used to produce readout signals. The method was able to detect 10 copies of SARS-CoV-2 RNA per microliter of RNA extract. The amplification and detection can be achieved within 30 min, which is particularly valuable for POC testing and on-site analysis.

RPA is compatible with CRISPR technology because both require similar optimal reaction temperatures. Zhang and coworkers previously developed an RPA-Cas13-mediated CRISPR platform, named SHERLOCK (specific high-sensitivity enzymatic reporter unlocking),[56] and demonstrated its application to the detection of dengue and Zika viruses. They recently released a SHERLOCK protocol[54] for the detection of SARS-CoV-2. After the extraction of RNA from SARS-CoV-2, they used reverse transcription RPA to exponentially amplify SARS-CoV-2 RNA, generating dsDNA products. After the RPA reaction at 42 °C for 25 min, an aliquot of the RPA solution was used for the detection of dsDNA products using Cas13. Because Cas13 can only detect RNA targets, they added a step to transcribe dsDNA to RNA using T7 RNA polymerase. Through collateral cleavage, Cas13 then cleaved a lateral-flow reporter, which allowed the use of the lateral flow strip to generate final readout signals. The detection of 10 copy/μL was demonstrated using the extracted RNA samples. The amplification and detection can be completed within about 1 h.

Although the compatibility of RPA and CRISPR technology has been proven by using the DETECTR platform for one-step detection of human papillomavirus (HPV),[53] a similar single-tube RT-RPA-Cas assay for SARS-CoV-2 detection has not been reported in a peer-reviewed journal. A single-tube method would simplify the operation procedures, making the method more suitable for on-site detection. Single-tube methods would also shorten the turnaround time and eliminate the amplicon contamination from the working environment. However, each step involved in the assay, including the reverse transcription, isothermal amplification, and CRISPR-mediated detection, requires at least one enzyme. The main challenge of achieving all reactions in a single tube is to find a compromise in the conditions that allow all enzymes to work efficiently. We have designed an RT-RPA-Cas12 assay for the detection of SARS-CoV-2 RNA. All the reactions take place in a single tube and under an isothermal condition. First, the target RNA is reverse transcribed into cDNA which is then amplified by RPA. CRISPR/Cas12 scans the dsDNA copies produced by RPA and specifically binds to a predesigned sequence complementary to the crRNA. This binding activates Cas12 to cleave single-stranded signaling reporters which generates fluorescence. We are able to detect SARS-CoV-2 RNA within 30 min.

Recent research has shown that the CRISPR technology has great potential in POC detection of SARS-CoV-2, although it has not yet been used for clinical diagnosis. Only a few CRISPR-based methods have been applied to the analysis of human samples. Analyses of extracted RNA from 78 respiratory samples (36 COVID-19 confirmed positives) using SARS-CoV-2 DETECTR have achieved a 95% sensitivity and 100% specificity.[55] More validation is required before using CRISPR-based techniques for diagnosis of COVID-19. On-site and POC tests also require instrument-free sample treatment techniques to be compatible with the CRISPR diagnostics.

DETECTION OF VIRAL PROTEINS

In addition to the detection of viral nucleic acids in a patient sample, the detection of viral proteins can provide complementary information to confirm the presence of the virus. Unlike exponential amplification of nucleic acids that can produce false positives arising from unintended amplification of contaminants, proteins cannot be directly amplified which reduces the risk of false positives. Conversely, because proteins cannot be directly amplified, the detection of minute amounts of proteins challenges analytical chemistry, demanding ultrasensitive detection techniques.

A total of 29 proteins of SARS-CoV-2 are known, including 4 structural proteins (E, M, N, and S) and 25 nonstructural proteins. High-abundance proteins are ideal targets for the detection of SARS-CoV-2. Two possible viral protein targets for the detection of SARS-CoV-2 are the N and S proteins. The N protein of SARS-CoV, which has a high degree of homology to SARS-CoV-2,[57] was shown to be produced in high amounts in SARS-CoV infections.[58,59] Although the nucleocapsid is an internal structural protein of the virus, it is found to be present in serum samples of SARS-CoV patients.[60]

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There is no peer-reviewed report showing the presence of nucleocapsid of SARS-CoV-2 in human sera despite the similarity of nucleocapsids of SARS-CoV and SARS-CoV-2 viruses.[61] Analysis of the transcriptome of SARS-CoV-2 shows that the transcript for the N protein forms a large proportion and may suggest that the N protein is also in high abundance in SARS-CoV-2 infections. However, there are no direct quantitative analyses of the proteome of SARS-CoV-2 as yet. On the other hand, the S protein is the preferred target for the diagnosis of COVID-19 because it is required for viral entry into host cells and therefore contributes directly to the virulence of SARS-CoV-2.[62] Although the presence of these proteins does not necessarily indicate active infection, Li et al.[60] was able to detect the N antigen of SARS-CoV in as long as 25 days after the onset of symptoms.

Currently, there are some affinity ligands available for both the S and N proteins. Monoclonal antibodies against the S1 subunit of the S protein[63] and against the N protein[64,65] of SARS-CoV-2 have been developed. An enzyme-linked immunosorbent assay (ELISA) kit is available for the direct detection of SARS-CoV-2 antigens.[66] The kit was reported to successfully detect recombinant N protein added to human serum with an LOD of 1 ng/mL. The N protein of SARS-CoV has been detected in human serum at a concentration of around 3 ng/mL.[67]

A major challenge when developing techniques that detect viral proteins is the lack of available antibodies against each of the proteins of SARS-CoV-2. One solution is to develop alternative affinity ligands, other than antibodies. Song et al. recently released a preprint describing selection of aptamers that recognize the receptor binding domain (RBD) of the S protein.[68] The authors discovered two DNA aptamers of 51 nt (K_d = 5.8 nM) and 67 nt (K_d = 19.9 nM) in length. Zhang et al. developed a peptide blocker that binds strongly (K_d = 47 nM) to RBD of the S protein.[69] Both the aptamers and the peptide can be biotinylated, which is conducive to their potential use as affinity ligands to develop diagnostic assays. The quicker production of aptamers and synthetic peptides relative to the production of antibodies could allow improved accessibility. Aptamers targeting the N protein of SARS-CoV have been modified to detect the N protein of SARS-CoV-2.[70] Although the N protein of SARS-CoV-2 and SARS-CoV are 90% identical (Table S5), the cross-reactivity with SARS-CoV may not be a major issue because there are no new SARS-CoV cases.

The S, E, M, and N proteins of SARS-CoV-2 share 70−95% sequence identity with those of SARS-CoV and 30−50% sequence identity with those of MERS-CoV (Table S5).[71] Epitopes specific to each virus can be carefully chosen to reduce cross-reactivity of assays for specific proteins.[71]

CHALLENGES AND RESEARCH NEEDS

Tremendous progress has been made in molecular diagnosis of COVID-19, with many assays developed in a very short time. However, the current capacity of testing cannot meet unprecedented global demand for rapid molecular diagnosis. Several areas of research are needed, from resolving the issues of false negative results to the development and validation of faster and easy-to-implement diagnostic assays.

Issues of False Negatives from Testing of Viral RNA. False negative results of actual COVID-19 patients could lead to detrimental effects, such as delayed care for severely ill patients and increased risk of transmission. The WHO detailed several specific reasons that cause false negative results.[28] In general, both the analysis of a patient's sample and the sample itself can be the source of a false negative result.

Although SARS-CoV-2 tends to initiate infection and viral entry in the oral or nasal cavities, during active infection the virus spreads to the lower respiratory system where it establishes and replicates. The viral load in a specimen varies with the time of infection and the site from which the specimen is collected. For example, viral load varies between nasal and oral swabs depending on collection date after onset of symptoms.[23] Upper respiratory viral titers are reported to be higher earlier in infection but change over time.[72] The dynamic viral loads in different sites during the progression of the disease complicate the specimen collection, contributing to false negative results.

A number of factors relating to sample handling and treatment can also contribute to false negative results. These may include improper collection of specimens, loss or degradation of the target RNA during shipping and storage of specimens, inefficient extraction of RNA from the specimens (e.g., nasal swabs), inadequate purification of RNA, and inefficient removal of sample matrix and impurities. Collection, storage, handling, and treatment of samples are critical for accurate and meaningful diagnosis of COVID-19.

Although positive RT-PCR results are indicative of active infection with SARS-CoV-2, negative results do not exclude SARS-CoV-2 infection. Molecular diagnosis should be used in combination with other diagnostic information, including clinical observation, patient history of exposure, and epidemiological tracing information. For example, a combination of computed tomography (CT) X-ray scan of the chest, and RT-PCR analysis was recommended for clinical diagnosis of COVID-19. The combined use of chest CT scan with RT-PCR analysis improved clinical sensitivity to 97% (580/601).[29]

As recommended by the WHO, the most commonly collected and analyzed upper respiratory specimens are nasopharyngeal and oropharyngeal swabs. However, testing other specimens may provide complementary molecular information. For example, if saliva samples provide valid diagnostic values,[73,74] analysis of saliva could open new opportunities for POC testing. Processing and analysis of saliva samples are less challenging than the processes for nasopharyngeal and oropharyngeal swabs, particularly for POC testing and at resource-limited settings.

Needs for Identifying the Virus and Its Mutations. Genome sequencing played a crucial role in the identification of the SARS-CoV-2 virus as the causative agent of COVID-19. There are more than 100 pathogens causing community-acquired pneumonia, and among these 30% are viruses.[75] Common symptoms, such as fever and cough, and conventional diagnostics, such as chest CT imaging, cannot identify or differentiate the causative agents. Traditional microbiological methods of separation and culture of microorganisms from patients' specimens are slow and tedious. The need for rapid molecular identification presented next generation sequencing (NGS) with the opportunity to achieve comprehensive molecular diagnosis of COVID-19. In addition to confirming suspected COVID-19 cases, NGS has the ability to determine cross-infections by multiple respiratory viruses and identify these viruses from a single analysis.

Available NGS methods commonly used for sequencing bacterial rRNA must be modified for sequencing the metagenome of viruses because viruses do not have rRNA.

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Genome BLAST analysis of the generated sequencing data takes more than 30 min and requires appropriate computing tools.[76] Major NGS technologies/platforms are summarized in Supporting Information Table S6. For example, nanopore direct sequencing, a representative technology of third-generation sequencing, has been applied to the identification of multiple viral genomes in clinical specimens.[11,77] The technology enables direct measurement of RNA molecules without the need of conversion to cDNA.

High-throughput sequencing is also necessary to monitor mutations in the SARS-CoV-2 genome, which is important for understanding the evolution of the virus and its transmission between animal hosts.[78,79] In addition, updated viral genome information is required to assess whether the primers and probes designed for the current RT-PCR assays will still be suitable for the detection of mutated virus strains. If necessary, the sequences of primers and probes for RT-PCR can be adjusted accordingly. The Global Initiative on Sharing All Influenza Data (GISAID)[20] and the China Biological Information Center (CNCB)[80] (Beijing Institute of Genomics, Chinese Academy of Sciences) have released more than 46 000 SARS-CoV-2 genome sequences by mid-June. The continuous updates and sharing of the genome data help international scientific communities to improve the analytical specificity of nucleic acid detection of SARS-CoV-2.

Opportunities for Improvement of RT-PCR Assays. RT-PCR is widely available and accepted as a standard molecular diagnostic tool. The viral RNA extracted and purified from patients' specimens can be detected after less than 40 cycles of exponential amplification. Scientists from around the world have developed many complementary RT-PCR assays, targeting multiple genes of SARS-CoV-2. Open sharing of these RT-PCR protocols and the primer/probe designs has offered opportunities to assess, compare, and improve the sensitivity and specificity of these RT-PCR assays. The availability of the rich genome data enables future reassessment of these RT-PCR assays to ensure their suitability for detecting mutated virus strains. Although RT-PCR technology is well-established, achieving accurate and valid results requires good laboratory practice, from the preparation and manufacturing of RT-PCR test kits to the analysis of samples. Lessons must be learned from the delayed deployment of the "CDC test kit...because it contained a faulty reagent".[81]

Opportunities for improvements also exist in the development of new RT-PCR platforms, better tolerance of matrix effects, and compatibility with simpler or minimal sample treatment procedures. One example of new platforms is droplet digital RT-PCR.[82] Droplet digital RT-PCR uses the same primers, probes, and reagents as conventional RT-PCR, except that the bulk reaction solution is divided into thousands of nanoliter-sized microdroplets or partitions. The formation of the partitions is designed such that each partition contains either one or no target sequence. Amplification occurs in each partition that has a target. Counting the number of positive partitions provides results of the total number of copies of the target present in the original sample. The digital format follows Poisson's statistics. Motivation for the development and further refinement of droplet digital RT-PCR comes from two of its attractive features. First, the partitions efficiently reduce template competition for primers. Second, the nanoliter volume of the isolated droplet reactors dramatically increases the local effective concentration of the target, favoring reaction kinetics and efficiency. Both of these features can lead to lower detection limits.[83]

RT-PCR assays are typically complete within 1−3 h. However, most of the RT-PCR assays require extraction of the RNA from patient's samples in combination with a purification step to concentrate the RNA and remove the excess solvents and sample matrix. Although the extraction and purification procedures are usually automated, they are time-consuming and require that an automated instrument be available to conduct them, which constrains the capacity of widespread testing. Confronting these challenges requires modifications and improvements of RT-PCR methods to be amenable for direct sample analysis without any extraction or with only minimal sample treatment. Alternative methods are also needed to ease the global demands for the same testing reagents.

Detection of viral nucleic acids without extraction procedures is exemplified in alternative techniques to RT-PCR, such as nucleic acid sequence-based amplification (NASBA),[84] RT-LAMP,[85] and CRISPR. To circumvent extraction, researchers typically heat samples in the presence of reagents that minimize the loss or degradation of the targets. For example, Heating Unextracted Diagnostic Samples to Obliterate Nucleases (HUDSON),[86] uses heat and chemical treatments to inactivate RNases in clinical specimens for the direct detection of viral nucleic acids. HUDSON was used in combination with CRISPR-based diagnostics for the detection of Zika virus in saliva down to 0.9 aM. SARS-CoV-2 has also been directly detected in nasal and pharyngeal swab samples as described in a recent report.[85] The specimens were placed in Universal Transport Media supplemented with proteinase K, and the solution was subjected to a 5 min heat treatment at 95 °C. RNA in the solution was subsequently amplified for detection using RT-LAMP. However, a recent study[34] has suggested that heat treatment to release RNA may adversely impact the ability of RT-PCR to detect specimens containing low viral loads, which can contribute to high false negative rates.

Needs for Improvement and Validation of POC Diagnostics of Viral RNA. Urgent demand for rapid and large-scale testing for COVID-19 requires new diagnostic tools that are suitable for POC settings. Development of POC tests must confront the following challenges commonly faced by on-site detection in a resource-limited setting: (1) only minute amounts of the target RNA may be present in individual samples, which requires significant signal amplification; (2) lack of sophisticated instrumentation or temperature control limits the amplification techniques to be preferably isothermal; (3) signals generated from the amplification reactions must be readily detectable; (4) specimen handling must be minimal to avoid operator exposure to the virus, which means that the assay is best performed in a single tube or in a closed compartment without the need for repeated opening; (5) the tests should be easily performed by personnel without extensive training; (6) time of analysis should be reasonably short; and (7) validation of the POC assays should be vigorously conducted with the analyses of actual clinical specimens. Ultimately, POC protocols should be simple so that nonlaboratory staff or even patients can perform the tests in less controlled testing environments rather than in analytical laboratories.[16]

A notable example using isothermal amplification is ID NOW-COVID-19 (Abbott), which received Emergency Use

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Authorization (EUA) approval from the United States FDA. According to the manufacturer, the technique requires only 5−13 min to generate positive results from samples of COVID-19 patients. However, concerns have been raised over the accuracy of ID NOW-COVID-19 for practical use.[87] The false negative rate is significantly increased for the analysis of patient samples stored in viral transport media, and the cause is still under investigation. A few studies have also shown that ID NOW-COVID-19 generated high false-negative results when the viral loads in samples are low.[88−90] A comparison between ID NOW-COVID-19 and RT-PCR (Xpert Xpress) showed that the positive detection rate of ID NOW was less than 55% when the RT-PCR threshold cycles for detecting N2 using Xpert Xpress were higher than 33.7 cycles.[88]

The most exciting and promising recent developments come from applications of isothermal amplification strategies and their incorporation with CRISPR techniques to achieve POC detection of SARS-CoV-2. Isothermal amplification enables rapid (10−60 min) amplification of nucleic acids at a constant temperature (e.g., 37−65 °C) without the requirement of thermal cycling. These features make isothermal amplification techniques suitable for POC testing.

RT-LAMP and RT-RPA, as alternatives to RT-PCR, have been developed to achieve similar levels of detection for viral RNA. Incorporation with CRISPR techniques, such as SHERLOCK and DETECTR, improves the specificity and sensitivity of the isothermal amplification assays. We anticipate an integration of these complementary techniques into a format in which the various reactions take place in a single tube/device. A simple format of "sample in and answer out" is desirable for POC testing.

There are opportunities to develop POC colorimetric assays for the detection of SARS-CoV-2. Colorimetric assays using gold nanoparticles (AuNPs) have previously been developed to facilitate CRISPR-based detection of hepatitis B virus.[91] AuNPs are simple to synthesize,[92] and the AuNP surface can be modified with hundreds of nucleic acids.[93−95] A color change resulting from aggregation of AuNPs is a simple readout and can be incorporated with both isothermal amplification and CRISPR techniques to achieve POC testing. This single-tube reaction format would minimize operation error and avoid cross-contamination.

Requirement for Biosafety. One major challenge in developing diagnostic assays for COVID-19 is the necessity to work directly with SARS-CoV-2. Optimization and validation processes often require direct handling of patient samples and the virus itself. Due to the high transmissibility of SARS-CoV-2,[96] working with viral cultures can be of high risk to laboratory personnel and must be done in Biosafety Level 3 laboratories, although analysis of viral RNA samples can be handled in Biosafety Level 2 facilities. Furthermore, expressing the S protein on mammalian cells is difficult due to its high degree of glycosylation.[97] To solve these logistical issues, Nie et al.[98] developed a pseudovirus consisting of a vesicular stomatitis virus that expressed the S protein of SARS-CoV-2 on its surface. Use of this pseudovirus in place of SARS-CoV-2 is a great opportunity to simplify and accelerate the development of assays for viral proteins.

Needs for Studying Viral Proteins. Unlike nucleic acids, proteins cannot be directly amplified. Without amplification, direct detection of trace amounts of viral proteins is challenging because of an inadequate limit of detection (Table S7). ELISA and nucleic acid mediated assays offer substantial amplification of detection signals, enabling indirect detection of specific proteins.[99,100] These techniques require affinity ligands, such as antibodies, receptors, aptamers, and peptides, to bind with the specific viral proteins. The binding affinity and specificity are critical to the outcome of affinity assays. Until strongly binding and highly specific affinity ligands are widely available, the detection of viral proteins remains challenging for the diagnosis of COVID-19.

The SARS-CoV-2 genome encodes four structural proteins (E, M, N, and S) and 25 nonstructural proteins. Characterizing and understanding the abundance (concentrations), structures, binding properties (affinity and specificity), and functions of these proteins in SARS-CoV-2 require diverse analytical techniques. Mass spectrometry and proteomic techniques will play important roles in the characterization and quantitative determination of viral proteins.

Needs for Improving Serological Tests of Antibodies. Serology for the purpose of diagnosing COVID-19 is not as viable as detecting viral RNA because of the late presentation of antibodies. Serum levels of antibodies against SARS-CoV-2 infection display a slower profile than that of viral loads in respiratory specimens and are composed of two phases. Antibody titers are low or undetectable at the symptom onset and then rise to detectable levels after 3−5 days.[101] From the onset of symptoms, IgM is initially detected in as soon as 3−5 days followed by IgG, detectable in as soon as 4 and up to 14 days.[102−104] IgM and IgG were detected at median times of 5 and 14 days,[104] and peaks of IgM and IgG antibodies have both been detected weeks after the onset of symptoms.[17]

Despite many serological test kits in the market, a number of challenges have hampered the confidence of the available COVID-19 antibody tests.[18,19,101] Main challenges include: (1) current serological tests for COVID-19 do not measure neutralizing antibodies and therefore cannot conclude whether a previously infected person has sufficient neutralizing antibodies for immunity; (2) most POC serological tests for COVID-19, such as lateral flow immunoassays, only indicate a yes or no answer but do not quantify the actual concentrations of antibodies; and (3) many of the POC antibody tests have poor clinical specificity, leading to high false positive results.

Environmental Surveillance by the Analysis of Sewage Wastewater. Several studies have confirmed that SARS-CoV-2 is discharged in feces of COVID-19 patients.[22,72,105−108] Community sewage and wastewater samples can be analyzed for the presence of viral components, e.g., viral RNA of SARS-CoV-2, which provides surveillance information on the community level.

Medema et al.[109] reported the detection of three regions in the N gene (N1−N3) and the E gene of SARS-CoV-2 in sewage of six communities in The Netherlands. They detected 2.6−30 gene copies of SARS-CoV-2 per mL in three of the six communities in early March. In one community, the N3 gene was detected 6 days before the first reported case. As COVID-19 spread in The Netherlands during March, the viral gene copies in sewage increased and correlated with the increasing prevalence of COVID-19 cases. Subsequent reports from Australia, France, Israel, Italy, Spain, and the U.S. have also shown the feasibility of detecting viral RNA in wastewater and sewage for environmental surveillance.[110−112] Also called wastewater-based epidemiology,[113,114] environmental surveillance provides community-wide public health information on the progress of the disease and may serve as an early warning of re-emergence of COVID-19 in communities.

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In summary, molecular diagnostic tools and assays are crucial for clinical diagnosis, public health surveillance, and mitigation strategies to contain the spread of COVID-19. SARS-CoV-2 virus, the causative infectious agent of this pandemic, has infected millions of people around the world, and the number of COVID-19 cases continues to rise.[115] There are critical needs and tremendous opportunities for analytical chemists to collaborate with multidisciplinary scientists, clinicians, public health practitioners, and engineers in a collective effort to achieve rapid and accurate diagnosis of COVID-19, improve our understanding of SARS-CoV-2 at the molecular level, and contribute to the development of preventive measures, therapeutics, and vaccines.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acs.analchem.0c02060.

Tables S1−S7 summarize the molecular weights of the S, N, M, and E proteins and their copy numbers in a virion, sequence alignment of these structural proteins comparing SARS-CoV-2 with SARS-CoV and MERS-CoV, available extraction kits, Emergency Use Authorization detection kits, next generation sequencing techniques, temporal trends of viral RNA and antibody levels in patients, and typical limits of detection (PDF)

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Notes

The authors declare no competing financial interest.

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