Review paper

An outlook on coronavirus disease 2019 detection methods

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ABSTRACT

Diagnostic testing plays a fundamental role in the mitigation and containment of coronavirus disease 2019 (COVID-19), as it enables immediate quarantine of those who are infected and contagious and is essential for the epidemiological characterization of the virus and estimating the number of infected cases worldwide. Confirmation of viral infections, such as COVID-19, can be achieved through two general approaches: nucleic acid amplification tests (NAATs) or molecular tests, and serological or antibody-based tests. The genetic material of the pathogen is detected in NAAT, and in serological tests, host antibodies produced in response to the pathogen are identified. Other methods of diagnosing COVID-19 include radiological imaging of the lungs and in vitro detection of viral antigens. This review covers different approaches available to diagnosing COVID-19 by outlining their advantages and short-comings, as well as appropriate indications for more accurate testing.

1. Introduction

In March 2020, the World Health Organization announced a global pandemic due to the occurrence of coronavirus disease 2019 (COVID-19) caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), one of the seven known coronaviruses capable of causing human infections [1]. The clinical symptoms of COVID-19 are nonspecific and range from asymptomatic to fatal pneumonia. Common symptoms include fever, myalgia (muscle pain), cough, shortness of breath, and sudden appearance of olfaction and taste disturbances [2,3]. Headaches, nausea, dizziness, diarrhea, and abdominal pain are less common in patients infected with SARS-CoV-2 [2]. Infected individuals may develop symptoms 2–14 days after infection, depending on the age and immune system of the patient [4].

COVID-19 is highly contagious, and the speed and extent to which it spreads are not merely due to transmission from symptomatic persons. Several reports have shown that the transmission of COVID-19 can occur in individuals who are pre-symptomatic (when the virus is detected before the onset of symptoms) [5] or

asymptomatic (when the virus is detectable with no symptoms) [6]. In addition, vaccinated individuals can be carriers of SARS-CoV-2 while remaining asymptomatic [7], which makes the containment of the virus more challenging. Therefore, early detection of the disease and isolation of infected individuals from the healthy population are imperative to addressing the COVID-19 pandemic.

Current COVID-19 testing techniques are classified into two general groups: nucleic acid amplification tests (NAAT) or molecular tests, and serological tests, also known as antibody-based tests. Molecular tests detect the genetic material of the virus in respiratory secretions, oropharyngeal and nasopharyngeal swab samples, as well as saliva by targeting one or more parts of the SARS-CoV-2 genome. Some of these targets include spike (S), which is composed of two subunits (S1 + S2), nucleocapsid (N), envelope (E) gene, and regions in the first open reading frame of the RNA-dependent RNA polymerase (RdRp) gene. Currently, reverse transcription quantitative polymerase chain reaction (RT-qPCR) is considered as the "gold standard" and is consistently used to diagnose COVID-19. Alternative amplification techniques include recombinase polymerase amplification (RPA) and loop-mediated isothermal amplification (LAMP), which have been developed in the past decade, and are timely, simpler, and less expensive than RT-qPCR.

In addition to NAAT, COVID-19 diagnostic tests that detect viral proteins (antigens) and antiviral antibodies have been developed. Serological tests measure seroconversion in plasma/serum via

different types of assays such as enzyme-linked immunosorbent assay (ELISA), chemiluminescent immunoassay (CLIA), colloidal gold immunochromatographic assay (GICA), and lateral flow immunoassay (LFIA). However, because seroconversion of infected individuals depends on the stage of infection, serological tests may not detect antibodies in those with active infection and are more appropriate for individuals who have been pre-exposed to SARS-CoV-2. In some instances, imaging of the lungs can be used to diagnose patients with COVID-19, which is also appropriate for assessing the severity of the disease. Computed tomography (CT), chest radiography (CXR), and lung ultrasound (LUS) are common imaging modalities for diagnosis of COVID-19. Tests based on imaging of the lungs have some shortcomings, such as being limited to healthcare settings and requiring professional radiologists to assess the results. Moreover, the results are time dependent and can vary depending on the stage of infection. Thus, imaging of the lungs cannot always rule out the probability of SARS-CoV-2 infection.

In this review, various testing techniques currently available for the diagnosis of COVID-19 are described by outlining their merits and demerits as well as suitable indications for a more accurate diagnosis. The COVID-19 pandemic may not be the last pandemic of the century. Therefore, a review that highlights different diagnostic methods for monitoring SARS-CoV-2 infection may be useful for selecting appropriate diagnostic techniques.

2. NAAT

2.1. RT-qPCR

COVID-19 can be confirmed by the identification of SARS-CoV-2 genome using RT-qPCR. The process involves respiratory sample collection, RNA extraction and purification, followed by RT-qPCR. The reverse transcriptase enzyme transcribes viral RNA into complementary DNA (cDNA), and the resulting cDNA undergoes qPCR to amplify the target gene [8]. Since the identification of SARS-CoV-2, numerous RT-qPCR assays that target different regions of the SARS-CoV-2 genome with different limits of detection (LOD) have been developed and used worldwide (Table 1) [9—16]. LOD is defined as the smallest amount of analyte (RNA copies in the case of SARS-CoV-2 molecular tests) detected in a sample, and the lower the LOD, the higher the analytical sensitivity of the test. The first RT-qPCR assays targeting the RdRp, E, and N genes of SARS-CoV-2 were developed by Corman et al. [9]. This TaqMan-based assay had the highest sensitivity for the RdRp and E

genes, with an LOD of 3.6 RNA copies/reaction and 3.9 RNA copies/reaction, respectively, whereas the N gene had the highest LOD (8.3 RNA copies/reaction).

Another RT-qPCR test is the RdRp/helicase (Hel) assay developed by Chan et al. [10], which exhibits greater sensitivity and specificity than the RdRp-P2 assay, one of the first assays that were implemented in most European laboratories to diagnose patients with COVID-19. However, the RdRp-P2 assay lacked sufficient specificity and cross-reacted with other related coronaviruses and common respiratory viruses. In addition, the RdRp-P2 assay was designed and validated using spiked (synthetic) samples. Unlike the RdRp-P2 assay, the RdRp/Hel assay was much more sensitive and did not exhibit cross-reactivity in vitro and in patient specimens, making it a useful platform for distinguishing SARS-CoV-2 from other respiratory pathogens [10].

The pan-coronavirus assay developed by Xiu et al. [11] is a semi-nested RT-qPCR assay with two pairs of degenerate primers that target the conserved RdRp gene and universally amplify coronaviruses within four genera. The efficacy of this assay was assessed using human and animal coronaviruses and other non-coronavirus respiratory viruses. The pan-coronavirus assay accurately identified different coronaviruses without cross-reactivity with other non-target pathogens with detection limits ranging from 4 to 400 copies/reaction, depending on the coronavirus species. Furthermore, the same procedure can be applied to detect previously identified coronaviruses or any future emerging viruses from the *Orthocoronavirinae* subfamily [11].

Reports based on the sensitivity of RT-qPCR assays that target different regions of SARS-CoV-2 genome have shown that the sensitivity of RdRp gene primer-probe assays is less than that of E and N gene primer-probes [17]. One way to resolve this issue is to use different PCR systems. The diagnostic efficiency of RT-qPCR tests can be augmented by utilizing multiplex real-time RT-qPCR tests, which enables the simultaneous detection of more than one target sequence. Petrillo et al. [12] developed a multiplex RT-qPCR method for the simultaneous detection of two regions of N genes (*N1* and *N2*) using the human RNAse P gene as an internal control. This assay showed 100% sensitivity and a detection rate of <5 copies/reaction. Further validation was carried out using swab diluent from human nasopharyngeal swabs without RNA extraction, and using this multiplex RT-qPCR assay, 17/20 of positive samples were detected [12]. Another multiplex RT-qPCR test is the assay developed by Ishige et al. [13], which simultaneously detects the E and N genes of SARS-CoV-2 and utilizes the human *ABL1* gene

Table 1
Different reverse transcription quantitative polymerase chain reaction (RT-qPCR) assays available for severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) detection.

Test	Sample source	Target	LOD	Refs.
RT-qPCR (RdRp, E, and N assay)	Throat, sputum, and nose swabs	RdRp, E, and N genes	RdRp: 3.6 RNA copies/reaction E: 3.9 RNA copies/reaction N: 8.3 RNA copies/reaction	[9]
RT-qPCR (RdRp-P2 assay)	Respiratory specimens, saliva, sputum, plasma, urine samples and stool/rectal swabs	RdRp gene	18 $TCID_{50}$/mL RNA from culture lysate, 3.6 RNA copies/reaction (in vitro viral RNA transcripts)	[10]
RT-qPCR (RdRp/Hel assay)	Respiratory specimens, saliva, sputum, plasma, urine specimens and stool/rectal swabs	RdRp/Hel	1.8 $TCID_{50}$/mL with genomic RNA from culture lysate, 11.2 RNA copies/reaction (in vitro viral RNA transcripts)	[10]
RT-qPCR (pan-CoV assay)	Nasopharyngeal swabs, saliva specimens, and rectal swab	RdRp gene	4 to 400 RNA copies/reaction, depending on the coronavirus	[11]
Multiplex RT-qPCR	Nasopharyngeal swabs	N gene (N1 and N2)	<5 RNA copies/reaction	[12]
Multiplex RT-qPCR	Nasopharyngeal and sputum swabs	N and E genes	<25 RNA copies/reaction	[13]
OSN-qRT-PCR	Respiratory specimens	ORF1ab and N genes	1 RNA copy/reaction	[14]
RT-qPCR (nsp1 assay)	Nasopharyngeal and saliva samples	nsp1 gene	18 $TCID_{50}$/mL viral RNA	[15]
RT-qPCR (nsp2 assay)	Respiratory specimens, saliva, plasma, urine samples, and stool/rectal swabs	nsp2 gene	1.8 $TCID_{50}$/mL viral RNA	[16]

LOD: limit of detection; RdRp: RNA dependent RNA polymerase; E: envelope; N: nucleocapsid; $TCID_{50}$: 50% tissue culture infective dose; Hel: helicase; pan-CoV: pan-coronavirus; OSN: one-step single-tube nested; ORF: open reading frame; nsp: non-structural protein.

as an internal control to check the quality of the samples, RNA extraction, and RT-qPCR amplification steps. The LOD of this assay was 100% up to 25 copies/reaction and 95% up to 21 copies/reaction.

Although RT-qPCR is a highly sensitive technique and is considered as the reference standard for diagnosing patients with COVID-19, false-negative results have been reported in nearly 30% of patients, which may arise from low viral load [18]. One way to prevent misdiagnosis due to low viral load is by using RT-qPCR assays with higher sensitivity. Wang et al. [14] established a very accurate and selective one-step single-tube nested quantitative real-time PCR (OSN-qRT-PCR) for the detection of SARS-CoV-2 by targeting its ORF1ab and N genes with a single-copy detection limit. Furthermore, the OSN-qRT-PCR assay exhibited 100% specificity since it did not exhibit cross-reactivity with other human coronaviruses and 38 other pathogenic bacteria or viruses. Due to its high sensitivity and selectivity, the OSN-qRT-PCR assay might be more suitable for diagnosing patients with low viral loads.

Genetic recombination and mutations in the viral genome of SARS-CoV-2 can sometimes give rise to false-negative results. Similar to other RNA viruses, SARS-CoV-2 is highly susceptible to mutations (approximately 10^{-4} nucleotide substitutions per site per year) [19]. Natural mutations in the SARS-CoV-2 genome can impact the accuracy of RT-qPCR assays that target ORF1ab, N, and E genes. In this regard, several targets can be incorporated to circumvent the bottleneck of misdiagnosis. Furthermore, additional targets, such as nonstructural proteins (nsps) of SARS-CoV-2, can be used. An RT-qPCR assay developed by Chan et al. [15] targets nonstructural protein 1 (nsp1), a highly expressed region found at the 5' end of the SARS-CoV-2 genome. This assay demonstrated high analytical sensitivity and specificity with an LOD of 18 50% tissue culture infective dose ($TCID_{50}$)/mL and did not show cross-reactivity with other common coronaviruses or respiratory viruses. Therefore, nsp1 gene can be used as an alternative target for SARS-CoV-2 detection. Another RT-qPCR assay is the SARS-CoV-2-specific nsp2 assay, which has similar sensitivity and specificity to the SARS-CoV-2 RdRp/Hel assay [10] when validated using viral samples and clinical specimens [16]. In addition, the test results can be obtained within an hour, which is shorter than the turnaround time of the RdRp/Hel assay. Another advantage of this assay is that the nsp2 gene is not present in other human pathogenic coronaviruses and can be an attractive target for specific RT-qPCR tests.

Although RT-qPCR is commonly used to diagnose COVID-19, the turnaround time is long and requires expensive tools, trained personnel, and a stable power supply. In addition, the results may be affected by human errors, incorrect sample collection, and technical issues, which can give rise to false-negative or false-positive results. Consequently, individuals suspected of having COVID-19 may not be diagnosed in time and create a risk for the unaffected population. Therefore, RT-qPCR tests should not be the only means of diagnosing patients with COVID-19.

2.2. Isothermal amplification

2.2.1. LAMP

LAMP is a robust and sensitive nucleic acid amplification technique that amplifies RNA and DNA with high efficiency and specificity under isothermal conditions (e.g., in a heat block), eliminating the need for costly thermocyclers or real-time PCR [20]. When LAMP is combined with reverse transcription (RT-LAMP), RNA sequences can be readily amplified with high efficiency and sensitivity. RT-LAMP assays use DNA polymerase and 4–6 specially designed primers that simultaneously amplify 6–8 sequences within the target gene in less than an hour. This technique has been used to detect various pathogens, such as influenza [21], severe

acute respiratory syndrome (SARS) [22], and Middle East respiratory syndrome (MERS) [23]. A plethora of LAMP-based assays have been designed that target different parts of the SARS-CoV-2 genome. Some of these assays are listed in Table 2 [24–33]. The results of these LAMP strategies are somewhat consistent with those of the RT-PCR standard tests for SARS-CoV-2 detection, with a sensitivity range of 90%–100%.

Lamb et al. [24] established a protocol for the detection of SARS-CoV-2 within 30 min of experimentation and an LOD of 304 RNA copies/reaction using LAMP. However, the assay was validated using simulated patient samples, where the samples were spiked with viral RNA. Similarly, Zhang et al. [25] detected SARS-CoV-2 from purified RNA or patient cell lysis samples using a colorimetric LAMP-based assay. The results were further analyzed and approved using RNA samples extracted from the respiratory swabs of patients with COVID-19. The performance of this test was reported to be comparable to that of commercial RT-qPCR tests. However, the test performance was validated on only 7 patients.

The RT-LAMP approach developed by Jiang et al. [26] is a sensitive and specific assay that has been validated in a larger number of COVID-19 patients and negative patients. The analytical sensitivity of this assay has been reported to be 500 copies/mL with an experimental time of less than 30 min. The specificity of this assay has been estimated to be 100% using a variety of virus, fungal, and bacterial species, as well as human DNA. This rapid RT-LAMP assay may have potential applications for large-scale screening of patients because it does not require a complex testing infrastructure.

Saliva specimens can also be used for SARS-CoV-2 detection through RT-LAMP. Saliva collection is simple and does not require invasive procedures. However, saliva is a complicated clinical matrix given its inconsistency in viscosity and pH. Lalli et al. [27] have resolved this issue by providing multiple saliva pretreatment procedures that are well-suited for RT-LAMP and RT-qPCR and do not require the RNA extraction step. The efficacy of these procedures has been assessed using simulated saliva samples, and both RT-LAMP and RT-qPCR have demonstrated high accuracy without cross-reactivity with other related coronaviruses. Another group of researchers have developed a single-tube colorimetric RT-LAMP test that can be performed within 30 min of experimentation and is also well-suited for saliva samples [28]. The assay can be performed without the RNA extraction step; however, purified RNA samples from saliva can highly sensitize the test results. Therefore, RT-LAMP assays that use saliva may also be a promising method to test patients with COVID-19.

Another LAMP-based assay is the isothermal LAMP-based method for COVID-19 (iLACO) that targets the ORF1ab of SARS-CoV-2 [29]. The LOD of this assay was 10 copies of SARS-CoV-2 with an experimentation time of 15–40 min using RNA samples from COVID-19 positive patients. Similar to most RT-LAMP assays, iLACO has a pH-based colorimetric readout. However, this assay can only detect the ORF1ab gene; therefore, it cannot ensure sufficient sensitivity for the detection of SARS-CoV-2 and might lead to inaccurate results.

Traditional monitoring techniques, such as gel electrophoresis, pH indicators, and SYBR dyes, are nonspecific and can sometimes elicit false-positive results when using LAMP assays. LAMP techniques can be multiplexed to enhance sensitivity and specificity. The multiplexing LAMP approach is highly amenable to low-income settings, where the infrastructure to support complex diagnostic testing is lacking. Zhu et al. [30] designed a multiplex RT-LAMP assay that incorporates a nanoparticle-based lateral flow biosensor (LFB) (mRT-LAMP-LFB) targeting the ORF1ab and N genes of SARS-CoV-2. The LOD of this assay was 12 copies (for each target)/reaction, with no cross-reactivity with non-SARS-CoV-2 targets. Furthermore, the assay demonstrated high specificity and

Table 2
Different isothermal amplification assays currently available for SARS-CoV-2 detection.

Test	Sample source	Target	LOD	Refs.
RT-LAMP	Spiked saliva, urine, serum, oropharyngeal, and nasopharyngeal swabs	ORF1ab and N genes	304 RNA copies/reaction	[24]
RT-LAMP	Respiratory specimens	ORF1a and N genes	120 RNA copies/reaction	[25]
RT-LAMP	Simulated samples and sputum	ORF1ab and N genes	500 RNA copies/mL	[26]
RT-LAMP	Spiked saliva samples	ORF1ab and N genes	<100 RNA copies/reaction	[27]
RT-LAMP	Spiked saliva samples	N gene	<100 RNA copies	[28]
RT-LAMP iLACO	Respiratory samples	ORF1ab gene	10 RNA copies/μL	[29]
Multiplex RT-LAMP	Oropharynx swab samples	ORF1ab and N genes	12 RNA copies/reaction for both ORF1ab and N genes	[30]
RT-LAMP	Nasal fluid swabs spiked with SARS-CoV-2	ORF1ab, S, ORF8, and N genes	50 RNA copies/μL in the VTM solution 5000 RNA copies/μL in the nasal solution	[31]
Single strand RPA	Buffer spiked samples, clinical saliva samples, and nasopharyngeal swabs in VTM or water	Genomic RNA	4 to 8 RNA copies per 50 μL reaction	[32]
COVID-19 Penn-RAMP	Spiked nasal swabs	ORF1ab gene	7 RNA copies/reaction	[33]

RT-LAMP: reverse transcriptional loop-mediated isothermal amplification; iLACO: isothermal LAMP based method for coronavirus disease 2019; VTM: viral transport medium; RPA: recombinase polymerase amplification; Penn-RAMP: University of Pennsylvania rapid amplification assay.

sensitivity (100%) since it could correctly diagnose all COVID-19 clinical specimens using RT-qPCR as a reference.

Ganguli et al. [31] established an RT-LAMP assay for point-of-care (POC) testing using an additively 3D manufactured cartridge and a smartphone-based instrument. This assay was validated using nasal fluid swabs spiked with SARS-CoV-2. The swabs were transferred to a viral transport medium (VTM), and the VTM was used to perform the RT-LAMP test, thus bypassing the RNA extraction step. The results of this assay were obtained in 30 min with an LOD of 50 RNA copies/μL in VTM solution without using other equipment for mixing, amplification, and readout. Therefore, it can be a suitable option for the portable, fast, and scalable diagnosis of COVID-19.

Unlike PCR, isothermal nucleic acid amplification can be performed at a constant temperature and is, therefore, more amenable to low-resource settings and places lacking PCR equipment. However, RT-LAMP techniques may lack sufficient specificity due to the presence of multiple primers, increasing the likelihood of nonspecific by-product production. Furthermore, false-negative results have been reported in some cases due to insufficient viral load, which leads to ineffective amplification of the target gene [29]. Therefore, the sensitivity and specificity of these tests should be further assessed using a variety of samples with different viral loads.

2.2.2. RPA

RPA is a more recent and faster LAMP approach. To amplify RNA targets, reverse transcriptase can be incorporated into RPA (RT-RPA). The benefits of RPA are its optimal temperature range (37–42 °C) and its short experimentation time (approximately 10 min). PCR primers could also be used for the RPA assays. Kim et al. [32] employed the single-strand RPA (ssRPA) method for the detection of SARS-CoV-2. The ssRPA method includes amplification of double-stranded DNA, conversion to ssDNA, and sequence-specific hybridization-based readout. ssRPA showed 100% specificity and sensitivity in all types of samples, such as buffer-spiked samples, nasopharyngeal swabs in water, and saliva samples.

RPA and LAMP can be combined into a two-stage amplification procedure referred to as RAMP. The COVID-19 University of Pennsylvania rapid amplification assay designed by El-Tholoth et al. [33] is a nested nucleic acid amplification method that can be performed with minimal sample processing in closed tubes and is compatible with colorimetric or fluorescence readout. This assay takes

advantage of two stages of isothermal amplification, thereby eliminating false-negative results and increasing sensitivity. The first stage is the RPA carried out in the cap of the tube at 38 °C, and the second stage is the LAMP which takes place inside the tube at 63 °C. The LOD of this assay has been reported to be 7 copies of viral RNA/reaction and is more sensitive than conventional RT-qPCR assays that use purified targets or LAMP assays without RPA.

2.3. Clustered regularly interspersed short palindromic repeats (CRISPR)

In addition to RT-qPCR and isothermal amplification techniques, another strategy for SARS-CoV-2 detection is CRISPR. Several research groups have established CRISPR-based techniques for the detection of SARS-CoV-2 (Table 3) [34–40]. CRISPR-associated (Cas) proteins recognize and cut foreign RNA or DNA. These systems are widely employed in genome editing applications due to their high accuracy and precision. Among the Cas proteins, Cas12a and Cas13a have been used to diagnose diseases due to their nuclease activities [41,42]. In a method called specific high sensitivity enzymatic reporter unlocking (SHERLOCK), Cas13a is used for RNA or DNA detection [42]. In this method, the target sequence is amplified by RT-RPA and T7 transcription, obviating the need for sophisticated PCR machines. When reverse transcriptase is incorporated for the conversion of RNA into DNA, DNA detection is possible. Cas13a is activated after binding to the target sequence. Active Cas13a is an RNase that nonspecifically cuts a reporter RNA, producing a fluorescent signal that can signify the presence of the viral RNA [42].

Zhang et al. [34] were among the first groups to use the SHERLOCK system to identify the ORF1ab and S genes of SARS-CoV-2. The LOD of this assay has been reported to be 10–100 copies of RNA/μL of input and the results can be obtained within an hour using a lateral flow dipstick. Similarly, Joung et al. [35] established a diagnostic platform called SHERLOCK testing in one pot, which is a single-step reaction that runs for an hour with an LOD of 100 copies of SARS-CoV-2 per sample. Moreover, the sensitivity of this assay was reported to be 97%, with a specificity of 100%. Another assay is the isothermal CRISPR-based diagnostic test for COVID-19 called CRISPR-COVID [36]. CRISPR-COVID is a rapid assay based on Cas13a. It incorporates RPA-mediated and CRISPR/Cas-mediated enzymatic signal amplification to enhance sensitivity. Moreover, it has a near

Table 3
Different clustered regularly interspaced short palindromic repeats (CRISPR) assays currently available for SARS-CoV-2 detection.

Test	Sample source	Target	LOD	Refs.
CRISPR-Cas13a/RPA-LFA (SHERLOCK)	Synthetic SARS-CoV-2 RNA samples	S and ORF1ab genes	10 to 100 RNA copies/μL of input	[34]
SHERLOCK testing in one pot	Synthetic SARS-CoV-2 RNA samples	N gene	100 RNA copies/reaction	[35]
CRISPR-Cas13a	Nasopharyngeal swab and bronchoalveolar lavage fluid	ORF1ab and N genes	1.25 to 7.5 RNA copies/reaction	[36]
CRISPR-Cas12a/RT-RPA (DETECTR)	Simulated saliva samples	ORF1ab gene	10 RNA copies/μL of input	[37]
AIOD-CRISPR (DETECTR)	Synthetic SARS-CoV-2 RNA samples	N gene	4.6 RNA copies/μL of input	[38]
LAMP, CRISPR-Cas12a (DETECTR)	Synthetic SARS-CoV-2 RNA samples	E and N genes	10 RNA copies/μL of input	[39]
CRISPR-Cas12a (CRISPR-FDS)	Nasal swabs spiked with SARS-CoV-2	ORF1ab and N genes	2 RNA copies/reaction	[40]

CRISPR: clustered regularly interspaced short palindromic repeats; Cas: CRISPR-associated; RPA-LFA: recombinase polymerase amplification lateral flow assay; SHERLOCK: specific high sensitivity enzymatic reporter unlocking; STOP: SHERLOCK testing in one pot; DETECTR: DNA endonuclease targeted CRISPR *trans*-reporter; AIOD: All-in-One Dual. LAMP: loop-mediated isothermal amplification; FDS: fluorescent detection system.

single-copy detection limit and no cross-reactivity with other pathogens and works under isothermal conditions, thus eliminating the need for complex instruments such as thermal cyclers.

In addition to SHERLOCK, there are other CRISPR-based detection methods, such as DNA endonuclease targeted CRISPR *trans*-reporter (DETECTR) [43]. In the DETECTR method, Cas12a ssDNase activation is joined with isothermal amplification for DNA detection. First, viral RNA is converted to DNA by reverse transcriptase and amplified via LAMP or RPA. Cas12a gets activated by binding to the target sequences within the amplified DNA and cleaves the ssDNA reporter to yield a fluorophore that indicates the presence of the virus.

Several research groups have used DETECR to identify SARS-CoV-2. Lucia et al. [37] detected the RdRp and ORF1ab genes of SARS-CoV-2 using the DETECR method. The LOD of the ORF1ab gene was reported to be 10 copies/μL of input using simulated saliva samples. The "All-in-One Dual CRISPR-Cas12a (AIOD-CRISPR)" assay developed by Ding et al. [38] takes advantage of RT-RPA and CRISPR-based detection methods and works in a single-tube reaction and at a single incubation temperature. The LOD of this assay was estimated to be 4.6 copies of RNA/μL of input with an incubation time of 40 min and no cross-reactivity with other non-targeted pathogens. Furthermore, AIOD-CRISPR is also useful for diagnosing patients with human immunodeficiency virus type 1 and can detect 1.2 copies of DNA within 1 min.

Broughton et al. [39] reported a robust and easy-to-implement method that incorporates LAMP (instead of RPA) and CRISPR-Cas12a for the detection of SARS-CoV-2 with a sample-to-result time of 30–40 min and comparable performance to RT-qPCR tests. In this method, viral RNA was extracted from patient samples and amplified via RT-LAMP. Subsequently, the amplicon solution and CRISPR/Cas12 reagents were added together, and the results were analyzed using a colorimetric lateral flow dipstick. The LOD of this assay was reported to be 10 copies/μL reaction with no cross-reactivity with non-SARS-CoV-2 viruses using the N gene as target.

Another fast and sensitive CRISPR-Cas12a test is the "CRISPR-based fluorescent detection system (CRISPR-FDS)" assay. This assay uses a single-step RT-PCR or RT-RPA method to amplify the target gene using spiked nasal swabs [40]. For fluorescence detection of viral RNA, the synthesized amplicons were moved to the RNA/Cas12a based CRISPR system. The advantages of the CRISPR-FDS assay include a better sensitivity than the RT-qPCR tests employed in clinical settings since the CRISPR-FDS assay can detect samples estimated to contain as few as 2 RNA copies, whereas the RT-qPCR assay failed to produce a noticeable target signal in samples with less than 5 copies of the amplified RNA. Furthermore, the sample-to-result time of this assay is approximately 50 min, which is shorter than that of the RT-qPCR test. Overall, the CRISPR-FDS assay is a suitable choice for high-throughput COVID-19 screening.

2.4. Sequencing-based detection method

An alternative method to diagnose COVID-19 is through sequencing platforms. The sequencing-based detection method allows for pathogen identification and monitoring of viral mutations that can increase transmissibility. The initial discovery of SARS-CoV-2 was through metagenomic next-generation sequencing [44]. RNA extracted from respiratory specimens was reversely transcribed to cDNA and amplified by PCR. The amplified DNA was sequenced using labeled nucleotides, and the target sequence was detected during polymerase elongation by monitoring the fluorescence signal. The sequences were then aligned with databases to analyze overlapping DNA regions [44].

Sequencing protocols based on nanopore target sequencing (NTS) have been developed to detect SARS-CoV-2 and other respiratory viruses. With the use of NTS, SARS-CoV-2 can be detected with a higher sensitivity than standard RT-qPCR. Wang et al. [45] simultaneously targeted the SARS-CoV-2 genome and other respiratory viruses using NTS. The LOD was recorded as 10 RNA copies/mL. In addition, using this method, simultaneous detection of mutations is possible. However, the process takes 6–10 h and the turnaround time is longer than that of RT-qPCR. Therefore, NTS cannot be used for mass screening of COVID-19, but it can be used to complement RT-qPCR and detect viral mutations. A conceptual summary of the COVID-19 molecular tests and sample preparation is shown in Fig. 1.

3. Antibody-based (serological) assays

Innate and adaptive immune systems play a fundamental role in ameliorating disease severity. Adaptive immunity involves B and T cells. After virus entry, a robust B cell response is triggered, leading to antiviral antibody secretion. The T cells recognize viral antigens through T cell receptors (TCRs) and eradicate virus-infected cells, expediting the production of virus-specific antibodies. T cell activation leads to clonal expansion, enabling the proliferation of T cells with uniform TCRs and identical antigen recognition abilities [46]. Using next-generation sequencing technologies, TCR repertoires can be analyzed. Currently, little is known about differences in TCR repertoires among patients with COVID-19. However, mild to severe cases of COVID-19 have been reported to exhibit diverse TCR repertoire profiles [47].

Serological or antibody tests detect antibodies against viral proteins present in the host and can be used as a complement to NAAT. Current COVID-19 serological tests detect antibodies produced in response to the N protein, S1 and S2 subunits, and the receptor-binding domain (RBD) of the S protein [48]. RBD attaches to the host angiotensin-converting enzyme 2 receptor and allows the virus to gain entry into the host cells. Therefore, the S protein plays an essential role in triggering immune responses and is considered as one of the most studied targets [49]. The N protein of
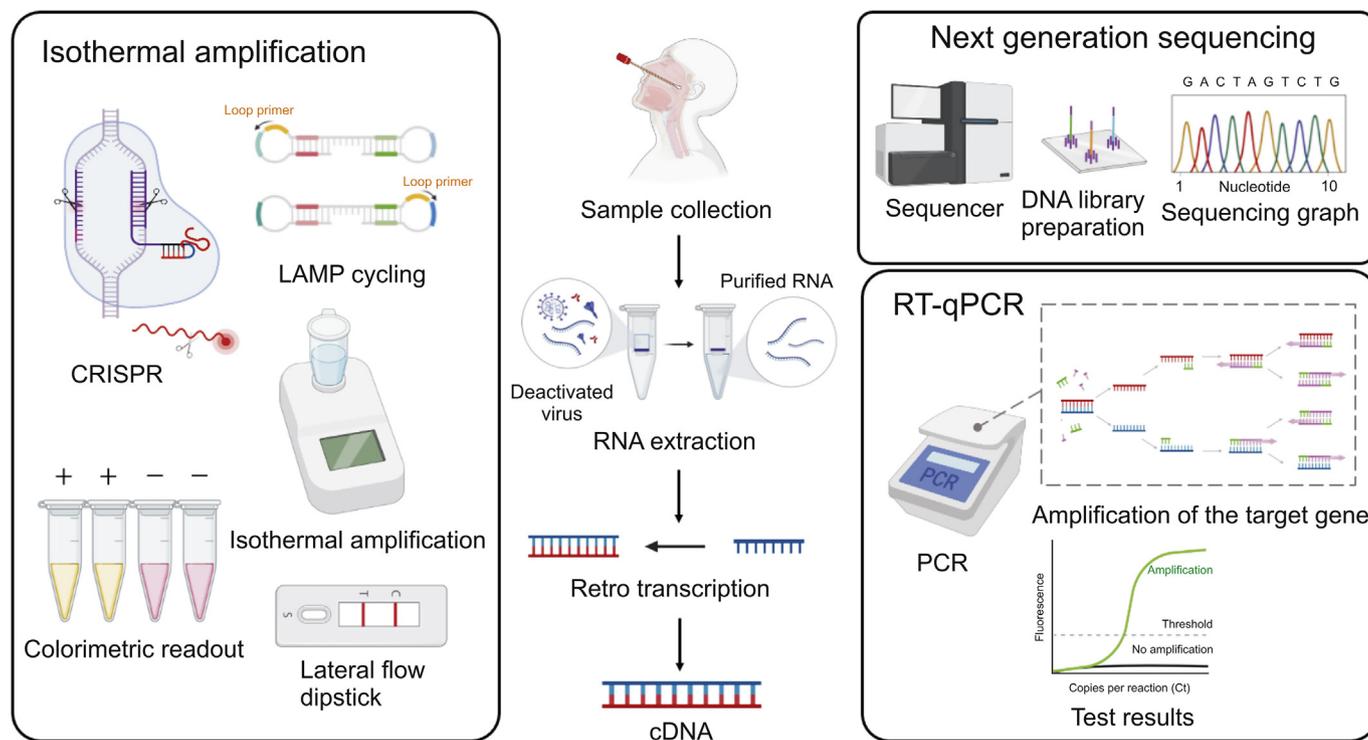
Fig. 1. Coronavirus disease 2019 molecular tests. First, RNA is extracted from patient samples and transcribed into cDNA. The cDNA can be used for RT-qPCR, where the cDNA is exponentially amplified via qPCR, and the test results are provided in real time or go through RT-LAMP, which can be accompanied by CRISPR, and the results can be analyzed through colorimetric readout or using a lateral flow dipstick. Sequencing (upper right) can also be employed to detect SARS-CoV-2. cDNA: complementary DNA; RT-qPCR: reverse transcription quantitative polymerase chain reaction; RT-LAMP: reverse transcriptional loop-mediated isothermal amplification; CRISPR: clustered regularly interspersed short palindromic repeats; SARS-CoV-2: severe acute respiratory syndrome coronavirus 2.

SARS-CoV-2 has been reported to be more immunogenic and triggers the production of antibodies earlier than the S protein [50]. In addition, a study has shown that assays targeting the S protein of SARS-CoV-2 show more cross-reactivity with antibodies produced against other related coronaviruses compared to assays targeting the N protein [51]. However, it remains unclear which antigen is superior in terms of the production of associated antibodies.

In the 2002/2003 SARS pandemic, the presence of antiviral IgM and IgG antibodies was found to be an indication of viral infection [52]. Because SARS-CoV-2 resembles SARS, the identification of IgM and IgG can assist the diagnosis of COVID-19. The kinetics of COVID-19 antibodies is inconstant. Typically, IgM is the first antibody produced during an infection and is linked to a primary immune response [53]. Therefore, the detection of IgM antibodies is utilized to determine recent infections. IgG antibodies, on the other hand, are known to be produced in later stages of infection and have a key role in long-term immunity and immunological memory [53]. One study reported that both IgM and IgG can be identified within day 4 after symptom onset in patients with COVID-19 [54]. The levels of IgM antibody declined at week 3 of infection, whereas IgG antibody levels remained the same and started to decline around day 28 after the onset of symptoms. IgA antibodies can be detected even earlier than IgM and IgG antibodies. However, IgA cannot be a good marker for SARS-CoV-2 detection, as it cross-reacts with other related viruses [55,56]. Generally, the specificity of the IgG antibody is higher than that of IgM [57], and its levels are higher in acute cases of COVID-19 than in mild cases. Thus, IgG levels are linked to disease severity [54]. Antibody titers differ among patients with COVID-19, depending on the disease severity. Earlier seroconversion and higher titers of IgM, IgG, and IgA have been reported in acute cases compared to mild to moderate COVID-19 cases [58,59]. Interestingly,

a study has shown that individuals pre-exposed to SARS-CoV-2 and who received one dose of the messenger RNA vaccine showed comparable immunogenicity and higher antibody titers than a naïve individual who received two doses of the same vaccine [60].

CLIA, LFIA, ELISA, and GICA are well-established methods for the evaluation and quantification of SARS-CoV-2 IgG and IgM antibodies (Table 4) [61–65]. Roy et al. [61] developed a robust quantitative ELISA assay that can detect and quantify IgA, IgM, and IgG antibodies produced in response to RBD. The sensitivity of this assay was 94.12% for IgA, 82.35% for IgG, and 77.94% for IgM. IgA had the highest sensitivity throughout whereas IgM and IgG sensitivity increased at later intervals. In another study, Xiang et al. [62] tested COVID-19 patients serum samples using ELISA and GICA to detect specific IgG and IgM antibodies. The sensitivity of the combined ELISA for IgM and IgG detection was 87.3%, whereas the sensitivity of the combined for GICA for IgM detection and IgG was 82.4%. Similarly, Cai et al. [63] developed a peptide-based luminescent immunoassay to detect antiviral IgG and IgM antibodies and evaluated it using sera from patients with COVID-19. The positive rate of IgG was 71.4%, while IgM had a positive rate of 57.2%. Combining these two antibodies improved the detection rate to 81.5%. Therefore, antibody tests that simultaneously detect IgM and IgG are more sensitive than tests that detect IgM or IgG separately.

LFIA tests are commonly used as POC antibody tests with a sample-to-result time of <30 min. Li et al. [64] designed a fast and easy-to-use POC LFIA to detect IgM and IgG antibodies from blood samples of COVID-19 patients in 15 min. The sensitivity of this assay was 88.66%, with a specificity of 90.63% for the combined IgM-IgG assay which is higher than separate IgM or IgG tests. Another group developed a colloidal gold-based immunochromatographic strip assay for the detection of IgG and IgM antibodies produced by

Table 4
Different serological assays available for SARS-CoV-2 detection.

Test	Sample source	Target	Sensitivity (%)	Refs.
ELISA	Serum samples	IgM, IgG or IgA	IgM: 77.94 IgG: 82.35 IgA: 94.12	[61]
ELISA	Serum samples	IgM and/or IgG	IgM:44.4 IgG: 82.54 Combined detection: 87.3	[62]
Gold-ICG assay	Serum samples	IgM and/or IgG	IgM: 57.1 IgG:81.3 Combined detection: 82.4	[62]
Peptide-based magnetic CLIA	Serum samples	IgM and/or IgG	IgM: 57.2 IgG: 71.4 Combined detection: 81.52	[63]
LFIA	Finger prick blood, serum, and blood plasma samples	IgM and IgG	Combined detection: 88.66—94.83	[64]
Colloidal gold-based ICG strip assay	Blood plasma	IgM and/or IgG	IgM: 11.1—74.2 IgG: 3.6—96.8 Combined detection: 11.1—96.8	[65]

ELISA: enzyme linked immunosorbent; ICG: immunochromatographic; CLIA: chemiluminescent immunoassay; LFIA: latera flow immunoassay.

recombinant SARS-CoV-2 N and S proteins [65]. The sensitivity of this antibody assay was measured in the early, intermediate, and late stages of infection. At the initial stages of infection, the positive rates for IgG and IgM were relatively low and started to increase as the disease progressed gradually. Thus, the sensitivity of antibody tests increases with respect to the stage of infection.

Antibody tests require minimal equipment and are relatively easier and faster than molecular tests. However, the latter offers the most sensitive detection for diagnosing patients with COVID-19. In addition, antibody tests are more applicable for diagnosing severe cases of COVID-19 or those who have been pre-exposed to the disease. Therefore, they can be used as a complement to molecular tests.

4. Alternative methods

4.1. Antigen tests

Antigen tests detect viral antigens or fragments of viral proteins rather than the viral genome. These tests have been commercially available for several years. Because they are target-specific, antigen tests are more accurate than antibody tests in diagnosing COVID-19. The most frequently used antigen test for SARS-CoV-2 detection is the fluorescence immunochromatographic (FIA) assay. The FIA assay developed by Diao et al. [66] detected the N antigen of SARS-CoV-2 in 10 min using nasopharyngeal swabs and urine samples. The sensitivity of this assay has been verified by RT-qPCR and has been reported to be 68% with 100% specificity. Although antigen tests are simple and only take a few minutes for the results to be ready, their accuracy is insufficient because they may not be able to detect active SARS-CoV-2 infection and have a higher tendency to elicit false-negative results. This may be due to inadequate viral loads or inconsistency in viral loads in patients. Antigen tests are more suitable for testing symptomatic and acute patients with adequate viral loads [67]. Therefore, further research is required to determine the efficacy of antigen detection methods.

4.2. Electrochemical approach

Electrochemical biosensors have great potential for the development of quick and sensitive COVID-19 diagnostic tests. A graphene-based field-effect transistor (FET) biosensor has been established for the detection of SARS-CoV-2, in which graphene sheets coated with antibodies against SARS-CoV-2 S protein act as a sensor to detect signal production in clinical samples upon SARS-CoV-2 binding [68]. The FET biosensor detected SARS-CoV-2 in clinical samples with an LOD of 242 copies/mL and distinguished the SARS-CoV-2 S protein from that of MERS without requiring sample pretreatment or labeling. Another biosensor-based test is the assay developed by Mavrikou et al. [69], which targets the S1 protein of SARS-CoV-2 and is connected to a portable readout device that is operated through a smartphone/tablet. The results can be obtained in as short as 3 min with an LOD of 1 fg/mL and a semi-linear range of response between 10 fg and 1 μg/mL. Due to its short sample-to-result time and the lack of prior sample processing, this biosensor can be applied for large-scale screening of patients with COVID-19 [69]. A similar biosensor device called "eCoVSens" has been constructed that targets the S1 protein of SARS-CoV-2, and its efficacy has been compared with that of a commercial potentiostat sensor [70]. The LOD of eCoVSens and commercial potentiostat was 90 and 120 fM, respectively, using saliva samples. These biosensors can have potential applications for the timely and easy-to-use diagnosis of patients with COVID-19 on a large scale.

Aptamer-based assays are a promising approach for the fabrication of COVID-19 biosensors. Aptamers are ssDNA or RNA oligonucleotides with high selectivity and specificity. They are considered as "artificial antibodies" and can detect various molecules with high selectivity and affinity; therefore, aptamers can be used in various applications including disease diagnosis [71]. Aptamers have previously been used to detect influenza virus, hepatitis C, hepatitis B virus, and SARS-CoV [72—75]. Aptamer-based assays for detection of SARS-CoV-2 have been also established. Zhang et al. [76] developed DNA aptamers specific to the SARS-CoV-2 N protein. These aptamers possess a high affinity for the SARS-CoV-2 N protein (K_d=0.49 nM) and can bind to it sequentially in a sandwich-type manner. These DNA aptamers can be utilized in serological assays such as GICA and ELISA to aid the detection of SARS-CoV-2 N protein present in human serum, sputum, or urine. Therefore, aptamers can be a suitable substitute for antibody-based assays because of their enhanced sensitivity, cost-effectiveness, and equal specificity. However, its clinical performance needs to be further evaluated.

4.3. Imaging of the lungs

Background chest CT is an essential complement to RT-qPCR tests and is faster and simpler than molecular tests and provides necessary information on the severity of the disease, an advantage that molecular tests cannot provide. However, CT results are variable and depend on the stage of infection. Commonly seen CT features in COVID-19 patients include
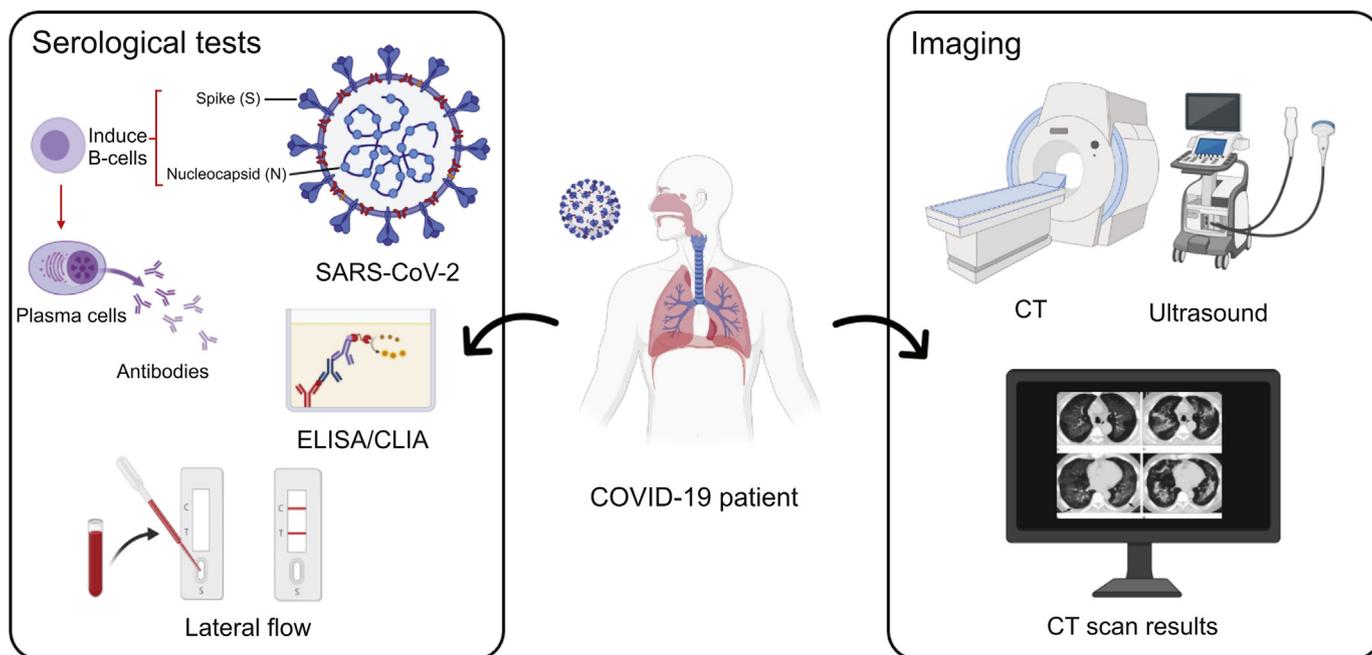
Fig. 2. COVID-19 serological and imaging tests. Once infected with SARS-CoV-2, the production of antibodies by B cells is induced. Serological tests, such as ELISA and chemiluminescent immunoassay (CLIA), can be utilized to detect and quantify antibodies produced in response to SARS-CoV-2. An individual with COVID-19 can also be diagnosed using imaging modalities, such as CT and lung ultrasound.

bilateral ground-glass opacities, primarily in the peripheral and lower lobes, similar to those seen in patients with SARS infection [77,78]. Studies comparing chest CT results of patients with COVID-19 with non-COVID-19 viral pneumonia such as influenza have indicated that ground-glass opacities and vascular thickening are more likely to be seen in COVID-19 CT results, whereas tree-in-bud sign, nodules, and peripheral effusion are less likely to be seen [79,80].

Patients with negative RT-qPCR results have been reported to sometimes have chest CT results showing irregularities consistent with those of COVID-19 [81]. However, chest CT of patients with COVID-19 may be negative at initial presentation, and its accuracy is limited since CT signs improve around 6—12 days after initial symptoms [78]. CXR and LUS have been used in a very limited number of COVID-19 cases. CXR and CT characteristics are comparable in patients with COVID-19, with both showing ground-glass opacities and/or bilateral peripheral consolidation [82]. Similar to CT, CXR signs develop in the later stages of infection around 10—12 days after symptom onset. The LUS features of patients with COVID-19 are irregular or interrupted pleural line thickening with small subpleural consolidations and B lines [83]. Imaging of the lungs plays a pivotal role in the management of SARS-CoV-2 infection. However, further investigation is needed to assess the utility of LUS and CXR in the diagnosis of COVID-19 patients. Fig. 2 shows a summary of the serological and imaging approaches used for the diagnosis of COVID-19.

5. Conclusion

The rate with which COVID-19 has spread worldwide explains why there is a pressing need for long-term investment in diagnostic testing. Many testing platforms have been developed for SARS-CoV-2 detection, and their number is rapidly increasing. Each testing technique has several advantages including some caveats. As a result, taking more than one test is crucial to ensuring the accurate diagnosis of patients with COVID-19 rather than relying on

a single detection method. RT-qPCR is a well-established method and is the most reliable and frequently used test for the diagnosis of COVID-19. However, RT-qPCR tests are time-consuming and can sometimes elicit false-negative or false-positive results due to improper sampling, timing of sample collection, and technical errors. Therefore, alternative testing platforms, such as an antibody or antigen-based test, should be used as a complement to molecular tests and to confirm suspected cases and take appropriate medical actions. Although vaccines are the only solution to ultimately ending the COVID-19 pandemic, the development of robust, cost-effective, and reliable tests to diagnose current and future emerging pathogens, such as COVID-19, is imperative.

CRediT author statement

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Declaration of competing interest

The authors declare that there are no conflicts of interest.

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