

REVIEW

Emerging clinically tested detection methods for COVID-19

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At the time of writing, there were 486 761 597 global cases of COVID-19 with 6 142 735 confirmed deaths (World Health Organization, 4 April 2022). According to the scarcity of information about estimation of cases with mild or no symptoms, it is suggested that they could represent 25–80% of all infections. The majority of these cases remain untested, although they are infective. The molecular diagnosis of COVID-19 is based mainly on quantitative reverse transcription PCR. However, this approach faces several challenges related to the shortage of resources and people who are adequately trained to run the tests. Alternative testing methods, targeting effectively several viral compounds at different stages of the infection, have quickly emerged. However, universal systems that are specific, sensitive, affordable, easy, portable and scalable are still warranted. In this review, a comprehensive compilation of the methods available is provided.

Introduction

SARS-CoV-2

In late December 2019, many cases of pneumonia with an unknown origin appeared in Wuhan (China). After several weeks, a virus designated as severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified as the causal agent of coronavirus disease 2019 (COVID-19). Coronaviruses are a large family of enveloped and positive-strand RNA viruses present in different mammals and birds, causing sickness ranging from the common cold to severe acute respiratory syndrome (SARS), including SARS-CoV [1], the Middle East Respiratory Syndrome MERS-CoV [2] and the recent SARS-CoV-2 [3]. This recent virus has a

remarkable ability to spread, mainly as a result of its trimeric spike glycoprotein (Fig. 1), which can attach to the angiotensin I converting enzyme 2 (ACE2) receptor with a higher affinity compared to the previous SARS-CoV virus [4]. Furthermore, the spike protein of SARS-CoV-2 possesses a solvent-exposed ‘Furin-like cleavage site’, different from other SARS-like CoVs, which is proposed as one of the reasons for its higher infectivity and faster spreading [5]. ACE2 receptor and furin enzyme are present in several human organs such as the lungs, liver and small intestines, and therefore are very susceptible to be infected. The new variants of concern (VOCs) that

Abbreviations

Ab, antibody; ACE2, angiotensin I converting enzyme 2; Ag-RDT, antigen rapid detection tests; CLIA, chemiluminescence immunoassay; COVID-19, coronavirus disease 2019; CRISPR, clustered regularly interspaced short palindromic repeats; Ct, cycle threshold; dRT-PCR, digital reverse transcription polymerase chain reaction; FDA, Food and Drug Administration; LFIA, lateral flow immunoassay; MERS-CoV, Middle East respiratory syndrome; NAAT, nucleic acid amplification test; RT, reverse transcriptase; RT-LAMP, reverse transcription loop-mediated isothermal amplification; RT-qPCR, quantitative reverse transcription PCR; SARS-CoV, severe acute respiratory syndrome coronavirus; SARS-CoV-2, severe acute respiratory syndrome coronavirus 2; SENSR, sensitive splint-based one-step isothermal RNA detection; VOC, variant of concern; WHO, World Health Organization.

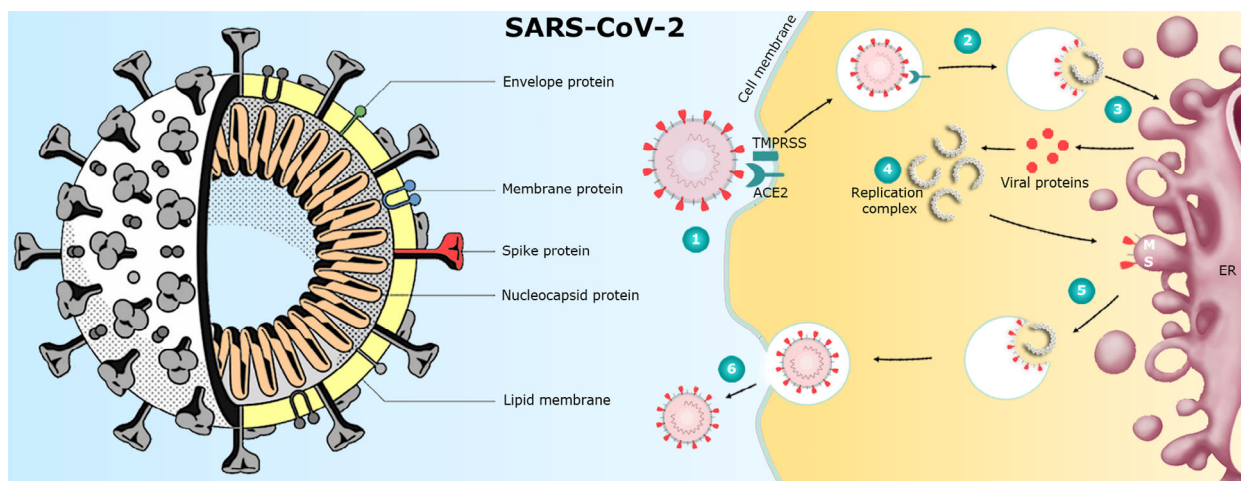


Fig. 1. The SARS-CoV-2 virus. The positive-sense, single-stranded approximately 30 kb RNA is enclosed in a pleomorphic particle of 60–140 nm diameter, constituted by four major structural proteins: spike (S), membrane (M), envelope (E) and nucleocapsid (N). Besides the structural proteins, the SARS-CoV-2 genome contains 15 non-structural proteins and eight accessory proteins, all of them playing a specific role in viral replication. Virus particle adapted from Knowlton [79] and distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0>). For cellular uptake (1), the virion binds to ACE2 cell-surface receptor through their Spike protein. TMPRSS2, surface serine protease assists the virion entrance (2). The virion releases its RNA (3) and the ORF1a and 1b are translated into non-structural proteins using the cell's machinery (4). Some of these proteins form the viral replication and transcription complex (5). *De novo* produced proteins and RNA are assembled into new virions in the Golgi and (6) secreted by exocytosis.

emerged during the pandemic, make the control of the disease rather difficult, mainly because of their higher transmissibility and severe clinical outcomes (especially in unvaccinated people). This is primarily a result of the incorporation of mutations in the spike glycoprotein, which ease their cellular internalization or more effectively avoid the immune response, amongst others [6].

Molecular diagnosis and types of tests

The detection of SARS-CoV-2 is currently achieved by three major strategies. Each one aims to detect an alternative analyte: (a) viral RNA, (b) viral proteins and (c) host antibodies (Abs) against the virus. A detailed description of their correct usage, sample timing, advantages and limitations is provided in Table 1.

The two first methods are suitable for detecting ongoing infections, whereas the third approach assesses whether the patient has been exposed to the virus. The first system is the reference method and can be the most sensitive because it can exploit the well-known exponential amplification processes based on enzymes [e.g. quantitative reverse transcription PCR (RT-qPCR)]. However, the extraction of the genetic material requires a specific elaboration of the sample. Also, it is important to note that the presence of RNA does not imply the existence of infectious viral particles [7]. The second approach is less sensitive and

requires a minimum concentration of viral particles, which can be detected using specific Abs and a readout system (e.g. fluorescence, electrochemical, colorimetric). The detection of viral proteins in the samples is a sign of active viral replication. The last method is an indirect approach that monitors the Igs generated during the infection. Thus, it might not be useful for the early detection of the virus because producing the required amount of Abs can take some time (6–14 days) [8], although it can be used to determine whether a person has already overcome the infection.

Based on all those strategies, companies and research centers are developing diagnostic systems worldwide. At the submission of this review, FIND, the global alliance for diagnostics (<http://www.finddx.org>), reported 1692 entries related to tests commercially available, or in development, for COVID-19.

Viral RNA approaches

Methods aiming to detect the RNA of the virus (specific regions in ORF1ab/RdRp, N, S and E genes) usually require the extraction of the genomic RNA and the removal of the different proteins that can interfere with the assay. The traditional method to extract the RNA involves guanidine isothiocyanate and organic solvents (phenol and chloroform) and is known as Trizol [9]. Nevertheless, the use of organic solvents might

Table 1. Molecular diagnosis and types of tests. Ab, antibody; LFIA, lateral flow immunoassay; ELISA, enzyme-linked immunosorbent assay; CLIA, chemiluminescence immunoassay; NAAT, nucleic acid amplification test; VOC, variant of concern; WHO, World Health Organization.

Detection method and type of sample	WHO recommended use and sample timing	Advantages	Limitations
Detection of viral RNA (NAAT test) <i>Nasopharyngeal, Oropharyngeal, Sputum, Saliva, Stool, Plasma</i>	Viral RNA may be detectable in the upper respiratory tract 1–3 days before the onset of symptoms Good for early-stage infections, especially in asymptomatic or mild cases. Combined nasopharyngeal and oropharyngeal swabs increase sensitivity and reliability Lower respiratory tract and faeces samples: second week after the onset of symptoms Good for patients with negative results from a upper respiratory tract sample and COVID-19 symptoms	RT-qPCR is the gold standard according to the WHO Well established technique High specificity and sensitivity Multiple and simultaneous target detection Easy adaptable to new sequences (VOC)	Costly, requires trained personnel and sophisticated equipment Needs validation to ensure reproducibility and reliability, especially because of the impact of the VOC mutations on the test performance (primers and sequence-specific probes design) Positive results (obtained during the convalescent phase) do not confirm active viral replication. It might be detected during months Results in 2–5 h
Detection of viral proteins (Antigen test) <i>Nasopharyngeal, Oropharyngeal, Sputum, Saliva</i>	Community screening of symptomatic people in the first 5–7 days from the onset of the symptoms Negative antigen test could be confirmed with a NAAT or, if this is not available, repeat the Antigen test (within 48 h) Detection and response to suspected COVID-19 outbreaks Screening of asymptomatic people in scenarios of high risk of COVID-19, such as healthcare professionals, COVID-19 patients' direct contacts, or other individuals in risk	Well established technique Fast (5–15 min) No specialized training required Low cost and scalability Depending on the sensitivity, positive detection can be accomplished from the beginning of the infection Can detect most infectious cases Obtaining positive results in the antigen test in many of the suspected cases is very indicative of a COVID-19 outbreak	Limited sensitivity and high false-negative rate It might require confirmation by NAAT Needs validation to ensure reproducibility and reliability, especially because of the impact of the VOC mutations on the test performance (Antigen modification could impair the immunocomplex formation) Better results in symptomatic patients with high viral loads, at the beginning of infection Little predictive value in communities with low or non-transmission (higher false-positive risk) Potentially affected by the emergence of antigenically-different VOC. The changes in structure and sequence in protein targets upon mutation could affect the reliability of the test Reproducibility problems False negatives because of an inadequate sampling (auto test) Qualitative information
Detection of host AbS against the virus (serological test) <i>Blood</i>	No used routinely for the diagnosis of COVID-19. High variability from patient to patient. Seroconversion could take weeks for patients with subclinical/mild infection If negative NAAT results are obtained from a patient in whom SARS-CoV-2 infection is strongly suspected, a paired serum specimen taken in the acute phase and one in the	Well established technique Fast (5–15 min for LFIA) <i>Point-of-care</i> for testing in place without the participation of specialized operators A sample (capillary blood) is easy to obtain with a low infection risk (no aerosol generation as in Nasopharyngeal sample taking) Useful for detecting past infections and research purposes and surveillance	LFIA has limited sensitivity and a high false-negative/positive rate compared with other methods (ELISA, CLIA) Average reproducibility Qualitative information Needs validation to ensure reproducibility and reliability, especially because of the impact of the VOC mutations on the test performance (Specific patient AbS against new variants can 'scape' if the antigen used is not appropriate)

Table 1. (Continued).

Detection method and type of sample	WHO recommended use and sample timing	Advantages	Limitations
	convalescent phase can be used retrospectively to determine whether the individual has had COVID-19	Moderate cost and scalability	Highly dependent on the immune system of each patient and the sampling time Indirect indicator of active infection False-positive risk because of cross-reaction with other human coronaviruses, or with pre-existing conditions (e.g. pregnancy, autoimmune diseases)

limit its use in different settings and, to overcome these limitations, a variety of extraction and purification kits have been developed. Direct RT-PCR from nasopharyngeal swabs may provide an alternative to RNA extraction, although limitations regarding the input volume, as well as an increased risk of RNA degradation and PCR inhibition, can lead to a loss of sensitivity [10,11]. Heat treatment prior to sample processing can affect the RNA quality [11,12]. Other factors that can affect RNA quality are the addition of detergents, transport media, the volume of the specimen used and the polymerase enzyme used [10,13–15]. It is worth mentioning some alternative methods such as BSA, proteinase K treatment or acid pH, which can be used to obtain pure RNA [16,17]. However, the biosafety implications of these alternative methods should be considered.

qRT-PCR, the gold standard approved by the World Health Organization (WHO)

The first method implemented to detect the virus was RT-qPCR (Fig. 2). It is the most widely used nucleic acid amplification test (NAAT) and the one endorsed by the WHO for detecting the virus. This approach requires the isolation of the viral RNA, followed by its conversion to the cDNA by a reverse transcriptase (RT). Then, exponential amplification of the DNA is carried out via the PCR. In this process, two primers bind the cDNA to initiate their elongation by the polymerase. Once this process is finished, a DNA duplex is obtained, which can be used again in the subsequent cycle. The detection of these new DNA duplexes can be achieved using a dye that lights up when intercalated in the duplex, or a DNA-base probe that recognizes specific regions on DNA (Fig. 2). However, one critical issue is the selection of the primers and the DNA probe, which might affect the sensitivity and specificity of the method. In this regard, several studies have compared different sets of primers

used in clinical, research and hospital laboratories [18,19]. In this sense, it is worth emphasizing that using the same set of primers, at least in a regional context, is desirable for comparing the results properly. Also, continuous surveillance of primer and probes sets sequences for detecting SARS-CoV-2 evolving RNA is advisable. For that reason, global, updated and exhaustive resources that help with this purpose are very welcome. In this sense, the CoVrimer webserver can be used to search for and align existing or newly designed conserved/degenerate primer pair sequences against the viral genome and assess the mutation load of both primers and amplicons [19]. Also, FIND, the global alliance for diagnostics (<http://www.finddx.org>), compiles data from communications and published reports by manufacturers (including those listed for emergency use by WHO) and provides relevant information regarding the impact of a VOC in the PCR (Table 2) and antigen testing. For the majority of the COVID-19 molecular tests (PCR), the overall sensitivity of detection is not expected to be impacted by the VOC specific genotype as long as these tests have multiple gene targets. Thus, if one gene target is not detected as a result of mutations or deletions, the other targets are still recognized. However, all these recommendations imply a great effort in terms of material and personal resources. To mitigate this, an alternative pooled-sample PCR analysis has been proposed. A deconvoluted individualized analysis of this specific group should only be performed if a pooled test is positive [20]. In general, in low-incidence areas, pooling may enable a laboratory to use a larger optimal pool size [21]. However, this strategy is not applicable in high-incidence areas because, as COVID-19 increases, the cost savings of a pooling strategy decreases. In this scenario, more pooled tests will be positive, and those specimens will need to be retested individually. Complementarily, novel approaches based on a modified PCR process and/or easier read-out systems can also be useful for reducing the cost of the process [22].

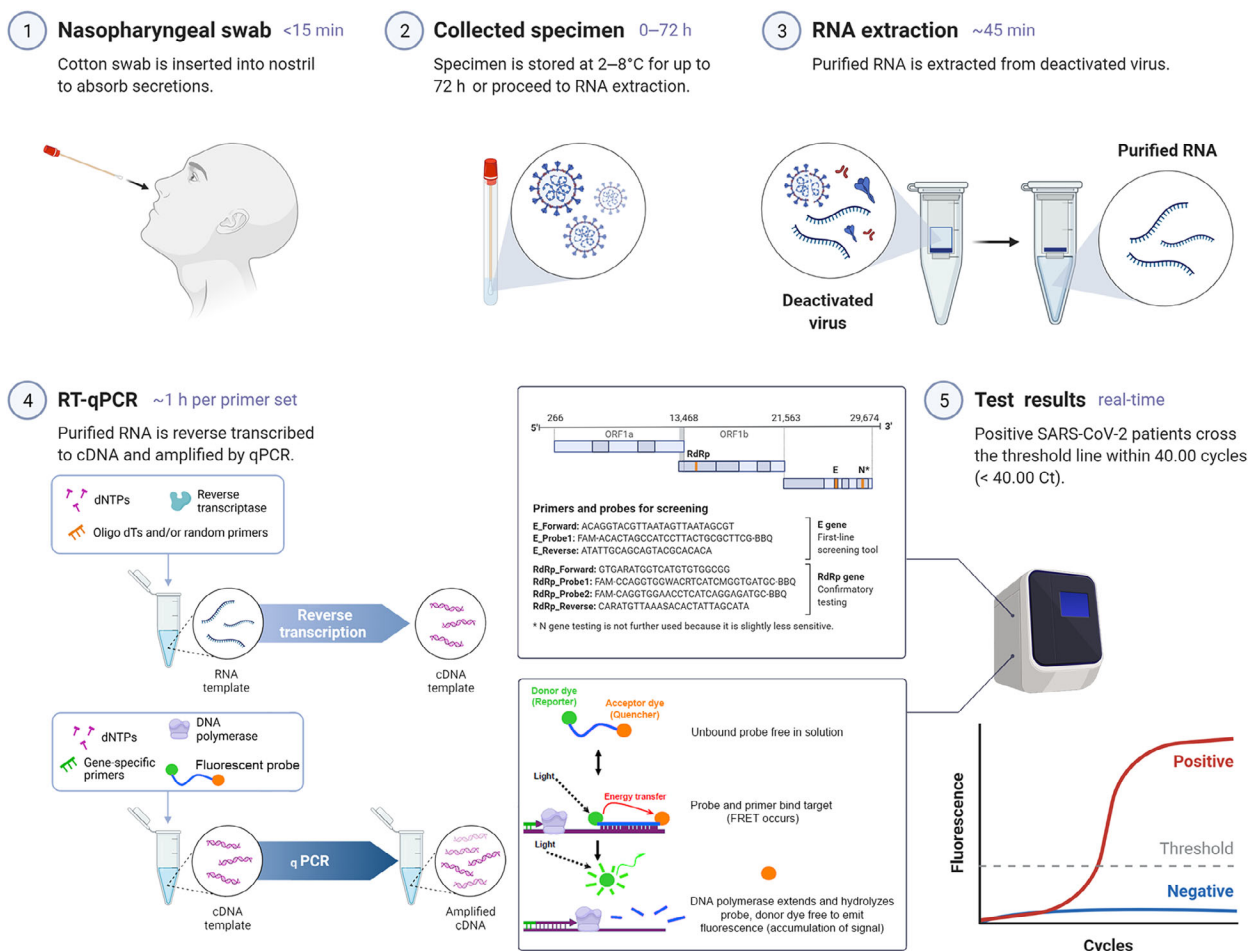


Fig. 2. The RT-qPCR detection method for SARS-CoV-2. RNA from a SARS-CoV-2 sample is first extracted from the virus (steps 1–3) and converted to its cDNA sequence by a process called reverse transcription (RT, step 4, upper right). The DNA thus transcribed is amplified by qPCR (step 4, bottom right). With qPCR, fluorescent dyes are used to label PCR products during thermal cycling (step 4, left). The qPCR machine measures the intensity of fluorescence emitted by the probe at each cycle (step 5). During the first cycles, there is insufficient fluorescence for detection, although the reaction rapidly produces more and more amplicons and the fluorescence builds up. A qPCR curve typically has an exponential phase followed by a plateau phase. The Ct measure is a determined PCR cycle and represents the basic result of a qPCR experience. It is taken in the exponential phase, where the curve is linear. The threshold (grey line) is placed in the linear phase, and the Ct is measured where the PCR curve crosses the threshold. The threshold is different for every qPCR assay (every gene tested) and is the same for all samples tested with this gene. The principle of the qPCR is based on the fact that, at each PCR cycle, the number of PCR products doubles. If there is a difference of two cycles between two reactions, we can say that there are four-fold more copies in the reaction with the lower Ct value compared to the other reaction. Adapted from 'COVID-19 Diagnostic Test through RT-PCR' and 'One-step vs. Two-step RT-PCR' via BIORENDER (<https://biorender.com>).

The viral load in patients, as measured by RT-qPCR, varies significantly depending on the type of sample and the time after the onset of symptoms [23]. A nasopharyngeal sample provides the most sensitive results, whereas sputum in subjects with a productive cough also possesses a high diagnostic power compared to the oral cavity or salivary glands [24]. Most patients present viral RNA in stool samples over 3 weeks, even without symptoms, suggesting an active replication of the virus in the gastrointestinal tract [25]. On the other

hand, urine and blood samples rarely test positive for viral RNA [26,27]. For these reasons, different authorities, such as the Food and Drug Administration (FDA), recommend analyzing multiple specimens from the same patient to increase the reliability of the test.

In general, the peak concentration of the virus is reached before day 5, with or without symptomatology, and the viral load is proportional to transmission risk [28]. Therefore, a patient's infectivity is expected to be the highest during this period [29]. However, the correlation

Table 2. Commercial Kits suitable for all SARS-CoV-2 VOC detection. Information was obtained from <http://www.finddx.org/> and/or directly from the company webpage. US FDA EUA, Emergency Use Authorization by the US Food and Drug Administration; CE-IVD, European CE Marking for In Vitro Diagnostic device; RUO, research use only; VOC, variant of concern.

Company name	Test name	Regulatory approval	Test target	S gene dropout
Illumina	Illumina COVIDSeq Test	US FDA EUA	Unknown	No expected S-gene drop out (based on <i>in silico</i> analyses)
Gold Standard Diagnostics	GSD NovaType IV SARS-CoV-2	CE-IVD	S gene	Only 0.84% of published SARS-CoV-2 genomes carrying the S gene targets K417N, L452R, E484K and E484 might affect the performance of the test (based on <i>in silico</i> analyses)
PerkinElmer	NEXTFLEX® Variant-Seq™ SARS-CoV-2 kit V2	RUO	All genes	No expected S-gene drop out. The kit has been designed to detect all mutations associated with SARS-CoV-2, including those specific to the omicron variant, in a PCR-positive sample
PerkinElmer	PKamp™ VariantDetect™ SARS-CoV-2 RT-PCR Assay	RUO	S gene	No expected S-gene drop out. The assay allows to perform secondary testing on positive COVID-19 samples to identify mutations in the SARS-CoV-2 virus in the samples, including those specific for VOC Alpha, Beta, Gamma, Delta and Omicron
QIAGEN GmbH	QIAseq DIRECT SARS-CoV-2 Kit	RUO	All genes	No expected S-gene drop out. The protocol utilizes a streamlined, workflow for enrichment and library prep of the SARS-CoV-2 virus genome. This procedure improves robustness, uniformity of coverage prior to library amplification and indexing
PathogenDx	DetectX-Rv	US FDA EUA	N gene	Not applicable

between viral load and the severity of the illness is still controversial and is not a clear predictor of disease outcome [30], although there is a clear correlation between cycle threshold (Ct) and viral RNA copies in the samples [31]. Nevertheless, in the SARS-CoV-2 model, the use of Ct values as precise indicators of the viral titer in nasopharyngeal samples is controversial [32], particularly when the objective is to correlate with transmissibility and/or clinical symptoms or outcome prediction. This is a result of several factors, such as the sample's source or inadequate sample handling and transportation. In this sense, if the sample collected is heterogeneous, it might not reflect the specific virus titer in the patient's nasopharynx. Also, the degradation of the sample can dramatically alter the results. Besides these concerns, different PCR platforms use alternative genes as targets for detecting SARS-CoV-2, leading to distinct Ct values for the same sample. Therefore, the Ct value should be considered, in this case, as a relative value. Although, there are several approaches developed to quantify the viral load, they are not implemented yet in the routine diagnosis of SARS-CoV-2. However, from an epidemiological point of view, the Ct values in the nasopharyngeal samples could be analyzed by ranges, which could be correlated as a surrogate marker of viral load; for example: Cts < 20 (very high viral load), Cts ~ 20–25 (high viral load), Cts < 30 (moderate to low viral load) and Cts 30–33 (very low transmitting risk or none).

False positives and negatives in RT-qPCR

Nucleic acid-based tests face important limitations in terms of false positive or negative results. The false positives are usually related to suboptimum specimen handling or inadequate reagents. Also, the high sensitivity of these techniques could be an Achilles' heel because minimal contamination can alter the results. This can originate from non-amplified nucleic acids (other clinical samples, positive controls, contaminated equipment or reagents) or amplified nucleic acids (amplicons) that can be carried over from a previous reaction [33]. To minimize false positives, good laboratory practice, clean working areas, and sterilization of the materials and surfaces are encouraged. Anyhow, false positives are a rare occurrence in molecular biology laboratories, and their consequences for the containment of COVID-19 are minimal compared to false negatives, for which the rate can be as high as 23% [34] and have a significant impact on the spread of the disease. For example, it has been observed that patients might present negative results, despite showing clinical symptoms such as fever or cough [35]. This might be because of the low viral load, the use of unoptimized primers and probes, or inappropriate handling of the samples, amongst others. Indeed, up to 58% of COVID-19 patients may have initial false negative RT-PCR results [36]. Thus, it is really

advisable to re-test patients suspected of SARS-CoV-2 infection, despite their negative RT-qPCR.

Alternative amplification methods

Digital RT-PCR

Dong *et al.* [37] have developed a more sensitive alternative using digital RT-PCR (dRT-PCR). In this case, the measurements are performed by dividing the sample into partitions (either chip-based or more often droplet-based), such that there are either zero or one or more target molecules present in any individual reaction. Each partition is analyzed after end-point PCR cycling for the presence (positive reaction) or absence (negative reaction) of a fluorescence signal (probe-based detection), and the absolute number of molecules present in the sample is calculated. It does not rely on a standard curve for sample target quantification and, in this case, each sample's fluorescence is measured at the end, reducing error and improving precision. Interestingly, dPCR is less sensitive to varying amplification efficiencies, the presence of PCR inhibitors or primer-template mismatch than qPCR [38]. After the measurements, Poisson statistical analysis of the numbers of positive and negative droplets yields the absolute quantitation of the target sequence. The dRT-PCR had a detection limit of approximately 2 viral RNA copies per reaction and allowed the diagnosis of the disease at the initial stages, with a low viral load. Similar conclusions were obtained in other laboratories, reporting a limit of detection at least 10-fold better than that of RT-PCR and a superior performance for the detection of asymptomatic cases of COVID-19 [39,40]. Amongst others, interesting reviews were published about the use of dPCR in COVID-19 diagnosis [41] or in the monitoring of the disease course by correlating the viral RNA plasma levels with the disease severity or progression [42]. Despite the significant advantages of dPCR over qPCR, there are still some limitations that prevent its universal application in clinical laboratories, such as its higher cost or the unavailability of commercial instruments and master mix reagents.

Isothermal amplification

Despite the excellent results obtained through PCR-based systems, one of their limitations is the use of PCR machines, which can be costly and require specialized personnel. For these reasons, a variety of isothermal amplification processes have been explored. They require a specific combination of enzymes and primers to amplify the nucleic acids, but the process can be

done at a fixed temperature. Thus, a basic thermoblock, or a water bath, can be used. Additionally, these isothermal amplification strategies have been optimized to allow an easy readout using fluorescent or colorimetric dyes. Therefore, they can be implemented in almost every healthcare facility (taking advantage of the color change inspection for the visual readout) and some approaches have been recently approved for personal home testing as 'Point-of-Care' devices.

RT-LAMP—Reverse transcription loop-mediated isothermal amplification (RT-LAMP) combines LAMP with a reverse transcription initial step to detect RNA. The method is based on auto cycling strand displacement DNA synthesis. The LAMP reaction involves four specific primers recognizing distinct target sequences on the template strand, making RT-LAMP a highly specific method. These primers are used in the presence of a DNA polymerase with strand displacement ability, which releases the newly formed DNA strands. One major challenge preventing LAMP from being used as a universal detection method for COVID-19 is the design of robust LAMP primers. The classical empirical testing of primers often yields sub-optimal results. In this sense, some algorithms can be used to improve their design, reducing the overall screening and optimization time [43]. A PCR can take around 3–4 h in total, whereas RT-LAMP takes less than 1 h (only for experimental routines avoiding RNA extraction [43,44]) and is performed at a temperature between 60 and 65 °C. The approach is very well known, and has been implemented to detect SARS-CoV-2 [45], providing a sensibility and accuracy similar to RT-qPCR. The lowest limit of detection of RT-LAMP is 3 SARS-CoV-2 RNA copies/reaction [46]. Interestingly, the approach could be combined with other molecular amplification methods such as the recombinase polymerase amplification technique [47] or the clustered regularly interspaced short palindromic repeat (CRISPR) system [48,49].

Kellner *et al.* [50] describe a method called 'HomeDip-LAMP' where SARS-CoV-2 RNA capture by magnetic beads (enrichment) is directly subjected to RT-LAMP amplification with colorimetric readout. In this approach, RNA isolation is avoided, the enzyme is likely to be prepared in any molecular biology laboratory and the reaction does not require sophisticated laboratory instruments. The result can be obtained in 35 min, with a sensitivity comparable to RT-PCR for a medium viral load in the samples.

There are a few RT-LAMP developments that have been approved by the FDA, such as the AQ-TOP™

COVID-19 Rapid Detection Kit (SEASUN Biomaterials Inc., Daejeon, Korea), the Color SARS-CoV-2 RT-LAMP Diagnostic Assay (Color Health, Burlingame, CA, USA) or Lucira COVID-19 All-In-One Test Kit Labeling (Lucira Health, Emeryville, CA, USA). The last one comprises a qualitative test that is very easy to use and detects SARS-CoV-2 RNA using a nasal swab from individuals with suspected COVID-19, in less than 30 min. After its extraction at room temperature, the RNA accesses a fluidic module, solubilizing the lyophilized reagents needed for the amplification of the sample and the test controls. An internal electronic heating element is automatically activated upon chamber filling, triggering the reaction. A successful amplification varies the pH, driving a color change of the halochromic agents within the reaction mixture, which is detected in real-time and analyzed via an on-board microprocessor with a diagnostic algorithm. The results are displayed as either positive, negative or invalid by a LED indicator. This valuable system claims a sensitivity of 100% on samples with equivalent qRT-PCR Ct values ≤ 37.5 , albeit it is not exempt from false negative results because of an improperly collected or handled specimen or when low levels of viruses are present. For the moment, this test has not been evaluated for patients without symptoms of COVID-19.

Sensitive splint-based one-step isothermal RNA detection (SENSR)—Complementarily to LAMP, Woo *et al.* [51] developed another isothermal approach for the detection of SARS-CoV-2, based on two enzymatic reactions. The process named SENSR could be used to detect RNA sequences in 30 min at a very low concentration (0.1 aM) using a fluorometer. The first reaction involves ligating two RNA molecules, templated by the target sequence. Then, a T7 RNA polymerase transcribes the sequence leading to the production of an RNA aptamer. This molecule recognizes a dye, which fluorescence increases significantly upon binding. The overall procedure implies the combination of the reagents at 37 °C, in the presence of the target RNA sequence and quantification of the fluorescence. The system has been tested for the detection of SARS-CoV-2 and different microbial RNAs, including bacteria and other viruses such as MERS-CoV and influenza A. In addition, the combination of two different aptamers and dyes in the same system allowed the detection of two pathogens simultaneously.

CRISPR-based methods

CRISPR is currently considered the most potent gene-editing tool, including some outstanding examples in

recent years. Interestingly, the ribonucleoproteins involved in that process have been applied for other purposes, from gene regulation at the mRNA level to detection systems. In this sense, Joung *et al.* [52] have developed a viral RNA detection system in one pot known as SHERLOCK. This approach requires the combination of (a) an isothermal amplification method (RT-recombinase polymerase amplification), (b) a thermostable Cas protein (LwaCas12b) to target the RNA sequence and (c) a lateral flow strip to ease the readout of the assay. Using this strategy, it was possible to detect RNA sequences present in SARS-CoV-2 patient samples from 10 to 200 aM (10–100 RNA molecules· μL^{-1}). Remarkably, the process can be completed in almost 1 h after the RNA extraction.

A related approach (so-called DETECTR) has been developed by Broughton *et al.* [48] and evaluated using clinical samples. In this case, the isothermal amplification was RT-LAMP, the nuclease employed to detect the sequence of the virus was Cas12 and a lateral flow strip was used to visualize the result. The primers used for the amplification are based on regions already validated by WHO and the US Centers for Disease Control and Prevention, but adapted for LAMP. The detection process takes around 30 min, from the extracted RNA, and can detect 10 copies· μL^{-1} with identical specificity and 90% sensitivity compared to the US Centers for Disease Control and Prevention qRT-PCR method.

Yoshimi *et al.* [53] have developed the CONAN system (Cas3-Operated Nucleic Acid detectionN), using, for the first time, a CRISPR class I and type I-E system. Similar to DETECTR, the system uses the non-specific cleavage activity on single stranded DNA of Cas3 upon the specific activation of the enzyme once viral RNA is localized. The method was validated by confirming the results in nine out of ten cases of RT-PCR positive patients (90% reliability) with a sensitivity of around 100 copies per reaction, and a positive was detected among 21 negative samples previously tested by RT-PCR.

An alternative method, called Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic acids (CARMEN), has been reported by Ackerman *et al.* [54] This approach combines genetic diagnosis mediated by Cas13 with microfluidics, allowing the simultaneous detection of hundreds of viruses in multiple samples or the same virus in more than 1000 different clinical samples. This system enables robust testing of > 4500 clustered regularly interspaced short palindromic repeats RNA-target pairs ('individual test') on a single array, with replicates and controls. Thus, the clinical samples must be processed first to

extract the RNA and transcribe it into cDNA. Then, a collection of inputs (samples) is prepared in the form of droplet emulsions, which organize themselves in the wells of the array, creating all possible pairwise combinations in replicates. Once in the wells, the samples encounter the different molecular components of the detection system. The readout is based on fluorescence (1050 different combinations only by mixing four dyes in different proportions) and has attomolar sensitivity and a reliability similar to next-generation sequencing. Remarkably, the cost of the system is > 300 times less than that of SHERLOCK. The capabilities of this technique are huge; however, it requires highly specialized personnel, which can limit its implementation in healthcare facilities.

Recently, a quantitative method has been developed also based on Cas13, with a sensitivity of ~ 100 copies- μL^{-1} in 30 min. The method does not require pre-amplification of the viral RNA and the readout could be performed with a mobile phone prepared to detect the changes in fluorescence intensity upon SARS-CoV-2 detection [55]. However, only five clinical samples have been tested with this approach. Similarly, López-Valls *et al.* [56] described CASCADE, an approach combining recombinase polymerase amplification, Cas13 and gold nanoparticles that could detect the presence of the virus with the naked eye in 30 min.

Detection strategies based on Abs

Currently, there are 967 immunoassays for COVID-19 commercially available or in development (<https://www.finddx.org>). Among them, only a small number pursue the detection of the virus using targets other than the genomic RNA, which are known as virus antigens. The vast majority are designed to detect the neutralizing Abs generated by patients. Those systems could become powerful point-of-care devices if they are properly validated. The majority of the tests are based on lateral flow immunoassay devices. As in other molecular techniques, there are several factors affecting the qualitative nature of these tests [57]. Briefly, manufacturing issues, operator errors, environmental factors, sample manipulation and cross-reactivity problems are some of the elements contributing to the potential alteration of the results.

Detection of viral proteins

The exquisite affinity and specificity of the antigen–Ab interaction enable the detection of the virus without further processing the sample. Thus, the diagnosis is based on the presence of a protein of the virus in body

fluids (mostly in secretions of the upper respiratory tract). This interaction can be evaluated by different methods, where the most established is the ELISA (Fig. 3); specifically, the sandwich ELISA, where two different Abs are used to detect the target. The method is stepwise and, in the first step, a reaction substrate (e.g. a plate, a nitrocellulose strip) is coated with the capture Ab, raised against the antigen of interest. Next, the sample is added, and any antigen present will bind to the capture Ab. In the third step, a detection Ab is added. This Ab is usually linked to an enzyme or labeled with a fluorescent dye. The detection Ab also binds to the target antigen already bound in the previous step. Finally, a substrate is added, and the enzyme mediates its transformation into a chromogenic colored product, which can be measured using a spectrophotometer. By contrast, if the Ab was conjugated to a dye, a direct measurement using a Fluorometer could be performed.

The WHO and the Pan American Health Organization comment that these assays have acceptable specificity and can be used as a confirmation criterion (in conjunction with the case definition, the clinical history and the epidemiological history) and make public health decisions [58]. However, the dynamics of production and secretion of these proteins (antigens) have not been established; therefore, a negative result (at any stage of infection) should not be used as a criterion to rule out a case and, consequently, other criteria must be taken into account. According to the WHO and other validation sources [59], the sensitivity for this test might vary from 34% to 94%, based on experience with antigen-based tests for other respiratory viruses such as Influenza.

FIND (<https://www.finddx.org>) lists 577 commercialized SARS-CoV-2 antigen rapid detection tests (Ag-RDT). Those systems consist of a lateral flow immunotest, which recognizes the SARS-CoV-2 nucleocapsid or spike proteins in 15–30 min, from a nasopharyngeal or saliva sample (Fig. 4). This test is qualitative and helps diagnose COVID-19 in people with a moderate to high viral load. Ag-RDTs are most reliable in patients with high viral loads (Ct values ≤ 25 or $> 10^6$ genomic virus copies- mL^{-1}), which usually appear in the pre-symptomatic (1–3 days before onset of symptoms) and early symptomatic phases (within the first 5–7 days of illness) [60]. A negative result cannot discard the infection, although their use in hospitals or other non-specialized contexts could be beneficial for early diagnosis and the isolation of the most infectious patients and their contacts [61]. According to WHO, very few SARS-CoV-2 Ag-RDTs have undergone rigorous regulatory review. Indeed,

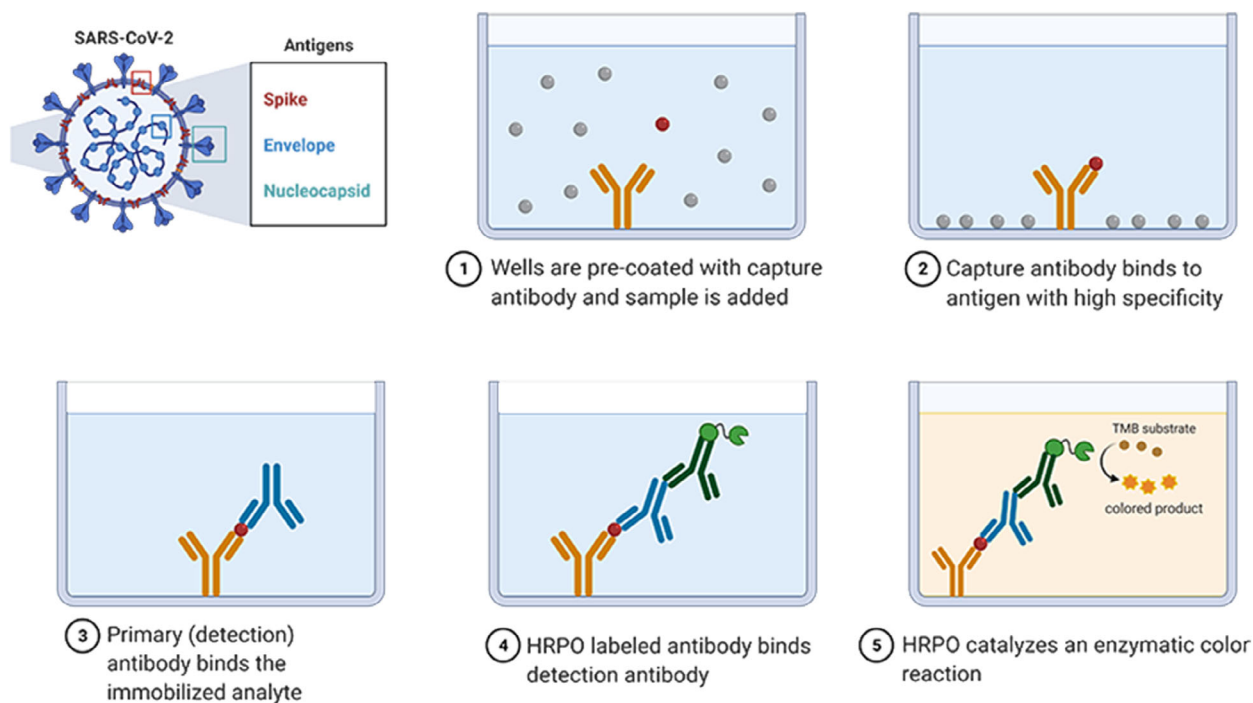


Fig. 3. Scheme for an ELISA sandwich assay used for the detection of specific SARS-CoV-2 antigens. The principle is as follows: (1) The well is coated with a capture Ab that specifically recognizes a SARS-CoV-2 antigen; (2) the patient sample is added, and any antigen present binds to capture Ab; (3) detecting Ab is added, which binds to the same antigen, but in a different location (epitope); (4) enzyme-linked secondary Ab is added, which binds to detecting Ab; (5) substrate is added, and is converted by enzymes to a detectable form (in this example, colorimetric). Partially adapted from 'Sandwich ELISA' via BIORENDER (<https://biorender.com>).

only 38 tests have FDA Emergency Use Authorization and another five have been approved by the WHO.

Detection of host Abs

The SARS-CoV-2 virus is an emerging virus and the immune response against this pathogen remains poorly understood in terms of clinical value or protective response duration. Regarding the humoral immune response, the SARS-CoV-2 virus elicits an Ab response just a few days after infection. The infected individuals start producing specific Abs (IgM, IgG and IgA), and this process is called seroconversion. IgM and IgA, are detectable during the first week, peaking in the second week after symptom onset and persist for more than 1 month [62]. IgG appears later but continues circulating in the blood, whereas IgM/A levels drop [63]. Its total persistence needs to be determined, although it usually reflects a previous infection.

Several researchers and institutions point out that the combination of PCR and Ab detection increases the detection percentage by up to 100% from 15 days after the onset of symptoms [64–66]. The quantitative data of Abs also can be used for the stratification of

patients between critical and non-critical groups. In addition, IgM/IgA determination can also be an alternative method for COVID-19 ongoing infection. However, we cannot rule out the possibility of detecting IgM in the blood even 2 months after the initial infection [67]. Among the tests, IgG-based ones perform better than IgM, although a better choice appears to be their combined use [68]. Also, the ELISA and lateral flow immunoassay (LFIA) show a better specificity (around 99%), whereas ELISA- and chemiluminescence immunoassay (CLIA)-based methods provide a better sensitivity (90–94%). Antibody detection faces other problems. If the inactivation of the sample for biosafety reasons is required, this may reduce the Ab levels by an average of 50%. Furthermore, false positive results as a result of cross-reaction cannot be entirely avoided because of the genetic and structural homology with other coronaviruses [69]. Another critical issue is that the absence of a detectable Ab response at a particular moment does not mean that the person already had the disease. In this sense, A T-cell immunity against SARS and SARS-CoV-2 has been observed. Also, previous infection with betacoronaviruses induces a long-term T-cell

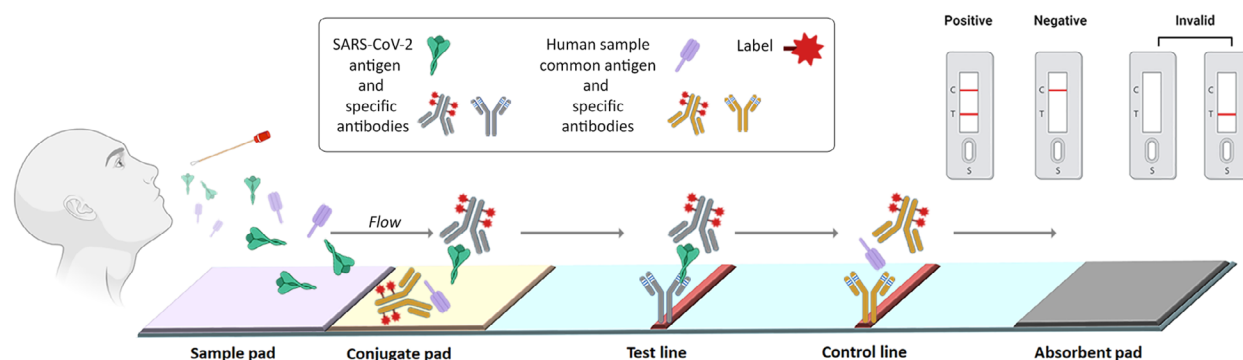


Fig. 4. Scheme for a SARS-CoV-2 antigen rapid detection tests (Ag-RDT) in a lateral flow strip with colorimetric readout. Ag-RDTs are similar to an ELISA test for antigens detection (Fig. 3), but in flow, in a nitrocellulose (NC) strip. The patient sample (Table 1) is chemically inactivated and loaded into a NC strip (in the sample pad). Driven by the flow, antigens move towards the conjugate pad, where the immunoreaction takes place and the complexes are formed. Two independent detection Abs recognize either (a) a SARS-CoV-2 antigen [test (T)], if any, or (b) a human antigen present in all well-taken samples [control (C)]. Both Abs are conjugated to gold nanoparticles (the label). Once the immunocomplexes reaches the T or the C line, respectively, a new Ab against a different epitope, but in the same antigen, retains the complex and stop its motion. This confinement in the NC lines results in a red signal, and the test could be interpreted on the basis of the instructions, for a period of around 15 min. These test are qualitative, and so even a weak T line together with a positive C line is indicative of COVID-19 infection. Partially adapted from 'COVID-19 Diagnostic Test through RT-PCR' and 'COVID-19 Serologic Diagnostic Test through Antibody Detection' via BIORENDER (<https://biorender.com>).

immunity against N protein that could help to neutralize COVID-19 infections [70]. According to the WHO, sensitivity is generally higher for laboratory versus point-of-care tests and depends on many factors such as the laboratory cut-off for sensitivity versus specificity, the severity of the infection [71] or the antigen used [58,71]. There are many companies and laboratories producing and distributing antigenic SARS-CoV-2 related proteins or peptides for the serological test. The differences in these molecules need to be analyzed with respect to designing and interpreting the immunological test, and also because viral mutations make the test more likely to fail to detect new variants' antigens. Liu *et al.* [72] assessed an ELISA in 214 COVID-19 positive patients and 100 healthy donors from China using the N and S proteins. The study revealed that the S-based IgM ELISA was more sensitive than the N one. However, no significant differences were observed in the detection of IgG or total Abs (IgG and IgM). Also, the sensitivity of the assays depended on the day from onset of symptoms, providing higher values on day 10, and a significant decrease on day 35 in the case of IgM. Guo *et al.* [62] evaluated an ELISA for the N protein in plasma samples from 82 confirmed and 58 probable cases in China. In their study, IgM and IgA were detected during the first days of the disease (5 days from onset of symptoms), although the IgG was better detected after 2 weeks. Furthermore, the detection by IgM provided better results than the RT-qPCR after 5.5 days. In this sense, the method was used to assess the presence of Abs in a family,

where RT-qPCR could detect the virus in two out of six members of the family, and the ELISA in five out of six. Xiang *et al.* [73] employed 154 serum samples to assess commercially available ELISA and gold-immunochromatography assay kits to test 63 and 91 samples, respectively. In both cases, the specificity was 100%, with similar sensitivity (87% versus 84%).

A Cochrane review of SARS-CoV-2 Ab testing, including 57 publications with 15 976 samples (8526 confirmed COVID-19 cases), clearly shows that diagnostic accuracy also depends on the test's timing [64]. The maximum sensitivity (IgG/IgM tests) was 96% on days 22–35 after symptom onset. For IgG alone, the maximum sensitivity was 88.2% on days 15–21 after symptom onset. It was declared that there are insufficient studies to estimate the sensitivity of tests beyond 35 days post-symptom onset, casting doubt over the utility of these tests for seroprevalence surveys for public health management purposes.

In addition, in some cases, it might be relevant to use a SARS-CoV-2 serum neutralization test for assessing Ab-mediated protection in naturally infected and vaccinated individuals. This assay detects only Abs with viral-neutralizing capacity. Among them, those able to recognize the spike protein RBD appear to have higher activity, followed by Abs that recognize the S1 domain, spike protein trimer and S2 subunit [74]. In this sense, Cerutti *et al.* [74] employed the Chorus SARS-CoV-2 'Neutralizing' Ab assay to detect the neutralizing Abs from 694 patient samples. The system allowed the identification of individuals who

have had COVID-19 or who have been vaccinated, and also assessed the actual neutralizing capacity of the samples analyzed. These tools are especially relevant for monitoring immune protection from emerging variants.

CLIA

Another technique employed to diagnose COVID-19 is the CLIA. It is very similar to ELISA, but, in this case, the enzyme used to generate the readout signal catalyzes a chemiluminescence reaction, in which photons are measured instead of a color change, as in the process involved in ELISA. CLIA provides better sensitivity than ELISA, with a wider dynamic range, and requires less time. Soleimani *et al.* [75] used a commercial CLIA kit to detect the presence of Abs in 276 serum samples, with 100 of them originating from negative COVID-19 individuals. The highest sensitivity (95.5%) is reached after 15–25 days from the onset of symptoms. Currently, there are several studies focused on the testing the reliability of this test, their equivalence ELISA and correlation with infection severity [76].

Colloidal gold-based lateral flow immunoassay

A complementary readout system for detecting IgM and IgG involves using gold nanoparticles and a nitrocellulose strip (Fig. 5). In this case, the nanoparticles and a defined line in the strip are modified with an Ab that recognizes IgM or IgG. The Abs present in a sample are labeled with the nanoparticles. Then, the

sample flows through the strip and, at the testing line, where the Abs are present, a reddish line is formed as a result of the color of the nanoparticles.

The main advantages of this approach are ease of use and portability. In this case, a small amount of blood is used, reducing the risk of transmission significantly. The results are obtained after 15 min and it does not require any equipment. On the other hand, this system is not suitable for quantification studies.

Pan *et al.* [77] evaluated 134 samples from 105 patients, and the results obtained were similar to those obtained with other immune-based approaches. Particularly, during the first week after symptoms, the sensitivity is very low, where IgM is slightly more sensitive than IgG, presenting a positive rate of 11.1% and 3.6%, respectively. During the second week, IgM is also more sensitive than IgG (78.6% versus 57.1%). Interestingly, the combination of IgM and IgG increases the positive rate up to 92.9%. On the other hand, after 2 weeks, IgG presents a higher positive rate (96.8%) than IgM (74.2%). Pan *et al.* [77] also examined the positive rate obtained from whole blood versus plasma samples, and the results showed that blood and plasma IgM present 100% consistency compared to IgG.

Similarly, Li *et al.* [78] also evaluated the sensitivity and specificity of a lateral flow assay combining commercial IgM and IgG Abs. Their system was tested in 397 COVID-19 positive and 128 negative patients from eight different hospitals. The sensitivity and specificity obtained were 88.66% and 90.63%, respectively.

These reports highlight that serological tests still present some drawbacks and limitations that need to

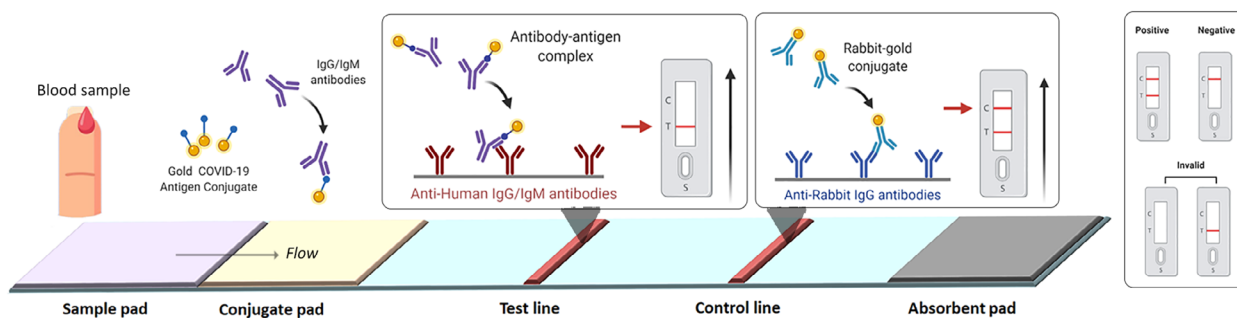


Fig. 5. General scheme for a serological test using a colloidal gold-based LFIA. LFIA are similar to an ELISA test for antibody detection but in flow, in a nitrocellulose (NC) strip. The patient sample (Table 1) is loaded into a NC strip (in the sample pad). Driven by the flow, Abs move towards the conjugate pad, where the immunoreaction takes place and the complex with a gold-labeled SARS-CoV-2 antigen is formed. Two independent capture Abs recognize either (a) a SARS-CoV-2 Ab bound to the labeled antigen (test, T), if any or (b) a non-human gold-labeled Ab (in this example, rabbit IgG as control, C). Once the immunocomplexes reaches the T or the C line, respectively, secondary Abs retains the complex and stop its motion. This confinement in the NC lines results in a red signal, and the test could be interpreted on the basis of the instructions, for a period of around 15 min. These test are qualitative, and so even a weak T line together with a positive C line is indicative of the presence of SARS-CoV-2 AbS in plasma. Adapted from 'COVID-19 Serologic Diagnostic Test through Antibody Detection' via BIORENDER (<https://biorender.com>).

be improved and standardized in seroprevalence studies during the pandemic.

Conclusions and outlook

To beat the pandemic, reliable, sensitive and fast diagnostic systems are desired. However, despite the great efforts made by companies and academia, the optimum system is not yet available. Currently, the gold standard is the RT-qPCR, although other methods based on alternative amplification techniques or Abs can provide complementary information in much less time. None of the methods reported is perfect, and their limitations should be considered by the health organizations and politicians with respect to applying the proper system in each particular scenario. For example, RT-qPCR can detect the presence of 100 copies of viral RNA/mL transport medium (among the best in class) from approximately the third day after the onset of symptoms in 3–4 h. On the other hand, antigen-based systems have a lower sensitivity but can be used to detect the presence of the infection after the first week in just 15 min. Despite that, new methods, such as SHERLOCK, CARMEN and RT-LAMP, aim to combine the precision and sensitivity of the PCR with the ease of use and portability of the lateral flow assay used in antigen-based systems, in 1 h or less. Despite these advances, almost every country or organization uses a different technique or protocol, making a fair epidemiological comparison difficult. The selection and interpretation of SARS-CoV-2 tests should be based on the context in which they are being used, including the prevalence of SARS-CoV-2 in the population tested. In addition, a robust and responsive testing global infrastructure is essential for stopping the spread of SARS-CoV-2.

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Conflict of interest

The authors declare no conflict of interest.

Author contributions

Both authors contributed to the concept of the article, the research and writing of all the sections, reviewed the text, and approved the final version of the manuscript submitted for publication.

Data availability statement

Data sharing is not applicable to this article as no new data were created or analysed in this study.

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