# CRISPR-based diagnostic approaches: Implications for rapid management of future pandemics (Review)

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*Abbreviations:* CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; AaCas12b, *Alicyclobacillus acidiphilus* Cas12b; AAVs, adeno-associated viruses; AsCas12a, *Acidaminococcus* sp. Cas12a; ASFV, African swine flu virus; AuNPs, gold nanoparticles; CARMEN, combinatorial array reactions for the multiplex evaluation of nucleic acids; CcaCas13b, *Capnocytophaga canimorsus* Cas13b; CDC, Centers for Disease Control and Prevention; crRNA, CRISPR RNA; gRNA, guide RNA; DENV, dengue virus; DETECTR, DNA endonuclease targeted CRISPR trans reporter; DISCoVER, diagnostics with coronavirus enzymatic reporting; DRs, direct repeats; dsDNA, double-stranded DNA; EiCsm6, Csm6 from *Enterococcus italicus*; FAM, fluorescein amidites; FIND-IT, fast integrated nuclease detection in tandem; FLUAV, Influenza A virus; HAVs, human-associated viruses; HEPN, higher eukaryotic and prokaryotic nucleotide; HEX, hexachloro-fluorescein; HIV, human immune deficiency virus; HOLMES, 1-h low-cost multipurpose highly efficient system; HPV, human papillomavirus; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; JEV, Japanese encephalitis virus; LAMP, loop-mediated isotherm amplification; LbaCas13a, *Lachnospiraceae* bacterium Cas13a; LbCas12a, *Lachnospiraceae* bacterium Cas12a; LbuCas13a, *Leptotrichia buccalis* Cas13a; LCMV, lymphocytic choriomeningitis virus; LsCsm6, Csm6 from *Lactobacillus salivarius*; LshCas13a, *Leptotrichia shahii* Cas13a; LwaCas13a, *Leptotrichia wadei* Cas13a; NASBA, nucleic acid sequence-based amplification; nt, nucleotide; PACMAN, prophylactic antiviral CRISPR in human cells; PAM, protospacer-associated motif; POC, point-of-care; PsmCas13b, *Prevotella* sp. MA2016 Cas13b; RNase, RNA endonuclease; RPA, recombinase polymerase amplification; RT-qPCR, reverse transcription-quantitative PCR; SARS-CoV, severe acute respiratory syndrome corona virus; ssDNA, single-stranded DNA; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; SHINE, streamlined highlighting of infections to navigate epidemics; SNP, single nucleotide polymorphism; ssRNA, single-stranded RNA; STOP, SHERLOCK test in one pot; tracrRNA, trans-activating CRISPR RNA; TtCsm6, Csm6 from *Thermus thermophilus*; ZIKV, zika virus

*Key words:* CRISPR, CRISPR-associated systems, CRISPR RNA, reporter, fluorescence-quencher, collateral activity, sensitivity, specificity, point-of-care devices, diagnostics

**Abstract.** Sudden viral outbreaks have increased in the early part of the 21st century, such as those of severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome corona virus, and SARS-CoV-2, owing to increased human access to wildlife habitats. Therefore, the likelihood of zoonotic transmission of human-associated viruses has increased. The emergence of severe acute respiratory syndrome coronavirus 2 in China and its spread worldwide within months have highlighted the need to be ready with advanced diagnostic and antiviral approaches to treat newly emerging diseases with minimal harm to human health. The gold-standard molecular diagnostic approaches currently used are time-consuming, require trained personnel and sophisticated equipment, and therefore cannot be used as point-of-care devices for widespread monitoring and surveillance. Clustered regularly interspaced short palindromic repeats (CRISPR)-associated (Cas) systems are widespread and have been reported in bacteria, archaea and bacteriophages. CRISPR-Cas systems are organized into CRISPR arrays and adjacent Cas proteins. The detection and in-depth biochemical characterization of class 2 type V and VI CRISPR-Cas systems and orthologous proteins such as Cas12 and Cas13 have led to the development of CRISPR-based diagnostic approaches, which have been used to detect viral diseases and distinguish between serotypes and subtypes. CRISPR-based diagnostic approaches detect human single nucleotide polymorphisms in samples from patients with cancer and are used as antiviral agents to detect and destroy viruses that contain RNA as a genome. CRISPR-based diagnostic approaches are likely to improve disease detection methods in the 21st century owing to their ease of development, low cost, reduced turnaround time, multiplexing and ease of deployment. The present review discusses the biochemical properties of Cas12 and Cas13 orthologs in viral disease detection and other applications. The present review expands the scope of CRISPR-based diagnostic approaches to detect diseases and fight viruses as antivirals.

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## 1. Introduction

The unexpected outbreak of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) has highlighted that the world was unprepared for a global pandemic of such a catastrophic nature. The emergence and recurrence of viral outbreaks, including severe acute respiratory syndrome coronavirus (SARS-CoV), Middle East respiratory syndrome corona virus, and SARS-CoV-2, have demonstrated the need to develop diagnostics and effective antivirals that can be used to alleviate the disease burden. Current diagnostic and antiviral development approaches are insufficient to control the onset of diseases at an early stage. In particular, in current technologies, the limitations include cost, processing time and the requirement for sophisticated equipment. In addition, diagnostic reagents, testing infrastructure and trained staff are under pressure when demand is high during the peak of a global pandemic (1-3). In the future, more outbreaks of human pathogens are expected due to the increasing access to wild habitats through man-made encroachments. Owing to the increased contact between wildlife and humans, the likelihood of future virus outbreaks is very high (4). There are only ~25 approved vaccines and effective antivirals to treat known viruses (2). Therefore, without developing efficient diagnostic approaches to detect emerging and recurrent viruses, and effective antiviral agents for early treatment in the initial stages of outbreaks, viruses will continue to pose a significant threat to human health (2).

The presence of numerous viruses as reservoirs in wild animal species, such as bats with pandemic potential, complicates preparation. The advent and success of modern genomics have accelerated the identification of new microbes, including viruses, in animal or insect species. A number of these viruses have pandemic or epidemic potential and can cause human diseases while infecting others (4-7). There is high sequence diversity among viruses; for example, there are three types of influenza (A, B and C). Within Influenza A, the virus has subtypes, including 18 hemagglutinins and 11 neuraminidases (8,9). Viruses have evolved at an increased rate because of higher mutation acquisition rates than that in humans. Mutation acquisition can render a virus tolerant to its host, cause acquisition of changes in diagnostic targets or dropout and development of resistance to therapy, as seen in SARS-CoV-2 variant B1.1.7, with the 69/70 mutation, which originated in the United Kingdom (2,10,11). Consequently, there is uncertainty regarding the virus species and variants in circulation during pandemics or epidemics. It is necessary that diagnostics and therapy do not require in-depth knowledge of viral biology and that the technologies for detecting viral mutations are robust and can be easily modified if necessary.

Molecular diagnostic tools based on the polymerase chain reaction (PCR) are considered the gold standard in traditional diagnostics. Programmability and usability are marked concerns associated with the traditional PCR-based diagnostic approaches. Although PCR-based assays require only sequence information to design primers or probes, laboratory infrastructure and requirements for trained personnel limit their use outside a well-equipped laboratory. Antigen-based tests offer alternatives that are easy to use but have lower specificity and sensitivity compared with the gold standard of PCR-based tests and are much slower to design and develop (1). The development of effective antiviral drugs requires an understanding of the viral and host target proteins. The utility of numerous small-molecule inhibitors has led to the approval of ~90 antiviral drugs for human use; however, identifying small molecules and reusing previously used inhibitors is a lengthy and time-consuming process (12). Recent global proteomics studies have reported novel SARS-CoV-2 targets that could be used to develop new treatment regimens against the virus. These studies provide new targets that can also be used to identify potent small molecules or to reuse known molecules with potent antiviral activity without in-depth knowledge of SARS-CoV-2 biology (13-16). Numerous nucleic acid-based antiviral drugs, including small interfering RNAs, have been successfully tested in cell cultures and animal models. Unfortunately, none of these drugs have been approved for treating viral diseases (17,18).

Therefore, the development of technologies that only require sequence information will improve future virus detection. One of the reported components of the bacterial adaptive immune system, CRISPR-Cas systems, offers opportunities for the rapid development of virus diagnostics. The successful use of CRISPR-Cas systems in genetic engineering and the treatment of certain hereditary human diseases has been previously reported (19). Furthermore, the continuous detection of new CRISPR-Cas systems and orthologous Cas proteins, and their in-depth molecular insights have expanded the scope of CRISPR-Cas systems in the urgent development of modern diagnostics (19,20). CRISPR-Cas systems have been used to develop virus diagnostics, including for the recently emerged SARS-CoV-2 (21). A number of these have been used on an emergency basis and may, in the future, be developed as point-of-care (POC) devices and as easy-to-use diagnostic tests for numerous human diseases (2).

## 2. CRISPR-Cas systems provide tools for modern diagnostics

In modern diagnostics, viral nucleic acids are detected using PCR. PCR can be either qualitative or quantitative, and is considered the gold standard (22). Reverse

transcription-quantitative PCR (RT-qPCR) is commonly used when quantification is required. RT-qPCR only requires knowledge of the viral genome sequence. However, the cost, time from sample to reaction, need for trained personnel and expensive laboratory equipment are significant barriers to using RT-qPCR for virus diagnostics (23).

In addition, certain other methods have been developed with advantages and limitations in performance, sensitivity, specificity, multiplexing ability, readouts and test throughput, which eliminating the need for costly thermal cycling or PCR, such as isothermal amplification which can be used instead of PCR (24,25). The most commonly used isothermal amplification methods are loop-mediated isothermal amplification (LAMP), recombinase polymerase amplification (RPA), nucleic acid sequence-based amplification (NASBA) and nicking enzyme amplification reaction (26-30).

CRISPR-based diagnostic tests are versatile and can detect viruses that contain DNA or RNA genomes. Several Cas proteins, reaction conditions, amplification methods and readouts have been used to demonstrate their versatility for diagnosing viral diseases (31-35) (Tables I and II). CRISPR-based diagnostics complement current diagnostic technologies because their sensitivity and specificity are similar to those of PCR methods. CRISPR-based tests are also easily adapted for fluorescence-based detection or visual displays using lateral flow assays (36-38).

Table I. Cas12 and Cas13 orthologues in clustered regularly interspaced short palindromic repeats-diagnostics.

| Essential features | Cas12 | Cas13 |
|---|---|---|
| Requirement of PAM | Required | Not required |
| Identity of PAM | TTTV | Not applicable |
| Cleavage type | Single staggered cut | Multiple cut sites |
| Type of target | ssDNA, dsDNA | ssRNA |
| Collateral cleavage activity | Present | Present |

Cas, clustered regularly interspaced short palindromic repeats-associated; ds, double-stranded; PAM, protospacer-associated motif; ss, single-stranded (34).

## 3. Cas proteins recognize target double-stranded DNA (dsDNA) and RNA mediated by single-stranded RNA (ssRNA)

CRISPR is organized into CRISPR arrays containing direct repeats (DRs) and spacer sequences (short sequences of viruses or plasmids) (39,40). The Cas proteins responsible for enzymatic activity are located near CRISPR arrays. CRISPR-Cas systems are divided into two classes, six types and numerous sub-types. Only class 2 Cas proteins are used in viral diagnostics or therapy because a single subunit protein with multiple domains acts as the effector required for nucleic acid cleavage, whereas multiple subunits are required in class 1. The Cas effectors Cas9, Cas12, and Cas13 interfere with target nucleic acids when complexed with mature CRISPR RNA (crRNA), also called guide RNA (gRNA) (Fig. 1). Each Cas effector protein possesses a different biochemical or catalytic activity that recognizes different nucleic acids for cleavage, which also determines their potential applications (Fig. 1; Tables I and II) (2).

Cas9 is the best-characterized Cas protein, and requires crRNA and trans-activating RNA (tracrRNA) for enzymatic activity. Although these ssRNAs are produced separately, they can be engineered into a single RNA molecule known as a single gRNA (Fig. 1). Cas9 proteins are used in epigenome and genome editing, among numerous other applications, which have been described previously (41,42). Cas9 enzymes are modular proteins, which comprise RuvC (an endonuclease domain named for an *Escherichia coli* protein involved in DNA repair)-like endonuclease and HNH (an endonuclease domain with catalytic histidine and asparagine residues) nuclease domains, which are critical for target cleavage (Fig. 1B). Cas9 requires a 30 nucleotide (nt) long protospacer sequence flanked by a protospacer-associated motif (PAM) (5'-NGG-3'). Cas9 cleaves dsDNA 3-nt upstream of the conserved PAM sequence. Consequently, host DNA repair enzymes repair the breakpoints, which leads to the incorporation of insertions or deletions (Fig. 1E) (42). CRISPR arrays and Cas proteins recognize and degrade foreign DNA, RNA and plasmids. All characterized Cas proteins identify target nucleic acids when guided by crRNAs (37,43-45).

The single subunit C2c2, or Cas13a, is an RNA endonuclease (RNase). The function of Cas13a is regulated by ssRNAs (37,43,46). Cas13a possesses dual RNase activity, which is required for crRNA processing and RNA-dependent RNA degradation (37,47). The two unique RNase activities of Cas13a provide flexibility for multiplex processing of pre-crRNA into crRNA and the loading of gRNA or a spacer sequence into the target sequence for cleavage, which is necessary for the sensitive detection of cellular RNAs or any RNA substrates (37). Cas13a proteins encompass two higher eukaryotic and prokaryotic nucleotide (HEPN) binding domains that are critical for RNA degradation (Fig. 1D) (43). Unlike Cas9 and Cas12, Cas13 does not require a conserved PAM (Table II). Cas13 proteins cleave ssRNA at multiple sites that do not depend on the crRNA position but on ssRNA secondary structures such as stems and loops known as hairpins (37,43,48). Initiation of CRISPR-mediated immunity requires processing of pre-crRNA into individual mature crRNAs composed of a single spacer. Pre-crRNAs harbor multiple spacers flanked by palindromic DRs (37,49-51).

Three possible steps are used alone or in combination by different CRISPR-Cas systems in the production of mature crRNAs, including: i) A dedicated endonuclease being required for crRNA processing or target cleavage (37); ii) integration of a host endonuclease with CRISPR-Cas proteins (37,52) or iii) an intrinsic RNase activity for effector function (37,47). The Cas13a homologs from three distinct branches of the Cas13 protein family including *Leptotrichia shahii* Cas13a (LshCas13a), *Leptotrichia buccalis* Cas13a (LbuCas13a), and *Listeria seeligeri* Cas13a cleave at the 5' end of the pre-crRNA,

Table II. Comparative features of Cas13 and Cas12 orthologs.

| Origin of Cas enzymes and associated essential features | Cas enzyme | | | | |
|---|---|---|---|---|---|
| | LwaCas13a | LbaCas13a | CcaCas13b | PsmCas13b | AsCas12a |
| Organism | *Leptotrichia wadei* | *Lachnospiraceae bacterium* NK4A179 | *Capnocytophaga canimorsus* | *Prevotella* sp. MA2016 | *Acidaminococcus* sp. BV3L6 |
| Target | ssRNA | ssRNA | ssRNA | ssRNA | ssDNA/dsDNA |
| Direct repeat orientation | 5' | 5' | 3' | 3' | 5' |
| Motif preference | Poly U/AU | Poly A/AC | Poly U/UA/UC | Poly A/GA | Not applicable |
| Spacer length, nt | 28 | 28 | 30 | 30 | 20 |
| Sensitivity, aM | ~$5x10^5$ | ~$1x10^9$ | ~$5x10^6$ | ~$5x10^8$ | ~$5x10^{10}$ |

Cas, clustered regularly interspaced short palindromic repeats-associated; ds, double-stranded; ss, single-stranded; A, adenosine; C, cytosine; G, guanosine; U, uracil; nt, nucleotide; PsmCas13b, *Prevotella* sp. MA2016 Cas13b; CcaCas13b, *Capnocytophaga canimorsus* Cas13b; LwaCas13a, *Leptotrichia wadei* Cas13a; LbaCas13a, *Lachnospiraceae* bacterium Cas13a; AsCas12a, *Acidaminococcus* sp. Cas12a (34).

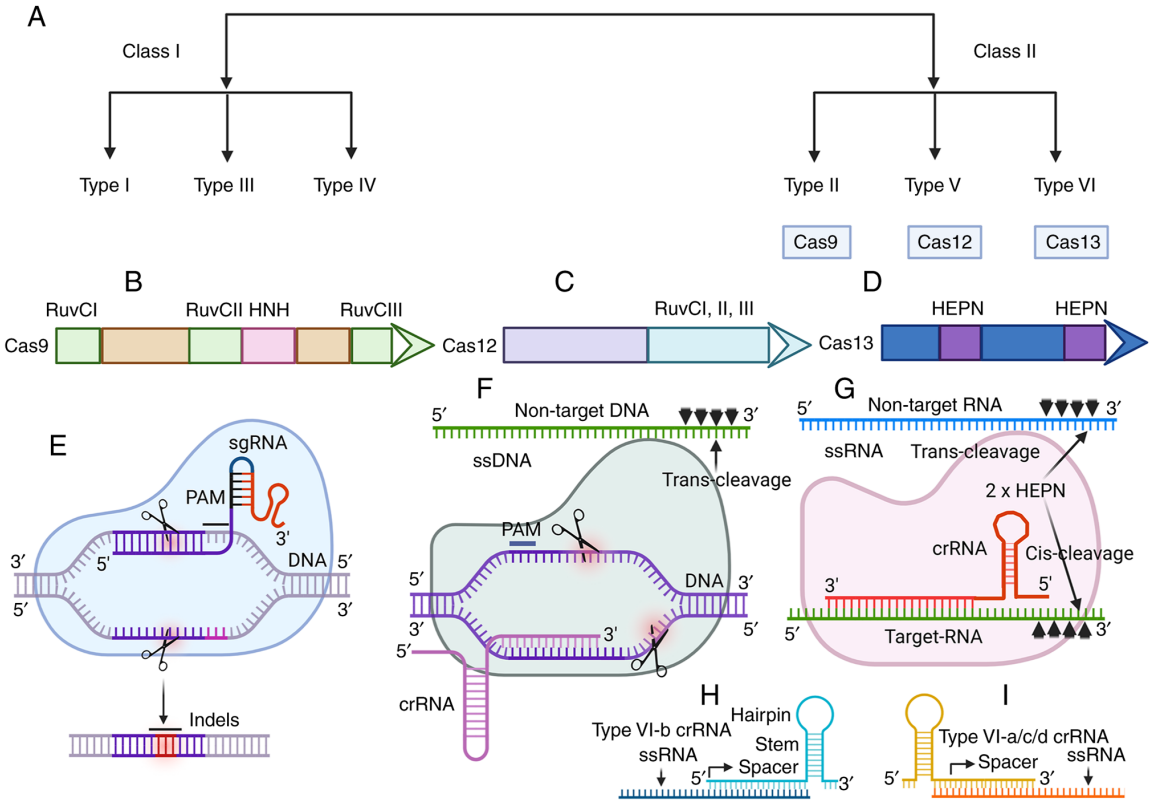


Figure 1. Cas12 and Cas13 Cas orthologs recognize dsDNA and ssRNA possessing ssDNA and ssRNA cleaving trans-collateral activity. (A) CRISPR-Cas systems can be divided into two classes and six types. The class II system encodes single subunit enzymes, such as Cas9, Cas12 and Cas13, that target nucleic acids for modifications. Schematic representations of (B) Cas9, (C) Cas12 and (D) Cas13. Cas9 contains three RuvC (I, II and III) and one catalytically active HNH domain required to induce DNA cleavage. Cas12 contains three RuvC (I, II and III) endonuclease domains. Cas13 has two HEPN-binding domains, as presented in the figure. (E) Cas9 is presented in complex with sgRNA and target DNA. The position of PAM, target binding and cleavage position are marked in the figure. (F) Cas12 is presented in complex with crRNA and a double-stranded DNA target. The position of PAM and the cleavage sites are also presented. Cas12 also possesses promiscuous ssDNA degrading activity. (G) Cas13 is presented in complex with crRNA and ssRNA targets. Cas13 also possesses ssRNA-degrading collateral activity when complexed with target RNA. (H) The crRNA structure demonstrated in type VI b-Cas orthologs. The pre-crRNA processing occurs at the 3' ends, which create spacers. (I) The most common crRNA structure reported in type a/c/d Cas orthologs in which processing of pre-crRNA occurs at the 5' end, creating spacers. This figure was generated using BioRender (www.biorender.com). CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; PAM, protospacer-associated motif; Indels, insertions or deletions; ss, single-stranded; sgRNA, single guide RNA; crRNA, CRISPR RNA; HEPN, higher eukaryotic and prokaryotic nucleotide; RuvC, an endonuclease domain named for an *Escherichia coli* protein involved in DNA repair; HNH, an endonuclease domain with catalytic histidine and asparagine residues.

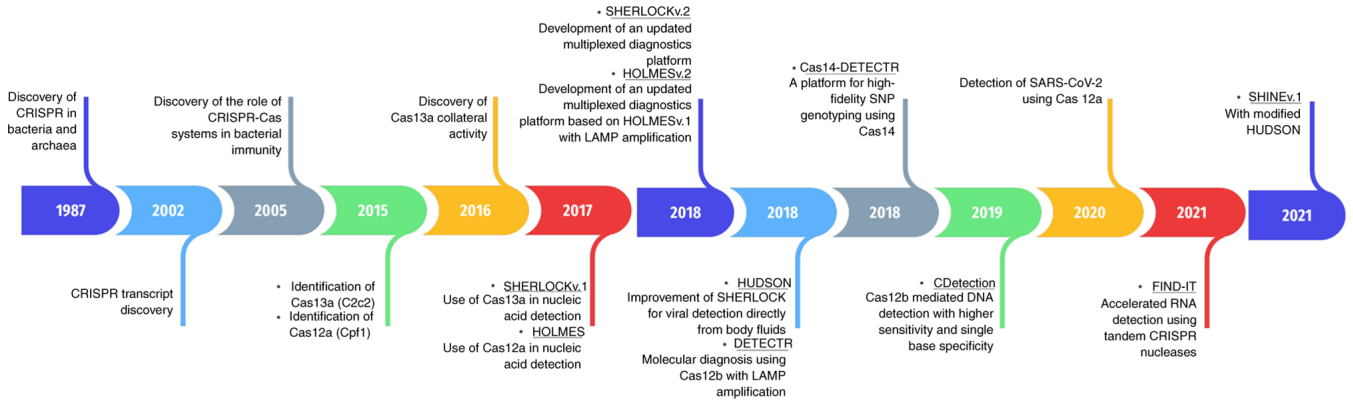


Figure 2. The discovery of Cas12 and Cas13 Cas orthologs has revolutionized the development of CRISPR-based diagnostics. Timeline of the major discoveries for CRISPR-Cas systems in nucleic acid detection. The discovery of CRISPR-Cas systems in bacteria and archaea, together with biochemical characterization of Cas12 and Cas13 ortholog enzymatic properties, has revolutionized the development of CRISPR-based diagnostics. The nonspecific trans-collateral activity of Cas12 and Cas13 in cleaving single-stranded DNA and single-stranded RNA has been exploited in the design and development of CRISPR-based diagnostic approaches for nucleic acid detection. CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; v.1, version 1; HOLMES, 1-h low-cost multipurpose highly efficient system; LAMP, loop-mediated isotherm amplification; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; DETECTR, deoxyribonucleic acid endonuclease targeted CRISPR trans reporter; SNP, single nucleotide polymorphism; SARS-CoV-2, severe acute respiratory syndrome corona virus 2; FIND-IT, fast integrated nuclease detection in tandem; SHINE, streamlined highlighting of infections to navigate epidemics.

which comprises consensus DRs and a conserved 20 nt spacer sequence (Fig. 1I). LshCas13a and LbuCas13a process crRNA three or five nucleotides upstream or downstream of the DRs and form hairpin structures depending on the Cas13a homolog (37,43). Changing the stem and reducing or inverting the hairpin in the DRs attenuates LbuCas13a processing of pre-crRNA. Four contiguous nucleotide mutations in a single-stranded region near or including the cleavable bond completely abolishes the activity of LshCas13a and LbuCas13a (37,49,50,53).

Similarly, LshCas13a requires 3' flanking sequences for DRs, stems and secondary hairpin structures in crRNA processing (43). Target identification using LshCas13a is sensitive to mutations in the protospacer region (target RNA sequence). The processing activity of LbuCas13a is independent of divalent cations (37,47). The mature crRNA binds to Cas proteins, generating an RNA-directed RNA-degrading complex for highly sequence-specific recognition and cleavage of target RNA (Fig. 1F and G) (37,42,44,45). LshCas13a and LbuCas13a have demonstrated non-specific promiscuous RNA-dependent RNA trans-degradation activity (Fig. 1G). LshCas13a has been reported to preferentially cleave uracil (U) residues in the ssRNA region of crRNA (37,43). crRNA processing and ssRNA target cleavage are two independent activities of the Cas13a proteins. ssRNA cleavage is ~80 times faster compared with crRNA processing. Cas13a proteins, including LshCas13a and LbuCas13a, contained two HEPN domains (Fig. 1D). Mutations in conserved arginine (R) or histidine residues abolish crRNA-directed target cleavage, while retaining crRNA processing and binding activity (37,43). In addition, mutational studies with LbuCas13a have reported a conserved R1079, which is required for crRNA processing; however, crRNA-driven RNA cleavage and binding activities remain unaffected. These observations indicated that Cas13a proteins possess distinct pre-crRNA processing and crRNA-directed cleavage activities (Fig. 1D) (37).

## 4. ssRNA or dsDNA target recognition-dependent collateral activity of Cas13 or Cas12 proteins

Cas12 and Cas13 proteins have demonstrated single-stranded DNA (ssDNA) and ssRNA-degrading collateral activity (cleavage of nonspecific/nontarget ssDNA or ssRNA molecules) (32,36) (Fig. 1F and G). The identification of CRISPR-Cas systems in bacteria and the development of Cas12- and Cas13-based diagnostics for the detection of viral diseases and other applications occurred over a ~35 year period (Fig. 2). ssRNA- and ssDNA-degrading collateral activities were used to detect viral RNA or DNA in a readable format when combined in a CRISPR reaction using a synthetic ssRNA or ssDNA molecule flanked by a fluorophore and quencher. Collateral cleavage of the fluorophore and quencher-labeled synthetic ssRNA or DNA physically separated the quencher from the fluorophore, which generated a fluorescent signal that could be assessed with a fluorimeter or fluorescence detector (Fig. 3) (37). In the second approach, synthetic reporters were flanked by fluorescein amidite (FAM) and biotin molecules. The FAMs were labeled with gold nanoparticles (AuNPs) for visualization. A streptavidin-coated lateral flow strip and anti-FAM antibody were used to visualize the cleavage of the target molecule. If the target was present in the sample, the lateral flow strip would present two bands at the streptavidin and anti-FAM antibody positions (Fig. 3) (43). LshCas13a and LbuCas13a comprise the trans-RNA-stimulated cleavage activity of ssRNA substrates. This is an essential feature of Cas13a proteins, which recognize specific RNAs from a pool or mixture of RNA molecules. Although numerous polymerase-based methods for nucleic acid amplification and subsequent detection are available, only a few, including NASBA, LAMP and RPA, can directly detect the target RNA without further improvements (37,54-57).

It has been reported that the RNA-directed trans-endonuclease activity of LbuCas13a can be used to cleave fluorophore quencher-tagged reporter RNA. Target RNA-induced trans-RNase activity resulted in enhanced

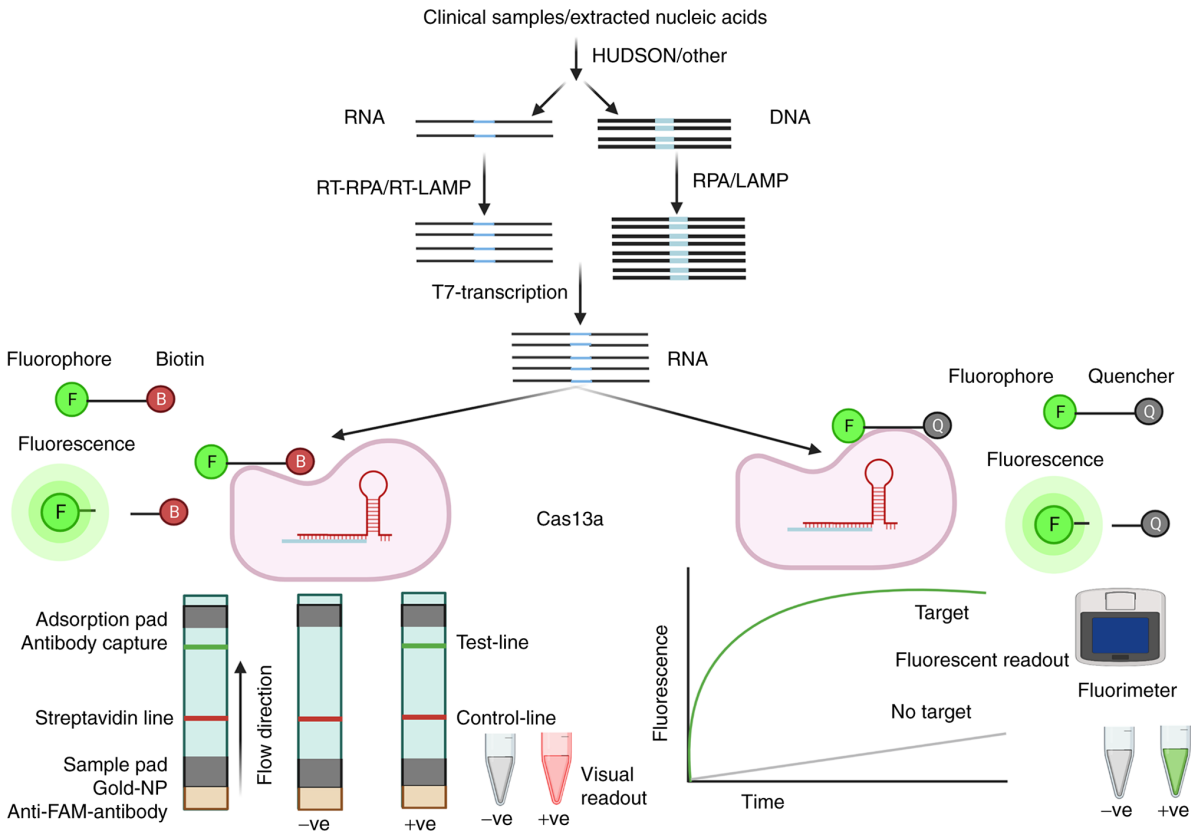


Figure 3. Exploitation of collateral RNA cleavage activity of Cas13a in CRISPR-based diagnostic approaches. Schematic representation of Cas13a-mediated detection of any target RNA or DNA molecule. The clinical sample is processed using methods, such as HUDSON or chemical and heat treatment, followed by extraction of nucleic acids. Target nucleic acids are pre-amplified using isothermal amplification RPA or LAMP. RNA targets are first reverse transcribed and amplified with RPA or LAMP. DNA targets are amplified directly. After amplification, the amplified targets must be transcribed using T7 RNA polymerase in case of Cas13-mediated detection. After amplification, detection is performed by adding CRISPR RNA, target and appropriate Cas enzymes. The signal is detected using either visual indicators in lateral flow strips or fluorescence monitoring in reaction tubes. Fluorescence signals can also be read with a fluorimeter for quantification. Numerous methods have been developed using clinical samples in which reaction reagents are simultaneously used in target amplification and detection. This figure was generated using BioRender (www.biorender.com). CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; LAMP, loop-mediated isotherm amplification; HUDSON, heating unextracted diagnostic samples to obliterate nucleases; FAM, fluorescein amidites; NP, nanoparticle; RT, reverse transcription; RPA, recombinase polymerase amplification, -ve, negative; +ve, positive.

fluorescence intensity within 30 min (37). Promiscuous trans-RNase cleavage activity could be detected by adding 1-10 pM of target RNA. The results indicated that LbuCas13a was a robust RNA-cleaving enzyme capable of 104 turnovers per recognized target. In the presence of ~0.02% target RNA, LbuCas13a demonstrated ~2,550% cleavage of the labeled reporter RNA substrate compared with the level of crRNA-directed cleavage. These results indicate that LbuCas13a has potent trans-RNA cleavage activity that can be used to detect ssRNA molecules present as genetic material in numerous viruses (37). The presence of two different RNase activities can also be exploited in the multiplex detection of RNA molecules with markedly increased signal intensity using a fluorescence-quencher-labeled RNA reporter for the easy detection of ssRNA viruses without target amplification (37).

## 5. Diagnostic technologies which exploit the collateral activity of Cas12 and Cas13-Cas proteins

*Trans-RNA cleaving collateral activity of Cas12 and Cas13, has been exploited in the development of CRISPR-based diagnostics*. The identification of the collateral activity of Cas13 proteins has led to the emergence of CRISPR-Cas-based diagnostic approaches to detect viral and human genomic variations. Characterization of Cas13 collateral activity led to the development of the specific high-sensitivity enzymatic reporter unlocking (SHERLOCK) version 1 (v.1) (32,34). LbuCas13a collateral activity is activated after crRNA binding and target recognition. Collateral activity has been detected using fluorophore-quencher-labeled ssRNA cleavage and the subsequent generation of a fluorescent signal (Fig. 3). A fluorescence reader or fluorimeter can be used for fluorescence signal detection. The collateral activity of Cas12a was revealed after discovering the collateral activity of Cas13a. Cas12a recognizes dsDNA and possesses ssDNA-cleaving trans-collateral activity (Fig. 1C and F). The discovery of Cas12 ended the T7-mediated *in vitro* transcription of the samples (32,36,58).

Two methods have been previously reported that utilize the collateral activity of Cas12, known as DNA endonuclease-targeted CRISPR trans reporter (DETECTR) (36) and 1 h low-cost, multipurpose, highly efficient system (HOLMES) (59). The dsDNA-activated collateral activity of Cas12a cleaves fluorescence-quencher-labeled ssDNA, which produces a fluorescence signal that is read with a fluorescence reader. The two approaches use different preamplification steps: DETECTR uses RPA, whereas

Table III. Characteristics of CRISPR-based diagnostic approaches.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| DETECTR | Cas12a. | RPA. | RPA for 10 min and CRISPR for 60-120 min. | Crude extraction. | Fluorescence . | Highly specific, fast, portable, low occurrence of false positive results, multiplexing can be used and can differentiate viral subtypes. | Off-target effects, limited scope and narrow target range. | HPV16 and HPV18 detection in human samples. | $1.0x10^{-18}$ | (36,60) |
| Cas14-DETECTR | Cas14 (Cas121). | PCR. | The assay time for PCR is NS and for CRISPR it is 120 min. | Crude extraction. | Fluorescence | High sensitivity, simple design, high specificity, multiplexing can be used, no PAM requirements, cost effective detection of pathogenic mutations, user friendly, uses less complicated sample processing, has a high fidelity in SNP detection. | Limited scope, narrow target range, requires extensive validation. | HECT and RLD domain containing E3 ubiquitin protein ligase 2 SNPs detection in human samples. | NS | (61,62) |

Table III. Continued.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| HOLMESv.1 | Cas12a. | PCR. | PCR for 88 min and CRISPR for 15 min. | Column based. | Fluorescence | High sensitivity, rapid PCR amplification, multiplexing can be used, high efficiency, rapid results and no *in vitro* transcription required | Limited scope and high cost. | Discrimination of SNPs in cell lines and human samples; Pseudorabies and JEV detection; and discrimination of viral strains | $1.0 \times 10^{-17}$ | (34,63-65) |
| CDetection | Cas12b. | RPA. | RPA for 10 min and CRISPR for 60-180 min. | Synthetic targets or crude extraction | Fluorescence | High sensitivity, single-base specificity, rapid results and cost-effective. | Limited scope, off-target effects, complex sample preparation and narrow target range. | HPV16 detection; ABO blood genotyping in human; and SNPs detection of breast cancer 1 gene and tumor protein p53. | $1.0 \times 10^{-18}$ | (66) |
| HOLMESv.2 | Cas12b. | LAMP. | LAMP for 40 min and CRISPR for 35 min or one pot for 120 min. | NS. | Fluorescence | High sensitivity and specificity, one-pot detection, enhanced multiplexing capabilities compared with | Sample preparation (temperature gradients were required for target amplification), off-target effects and high costs | Discrimination of SNP in cell lines; JEV detection; detection of human mRNA and circular RNA; and detection of DNA | $1.0 \times 10^{-17}$ | (34,63,65) |

Table III. Continued.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | HOLMESv.1., rapid results and is portable. The use of Cas12b in HOLMESv2 provides greater flexibility in target selection and the detection of multiple targets in a single reaction, which are important advantages over HOLMESv.1 | | methylation | | |
| SHER-LOCKv.1 | Cas13. | NASBA or RPA. | NASBA for 132 min or RPA for 120 min and CRISPR for 60-180 min. | Crude extraction or column based . | Fluorescence . | Ultra-sensitive and specific ($2x10^{-18}$ M), single molecule detection, can detect both DNA and RNA, reagents can be lyophilized without impacting specificity and sensitivity, | Sample preparation requires expertise in protein purification and RNA biology, multistep nucleic acid amplification which may affect precise target quantification, | ZIKV and DENV detection; bacterial detection (such as *E. coli*, *K. pneumoniae*, *P. aeruginosa*, *M. tuberculosis* and *S. aureus*); viral strain discrimination; and SNPs detection | $2.0x10^{-18}$ | (32,60) |

Table III. Continued.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | specificity of Cas13 can be enhanced by introducing synthetic mismatch, it is rapid, and it has a low cost. | it is less useful for precise gene expression profiling, and the absolute digital quantification of the target is not possible | | | |
| SHER-LOCKv.2 | Cas13. | RPA. | RPA for 60 min and CRISPR for 60-180 min or one pot for 60-180 min. | Crude extraction or column based . | Lateral flow or fluorescence . | High specificity for single nucleotides, high sensitivity ($8x10^{-21}$ M) and flexible detection using fluorescence and lateral flow assays. | More time-consuming, multistep nucleic acid amplification process, which may affect the precise target quantification and it is less applicable for precise gene expression profiling. | ZIKV and DENV detection; bacterial detection (such as *Escherichia coli, Klebsiella pneumoniae, Pseudomonas aeruginosa Mycobacterium tuberculosis* and *Staphylococcus aureus*); viral strain discrimination; and SNPs detection. | $8.0x10^{-21}$ | (34,38,60) |
| SHINE. | Cas13. | RPA. | One pot for 50 min. | Crude extraction. | Lateral flow or fluorescence. | High throughput, high sensitivity, | Requires preparation of multiple reaction | SARS-CoV-2 detection | $8.0x10^{-18}$ | (96) |

Table III. Continued.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | | high specificity, it is easy to use, it is less time consuming, it is simple (easy to use lyophilized reagents reduce assay time, target amplification and detection are performed in a single tube), portable and has versatile detection methods | mixtures and the handling of multiple samples | | | |
| STOPcovid | Cas12b. | LAMP. | One pot for 60 min. | Crude extraction. | Lateral flow or fluorescence. | High sensitivity, it is rapid, cost-effective and has a one-step reaction assay. | Limited to SARS-CoV-2 detection, has a limited sensitivity, there is a limited validation and requires development of simple and efficient sample processing steps | SARS-CoV-2 detection | $3.3x10^{-18}$ | (68,69) |

Table III. Continued.

| Name | Cas-enzyme | Preamplification | Assay-time | Preparation of sample | Readout | Advantages | Disadvantages | Applications | Load of detection, mol/l | (Refs.) |
|---|---|---|---|---|---|---|---|---|---|---|
| CARMEN | Cas13. | PCR or RPA. | RPA for 20 min and CRISPR for 180 min. | Column based . | Fluorescence . | High specificity, easy to use (single array can detect more than 100 viruses), high multiplexing capability and can be used for multiplex detection of multiple pathogens | Limited to Cas13a targets, limited validation and off-target effects | Detection of viruses; influenza A strain subtyping; and drug-resistant mutation detection in HIV | $9.0 \times 10^{-19}$ | (70,71) |

CARMEN, combinatorial array reactions for the multiplex evaluation of nucleic acids; CRISPR, clustered regularly interspaced short palindromic repeats; Cas, CRISPR-associated; CDetection, Cas12b-mediated DNA detection; DENV, Dengue virus; DETECTR, DNA endonuclease targeted CRISPR trans reporter; *E. coli*, *Escherichia coli*; HIV, human immune deficiency virus; HOLMES, 1-h low-cost multipurpose highly efficient system; HPV, human papillomavirus; JEV, Japanese encephalitis virus; *K. pneumoniae*, *Klebsiella pneumoniae*; LAMP, loop-mediated isotherm amplification; *M. tuberculosis*, *Mycobacterium tuberculosis*; NASBA, nucleic acid sequence-based amplification; NS, not specified; *P. aeruginosa*, *Pseudomonas aeruginosa*; PCR, polymerase chain reaction; RPA, recombinase polymerase amplification; *S. aureus*, *Staphylococcus aureus*; SARS-CoV-2, severe acute respiratory syndrome corona virus 2; SHERLOCK, specific high-sensitivity enzymatic reporter unlocking; SHINE, streamlined highlighting of infections to navigate epidemics; SNPs, single nucleotide polymorphisms; STOPcovid, SHERLOCK test in one pot coronavirus disease.

HOLMES uses PCR (Table III) (32,34,36,38,60-71). CRISPR-Cas-based approaches detect viruses, single nucleotide polymorphisms (SNPs) and human genome variations in DNA samples isolated from patients with cancer (72).

*Target pre-amplification coupled with CRISPR-based, highly sensitive detection of viruses using SHERLOCKv.1.* Bacterial CRISPR-Cas enzymes, such as LshCas13a and LbuCas13a, opened a new area of application for CRISPR-Cas in diagnostics, particularly in the detection of viruses with an ssRNA genome (37,43). LbuCas13a recognizes target RNA at the pM level (1-10 pM) (37). In addition, the newly characterized *Leptotrichia wadei* Cas13a (LwaCas13a) has been reported to exhibit potent collateral RNA cleavage activity, capable of detecting ~50 fM of target RNA without amplification (32). However, aM sensitivity is required, particularly for the *in vitro* detection of pathogens containing RNA or DNA (73-75). The two commonly used PCR-based technologies, droplet digital and RT-qPCR, have a high sensitivity (in aM). Achieving similar, aM, sensitivity using CRISPR-based approaches requires pre-amplification of the target substrate, which can be achieved with isothermal RPA (Fig. 3) (28).

RPA offers the highest sensitivity when coupled with T7 transcription. In RPA, RNA targets are first converted into DNA using reverse transcriptase and DNA targets are directly amplified. T7-mediated transcription of amplified DNA into RNA enables detection by LwaCas13a using a fluorescence-quencher-labeled RNA reporter, generating an amplified signal, which is referred to as SHERLOCKv.1 (32). SHERLOCKv.1 achieves aM sensitivity in the detection of DNA and RNA, similar to droplet digital PCR and RT-qPCR, with less inter-sample variability, and can be easily performed in a single reaction mix (32).

In addition, the utility of SHERLOCKv.1 for the detection of Zika virus (ZIKV) and Dengue virus (DENV) has been previously reported. SHERLOCKv.1 recognized ZIKV and DENV with a sensitivity of 2 aM. The lyophilized and rehydrated components of SHERLOCKv.1 detected unamplified target RNA at 20 fM. The lyophilized and rehydrated SHERLOCKv.1 component detected target RNA in an aqueous reaction with a sensitivity of 3 aM in combination with a pre-amplification step. However, it was reported that paper spotting and lyophilization of SHERLOCKv.1 reagents slightly reduced the sensitivity, to 20 aM. These features of SHERLOCKv.1 are ideal for the development of POC devices to diagnose various human-borne pathogens with high accuracy within 1 h. SHERLOCKv.1 can detect ZIKV RNA in clinical samples, such as urine, serum and saliva. SHERLOCKv.1 can also detect bacterial pathogens and can be used to identify clinical isolates (34). SHERLOCKv.1 has been reported to also identify clinical ZIKV strains harboring a single-nucleotide mutation with high specificity and sensitivity (Table III) (32). Cas13a-based tests can detect a single copy of the viral nucleic acid in 1 $\mu$l of sample. These diagnostic tests can easily be adapted for virus detection in remote areas without complex, laborious and costly instrumentation (76-78).

*HUDSON enables the development of SHERLOCKv.2 for the multiplex detection of viruses in a single reaction tube.* To directly detect viral RNA in samples from patients in a readable colorimetric or fluorescent format, heating unextracted diagnostic samples to obliterate nucleases (HUDSON) was developed for sample processing. SHERLOCKv.1, coupled with HUDSON [SHERLOCK version 2 (SHERLOCKv.2)], enabled the specific detection of multiple circulating isolates of DENV and ZIKV serotypes. SHERLOCKv.2 recognizes a single copy/$\mu$l of ZIKV and DENV reverse-transcribed cDNA from the samples of patients with 100% specificity and sensitivity, similar to the standard RT-qPCR tests explicitly designed for ZIKV and DENV viruses (79).

In addition, the utility of SHERLOCKv.2 in detecting ZIKV and DENV RNA in the samples or bodily fluids of patients without purification has been demonstrated (79). ZIKV and DENV viruses are excreted in the saliva and urine. Urine and saliva samples can be collected quickly, without invasive procedures. In SHERLOCKv.2, HUDSON-treated samples are added directly to the RPA reaction mixture without dilution or purification. Using HUDSON in SHERLOCKv.2 does not influence downstream amplification and detection of the target RNA. SHERLOCKv.2 easily detects spiked ZIKV RNA in blood, plasma, serum, saliva and urine samples with aM sensitivity. ZIKV particles been reported to have been spiked into urine, saliva, blood, plasma and serum to mimic clinical infections, and the total turnaround time was <2 h when colorimetric and fluorescent reading formats were combined. The sensitivity of SHERLOCKv.2 is comparable with that of ZIKV RNA in the samples of patients, that is, 1-1,000 copies/ml. The SHERLOCKv.2 also detects DENV RNA with high sensitivity and specificity in <1 h in both the saliva and serum. SHERLOCKv.2, adapted in a lateral flow assay format, can be easily used in a simple pipeline with minimal equipment requirements for diagnosing ZIKV and DENV viruses (79). In addition, the utility of SHERLOCKv.2 in identifying ZIKV and DENV serotypes has also been demonstrated (79) (Table III). SHERLOCKv.2 enables precise and sensitive detection of ZIKV and DENV serotypes due to the development of serotype-specific crRNAs (79). In addition, SHERLOCKv.2 can also target multiple flaviviruses (ZIKV, DENV, West Nile virus and Yellow fever virus) in a single reaction tube using engineered universal RPA primers and crRNAs (79). Numerous advances have been made using orthogonal Cas proteins to further increase the versatility of SHERLOCKv.2. New advances include the use of multiple fluorophores in a single reaction, quantification, visible reading in lateral flow format and a three-and-a-half-fold increase in sensitivity through the combination of the additional Cas protein, Csm6 (38).

*The discovery and biochemical characterization of tandem-acting Cas enzymes and orthologs enables the development of highly sensitive and multiplex CRISPR-based diagnostics.* The multiplexing-compatible Cas proteins were identified by biochemical characterization of Cas13a and Cas13b family members (Fig. 4B). The cleavage preferences of these proteins have been studied using homopolymer reporters, and the majority of these proteins preferred uridine (U), adenine (A) or a combination of bases (Table II) (38). Cleavage preferences were further improved through the optimization of buffers and design of crRNAs. Cas13b from *Prevotella* sp. MA2016 (PsmCas13b)

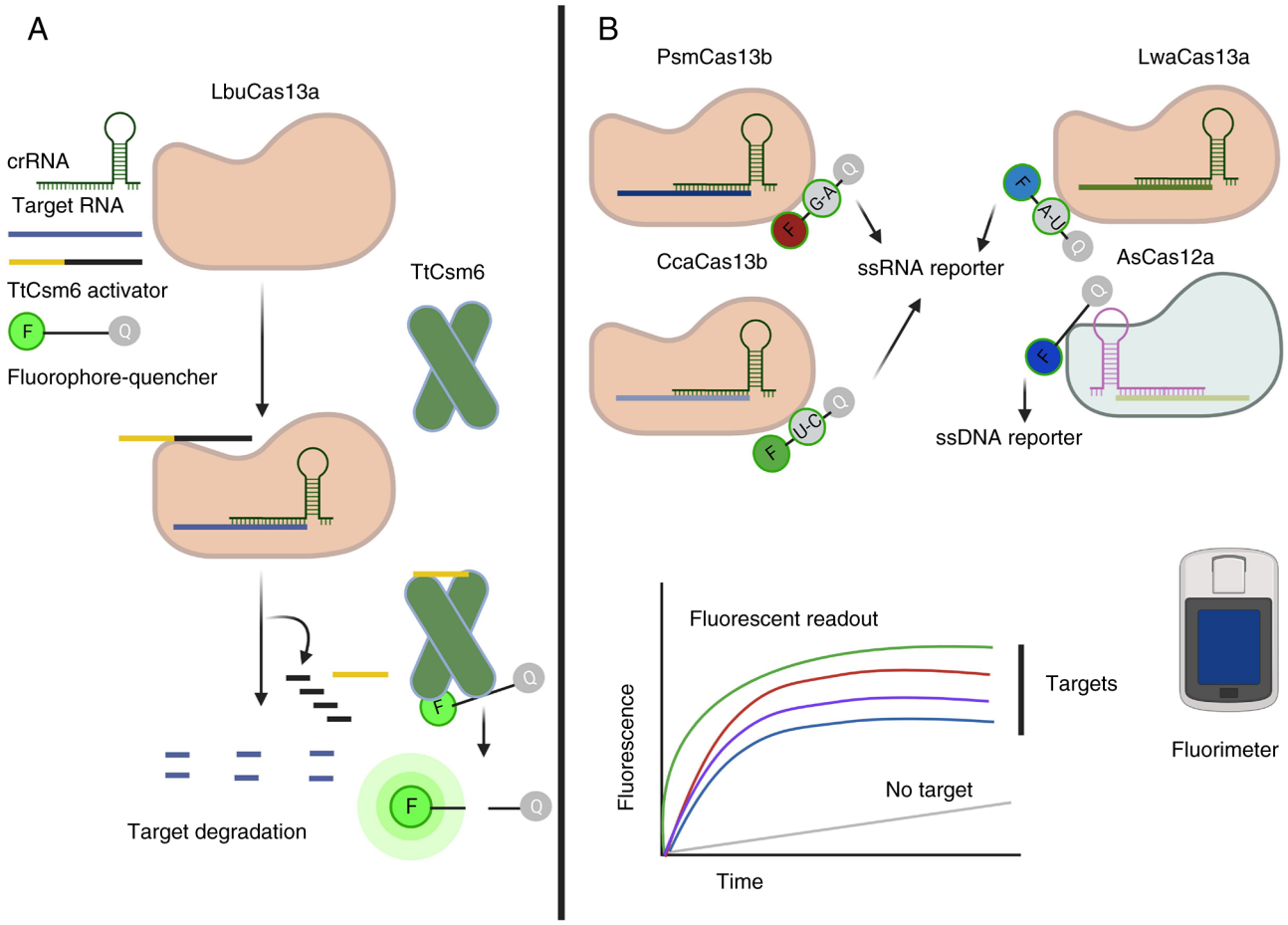


Figure 4. The discovery and characterization of tandem acting and orthogonal Cas enzymes have enhanced the multiplexing of CRISPR-based diagnostic approaches. (A) Orthogonal Cas enzymes can be used simultaneously, where target recognition by one enzyme activates its collateral activity, which produces byproducts that act as an activator for the second Cas enzyme. Activation produces a stable and robust signal that can be easily detected without a pre-amplification reaction. For example, LbuCas13a and TtCsm6 can be used to detect any RNA target. The collateral activity of LbuCas13a produces 2'-3' cyclic phosphates or linear oligonucleotides that act as an activator for TtCsm6. Stable activation of TtCsm6 produces a strong signal easily detected with a fluorimeter. (B) Orthologous Cas enzymes can be used together for multiplex detection of pathogen RNA and DNA, saving time and cost. Different Cas orthologs demonstrate different collateral activity on oligonucleotides, including dinucleotides and hexanucleotides. The figure presents four orthologous Cas enzymes used together in the presence of defined di- or oligonucleotide motifs that separate the quencher molecule. Recognition of these targets by the enzymes activates the collateral activity, which results in the generation of fluorescent signals that can be detected with a fluorimeter. This figure was generated using BioRender (www.biorender.com). Cas, clustered regularly interspaced short palindromic repeats-associated; crRNA, CRISPR RNA; ss, single-stranded; LbuCas13a, *Leptotrichia buccalis* Cas13a; TtCsm6, Csm6 from *Thermus thermophilus*; PsmCas13b, *Prevotella* sp. MA2016 Cas13b; CcaCas13b, *Capnocytophaga canimorsus* Cas13b; LwaCas13a, *Leptotrichia wadei* Cas13a; AsCas12a, *Acidaminococcus sp*. Cas12a.

is more sensitive to A during cleavage of homopolymer A compared with Cas13a from the *Lachnospiraceae* bacterium (LbaCas13a). The cleavage preferences of other Cas proteins were refined using dinucleotide motifs. Cleavage of RNA sequence motifs and dinucleotide recognition preferences confirmed that the activities of LwaCas13a, Cas13b from *Capnocytophaga canimorsus* (CcaCas13b), LbaCas13a and PsmCas13b could be measured independently using four dinucleotide reporters, namely, AU, UC, AC and GA, respectively. In addition, the cleavage preference of Cas proteins was further refined using a random library of RNA motifs. It was demonstrated that Cas proteins can efficiently recognize RNA oligomers of 6 nt, which further enhanced orthogonal usage and provided flexibility for multiplexing in a single reaction. Using these cleavage preferences, synthetic ssRNAs from ZIKV and DENV were detected in a single reaction using hexachlorofluorescein (HEX) and FAM channels (38).

By extending the utility of SHERLOCKv.2 to multiplex detection in a single reaction, the collateral cleavage activity of Cas12a was exploited. Cas12a isolated from *Acidaminococcus* sp. BV3L6 (AsCas12a) demonstrated weak collateral activity and required an input concentration >10 nanomoles (nM). Therefore, the combination of RPA pre-amplification with AsCas12a enabled the detection of a single molecule with a detection limit of 2 aM. Triplex detection was achieved using LwaCas13a [U reporter in the cyanine-5 (Cy5) channel], PsmCas13b (A reporter in the FAM channel) and AsCas12a (ssDNA reporter in the HEX channel). Combining these enzymes and reporters enabled recognition of three targets in a single reaction: A ssDNA target, ZIKV ssRNA and DENV ssRNA (38).

Furthermore, the recognition of four targets in a single reaction was achieved using the reporter cleavage activities of the orthogonal dinucleotide motifs of AsCas12a, LwaCas13a, PsmCas13b and CcaCas13b in HEX, FAM, Cy5 and TEX

channels, respectively (Fig. 4B) (38,69,80). By combining RPA, two DNA targets (*Pseudomonas aeruginosa* acyltransferase and *Staphylococcus aureus* thermonuclease genes) were detected in a single reaction with an aM sensitivity (38). In addition, multiplex SHERLOCKv.2 detects aM concentrations of ZIKV and DENV RNA diluted with PsmCas13b and LwaCas13a. The identification and characterization of orthogonal Cas proteins, RNA and dinucleotide cleavage preferences, enabled multiplex detection of DNA and ssRNA in a single reaction with aM sensitivity combined with pre-amplification using RPA (34,38,81). Other improvements have also been incorporated to make SHERLOCKv.2 more versatile, quantitative and sensitive. RPA primer concentrations were optimized, and it was reported that a primer concentration of 240 nM demonstrated correlation between signal and input and could detect sample concentrations as low as a single molecule in the aM range. A number of nucleic acid detection applications require a detection limit of one molecule/ml, for example, for human immunodeficiency virus (HIV) (38,82). Using LwaCas13a, PsmCas13b and the RPA reaction, a detection limit of 200 zM was achieved. Collateral RNase activity of Csm6 was exploited to further increase the sensitivity of SHERLOCKv.2 (38). Csm6 from *Enterococcus italicus* (EiCsm6) and *Lactobacillus salivarius* Csm6 (LsCsm6) requires RNA with 2',3'-cyclic phosphate ends (38). The collateral activity of LwaCas13a and PsmCas13b produces RNA with 2',3'-cyclic phosphate ends, which suggests that EiCsm6 and LsCsm6 can be used to increase the sensitivity of SHERLOCKv.2. The cleavage preference of *Thermus thermophilus* Csm6 (TtCsm6), EiCsm6, LsCsm6 and Csm6 over A- and C-rich reporters allowed measurement of LwaCas13a and Csm6 cleavage activity using two distinct fluorophores in different channels (FAM and HEX) of a fluorescent plate reader. LwaCas13a recognizes and cleaves poly U oligomer reporters, whereas Csm6 cleaves oligo-reporters containing poly-A. The combination of poly-A and poly-U produced a reporter recognized by Cas13a, which produced a reporter for Csm6 (Fig. 4A). Both enzymes were used in a single reaction to achieve enhanced signal generation, which could be easily detected using single-channel fluorescence (38). Increased addition of a Csm6-specific reporter correlated directly with increased signal intensity (Fig. 4A) (38).

*Cas13 orthologs have been used for the targeting, degradation, and subsequent detection of viruses*. LbaCas13a and PsmCas13b activities were used for coupled ssRNA virus targeting, degradation and subsequent detection (83), this study suggested the possibility of using Cas13 proteins in viral RNA degradation with subsequent detection as a viral treatment option. It has been reported that the majority of human-associated viruses (HAVs) that infect humans or related animals contain genomic regions recognized by Cas13-specific crRNAs (83). The efficiency and feasibility of using Cas13a and Cas13b for detection and degradation have been previously demonstrated against three ssRNA viruses, namely human stomatitis virus, influenza virus A and lymphocytic choriomeningitis virus (LCMV) (83). Both enzymes can attack these viruses in a cell culture model and can be detected with SHERLOCKv.2 by combining HUDSON, RPA-mediated pre-amplification and crRNA. The combination of viral targeting and detection based on Cas13 enzymes is called the Cas13-assisted restriction of viral expression and readout (83). These results suggested that the majority of HAVs can be targeted for degradation by incorporating specific crRNAs and Cas proteins.

*Cas12a comprises ssDNA-cleaving trans-collateral activity used for the specific and sensitive detection of HPV16 and HPV18 viruses*. Type V family proteins, such as Cas12a, harbor RNA-directed deoxyribonuclease (DNase) activity. The *Lachnospiraceae* bacterium ND2006 Cas12a (LbCas12a) recognizes dsDNA using a 20 nt gRNA. LbCas12a cleaves dsDNA using a single catalytic RuvC endonuclease domain, which generates 5' and 3'-staggered ends (Fig. 1C and F). Recognition of target DNA activates ssDNase activity and indiscriminately degrades ss-linear or circular DNA molecules (36,84). Cas12a uses a T-rich neighboring PAM motif to induce DNA cleavage and catalyzes the processing of its pre-crRNA to produce mature crRNA (Fig. 1F) (36,47,85-90). Using fluorophore quencher-labeled non-specific ssDNA, Cas12a-specific crRNA and the dsDNA target of Cas12a, Cas12a exhibited non-specific trans-ssDNA cleavage activity (Fig. 1F). Cas12a requires 15 nt of target complementary to the crRNA (36). Cas12a bound to the crRNA-DNA complex can degrade ~1,250 ssDNA molecules/s, which is equivalent to the diffusion limit. A 2-nt mismatch mutation in the gRNA and dsDNA target region results in an ~100-fold increase in the trans-degrading activity of LbCas12a (36). The majority of Cas12a orthologs contain a single RuvC catalytic domain (Fig. 1C). It has been previously demonstrated that Cas12a orthologs AsCas12a, *Francisella novicida* Cas12a and Cas12b from *Alicyclobacillus acidiphilus* (AaCas12b) possess ssDNA-degrading activity when assembled with crRNA and double-stranded activator DNA (36).

The use of LbCas12a for the detection of human papillomavirus 16 (HPV16) and HPV18 DNA has been previously demonstrated (36). LbCas12a detects HPV16 and HPV18 DNA with pM sensitivity. LbCas12a combined with RPA pre-amplification detected HPV16 and HPV18 DNA with aM sensitivity. LbCas12a efficiently discriminates HPV16 and HPV18 DNA from complex DNA samples purified from anal swabs with similar specificity and sensitivity to the, gold-standard, PCR assays (36).

## 6. SARS-CoV-2 detection using orthologous CRISPR-Cas proteins

RT-qPCR is considered to be the gold standard for SARS-CoV-2 detection. This requires trained personnel and special equipment to perform the tests. However, RT-qPCR cannot be performed under normal testing conditions, such as at room temperature or 37°C (91). To overcome these limitations, SHERLOCK was validated for the diagnosis of SARS-CoV-2. In SHERLOCK, detection is performed using either fluorescence readout or lateral flow assays. SHERLOCK was validated using 154 nasopharyngeal and throat swab samples, which indicated 100% specificity and sensitivity in the fluorescence reading and 100% specificity and 97% sensitivity in the lateral flow assay (92). Future advances in CRISPR-based diagnostics are expected to increase the sensitivity of lateral

flow assays (92). In particular, the utility of SHERLOCK for SARS-CoV-2 testing was further validated using 380 preoperative SARS-CoV-2 negative samples, in which SHERLOCK indicated 100% concordance with RT-qPCR (92). These results suggested that CRISPR-based diagnostics can successfully diagnose SARS-CoV-2 infection (92).

SHERLOCK uses two steps to detect bacterial or viral pathogens including SARS-CoV-2 (31,32,36). This involves RNA extraction, followed by CRISPR-Cas-based detection. The SHERLOCK test in one pot (STOP) streamlined and simplified RNA extraction and concentration using magnets, which can be easily combined with detection reactions. STOP coronavirus disease (covid) v.1 (STOPcovidv.1) used RNA extraction, reverse transcription and LAMP-mediated isothermal amplification of the target RNA (26). LAMP functions optimally at 55-70˚C; therefore, a thermostable Cas enzyme is required for detection. AaCas12b was combined with RT-LAMP to detect SARS-CoV-2 (93). STOPcovidv.1 used fluorescence or lateral flow strip-based display formats. It was validated with 202 positive and 200 negative nasopharyngeal swab specimens and demonstrated a sensitivity of 93.1% and a specificity of 98.5%, consistent with RT-qPCR (68). STOPcovidv.2 detected viral loads at 1/30 the level that can be detected by the Centers for Disease Control and Prevention (CDC) RT-qPCR, which is a standard test for detecting SARS-CoV-2 (68). The SARS-CoV-2 DETECTR was validated for the detection of SARS-CoV-2 in patients and artificial samples (31). RNA extracted from samples from patients was reverse-transcribed and amplified using LAMP at 60˚C. Detection was performed at 37˚C with LbCas12a using fluorescence or lateral flow strip-based detection, with 95% agreement with RT-qPCR results. DETECTR demonstrated good agreement with CDC-approved RT-qPCR and could be performed within 45 min. This technology can also be combined with microfluidic cartridges and freeze-dried reagents for POC applications outside clinical laboratories, such as in airports, public spaces, private and government clinics, and local emergency rooms (31).

LwaCas13a and EiCsm6 were used to detect, DENV, ZIKV and SARS-CoV-2 viruses. LwaCas13a and EiCsm6, in tandem with RPA or RT-LAMP pre-amplification, demonstrated sensitivities in the aM range (2 aM). Without pre-amplification, the combined use of LwaCas13a and EiCsm6 demonstrated sensitivity of 1 $\mu$M (38,94). To increase the sensitivity and speed of CRISPR-based diagnostics, which made the methods amenable to POC development, thermostable TtCsm6 and LbuCas13a were characterized, along with multiple crRNAs and linear artificial and stable A4U6 (four contiguous adenine and six uridine) activators of TtCsm6 (Fig. 4A) (94). The utility of LbuCas13a with TtCsm6, crRNA and an A4U6 activator and reporter was demonstrated using purified SARS-CoV-2 RNA and RNA extracted from nasal swab specimens. LbuCas13a and TtCsm6 detected SARS-CoV-2 at ~30 copies/$\mu$l in 20 min. A microfluidic cartridge with a light-emitting diode light source and detector was developed to make the method easier to use and more versatile. This device was called fast-integrated nuclease detection in tandem (FIND-IT). FIND-IT could detect SARS-COV-2 in samples with an RT-qPCR-determined cycle threshold value of ~33 (94).

During the peak of pandemics, continuous viral surveillance and monitoring is essential and requires an on-site POC device that can provide results within 1 h. Diagnostics with coronavirus enzymatic reporting (DISCoVER) was developed to test for SARS-CoV-2 using saliva as a sample. DISCoVER utilizes sample denaturation and inactivation (lysis and reduction) with simple reagents, such as tris(2-carboxyethyl) phosphine and ethylenediaminetetraacetic acid. In addition, DISCoVER uses LAMP followed by detection using LbuCas13a. DISCoVER contains a gravity-driven microfluidic device with sample inactivation, isothermal amplification and separate fluorescence detection of the control and positive samples. DISCoVER indicated ~95% sensitivity and 100% specificity for RT-qPCR-validated samples (95). Furthermore, DISCoVER has been reported to have detected ~40 copies/$\mu$l of the SARS-CoV-2 genome and demonstrated 100% positive predictive value and ~93% negative predictive value (95).

To develop a POC device that can be used outside well-equipped laboratory conditions, streamlined highlighting of infections to navigate epidemics v.1 (SHINEv.1) was developed (96). In SHINEv.1, a modified HUDSON is used for nasal swab and saliva processing. The modified HUDSON could inactivate the sample within 10 min. Isothermal amplification is performed in a single tube using RPA- and Cas13a-based detection methods. The sample values are recorded in a sealed tube using a smartphone. SHINEv.1, validated on 60 nasopharyngeal samples, demonstrated 90% sensitivity and 100% specificity for samples validated using the gold standard RT-qPCR, with a sample response time of 50 min (96).

Additionally, SHINEv.2 was designed to test for SARS-CoV-2 and different variants of concern, including α, β, γ, δ and o (67). A commercially available FastAmp virus and cell solution is used for isothermal amplification to effectively inactivate SARS-CoV-2 in combination with RNase inhibitors at ambient temperature. The need for refrigerated storage of reagents is eliminated using SHINEv.2. Optimized lyophilized reagents, including mannitol and sucrose, are used in the reaction. The reaction is performed at 37˚C using a heating block or body heat without compromising the sensitivity and specificity within 90 min. SHINEv.2, validated on nasopharyngeal specimens, demonstrated ~90% sensitivity and 100% specificity in a paper-based lateral flow strip, and different variants of concern could be identified visually (67).

In addition, a Cas12a-based test procedure was developed to distinguish between the α, β, γ and δ variants of concern (67). LbCas12a and AsCas12a are used for target recognition and signal generation, followed by detection of fluorescence signals. The assay distinguished the open reading frame 8a-L/S mutation in SARS-CoV-2. The CRISPR-Cas12a assay also efficiently detects all characteristic spike protein mutations, including (K-417-N/T, L-452-R/Q, T-478-K, E-484-K/Q, N-501-Y and D-614-G) to discriminate between the α, β, γ, δ, κ, λ and ε variants of SARS-CoV-2. The assay was validated using 32 positive samples of SARS-CoV-2 and demonstrated 100% agreement with sequencing results. This Cas12a-based multiplex assay can be easily adapted for genotyping different variants of SARS-CoV-2 in laboratories performing SARS-CoV-2 testing (67).

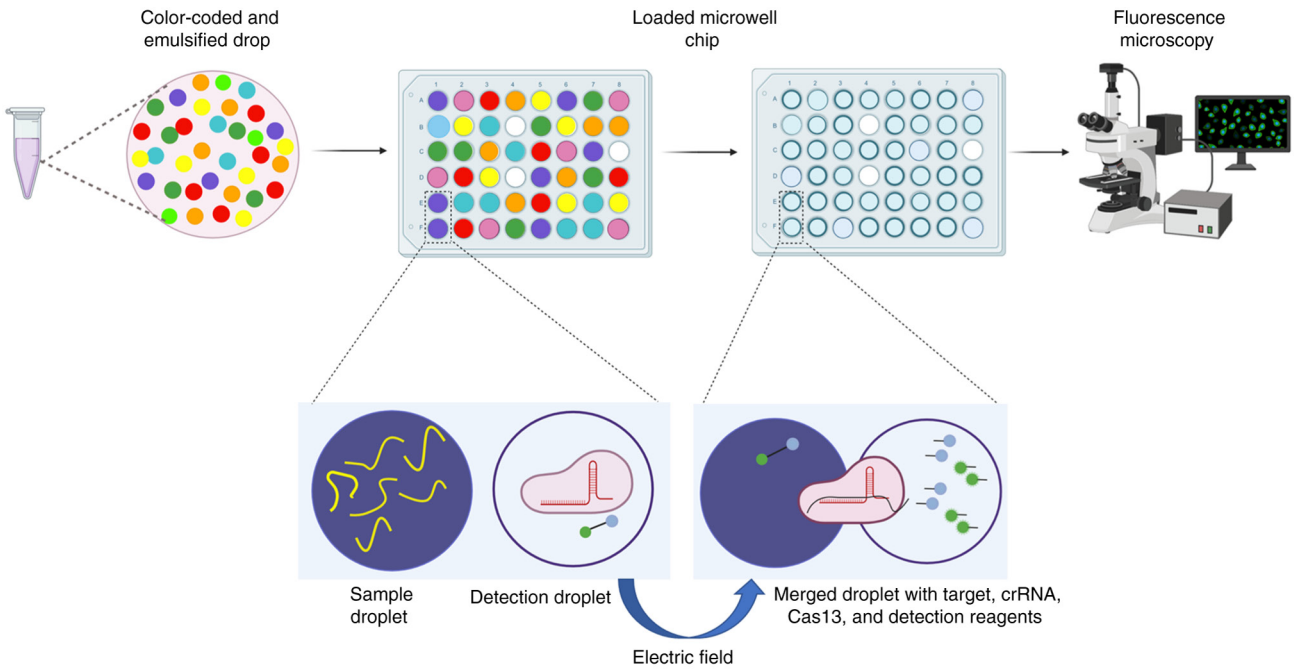


Figure 5. High throughput clustered regularly interspaced short palindromic repeats-based multiplex detection of targets. The combinatorial array reactions for the multiplex evaluation of nucleic acids uses a unique color code assigned to each PCR amplified target/sample or detection mix containing Cas13. The detection mixture comprises Cas13a, crRNA (sequence-specific) and fluorophore-quencher labeled reporter. Fluorous oil is used to emulsify color-coded solutions, which creates nl volume droplets. Samples and detection droplets are pooled together. Pooled droplets are then loaded into a microwell array chip in one step, creating all possible pairwise combinations. Fluorescence microscopy is used to identify droplet pairs in each well before they fuse using an electric field. The Cas13-based detection reaction is monitored using fluorescence microscopy. This figure was generated using BioRender (www.biorender.com). Cas, clustered regularly interspaced short palindromic repeats-associated; crRNA, CRISPR RNA.

## 7. Multiplexed high-throughput CRISPR-Cas-based virus detection

Multiplexing has additional benefits, as it can process multiple samples and targets in parallel. Multiplexing can save time and money, and is beneficial for monitoring virus outbreaks by distinguishing multiple strains and virus types (Figs. 4 and 5). It can distinguish the dominant circulating variants of viruses at any time point using variant-specific crRNAs (79). CRISPR-based multiplex assays have been developed for flaviviruses to distinguish between the four serotypes of DENV (79). Multiplexing of CRISPR assays requires the separate amplification of targets, followed by detection using Cas13 and crRNA (79). Microfluidic devices have been integrated into CRISPR diagnostics to increase the versatility and ease of use of CRISPR-based testing, while handling thousands of targets or samples. The handheld microfluidic device was first used to detect Ebola virus RNA in up to 24 samples without amplification (97). In addition, microwell arrays have been combined with CRISPR-based assays for the highly multiplexed detection of thousands of samples using a method known as combinatorial array reactions for the multiplex evaluation of nucleic acids (CARMEN) (70). CARMEN can process up to 5,000 samples simultaneously. In addition, it is modular in design, allowing the processing of thousands of samples for multiple targets or a few samples for all viral targets simultaneously. In CARMEN, re-amplified samples are used to produce nl volume droplets, and a pair is produced, which includes a sample droplet with the target and a detection droplet comprising crRNA, Cas enzyme, and detection reagents. Detection is performed by fusing the target and crRNA with a reagent on a microfluidic chip (Fig. 5). CARMEN-Cas can detect all known viruses or viruses suspected to infect humans in a single sample using a pan-viral detection test. Additionally, CARMEN can distinguish all known haemagglutinin 1-6 and neuraminidase 1-9 subtypes of Influenza A virus (FLUAV) (70). Multiplexing CRISPR-Cas-based detection technologies with highly automated microfluidic and microchip-based assays could improve high-throughput viral screening and surveillance.

## 8. Utilization of Cas13 and Cas12 for the specific and sensitive detection of a single virus

Several approaches have been developed to detect viral pathogens since validation of the SHERLOCK and DETECTR technologies (Table III) (98-102). The sudden appearance of SARS-CoV-2 in 2019 and the first publication of its genome sequence led to the development of several new methods for its detection (2,103). The Food and Drug Administration issued an emergency authorization for the use of CRISPR-based diagnostic technologies such as SHERLOCK and DETECTR to diagnose SARS-CoV-2 (2). The precise and sensitive detection of viral nucleic acids and their dependence on the complementarity between crRNA and target sequences has led to the development of Cas13- and Cas12-based assays that can detect a single nucleotide difference between viral isolates (Table III). A single-nucleotide mismatch between the crRNA and the target sequence does not entirely abolish target cleavage or collateral activity. However, introducing a single mismatch near a mutant site markedly abolishes the target

and ssRNA cleavage activity (32). This property of Cas13 and Cas12 has been exploited for the detection of ZIKV isolates, DENV serotypes and single-nucleotide mutations in HPV16 and HPV18 DNA (32,79). Cas13 has been used to identify two isolates of ZIKV and two common serotypes of DENV circulating in Asia and America (79). Cas12 has been used to identify HPV16 and HPV18 DNA SNP (66). In addition, Cas13 has been used to identify drug resistance mutations in several HIV genes. For example, 27 common mutations in HIV drug resistance have been verified, including six reverse transcriptase inhibitors and 21 integrase inhibitor mutations (70). The development of Cas13- and Cas12-based methods beyond the detection of viral nucleic acids has expanded the scope of CRISPR-Cas-based technologies. This will provide further impetus for developing CRISPR-Cas-based technologies for clinical use to identify various human diseases caused by bacteria, viruses and fungi (32,79).

## 9. Application of CRISPR-based diagnostics beyond the laboratory

To further expand the utility of CRISPR-based diagnostics as a POC device, numerous sample processing methods have been developed that are easier to use than previously developed CRISPR technologies, such as SHERLOCK and DETECTR, and do not require trained personnel or laboratory equipment. These methods utilize optimized lysis buffers, heat inactivation and chemical treatments for subsequent applications. Numerous samples such as saliva, nasal swabs, urine, blood, blood plasma and serum have been successfully processed (35). Modified HUDSON has been reported to inactivate the LSV virus and its viral inactivation efficiency has been demonstrated using a plaque assay (102).

During the processing of samples containing HPV DNA, a simple chemical (proteinase K) and heat inactivation method was effectively combined with the DETECTR assay to minimize the complex sample processing and ensure the safety of working personnel (36). Simple and easy-to-use room-temperature lysis buffers compatible with reverse transcription and LAMP-based pre-amplification of target RNA combined with magnetic bead-based RNA extraction containing concentrated SARS-CoV-2 nucleic acids are easy to use with the current detection methods, including STOPcovidv.2 (68).

Several approaches have been used for the minimal handling of processed samples, such as streamlined magnetic bead-based nucleic acid extraction, easy-to-use chemical reagents, and reverse transcription, pre-amplification and detection in a single tube. This is important to prevent contamination during sample handling after the initial processing. This can be accomplished in two ways: i) Physical separation of reaction components that can be mixed in a single tube; and ii) standardized reaction conditions that can eliminate pre-amplification or the identification of reaction conditions in which amplification and CRISPR-based cleavage can be performed together. With the former, there is still a need to manipulate the reaction tubes without opening them. Several modifications have also been used to separate amplification and CRISPR-based cleavage, such as using mineral oil (104), placing the reagents on the side of the tubes (105) or capping the reagent (106) followed by shaking after the initial amplification is complete. Cas12 orthologs of thermophilic origin were used in HOLMESv.2 and STOPcovidv.1and STOPcovidv.2, combining RT-LAMP amplification and cleavage in a single tube to detect Japanese encephalitis virus (JEV) and SAR-CoV-2 (63,68). In addition, SHERLOCK was combined with RT-RPA and Cas13a to enable amplification, cleavage and detection in a single reaction for the detection of SARS-CoV-2. Performing amplification and cleavage reactions in a single step requires optimization of the reaction conditions, reporter and enzymatic components (96). Finally, it has been demonstrated that the combination of crRNA and hyperactive LbuCas13a eliminated the need for amplification (107). These approaches efficiently detect viruses without manipulating the reaction conditions after being set up in a single tube, thus reducing the risk of contamination of the starting sample (107).

A portable and inexpensive fluorimeter and fluorescence reader were used to collect signals in either a lateral flow paper strip or colorimetric assay (Fig. 3). When detecting SARS-CoV-2 with Cas12a- or Cas13a-based methods, a green-fluorescent signal with a blue light-emitting diode has been reported (96,106-109). Colorimetric detection is easier to use because it does not require expensive laboratory equipment and is also easy to use at home. Lateral flow strips were combined with Cas13a cleavage and AuNP-tagged FAM-biotin using SHERLOCKv.2. The tests and control lines could be visualized with the naked eye on the lateral flow strip (Fig. 3) (38,79). A similar method was used to identify the African swine flu virus (ASFV) using Cas12a-based detection with pre-amplified samples (110). Compared with strip-based lateral flow tests, colorimetric or fluorescence-based detection in reaction vessels is more convenient and scalable and has a lower risk of contamination. An isothermal amplification-based assay that requires only nucleic acid purification and heat inactivation, such as LAMP, can detect pH-based, in-tube, color changes (111). However, LAMP-based colorimetric assays have not been reported as being used for Cas13-based detection. AuNP-tagged amplified ASFV DNA was detected by Cas12-based cleavage in a reaction tube, where cleavage of the target by Cas12 resulted in a color change in the disaggregated AuNPs, whereas the aggregated AuNPs were colorless. AuNP color is determined by proximity, which presents a visual color change when Cas12-based target cleavage occurs. Although not used for Cas13-based detection of ASFV, AuNP-based colorimetric assays can also be used for Cas13 (112).

## 10. Inhibiting viruses with CRISPR

CRISPR-Cas systems have evolved in half of bacteria and almost all archaea to protect them from invading bacteriophages and plasmids (113). Effective antiviral agents, antibodies, or other therapeutics are needed for effective treatment of viral diseases. Developing an effective treatment against viral diseases requires detailed mechanistic insights into viral biology and is time consuming. In contrast, CRISPR-based interventions require only genomic sequence information as the basis from which to inhibit viruses as a treatment option. CRISPR-Cas systems can target the genomes or genomic intermediates of viruses. Several approaches have

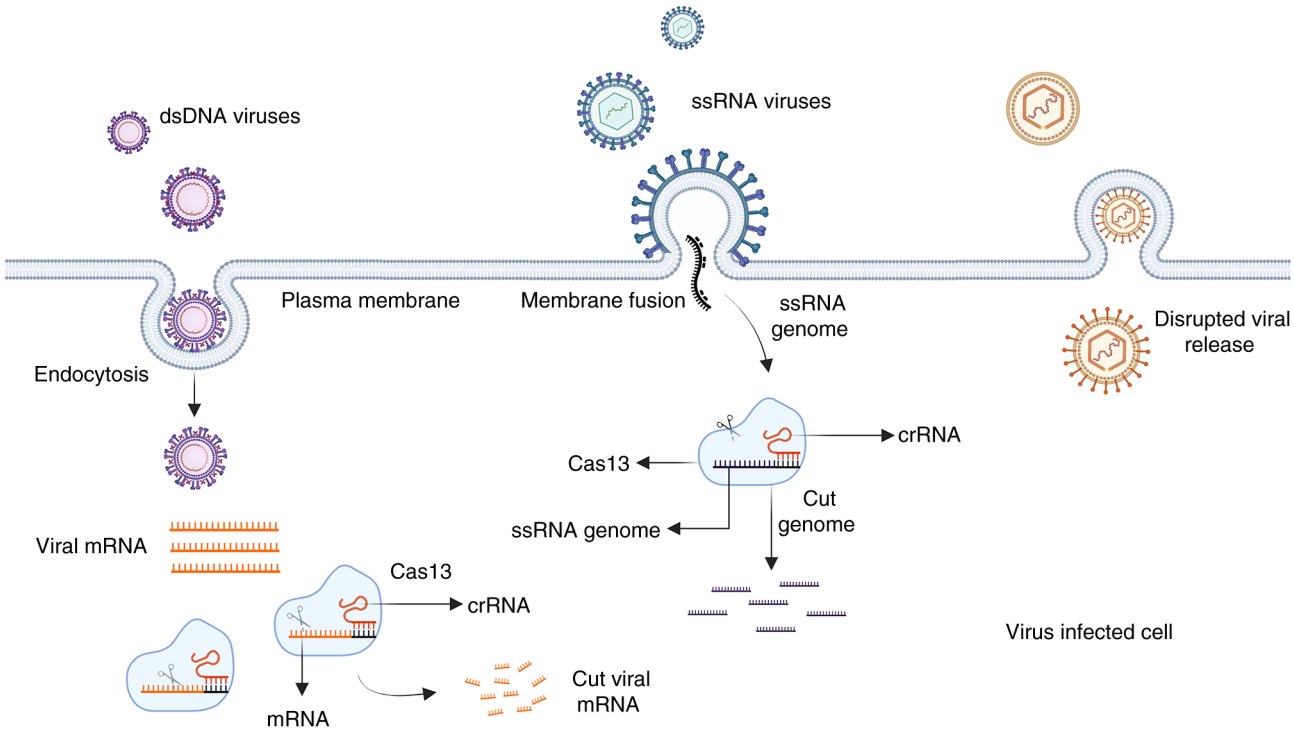


Figure 6. Inhibition of viral life cycle phases by Cas13. Cas13 inhibits viral replication by targeting the viral genome or genomic intermediates. Cas13 specifically targets ssRNA genomes or ssRNA intermediates produced during the life cycle of RNA or DNA viruses, including viral mRNAs. Targeting occurs within the cell once the nucleic acid is accessible to Cas13-crRNA complexes, resulting in inhibited virus release. This figure was generated using BioRender (www.biorender.com). Cas, clustered regularly interspaced short palindromic repeats-associated; ds, double-stranded; ss, singled-stranded; crRNA, CRISPR RNA.

been used to inhibit viruses by using different Cas orthologs, such as targeting viral mRNA or RNA genomes (2).

## 11. Cas13-based targeting of viral mRNA or RNA genome

Several viruses contain ssRNA in their genome, making Cas13 a powerful tool to treat them. Cas13a can be used to slow down replication and growth (Fig. 6). Previous studies have demonstrated the utility of Cas13a and Cas13b for reducing host mRNA using mammalian cells (114,115). These studies prompted research into the investigation of the reduction of ssRNA expression in LCMV, FLUAV and vesicular stomatitis viruses by Cas13. The study indicated that Cas13 efficiently inhibited RNA expression (83). These aforementioned studies identified the optimal crRNA design for effective targeting of ssRNA in mammalian cells: i) The secondary structure of crRNAs should be limited; ii) several cytosines should be present nearby; and iii) several Us or preferred nucleotides should be present upstream of the target (116).

Further studies have reported that Cas13a can inhibit the expression and infectivity of viruses, such as HIV-1 and DENV (Fig. 6). Cas13a inhibits the production of HIV-1 RNA and viral particles, RNA expression from reactivated proviruses and integrated DNA. Cas13a can also inhibit the infectivity and expression of DENV and its RNA when expressed with non-structural 3-targeted crRNA (2,117,118). Detailed characterization of Cas13 revealed that it could be used to combat viruses containing ssRNA genomes for which the sequence information is known (Fig. 6). SARS-CoV-2 emerged in December 2019 and its first genomic information was published in January 2020. The first Cas13-based method was reported in April 2020 to inhibit the virus both *in vitro* and *in vivo*. Prophylactic antiviral CRISPR in human cells (PACMAN) was developed to inhibit SARS-CoV-2 in human cells using smaller Cas13 orthologs known as Cas13d or CasRx (engineered ribonuclease effector derived from *Ruminococcus flavefaciens* XPD3002), which can be rapidly packaged into adeno-associated viruses (AAVs) (119-121). PACMAN has also been used to decrease FLUAV levels in human lung epithelial cells. In addition, crRNA was constructed against the conserved regions of coronaviruses capable of infecting humans, including SARS-CoV-2, and Cas13d activity was analyzed using the SARS-CoV-2 reporter assay system to assess the efficiency of crRNA to target and degrade SARS-CoV-2 sequences in human cells (119). In addition, further work has demonstrated that Cas13a, mRNA and virus-specific crRNAs packaged with a poly(β-amino ester)-based polymer can be delivered to *in vivo* mouse and hamster models using a nebulizer. The effectiveness of this approach was demonstrated using hamster and mouse models of SARS-CoV-2 and FLUAV infection. It was revealed that delivery of Cas13a and crRNAs resulted in decreased weight loss and viral load in the lung tissue, even though the effects observed were not pronounced (122).

The collateral cleavage activity of Cas13 orthologs has been widely used in viral diagnostics; however, its effect on viral RNA or cellular mRNA has not been well explored. Cas13-based cellular mRNA and viral RNA targeting has little or no collateral or off-target effects. In addition, it has also been reported that Cas13- and crRNA-mediated targeting of non-cytotoxic viruses does not affect cell

viability (83,114,115,120,122). Additionally, overexpression of Cas13a using a lentiviral transduction system and Cas13a-mediated targeting of overexpressed RNA in glioma cells demonstrated collateral activity (123). Transplanting these cells into a mouse model of intracranial glioblastoma resulted in effective tumor inhibition. The aforementioned studies indicated that characterization of the collateral activity of Cas13 against host mRNA and viral RNA still requires further development. Comprehensive insight into the collateral activity of Cas13 orthologs, their expression conditions and the concentration of the target RNA would be beneficial for the characterization of the therapeutic utility of Cas13.

Cas13-based antiviral approaches are potent and effective agents for combating viral infections. However, concerted efforts are needed to study their efficacy and safety requirements before realizing their clinical potential. Detailed molecular insights are needed to provide a way to design crRNAs that are less likely to promote the development of resistance in the target. This could be achieved by identifying the most conserved regions in the viral genomes. The enigmatic properties of Cas13 proteins, including their inherent quality of crRNA processing and maturation, facilitate multiplexing, which is critical for reducing the development of resistance during infection and preventing the inhibition of Cas13 enzymatic activity (46). A previous study indicated that no mutations in crRNAs were detected in cells treated with Cas13 and infected with LCMV or DENV2 viruses (117). However, insertions and deletions flanking crRNA-binding sites were observed in DENV2 isolated from the supernatant (83,117). Similar studies should be performed *in vitro* and *in vivo* to investigate the efficacy of Cas13-based treatments that would benefit from stringent crRNA design. The effectiveness of Cas13-based antiviral therapies will depend on the further investigations of the optimal delivery system and minimal or no off-target effects due to the expression of Cas13 and crRNAs in mammalian cells. The majority of experiments have been performed with Cas13 and its crRNA before viral infection, and the *in vivo* results indicated very low potency (2). Therefore, more detailed experiments and insights are required to demonstrate the effectiveness of Cas13-based treatment approaches for viral diseases. Furthermore, expression and inhibition by Cas13 should be equally effective at different stages of viral replication. Additionally, the potency of Cas13-based therapeutics should outperform newer antiviral approaches (2).

## 12. Conclusions and future perspectives

The identification of CRISPR-Cas proteins, such as Cas12 and Cas13, has provided a significant impetus for developing CRISPR-Cas-based diagnostics. Additional molecular insights into the ssRNA- and ssDNA-degrading collateral activity of these proteins have prompted the rapid development of CRISPR-based diagnostics for disease detection (34,36). Multiplexing, ease of use and use outside well-equipped laboratories have made these tests increasingly popular. Furthermore, the development of buffers, reagents and effective methods for inactivating viral samples that are compatible with pre-amplification and detection by Cas13 or Cas12 in a single tube makes CRISPR-based diagnostics viable as a POC device (2,35,68,96). Biochemical characterization of Cas13 and Cas12 orthologs with specific and robust fluorescence-quencher reporters have enabled multiplexing (Fig. 4B). The identification and characterization of Csm6-Cas type III proteins makes tandem utilization of Cas orthologs possible (Fig. 4A) (38). Detailed molecular knowledge of hyperactive LbuCas13a has increased the sensitivity of CRISPR-based diagnostics from aM to zM (107). The present review hypothesizes that CRISPR-based diagnostic tests will remain at the forefront of diagnosing viral and human-associated diseases in the coming decades.

The detection of smaller Cas enzymes would support the development of CRISPR-based diagnostics and therapeutics. The previously reported Cas14a (CasX) proteins are small and contain ~400-700 amino acids (61). CasX proteins contain a single RuvC endonuclease domain that is required for ssDNA cleavage. It recognizes ssDNA, which is directed by ssRNA, to induce ssDNA cleavage. This requires crRNAs and tracrRNAs for target recognition and cleavage. CasX recognizes and binds to the 20 nt target ssDNA. CasX does not require PAM; variations in the middle of the target sequence (seed) are the most sensitive, and changes in this region abolish cleavage. Recognition of the target by CasX gRNA on ssDNA activates ssDNA-cleaving collateral activity (61). The utility of Cas14a in the efficient detection of SNP was validated against Cas12a using a quencher-labeled ssDNA probe (61). In addition, finding CRISPR-Cas systems in bacteriophages could lead to the detection of the smaller counterparts of the Cas enzymes that could be quickly packaged in AAV particles, which could improve the scope of CRISPR-based diagnostic and therapeutic approaches to treat a variety of human genetic disorders (124). LbuCas13a has recently been demonstrated to manipulate phage genomes, and multiple phage genes can be simultaneously edited. Advances in the detection and in-depth characterization of these enzymes have expanded the scope of CRISPR-based diagnostics for the detection of human diseases (125,126).

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MS and MK conceptualized the project. MS reviewed the literature. MS and MK prepared the manuscript. MS, RS and NA designed and created the schematic representation. MS, MK, NA, RS, RR and PK edited the manuscript. All authors have read and approved the final version of the manuscript. Data authentication is not applicable.

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## Competing interests

The authors declare that they have no competing interests.

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