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Review

CRISPR Systems for COVID-19 Diagnosis

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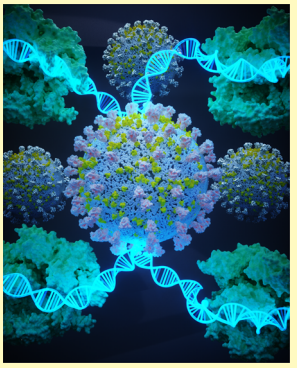
ABSTRACT: The emergence of the new coronavirus 2019 (COVID-19) was first seen in December 2019, which has spread rapidly and become a global pandemic. The number of cases of COVID-19 and its associated mortality have raised serious concerns worldwide. Early diagnosis of viral infection undoubtedly allows rapid intervention, disease management, and substantial control of the rapid spread of the disease. Currently, the standard approach for COVID-19 diagnosis globally is the RT-qPCR test; however, the limited access to kits and associated reagents, the need for specialized lab equipment, and the need for highly skilled personnel has led to a detection slowdown. Recently, the development of clustered regularly interspaced short palindromic repeats (CRISPR)-based diagnostic systems has reshaped molecular diagnosis. The benefits of the CRISPR system such as speed, precision, specificity, strength, efficiency, and versatility have inspired researchers to develop CRISPR-based diagnostic and therapeutic methods. With the global COVID-19 outbreak, different groups have begun to design and develop diagnostic and therapeutic programs based on the efficient CRISPR system. CRISPR-based COVID-19 diagnostic systems have advantages such as a high detection speed (i.e., 30 min from raw sample to reach a result), high sensitivity and precision, portability, and no need for specialized laboratory equipment. Here, we review contemporary studies on the detection of COVID-19 based on the CRISPR system.



KEYWORDS: *CRISPR, COVID-19, SARS-CoV-2, RT-qPCR, diagnosis*

The emergence of severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), also known as the new coronavirus 2019 (COVID-19), was first seen in Wuhan, China in December 2019. This devastating disease has caused a huge number of deaths worldwide, and the death toll is increasing by the day. Coronaviruses, a large human and animal pathogen, are a group of enveloped viruses and have an RNA genome. Because of the crown-like structure on their surface, these viruses are called coronaviruses (in Latin, "corona" means "halo" or "crown"). This group of viruses was first identified in chickens[1] and humans[2,3] in the 1930s and 1960s, respectively. These viruses constitute the largest group in the order of the Nidovirales. Coronaviridae, Arteriviridae, Mesoniviridae, and Roniviridae are the families of the Nidovirales. The family of Coronaviridae is classified into two groups, Coronavirinae and Torovirinae. The Coronavirinae subfamily is composed of alpha, beta, gamma, and delta groups which infect humans by alpha (229E, NL63) and beta (OC43, HKU1) groups. Middle east respiratory syndrome (MERS) and severe acute respiratory syndrome (SARS) coronaviruses are other human beta-group coronaviruses.[4,5] Coronaviruses in humans can cause infections in respiratory, gastrointestinal, and liver systems, leading to both mild symptoms such as colds and fatalities such as MERS. Moreover, the symptoms caused by these viruses are different in other animal species; for example, they cause infections in the upper respiratory tract of chickens, while they induce diarrhea in cows and pigs.[6−8] Animal-to-human transmission of the virus and vice versa was observed with the outbreak of acute respiratory syndrome (ARS) and MERS in 2002/2003 and 2012, respectively.[9,10]

The positive-sense RNA genome of coronaviruses is 26 to 32 kb in size and has a variable number of open reading frames (ORFs) ranging from 6 to 11. The first ORF, which encodes 16 nonstructural proteins, represents approximately 67% of the entire genome, while accessory and structural proteins are encoded by other ORFs.[9,11] Structural proteins encoded by the genome of these viruses include spike (S), nucleocapsid protein (N), membrane (M), small envelope protein (E), and hemagglutinin-esterase glycoprotein (HE; Figure 1I).[12] S proteins are virus-binding mediators to host receptors through domains that bind to the receptor.[7] M protein, with an approximate molecular weight of 25−30 kDa, is the virion's most abundant structural protein and is involved in defining

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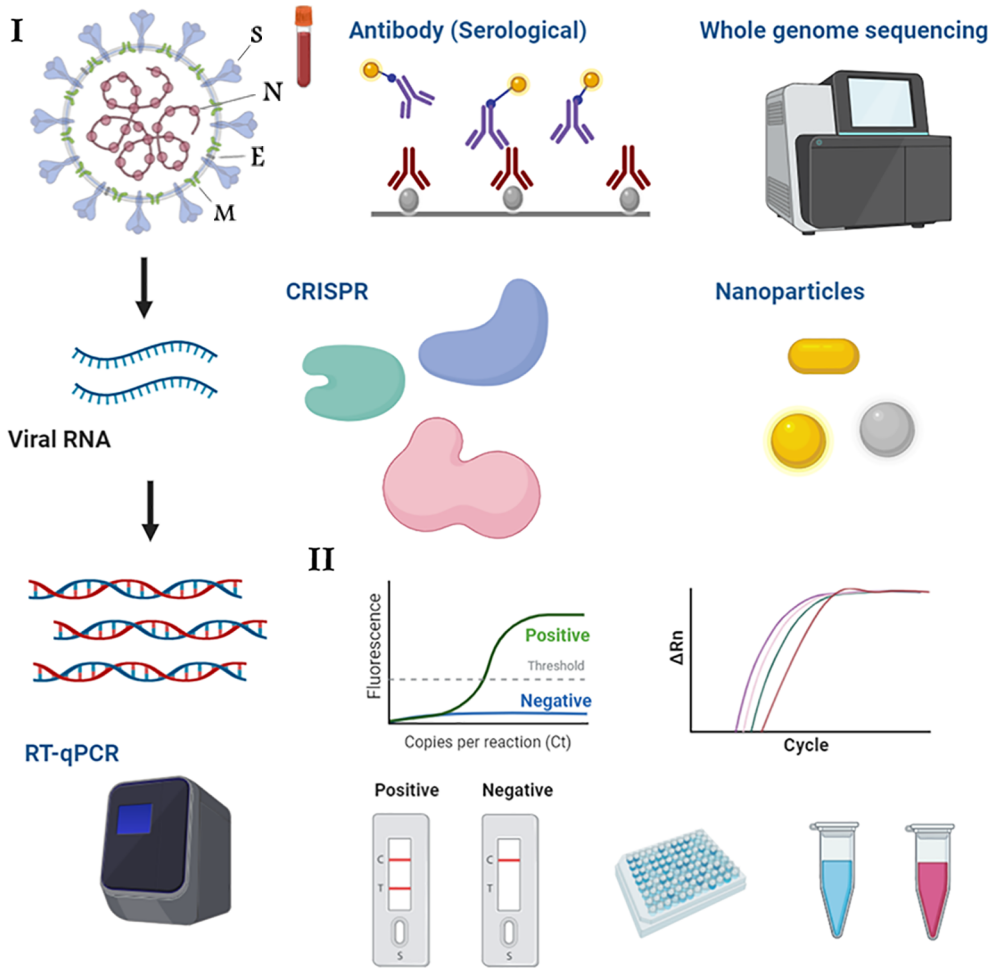

Figure 1. Schematic representation of various diagnosis tools for SARS-CoV-2. I. Types of methods used to diagnose SARS-CoV-2 and II. SARS-CoV-2 diagnostic methods results' readout ways.

the shape of the viral envelope.[13] The M and E proteins form a viral envelope together and interact to produce and release viral particles.[4,14] N protein is the only protein that binds to the RNA genome of the virus and triggering nucleocapsid formation.[15]

Early diagnosis of a viral infection may allow for rapid intervention, which can effectively minimize the risk of spreading the disease to others. PCR is one of the most common techniques used to detect viral nucleic acids and, due to its high sensitivity and accuracy, is used as the gold standard technique for diagnosing viral infections. The quantitative PCR (qPCR) test can be used to diagnose COVID-19, but limited access to qPCR equipment and materials may slow down the diagnosis process.[16−19] Loop-mediated isothermal amplification (LAMP) and recombinase polymerase amplification (RPA) are approaches that have emerged as effective and appealing alternatives to PCR due to their cheapness, high speed, and versatility.[20,21] The isothermal amplification feature, which is performed at a constant temperature and does not require thermal cycling, is one of the key aspects of RPA and LAMP and can also be performed with minimal tools and equipment.[22] CRISPR, discovered in the 1980s and eventually becoming a tool for genome editing, is an adaptive immune system in prokaryotic organisms such as bacteria and archaea that protects the organism against foreign and invasive genetic elements such as viruses and plasmids. This system, which has become currently a powerful genome editing system, relies on RNA-guided activity of Cas proteins.[23] In addition to genome editing, CRISPR/Cas technology has recently gained a lot of interest in the area of nucleic acid detection owing to its unique features. To date, different variants of the CRISPR systems have been used to design and develop simple, portable, precise, efficient, rapid, and inexpensive molecular detection methods. In general, there are two major parts in the CRISPR system: Cas endonuclease (to break the target genomic site) and guide RNA (to identify and direct Cas endonuclease to the target region).[24] Successful detection of nucleic acids by the dead Cas9 (dCas9) system (is a mutant form of Cas9 whose endonuclease activity is removed through point mutations in its endonuclease domains) has been reported several times.[25,26] Cas12 and Cas13 endonucleases have collateral cleavage activities that can be used to detect nucleic acid. For example, cutting a fluorescent reporter using the collateral cleavage activity of these endonucleases can produce a fluorescent signal that results in diagnosis. In addition, Cas9 endonuclease specific cleavage activities have been used to establish highly sensitive and accurate diagnostic tools for DNA detection. RPA and LAMP isothermal amplification approaches are typically used in CRISPR-based diagnostic works to amplify target genomic sequences.[27] However, in an attempt, a Cas13a-based diagnostic system was developed to

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detect SARS-CoV-2 that does not require preamplification of the virus genome.[28]

DETECTION OF SARS-COV-2 BASED ON MOLECULAR TECHNIQUES

Efficient detection of foreign genomes and pathogens in clinical specimens, as well as changes in their sequences, are critical to the diagnosis and management of specific diseases. Early and efficient detection of SARS-CoV-2 was first established by analysis of pathogenic sequences in respiratory tract samples, which is important to limit transmission of the virus and maintain disease control.[29] A variety of methods including RT-qPCR, sequencing-based methods, nanotechnology-based assays, immunological assays, and the CRISPR/Cas system have been used to diagnose SARS-CoV-2 (Figure 1I) and along with appropriate readout (Figure 1II) were shown in Figure 1. The medical nanotechnology revolution has opened a window of hope for the treatment and diagnosis of a wide range of diseases, especially cancer and viral diseases. Owing to the unique properties of nanomaterials and the effectiveness of medical nanotechnology against various infectious diseases such as HIV-1, influenza virus, HBV, and respiratory syncytial virus, researchers are urged to develop nanotechnology-based tools for SARS-CoV-2 disease management.[30,31] Rapid and specific diagnosis of SARS-CoV-2, targeted delivery of antiviral agents to different parts of the body, and preparation of nanobased disinfectants are some of the applications of nanotechnology in the management of SARS-CoV-2 disease.[32] Immunological tests can detect the presence of COVID-19 antivirus antibodies or viral antigens (viral structural proteins). Enzyme linked immunosorbent assays (ELISAs), chemiluminescence immunoassays (CLIAs), and lateral flow immunoassays (LFIAs) are immunological methods that have recently been used to diagnose SARS-CoV-2.[33] While diagnostic tests based on nucleic acid amplification are suitable for early stage diagnosis of viral diseases, immunological diagnostic tests allow the diagnosis of a past or ongoing infection, which provides a better understanding of the mechanism and dynamics of disease transmission.[34] In the diagnosis of COVID-19, immunological diagnostic tests mainly target the structural proteins of the virus. Since the S protein is the major transmembrane protein of SARS-CoV-2 and is highly immunogenic, this protein may be a very suitable option as an antigen in the diagnosis of SARS-Cov-2.[34,35] In addition, the receptor-binding domain (RBD) located along the structural S protein is a target for the detection of COVID-19-specific antibodies.[34]

One of the main ways to diagnose COVID-19 infection is to identify the SARS-CoV-2 RNA genome.[36,37] While molecular diagnostics can be developed rapidly and provide very high precision and sensitivity and also quantitative detection of SARS-CoV-2 nucleic acid, it still faces issues such as high cost, difficulty, and the need for highly skilled technicians and equipped centers. For example, an RT-PCR kit can cost more than $100 U.S., and sample analysis can take about 4 to 6 h, and more than 24 h from raw sample to the final result may be required. Information on the amount of detectable viral titer in the respiratory tract at various stages of SARS-CoV-2 infection is still expanding. The kinetics of viral load can also differ from person to person and depend on various factors such as the patient's epidemiological history, immune response, and effects of medication and treatment.[38,39] Hence, one of the most important limiting factors for molecular diagnosis is detection time.[40,41]

There have been significant advances in the molecular detection of COVID-19 since its outbreak. Although testing capability cannot currently address the global need for rapid COVID-19 diagnosis, fundamental issues such as false-negative results and the development of faster, highly sensitive, and affordable diagnostic procedures remain to be tackled. Delays in patient care, as well as an increased risk of asymptomatic spread to others, are concerns related to the false-negative results in SARS-CoV-2 patients. False negative results can be caused by factors such as sampling and sample analysis.[42] Depending on the time of infection and the place where the sample was taken, the viral load in a sample can be vary. For instance, following the appearance of illness, the viral load varies depending on the timing of collection between the nasal and oral swabs.[43] During the progression of the disease, the variation in viral load at different locations makes sampling more challenging and allows the production of false-negative results. Some other factors, such as mistakes in the collection of clinical samples, degradation of RNA, inefficient extraction of RNA, and insufficient purification of RNA, may contribute to false negative results. Positive RT-PCR test results demonstrate SARS-CoV-2infection, but negative RT-PCR results do not reject SARS-CoV-2 infection. Molecular diagnosis should be used in combination with other diagnostic information such as patient medical history, clinical observations, and epidemiological surveillance information to ensure the diagnosis of the disease.[42] The most common methods used in the clinic to detect foreign gene material include qPCR, next generation sequencing (NGS), and fluorescence in situ hybridization (FISH), which have changed the landscape of molecular detection. Despite fundamental improvements, the use of such methods still presents challenges. While the qPCR test is the primary tool utilized in clinical laboratories to recognize the causative agent of the CoVID-19 (e.g., SARS-CoV-2), it may face problems such as the need for highly trained technicians, advanced thermocyclers, and well-equipped laboratories.[44−46]

One of the valuable molecular tools used to study the function and regulation of genes, and specifically viral detection, is the in situ hybridization (ISH) technique. This technique uses hybridization probes (made of single- or double-stranded nucleic acids or synthetic oligonucleotides) to detect and localize a specific nucleic acid in a tissue sample or cell. By attaching a reporter to the complementary strand of the desired nucleic acid in a known labeling method, hybridization probes are produced. If fluorescence is used in the ISH technique, this technique is called FISH.[47,48] The FISH approach also offers a single-cell system to analyze the number of copies, replication, and gene rearrangement that may influence targeted therapy. The requirement for long-term high-temperature treatment to hybridize the FISH probe is time-consuming, as well as cell morphology weakening, which contributes to the possibility of missing spatial structure information. Additionally, toxicity caused by formamide used for denaturation of double-stranded DNA is another obstacle in the FISH procedure.[49] This technique also faces challenges such as false positive and negative results, incomplete hybridization, processing problems, nonspecific binding, and photobleaching.[48]

The massively parallel sequencing technology, known as next generation sequencing (NGS), has revolutionized

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molecular biology. The benefits of NGS technology including ultrahigh throughput, speed, and scalability have allowed researchers to conduct a broad variety of biological studies that were not feasible until this technology was developed. This technology can detect all kinds of nucleic acid sequences in a single sample, so that NGS can recognize all known viruses as well as new ones that have not yet been detected.[50] Nevertheless, the widespread use of NGS is constrained by concerns such as insufficient target enrichment, which raises costs as well as reduces sensitivity.[51,52] In reality, the biggest obstacles to converting NGS technology into a routine diagnostic test are the high cost and high processing time of achieving the final result from the raw sample. Compared to RT-PCR, NGS technology has a high cost and high turnaround time. Therefore, the difficulty and time-consuming nature of NGS-related methods restricts the usage of NGS when rapid results are required.[53]

CRISPR/CAS-BASED SYSTEMS FOR COVID-19 DETECTION

The CRISPR system is a simple, efficient, and reliable system that enables researchers to make desired changes in genomic sequences that may alter gene function. This system, which functions like a pair of molecular scissors and can cut DNA strands, is a family of DNA sequences found in prokaryotes such as bacteria and archaea.[54−57] Generally, the CRISPR system is divided into two main classes and six types.

The first class includes type I, III, and IV systems, while the second class includes type II, V, and VI systems. In class I, type I Cas3 nuclease is used to cut DNA, and type III Cas10 nuclease can cut RNA. In class II, type II systems use Cas9 endonuclease to cut DNA, and type V uses Cas12 to cut DNA. In addition, Cas13 nuclease is used in class II, type VI systems to make cuts in target RNA. The CRISPR system functions as the bacteria and archaea's adaptive immune system against foreign elements including viruses or plasmids.[58−62] Class II CRISPR systems are used widely for genomic manipulation and infectious disease diagnosis. For example, CRISPR/Cas12a, CRISPR/Cas13a, and CRISPR/Cas13b systems have been used in recent years to develop rapid and sensitive diagnostic methods for human pathogen (bacteria and viruses) detection.[63−67] A multiplex diagnostic system developed by Kellner et al.[68] recently incorporated nucleic acid preamplification with CRISPR/Cas enzymology to accurately identify the targeted nucleic acid sequences. The developed system, called specific high-sensitivity enzymatic reporter unlocking (SHERLOCK; Figure 2), can detect clinical sample nucleic acid sequences in a portable and ultrasensitive manner.[68] DNA endonuclease-targeted CRISPR trans reporter (DETECTR; Figure 2) is another CRISPR-based diagnostic system (CRISPR/Cas12) that detects viral infections rapidly (~30 min), inexpensively, and accurately.[60] The two aforementioned diagnostic systems (SHERLOCK and DETECTR), which have high specificity and sensitivity, are comparable to conventional diagnostic methods such as PCR but do not require expensive advanced equipment.[60,68] To the best of our knowledge, DETECTR and SHERLOCK diagnostic kits for SARS-CoV-2 detection have been approved and are commercially available.

PCR and other conventional approaches for amplifying DNA or RNA sequences for recognition are reliable but still demand nonportable tools that prevent their spread in the area of diagnosis.[69,70] Thus, in the near future, CRISPR based

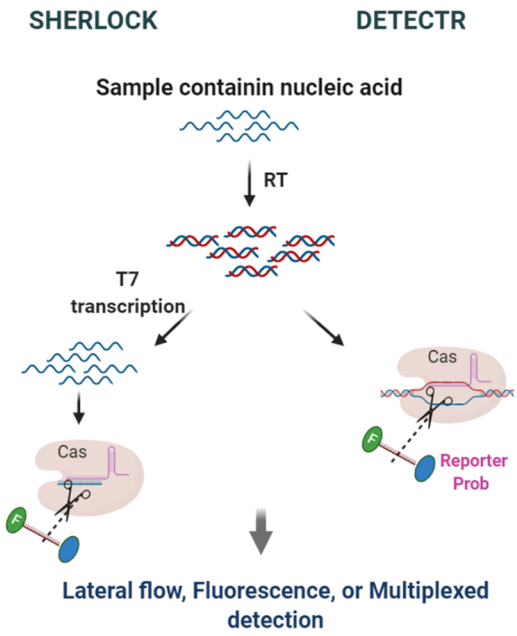

Figure 2. Schematic illustration of SHERLOCK and DETECTR workflow and the main mechanisms involved in CRISPR-based diagnosis systems.

detection systems may become widely available and replace conventional methods such as PCR. The application of CRISPR-based diagnosis systems in the molecular diagnostic field has grown, and a variety of CRISPR-based diagnostic tools for diagnosing infectious and noninfectious diseases have been created so far. Here, we review all the studies conducted to diagnose SARS-CoV-2 infection using the CRISPR/Cas system (Cas12, Cas13, Cas9, and Cas3; Figure 3).[71−74]

Table 1 summarizes studies on the development and design of CRISPR-based diagnostic systems for rapid and sensitive diagnosis of COVID-19.

DIAGNOSIS OF SARS-COV-2 BASED ON CRISPR/CAS12

Broughton et al.[16] developed a CRISPR/Cas12a-based precision technique, called SARS-CoV-2 DNA endonuclease-targeted CRISPR trans reporter (DETECTR), which allows for the simple and rapid diagnosis of SARS-CoV-2 RNA extracted from patient respiratory tract swab samples in less than 40 min. Actually, the developed method is the product of combining the CRISPR/Cas12a DETECTR system with isothermal amplification that simultaneously performs reverse transcription and isothermal amplification by loop-mediated replication (RT-LAMP) for purified RNA from nasopharyngeal or oropharyngeal swabs. Cas12a then detects predetermined viral sequences, whereupon the reporter molecule's cleavage confirms the presence of a virus. Eliminating the need for thermocycling and isothermal signal amplification offers significant benefits compared to qRT-PCR, such as fast turnaround time, target specificity for single nucleotides, integration with usable and user-friendly reporting formats like lateral flow strips, and no requirement for sophisticated laboratory systems. The established system was validated with reference samples and also United States patient samples including 36 patients with COVID-19 and 42 patients with other respiratory viral infections. This system is a high-speed and visual alternative to rRT-PCR for detecting SARS-

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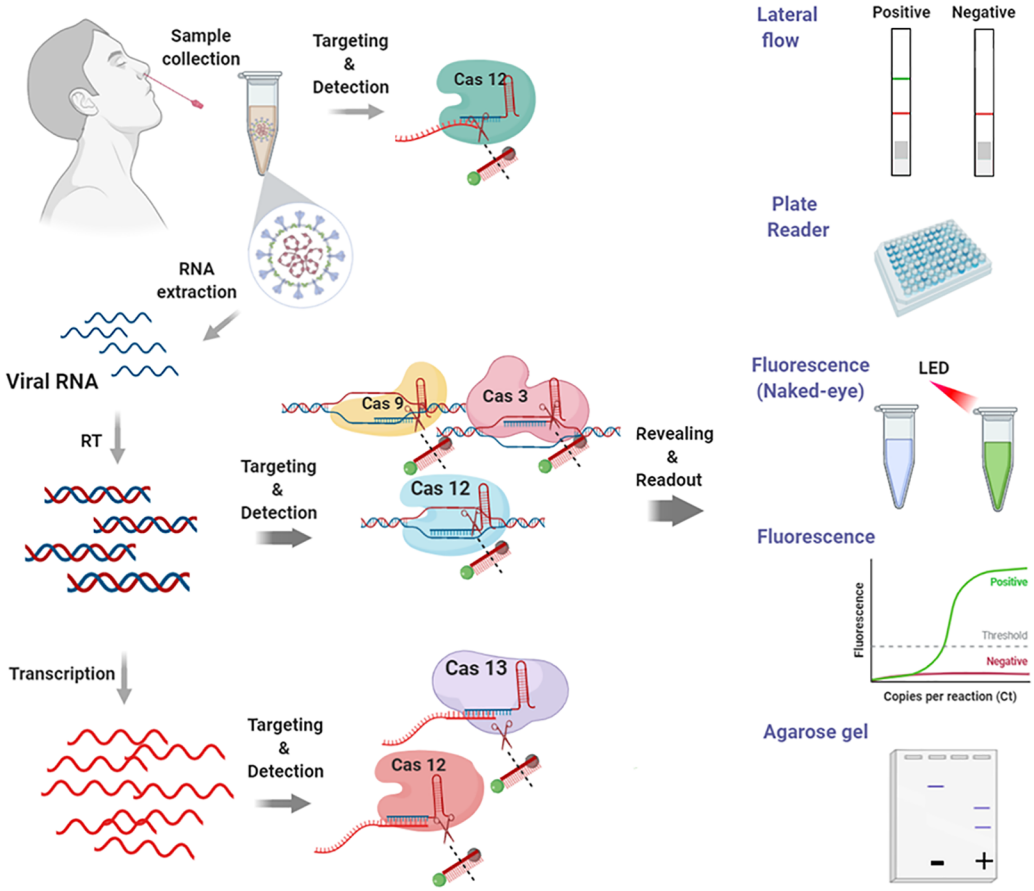

Figure 3. Schematic illustration of the most commonly used CRISPR systems, CRISPR-Cas (Cas3, Cas9, Cas12, and Cas13), for detection of SARS-CoV-2.

CoV-2 with 95% and 100% agreement for positive and negative prediction, respectively.[16]

The all-in-one dual CRISPR-Cas12a (AIOD-CRISPR) system was developed by Ding et al.[75] for faster, highly sensitive, highly specific and optical-based nucleic acid detection. This method uses dual crRNAs to efficiently detect the target genome sequence. The AIOD-CRISPR system integrates all the components needed for target nucleic acid amplification as well as CRISPR system-based detection into a single reaction that resulted in deletion of the need for separate amplification and transfer of amplified product. The AIOD-CRISPR system was designed to be used for the detection of SARS-CoV-2 and HIV-1. As both viral agents are retroviruses, the capability of the AIOD-CRISPR system to detect each of their nucleic acid states (DNA and RNA) was assessed and their nucleic acids were detected successfully.[75]

Among the various classes of CRISPR systems, the CRISPR/Cas12 system, an RNA-guided DNase, causes single-stranded DNA cleavage after identifying the target region. The CRISPR/Cas12 ability to create collateral cleavage in single-stranded DNA can be enjoyed to destroy single-stranded reporter molecules that generate fluorescent signals.[65] Thus, CRISPR-Cas12-related approaches can be used in real time as an in situ diagnostic tool for diagnosing SARS-CoV-2 infection. The CRISPR/Cas12a system was implemented in another attempt to identify the synthetic SARS-CoV-2 nucleic acid sequences reliably, sensitively, and in a rapid manner.[88] Synthetic sequences of the RdRp, ORF1b, and ORF1ab genes were considered as references in this work. The lack of actual samples of SARS-CoV-2 cases in this study may be criticized, but no real samples were used in this study due to the lack of reports of patients suffering from SARS-CoV-2 disease in the study area (South America). To mimic real samples, synthetic SARS-CoV-2 nucleic acid sequences were added to a healthy person's saliva sample. The use of saliva to diagnose SARS-CoV-2 is a reasonable approach as it is completely noninvasive and sampling is quick and easy. It was found that the CRISPR diagnostic system used in this research is not inactivated in saliva, so it is a promising tool for accurate and fast identify of real SARS-CoV-2 samples.[76]

Wang et al.[77] developed a rapid and accurate CRISPR/Cas12a-based system that allows reading with the naked eye (CRISPR/Cas12a-NER) to boost and accelerate the detection of the SARS-COV-2 Genome.[77] CRISPR/Cas12a-NER can rapidly, reliably, and sensitively detect at least 10 copies of a viral gene in 40 min without the demand for specialized instruments. The designed system consists of Cas12 protein, SARS-COV-2-specific crRNAs, and a single-stranded DNA molecule as a reporter (labeled with a green fluorescent off−on molecule). Where the genome of SARS-COV-2 is present in the sample and detected by the designed diagnostic system, the reporter molecule is cleaved by the Cas12 protein, resulting in green fluorescent light visible to the naked eye at 458 nm.[77]

Inexpensive, highly sensitive, precise, high-throughput, and highly effective methods are needed to control the disease and recognize even asymptomatic patients in any region. Studies have shown that, although SARS-COV-2 patients have no signs or presymptoms, they are strongly contagious and can infect

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Table 1. Rapid and Sensitive Detection of COVID-19 with CRISPR-Based Diagnostic Platforms

system	Cas type	test timing	advantages	shortcomings	ref
DETECTR	Cas12a	30−40 min	accurate, easy-to-implement, rapid turnaround time, no need for thermocycling, single nucleotide target specificity, and no need for complex laboratory infrastructure	needs for nucleic acid extraction, limited access to extraction, kits and reagents, needs for personal protective equipment	16
AIOD-CRISPR	Cas12a	40 min	rapid, highly sensitive, highly specific, one-pot reaction, no need for separate preamplification and amplified product transferring, visibility of results with the naked eye, nucleic acid detection in both DNA and RNA states, performable in one-step, single-molecule sensitive, and robust	needs for nucleic acid extraction, limited access to extraction, kits and reagents	75
CRISPR-Cas12 based	Cas12a	Less than 60 min	portable, sensitive, rapid, and low cost	patient samples are not used and requires certain kits	76
CRISPR/Cas12a-NER	Cas12a	45 min	portable, simple, sensitive, specific, no need for special instrument, rapid, and visibility of results with the naked eye	needs for nucleic acid extraction, limited access to extraction, kits and reagents	77
CRISPR-FDS	Cas12a	∼50 min	sensitive, robust, rapid, and can be done with available equipment	needs for nucleic acid extraction, not suitable for quantifying viral load	78
SHINE	Cas13a	50 min	sensitive, specific, single-step reaction, can be used outside of hospitals and laboratories, and no need for nucleic acid extraction		79
CONAN	Cas3	40 min	rapid, sensitive, low-cost, instrument-free, and single-base-pair discrimination	needs for nucleic acid extraction, limited access to extraction, kits and reagents	80
iSCAN	Cas12a	1 h	sensitive, specific, efficient, rapid, user-friendly, accurate, field-deployable, and suitable for large-scale	requires nucleic acid extraction, limited access to extraction, kits and reagents	81
CASdetec	Cas12b	1 h	no cross-reactivity, reduced false positive rate, and accuracy	needs for nucleic acid extraction, limited access to extraction, kits and reagents	82
VaNGuard	Cas12a	30 min	robust, rapid, sensitive, affordable, specific		83
CREST	Cas13a	∼2 h	scalable, low-cost, no need for specialized instrumentation, highly sensitive, easy to deploy	requires nucleic acid extraction, limited access to extraction, kits and reagents	84
STOPCovid	Cas12b	1 h	simple, suitable for point-of-care (POC) analysis, sensitive, low-cost, availability of test components, no need for RNA extraction		85
ITP-CRISPR	Cas12a	30 min	amenable to automation and the use of a minimum volume of reagents		86
SHERLOCK	Cas13a	less than 1 h	rapid, sensitive, and no need for sophisticated equipment	not fit to test clinical samples	87

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many others. Therefore, these conditions encourage researchers to establish fast, precise, and highly sensitive methods for SARS-COV-2 detection on a large-scale. In an attempt to meet these demands, a diagnostic system based on CRISPR was developed by Huang et al.[78] The method consists of CRISPR/Cas12a system and a fluorescent probe to diagnosis RT-PCR or RPA amplified amplicons, allowing sensitive and accurate detection in areas that do not have a real-time PCR system. It was observed that the developed system was capable of detecting positive SARS-COV-2 samples in about 50 min, with the limitation of detecting two copies of the target RNA sequences for each sample. However, in samples with less than five copies of the amplified target DNA, the qPCR test failed to produce a detectable signal. The use of CRISPR in detection has become very common due to the ability of Cas13 to bind to the target RNA molecule and Cas12 to bind to the target DNA molecule by the gRNA sequence to make a cleavage in the probe to produce the detection signal.[60,67] Most CRISPR-based diagnostic methods use paper strips to detect the output signal. Using paper strips to recognize single samples is a reasonable strategy since the tests do not require special equipment, but the limit of detection (LoD) value obtained is very low compared to fluorescence-based approaches. The CRISPR-FDS method developed by Huang et al.[78] could be conveniently implemented on 96-well microtiter plates that can be used in most well equipped laboratories with precision and ease using fluorescent plate readers.[78] Overall, the results of this study were comparable to the results of the RT-qPCR test performed in public laboratories; however, the CRISPR-FDS method provided more reliable results than those reported in a clinical setting when the same RT-qPCR assay was used. It should be stated that the CRISPR-FDS method has provided positive results for certain samples that have obtained negative results using the RT-qPCR test, although it is not possible to decide if these results are false positives for CRISPR-FDS or false negatives for RT-qPCR owing to a shortage of valid follow-up data. Therefore, the CRISPR-FDS method can be considered as a sensitive and powerful method for producing results using tools and available equipment for use in clinical laboratories as well as care facilities with appropriate equipment. The CRISPR/FDS system is designed so that it produces clear negative or positive results and cannot quantify virus titers like RT-qPCR, which is a drawback for the CRISPR/FDS system. However, given that the main goal is to develop a quick, accurate, and reliable COVID-19 detection system, this deficiency cannot be considered an important flaw.[78]

A diagnostic method consisting of a combination of CRISPR/Cas12a and RT-LAMP, called the in vitro specific CRISPR-based assay for nucleic acid diagnosis (iSCAN) system, was introduced in an attempt by Ali et al.[89] to overcome the shortcomings of SARS-CoV-2 detection.[89] The potential benefits of the iSCAN system include: (i) high speed, (ii) precision (because of the diagnosis reliance on SARS-CoV-2 nucleic acid sequences being detected and cut by Cas12), (iii) field-deployability (since it needs only the basic tools), and (iv) easy operation (since it provides easy access to test results by incorporating a colorimetric reaction with lateral immunochromatography flow). The iSCAN system is well suited for early identification of COVID-19 carriers so that they can be identified and quarantined early, thereby preventing the virus from spreading widely.[89] Another diagnostic tool based on CRISPR called Cas12b-mediated DNA detection (CDetection) was established for the detection of SARS-CoV-2 in an attempt by Guo et al.[82] By incorporating sample treatment protocols and nucleic acid amplification strategies with CDetection, they set up an integrated viral nucleic acid detection system—CASdetect (CRISPR-assisted detection). The CASdetect system's limit of detection was 1×10^4 copies/mL for the identification of the SARS-CoV-2 pseudovirus, without cross-reactivity to other human endemic coronaviruses.[82]

Nucleic acid amplification based molecular detection is currently the most reliable, fast, and inexpensive approach for SARS-CoV-2 disease diagnosis; quite a few companies and laboratories have developed rRT-PCR kits.[90] The rRT-PCR performance for SARS-CoV-2 detection presents a number of urgent challenges particularly with uncertain negative or positive results associated with the frequently encountered "gray zone" designated with high Ct value.[91−95] In addition to user errors such as inaccurate sampling, poor quality reagents, and uncalibrated instruments, ineffective RT reaction and PCR proliferation of patient specimens with very poor virus titers are probably main causes of inaccurate rRT-PCR readouts and unclear diagnosis. While the diagnosis can be verified through repeated sampling and control, troubleshooting efforts are time-consuming, and low-viral load specimens will not be detected in mild or asymptomatic patients or in advanced cases, therefore this leads to a false negative result that may raise concerns about fighting the disease.[96] The identification of nonspecific trans-cleavage activities in various Cas proteins, including Cas12, Cas13, and Cas14, contributed to the rise of the CRISPR-Diagnostics strategy (CRISPR-Dx).[60,66,67,97−102] The mechanism of action of the CRISPR-Dx technique is based, as demonstrated by the Cas12a-based HOLMES system,[103] on the efficient trans-cleavage activity of Cas12a (upon detection of target DNA) against single-stranded DNA labeled with a fluorophore quencher (FQ), whose fluorescence signal increases exponentially within a few minutes. On the basis of this mechanism, Huang et al.[96] developed a specific enhancer for the identification of nucleic acids amplified by PCR (SENA) to improve the accuracy and efficiency of detecting preamplified nucleic acid sequences of SARS-CoV-2. In summary, they first analyzed SARS-CoV-2 samples using rRT-PCR and then validated amplicons with unclear readouts by SENA.[96] The SENA diagnostic system was highly sensitive and specific and was able to detect false positives and negatives with a detection limit of two copies per reaction less than the corresponding rRT-PCR test. Initially, the amplicon sequences were determined from different rRT-PCR kits to design suitable cRNAs for the SENA, and then a specific crRNA was designed for each amplicon. Then, candidate crRNAs were screened on the SENA consisting of Cas12a, crRNA, FQ-reporter, and rRT-PCR products, and the best crRNAs were selected for further SENA testing.

Owing to the sampling distribution of Poisson, replica variations are very important when copies of templates are designed to be small (below 3−4 copies/Rx), close to the LoD for rRT-PCR, and very low (equal to and less than 1 copy/Rx).[104,105] To address the sampling issue, nine replicas were conducted for groups of RNA templates with a half and one copy/Rx, while six replicas were conducted in each of the other concentration levels. Following the rRT-PCR reaction, all amplicons were entered into three different SENA reactions with crRNAs related to O and N genes and both (N-SENA, O-SENA, and mix-SENA). By reducing RNA templates to less

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than three copies per reaction, it was found that Ct values exceeded 38 (cutoff for positive) in some replicas mainly related to the N gene, but when it drops below 40, it is considered to enter the gray zone. As the concentration of RNA templates decreases, Ct values increase steadily, with most replicas indicating that one or both Ct values enter the gray zone, and ultimately, they all become negative. Taking Ct = 38 as the cutoff for positive detection, the detection limit for O and N genes with a 95% confidence interval (CI) of this set of rRT-PCR was estimated to be $3.3 \leq 4.0 \leq 6.1$ and $4.0 \leq 4.1 \leq 4.4$, respectively. The rRT-PCR amplicons were then examined with the SENA system to measure the signals of fluorescence for each replica and after comparing the increase in fluorescence versus the rate of fluorescence change between samples at a given time relative to the negative control (FC), the FC parameter was defined as the ratio of FC values measured at the 10th and fifth minutes following the fluorescence reading starting. It has also been found that the effectiveness of rRT-PCR for both O and N genes is different from that of the SENA system with decreasing template concentration. The amplicons of the RNA template were investigated via NGS, and it was found that the findings were found to be consistent with O-SENA and mix-SENA results. In addition to avoiding false-positive and false-negative diagnoses, the highly sensitive SENA system can be useful in demonstrating that the virus has been cleared from recovered cases. Since the qPCR system is still the most popular molecular detection system, and the SENA system is remarkable for operational simplicity, the SENA system can be widely used to solve fuzziness troubles of qPCR and other molecular detection systems based on amplification of nucleic acids.[96]

Recent trends in effective CRISPR-based diagnostic systems demonstrate that the DTECTR diagnostic system can be applied as a convenient, inexpensive, and rapid substitute to qRT-PCR, avoiding the loss of sensitivity and specificity for molecular detection. A comparison of the DETECTR system with qRT-PCR for SARS-CoV-2 diagnosis in 378 patients revealed a 95% consensus in an attempt by Brandsma et al.[106] Clinical sample dilution assessments showed DETECTR's higher analytical sensitivity compared with qRT-PCR, but it was not verified in a majority of patients. The findings revealed that both DETECTR and qRT-PCR techniques were similarly sensitive for the SARS-CoV-2 diagnosis. In the DETECTR system, different gRNAs could be used simultaneously to obviate negative results owing to N gene mutations. The DETECTR system was 100% specific to detecting nucleic acid sequences of SARS-CoV-2 and therefore could not recognize other human coronaviruses. In addition, since the DETECTR system for SARS-CoV-2 diagnosis does not require specialized equipment and can be used as an independent qRT-PCR method in diagnostic laboratories, PCR systems in the laboratory can be used for other diagnostic works.[106]

CRISPR-based diagnostic systems are currently considered as field-deployable solutions. The CRISPR-Cas12/gRNA complex is a basic form of such systems. Cas12/gRNA is activated when it specifically binds through gRNA to the target DNA sequence and then nonspecifically cleaves the fluorophore-quencher pair-labeled single-stranded DNA reporter probe. It has recently been shown that electric field gradients can be used to control and accelerate this CRISPR-based diagnostic system by cofocusing the Cas12/gRNA complex, reporter, and target.[88] An appropriate electric field gradient could be obtained using isotachophoresis, a special ionic focusing technique, applied to a microfluidic chip. Overall, on-chip electric field control and microfluidics were combined to perform two very important steps: (1) automatic extraction of nucleic acid from primary biological samples (here, nasopharyngeal samples of patients with COVID-19 and healthy controls were used) and (2) application of an electric field to monitor and influence the rapid enzymatic activity of the CRISPR/Cas12 complex as soon as the target nucleic acid sequence is detected. This allows simultaneous combination of enzymatic reactions, preconcentration, and speed. This developed microfluidic system not only needs a small volume of reagents but also is open to automation. It was assessed for detecting SARS-CoV-2 nucleic acid in COVID-19 positive and healthy samples. Ultimately, as a remarkable difference compared to current COVID-19 test procedures, this process takes just 30 min from the raw sample to be completed.[107]

As discussed above, Cas12 has been used for the detection of SARS-CoV-2 in three states. Cas12 is able to detect the SARS-CoV-2 genome both before and after nucleic acid extraction, which schematically is shown in Figure 4.

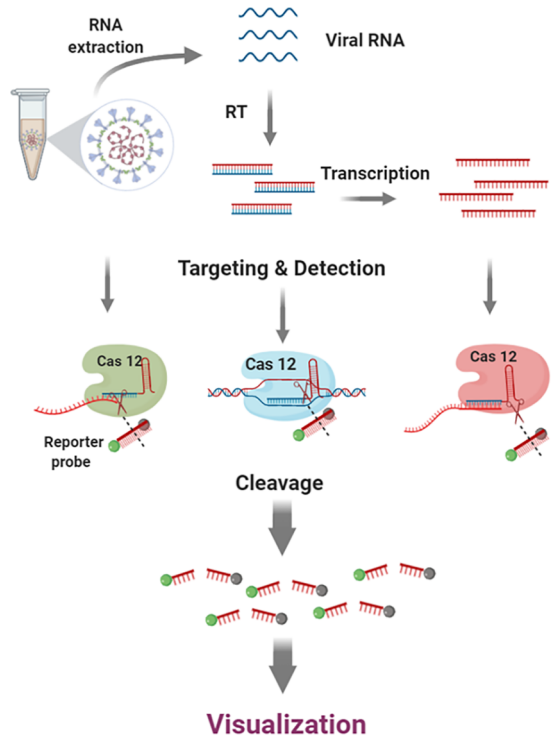

Figure 4. Overview of CRISPR/Cas12 based systems used for SARS-CoV-2 detection.

SARS-COV-2 DIAGNOSIS BASED ON CRISPR/CAS13

The CRISPR/Cas13 based diagnostic system's performance depends on the base pairing between crRNA and the target sequence, making such a system a highly accurate and programmable diagnostic tool.[66,108] Although such systems have a high level of sensitivity and diagnostic efficiency, they require nucleic acid extraction, which is often restricted in terms of access to the extraction kits, as well as multiple sample transfer steps that hinder ease of use. To eliminate the need for nucleic acid extraction, Myhrvold et al.[65] merged the heating

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unextracted diagnostic samples to obliterate nucleases (HUDSON) system with the SHERLOCK system. The developed system employs chemical reduction and heat to the degradation of nucleases that break down RNA and lysis viral particles.[65] Overall, it is practical to use the combined SHERLOCK and HUDSON system in laboratories with limited facilities, as only one heating system is needed. However, its scalability has been limited due to the need to prepare multiple reaction mixtures and transfer samples between them. A fast and sensitive system called SHINE (SHERLOCK and HUDSON integration to navigate epidemics) that did not require extraction of nucleic acid was established in a worthy attempt to detect the SARS-CoV-2 nucleic acid. Following the development of a SARS-CoV-2 test,[109] diagnostic steps based on the Cas13a system and SHERLOCK's amplification were combined, which shortened both the testing time and operator interventions. The SHINE system has been shown to be able to detect the SARS-CoV-2 genome in clinical samples treated with HUDSON in paper-based colorimetric or in-tube fluorescent readout methods that can be applied using portable devices and with a low probability of contamination of samples. To evaluate the performance of the SHINE system, 50 nasopharyngeal specimens (30 RT-qPCR-confirmed SARS-CoV-2 positive specimens and 20 SARS-CoV-2 negative specimens) were examined. The SHINE system was evaluated on six positive COVID-19 samples using the paper-based colorimetric method, resulting in the SARS-CoV-2 nucleic acid being identified in all six positive samples and not in negative control samples, indicating 100% concordance. The use of the SHINE system to test 50 samples using the in-tube fluorescence method resulted in SARS-CoV-2 nucleic acid identification in 27 out of 30 positive samples of SARS-CoV-2 and no false negative results within a sample-to-result period of 50 min, indicating significantly higher specificity (100%) and sensitivity (90%) in comparison with RT-qPCR.[79]

Metsky et al.[109] developed a series of test designs and experimental procedures for use in the diagnostic systems that focused on CRISPR and could be useful for continuous surveillance. The presented designs were developed to detect 67 species and subspecies of viruses, such as SARS-CoV-2. The algorithms developed by Metsky et al.[109] allow molecular detection assay designs, the output of which is also promising for improvement of diagnostics for the detection of viral species. On the basis of the CRISPR/Cas13a (SHERLOCK) system, they screened four of the existing designs for SARS-CoV-2 diagnosis and then thoroughly evaluated the best performing SARS-CoV-2 assays. This assay was used to demonstrate the sensitivity of SARS-CoV-2 synthetic sequences, which was 10 copies per microliter.[109]

While special attention has been paid to the development of diagnostic systems based on CRISPR for SARS-CoV-2 nucleic acid detection rapidly, such systems have not addressed mutations and genomic rearrangements related to the virus's nucleic acid. It is well-known that RNA viruses frequently mutate to avoid attacks from hosts' immune systems. Numerous genomes of SARS-CoV-2 have been sequenced, and different mutations have been identified, indicating that the coronavirus will constantly adapt to its host. Especially, such mutations have been reported in gene sequences that are focal areas for SARS-CoV-2 diagnostic tests, which can affect the efficiency of qRT-PCR assays.[110−113] Most noticeably, mutations in the gRNA binding site can trigger mismatches that affect the CRISPR/Cas system's ability to recognize the target area. It has been shown recently that when SARS-CoV-2 enters the body its RNA genome is edited by the deaminases including ADAR and APOBEC, which are part of the human immune system against viral attacks.[88] Changes in nucleotides during conversions of adenosine-to-inosine and cytosine-to-uracil can also affect the ability of the CRISPR/Cas system to recognize the virus.

Therefore, the variant nucleotide guard procedure was developed to overcome these challenges and increase the capacity and strength of the diagnostic system for specific and sensitive detection, as well as to identify mutated and altered nucleic acid regions of SARS-CoV-2.[83] The DETECTR system and different forms of Cas12a enzymes were initially studied, and it was found that enAsCas12a had the greatest tolerance in the CRISPR target area for single mismatches. Interestingly, enAsCas12a was highly specific to SARS-CoV-2 nucleic acid and also demonstrated no cross-reactivity with SARS-CoV and MERS-CoV, two other related coronaviruses. Additionally, various gRNAs were investigated, and all nucleases tested, except enRR, were found to show that S2 gRNA leads to higher trans-cleavage activity. Thus, the enCas12a-S2 gRNA has proven to be a powerful and very sensitive system for detecting SARS-CoV-2 compared to LbCas12a-N-Mam gRNA. Remarkably, this method can be applied using a dipstick within 30 min.[83]

A protocol called Cas13a-based, rugged, equitable, scalable testing (CREST) was developed by Rauch et al.[84] to overcome the scalable testing challenges of SARS-CoV-2 diagnosis, such as access to tools and materials, highly skilled operators, and investors. Based on CRISPR/Cas13a, this diagnostic protocol uses readily available reagents and equipment and is also highly sensitive to the detection of SARS-CoV-2. The CREST technique utilizes the sensitivity and convenience of a transcription-recognition reaction and also benefits from the PCR method's robustness and reliability. With management in ∼2 h, without the need for AC power or a sophisticated facility, CREST can be carried out from an RNA sample to a result.[84]

New alternatives to RT-qPCR have decreased RT-qPCR dependency for the detection of SARS-CoV-2, which focuses on the combination of isothermal amplification and CRISPR systems, such as the SHERLOCK system. With this in mind, a simple chemical method called SHERLOCK testing in one pot (STOP) was established in a valuable effort to diagnose SARS-CoV-2, which is suitable for use at the point of care and can be carried out within an hour. The STOPCovid method is comparable in sensitivity to RT-qPCR-based techniques and also has an LoD of 100 copies per reaction of the viral genome in the samples of saliva or NP. The test result is obtained using a lateral flow or fluorescence readout within 70 and 40 min, respectively. In addition, examination of NP specimens from COVID-19 patients for further testing of the STOPCovid method showed that 12 positive specimens and five negative specimens out of three replicates could be detected by STOPCovid. The STOPCovid method is a valuable technique for point-of-care diagnostic systems development for detecting SARS-Cov-2 and has the ability to assist in measures of test−trace−isolation to end COVID-19 spread and restore public health.[85] Zhang et al.[87] developed a protocol to improve and advance the diagnosis of COVID-19, using a CRISPR-based SHERLOCK method. Using artificial RNA fragments of the virus, they were able to identify COVID-19 target sequences in

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the 20 and 200 aM range (10−100 copies per microliter of input). The established protocol can be carried out in less than 1 h using RNA extracted from clinical samples, without the need for advanced laboratories.[87]

FNCAS9-BASED DIAGNOSIS OF SARS-COV-2

With the aim of rapid and accurate nucleic acid detection, Azhar et al.[114] developed the FnCas9 editor linked uniform detection assay (FELUDA) procedure, which uses a highly accurate enzymatic readout to diagnose nucleic acid sequences. Since the FELUDA approach does not require complicated instruments and can be an effective and convenient alternative to diagnostic methods such as PCR-based methods, it was examined for the diagnosis of SARS-CoV-2. It was observed that the use of FELUDA for the detection of SARS-CoV-2 using the specific FnCas9 RNP complex leads to clear signatures of the SARS-CoV-2 sequence in synthetic DNA. Surprisingly, the FELUDA method was able to differentiate between sequences of SARS-CoV-2 and SARS-CoV-1 which differed in one nucleotide. Ultimately, after effective detection of viral signatures from small amounts of total RNA nucleic acid collected from SARS-CoV-2 cases within 1 h, they verified the validity of such lateral flow tools as a fast, inexpensive, and machine-independent alternative to the existing detection procedures.[114]

CRISPR/CAS3-BASED DIAGNOSIS OF SARS-COV-2

Recently, several groups have documented that class I, type I CRISPR systems from *Escherichia coli* and *Thermobifida fusca*, both using Cas3 enzyme and the crRNA-bound complex, can trigger targeted cleavage of DNA in human cells by long-range deletions.[80,115,116] Yoshimi et al.[80] established an in vitro nucleic acid diagnostic tool based on Cas3, Cas3-operated nucleic acid detection N (CONAN). The CONAN tool is a sensitive, fast, and device-free diagnostic system for SARS-CoV-2 detection in combination with isothermal amplification methods. To assess the efficiency of the CONAN system in detecting nucleic acids of SARS-CoV-2, purified RNA nucleic acids from NP swabs, including 10 positive COVID-19 samples (confirmed by PCR) and 15 negative samples (confirmed by PCR), were evaluated using CONAN RT-LAMP and DETCTR RT-LAMP. Tests conducted using the CONAN RT-LAMP system resulted in SARS-Cov-2 detection in nine out of 10 positive samples as well as the detection of one sample from negative samples. In general, the SARS-CoV-2 detection rate by the CONAN RT-LAMP system was found to be comparable to the DETCTR RT-LAMP system.[80] Table 2 summarizes the characteristics of all Cas proteins used to diagnose SARS-CoV-2 infection, including Cas12, Cas13, FnCas9, and Cas3.

SUPERIORITIES AND SHORTCOMINGS OF CRISPR-BASED DIAGNOSTIC SYSTEMS

Similar to the Cas9 protein, Cas12a detects DNA sequences and makes double-stranded breaks in the target site. Cas12 endonuclease, especially LbCas12a, causes nonspecific cleavage of ssDNA molecules after binding to its target genomic region, which ultimately leads to degradation of other ssDNAs in the vicinity. This Cas12 activity led to the development of the DETECTR method and was used for molecular detection. The DETECTR is similar to RT-PCR in terms of accuracy but surpasses RT-PCR in speed to a final result. In addition, the

Table 2. Comparison of the Cas Proteins Properties Used in the Diagnosis of SARS-CoV-2

	CRISPR class	organism	length	target molecule	PAM	shortcomings	accuracy
Cas12	class II -type V	*Francisella novicida*, *Acidaminococcus* sp., *Lachnospiraceae* sp., *Prevotella* sp.	~1100−1300 amino acids	DNA (dsDNA or ssDNA)	Cas12a= TTTN Cas12b = TTN	target site must be near the PAM	ability to distinguish one nucleotide between targets
Cas13a	class II-type VI	*Leptotrichia shahii*, *Ruminococcus flavefaciens*, *Leptotrichia buccalis*	900−1300 amino acids	RNA (ssRNA)	protospacer flanking site (PFS)	can only be used for RNA targets—need to convert DNA to RNA to identify DNA targets—restriction of protein activity due to the secondary structure of RNA	ability to distinguish one nucleotide between targets
FnCas9	class II-type II	*Francisella novicida*	1629 amino acids	DNA	NGG	target site must be near the PAM	ability to single nucleotide variation detection
Cas3	class I-type I	*Escherichia coli*	700−1100 amino acids	DNA	AAG	perform collateral ssDNA cleavage only in sequences containing PAM	high specificity for single nucleotide discrimination

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collateral cleavage activity of the Cas13 protein also led to the development of the SHERLOCK method. This method uses the nonspecific activity of the Cas13 protein to cleave the fluorescent RNA reporter.[27,60,66] Both DETECTR and SHERLOCK methods can be adapted for detection by lateral flow dipsticks. In general, there are challenges in the diagnosis of the COVID-19 virus, such as access to staff safety facilities, collection of samples, extraction of virus genomes, and access to extraction kits and reagents in both RT-PCR and CRISPR-based diagnostic methods. However, one of the advantages of CRISPR-based diagnostic systems over RT-PCR is the use of isothermal amplification methods that eliminate the need for thermocycling.[117] Other advantages are high speed to achieve the final result, specificity to target single nucleotides, no need for specialized laboratory equipment, and the use of accessible reporting techniques such as lateral flow strips. The ability of the CRISPR systems to quickly recognize a variety of infectious diseases caused by emerging viruses such as coronavirus is one of the significant capabilities of this system. In addition, the CRISPR system is able to detect coronavirus mutant types with high accuracy. In CRISPR-based diagnostics, since protected areas are used to design the gRNA, the diagnostic platform will be able to diagnose the disease even if the virus genome is mutated. Variations in viral load at different stages of the disease is another challenge; that is, when the viral load is low, conventional diagnostic tests may not be able to detect the viral infection and may need to increase the viral load, which results in false negative results. However, CRISPR-based diagnostic systems rely on the detection of the viral genome, so they are able to diagnose the infection at every stage of the disease and do not require additional confirmatory tests.[24,109,118] In addition, the multiplexing ability of the CRISPR system allows it to distinguish between multiple viral pathogens or even different viral serotypes in the same sample.[65] Off-target occurrence, where nonspecific binding of gRNA to the virus genome leads to misinterpretation of results, is one of the main challenges of the CRISPR system. Therefore, in order to reduce the off-target effects, it is necessary to use specialized tools for gRNA design to select the best one(s).[24]

OVERVIEW ON CAS PROTEINS USED IN SARS-COV-2 DETECTION

Special attention has recently been drawn to the CRISPR revolution for the early detection of SARS-CoV-2, which is still rapidly spreading and has affected millions of people worldwide. In general, diagnostic systems based on CRISPR consist of two main elements: (1) Cas protein-guide RNA sequence and (2) modified nucleic acids used as reporters. Reporters generate a visual signal when they cut, which is used in detection. Once the specified nucleic acid sequences are detected and cut, reporter molecules are subsequently cut and generate a visual signal. Cas proteins used by different groups to recognize the nucleic acid of SARS-CoV-2 include Cas3, FnCas9, Cas12a and Cas12b, and Cas13a. Particularly, most CRISPR-based SARS-CoV-2 diagnostic studies have enjoyed Cas12 protein. The Cas12 protein belongs to the class II CRISPR systems which recognize and cleave DNA (dsDNA or ssDNA) sequences. This nuclease has high specificity for dsDNA sequences so that it can distinguish very similar dsDNA sequences from each other, while it does not have this ability for ssDNA sequences. Cas12a and Cas12b are the two subtypes of the Cas12 protein commonly used in CRISPR-based diagnosis.[68,119] Cas13 protein is related to class 2 CRISPR systems and is capable of recognizing and cutting RNA sequences. DNA sequences can be converted to RNA using the T7 promoter to detect DNA sequences using Cas13 nuclease.[66] This nuclease does not require a PAM sequence, which makes Cas13 more flexible. However, some of Cas13 orthologs require a protospacer flanking site (PFS). There are four types of Cas13 protein, including Cas13a−d; Cas13a and Cas13b are commonly used in engineering and diagnostics. Like Cas12, the Cas13 protein has collateral activity which is used for diagnostic applications such as reporter molecule cutting.[66,120,121] Cas9 endonuclease is also member of class II CRISPR systems and is commonly used in genome editing. This enzyme specifically identifies and cleaves DNA sequences through the guide sequence. Recently, the *Francisella novicida*-derived Cas9 protein called FnCas9 has been used to detect SARS-CoV-2. Since the FnCas9 protein requires a PAM sequence, PAM can be engineered into the primers to target and identify non-PAM regions. The FnCas9 enzyme is capable of cutting DNA sequences within the 10−50 °C temperature range. Considering the sensitivity of FnCas9 to mismatch, it can be used to detect single nucleotide variations.[114,122] Cas3 endonuclease belongs to class I CRISPR systems which are capable of identifying and cutting DNA sequences. This protein has a length of approximately 1100 amino acids and requires a PAM sequence to cut the target site.[80]

CONCLUSIONS AND PERSPECTIVES

The increasing number of deaths caused by the COVID-19 outbreak has caused major concern worldwide. One of the confounding facets of COVID-19 is that it presents a wide range of symptoms from patient to patient. Therefore, highly sensitive, specific, and precise approaches need to be established for early detection and thus better management of COVID-19. The discovery of the gene-editing toolkit known as CRISPR has reshaped biotechnology and biotechnology-based medical diagnostic tests. One of the appealing features of CRISPR is that it can be programmed to target almost any region of interest within the desired genome. Known for its genome manipulation applications, the CRISPR technique possesses a broad range of other applications and has been effectively used to diagnose SARS-CoV-2 in recent studies. Speed, accuracy, power, low cost, sensitivity, and versatility are the main features of this promising tool in the quest to banish disease from humans. Recently, CRISPR-based diagnostic systems have been developed that include the use of Cas12a and Cas13 enzymes. Like Cas9, the Cas12a nuclease binds to the desired genomic area via a gRNA and makes the break. In practice, however, the difference between Cas12 and Cas9 is that when Cas12 begins to cleave the target DNA, it also starts to cleave nonspecifically neighboring single-stranded DNA, so that a cleaved fluorescent reporter around the target genome could be detected. Cas13 nuclease has a similar function to Cas12a, but unlike Cas12a it acts on RNA sequences. A CRISPR/Cas13-based system can be programmed to target SARS-CoV-2 RNA sequences where the Cas13 nuclease can attack via an RNA guide and cleave the target region to destroy viruses. Following the outbreak of COVID-19 disease, different groups have developed various diagnostic systems to diagnose SARS-CoV-2 nucleic acid using the nonselective cutting activity of Cas12a and Cas13 nucleases. Quickly obtaining results is one of the great advantages of CRISPR-based diagnostic systems. Another exciting fact of employing

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CRISPR-based diagnostic systems is that they do not need sophisticated laboratory equipment, so they can be easily used even in areas with poor economic conditions. Due to the programmability of the CRISPR system, it can be harnessed in many fields. For example, the CRISPR system can be used to identify key factors involved in the pathogenicity of SARS-CoV-2 in the host cells, which could be a touchstone in understanding the mechanism of COVID-19 pathogenesis and in finding the drug. However, there are challenges and problems in using the CRISPR/Cas system for nucleic acid detection. For example, one of the common problems in CRISPR/Cas mediated detection is the limited number of detectable sequences. Cas proteins recognize target sequences on the desired nucleic acid through the guide RNA that must complement the target region. On the other hand, depending on the type of Cas protein used, the presence of a sequence called PAM is critical to create a functional correct form between the guide RNA/Cas complex and the target region. Another important issue is mismatch tolerance, in which the tolerance of mismatches between the spacer sequence and the target sequence depends on the Cas protein family. The number of mismatches as well as their location relative to the PAM sequence are determinants of the overall mismatch tolerance, so mismatches farther from the PAM sequence are more tolerated. The Cas13 protein family requires PFS and is capable of PAM-free detection. Standardization is also a very important factor in effective detection with the CRISPR system. Protocols need to be standardized to ensure that all operators achieve the same result. Since the level of nucleic acid concentration in patient samples is associated with disease progression, in addition to qualitative diagnosis, quantitative diagnosis is also very important. CRISPR-based diagnostic platforms such as SHERLOCK, DETECTR, and HOLMES are only capable of qualitative detection and cannot quantify the desired nucleic acid content. Many CRISPR-based diagnostic systems still require amplification of the target nucleic acid prior to detection. Conventional methods for nucleic acid amplification, such as PCR, are not suitable for use in POC systems due to the need for thermocycling as well as the need for several temperature steps leading to prolonged reactions. In these cases, isothermal amplification methods can be used in the absence of equipment and skilled operators. LAMP and RPA techniques are the most common isothermal amplification systems used in POC diagnostic systems. The low temperature requirement is one of the advantages of the RPA technique over LAMP. Problems such as dimer primer formation as well as nonspecific replication products are caused by low temperatures. However, if the CRISPR system with high specificity for its target area is used, these nonspecific and undesirable products will not interfere with the reaction and ultimately the reaction result.

The defensive feature of CRISPR can be applied for the design and establishment of effective antiviruses against SARS-CoV-2. This approach can be used to create antivirals against other possible new viruses that we may encounter in the future, allowing us to respond quickly to pandemics hereafter. Moreover, using the easy reprogramming capability of the CRISPR system, multiple guide RNAs can be used to ensure that the target sequence is identified even if the virus mutates. In addition to being a hope for coronavirus treatment, CRISPR is ambitiously in the race for diagnostics and testing and will likely leave an effective and lasting impact on the current pandemic.

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Notes

The authors declare no competing financial interest.

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