CRISPR-Cas, Argonaute proteins and the emerging landscape of amplification-free diagnostics

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A B S T R A C T

Polymerase Chain Reaction (PCR) is the reigning gold standard for molecular diagnostics. However, the SARS-CoV-2 pandemic reveals an urgent need for new diagnostics that provide users with immediate results without complex procedures or sophisticated equipment. These new demands have stimulated a tsunami of innovations that improve turnaround times without compromising the specificity and sensitivity that has established PCR as the paragon of diagnostics. Here we briefly introduce the origins of PCR and isothermal amplification, before turning to the emergence of CRISPR-Cas and Argonaute proteins, which are being coupled to fluorimeters, spectrometers, microfluidic devices, field-effect transistors, and amperometric biosensors, for a new generation of nucleic acid-based diagnostics.

1. Introduction

Eureka moments are rare in science, but that's how Kary Mullis described his conception of the polymerase chain reaction (PCR) [1]. While the idea for PCR arrived in an instant, the development of this assay required time and optimization. Initially, PCR was slow, insensitive, error-prone, labor-intensive, and did not involve thermal cycling. However, in March of 1985, Mullis and his team demonstrated that PCR could be used to detect mutations responsible for sickle cell anemia [2,3]. This proof-of-principle stimulated a flurry of innovations, including the use of thermostable polymerases, proofreading enzymes, incorporation of reverse transcriptases that enable detection of RNA, and the development of automated thermocyclers, which dramatically increase sensitivity and reduce contamination (Fig. 1). Now, after almost forty years of development, PCR continues to serve as the backbone for most molecular diagnostics. However, thermocyclers and the technical expertise necessary to perform these reactions have largely confined PCR to centralized testing facilities. Moreover, once samples have been transported to the lab, PCR generally requires about an hour to complete. While these requirements are compatible with diagnostic testing in most situations, the SARS-CoV-2 pandemic reveals an urgent need for innovations that enable fast, reliable, and scalable detection of viruses without technical requirements or time limitations intrinsic to PCR.

Isothermal amplification methods (e.g., LAMP, RPA, NASBA, etc.) use a variety of different strategies to eliminate the need for thermocyclers, and as such, these methods have garnered considerable interest for distributed diagnostics (Fig. 1) [4,5]. In addition, isothermal amplification methods have been coupled to colorimetric or fluorescent indicators that enable simple and rapid molecular detection. While isothermal amplification methods are sensitive and fast, they are prone to mispriming or template-independent amplification, which increases the frequency of false positives. Therefore, amplified nucleic acids generated by isothermal amplification often require confirmation by more specific methods, including CRISPR- and Argonaute-based diagnostics or high-throughput sequencing [6-22].

CRISPR-based diagnostics (CRISPR-dx) refers to a growing repertoire of CRISPR (clusters of regularly interspaced short palindromic repeats)

RNA-guided nucleases that have been creatively repurposed for sequence-specific detection of RNA or DNA targets [23,24]. The sequence specificity of CRISPR RNA-guided nucleases, which naturally provide bacteria and archaea with defense from invading genetic parasites, are emerging as programable new diagnostics [23-27]. Similarly, prokaryotic Argonautes (pAgos) are a phylogenetically and functionally diverse family of RNA- and DNA-guided proteins [28,29]. While some pAgo variants directly cleave bound targets, others lack nuclease activity and recruit *trans*-acting effectors with diverse enzymatic activities, reminiscent of the diversity observed in CRISPR systems [30-36]. The biological roles of pAgo are diverse, though several have been shown to function as immune systems while others facilitate de-concatenation of circular chromosomes generated during DNA replication [30,33,36-39]. However, like PCR, isothermal amplification, or almost any other emerging technology, the pioneering publications that demonstrate proof-of-principle for CRISPR-dx and pAgo-dx were not immediately competitive with technologies that have been developed over decades (Fig. 1) [12,14,40,41]. Here, we provide an overview of emerging CRISPR-dx and pAgo-dx technologies and highlight new advances that couple these technologies with innovative detection methodologies that aim to increase bedside accessibility, multiplexable detection, and sensitivity. Finally, we suggest standardized reporting metrics that will facilitate objective comparisons between diagnostic platforms.

2. Next-generation programmable diagnostics

2.1. Target-activated collateral nucleases

All CRISPR systems rely on RNA guides for target recognition. In most CRISPR systems, the RNA delivers a CRISPR-associated (Cas) nuclease to complementary DNA targets. However, in 2016, Cas13 (formerly C2c2) proteins were shown to be RNA-guided RNases [40,41]. Cas13 cleaves complementary RNA targets, but target recognition also triggers Cas13 to cleave any RNA in solution, which is frequently referred to as "collateral cleavage" (Fig. 2A) [41]. In nature, collateral cleavage of cellular RNAs results in senescence of the host cell, which limits the spread of the viral infection [42-44]. Like most CRISPR systems, Cas13 can be easily reprogrammed to target any sequence by design. Therefore, East-Seletsky *et al.,* repurposed Cas13 for sequence-specific detection of RNA by using the target activated collateral nuclease activity to liberate a fluorophore from an RNA tethered quencher, which is a clever adaptation of hydrolysis probes (e.g., TaqMan™) that were originally designed to increase the specificity of quantitative PCR [40,45]. This initial work on Cas13-based diagnostics led to a burst of programmable diagnostics that all rely on the collateral cleavage of single-stranded RNA or single-stranded DNA by Cas13 or

Cas12, respectively (Fig. 2A) [10-12,46,47]. Recently, TnpB proteins, the evolutionary precursors to Cas12, have been shown to be RNA-guided DNA-binding proteins with collateral nuclease activity [47,48]. Presumably, these nucleases will also be repurposed for molecular diagnostics.

The sensitivity of CRISPR-based diagnostics that rely on collateral cleavage of fluorescent nucleic acid reporters by target-activated Cas12 and Cas13 may be limited to 100 fM ($6x10^4$ copies/μL) of target RNA or DNA detectable within a 1-hour assay [49,50]. These reactions are constrained by substrate affinity (K_M), the reaction rate (k_{cat}), and the background fluorescent signal [49-51]. Several innovations have helped improve sensitivity including: i) pre-amplification of the target nucleic acid via isothermal amplification [10-12,14,18,20-22,52-57], ii) target concentration [22,58,59], iii) more sensitive collateral cleavage detection methods [60-72], and iv) design of protein circuits or signaling cascades [11,15,73-79]. Pre-amplification methods improve the sensitivity by six orders of magnitude and this work has been reviewed elsewhere [24-27]. Here we focus on the integration of CRISPR-Cas and Ago-based detection methods with technologies that enable rapid and reliable results. Further, we discuss the design of CRISPR signaling cascades and feedback amplification circuits that drastically increase sensitivity.

2.1.1. Digital enzymology

Digital enzymology measures absolute quantities by encapsulating nucleic acids in discrete droplets (Fig. 2B). This approach has been used to enhance the sensitivity of Cas13-based collateral nuclease assays from 50 pM to 5 fM (a 10,000-fold improvement) and reduces the time to detection from 30 min to 5 min [66]. Separation of the sample into "micro-containers" results in a statistical distribution of the target nucleic acids among the droplets. This method offers three advantages over ensemble experiments [80,81]. First, the instrumentation for digital measurements must only distinguish between a high (>1) or low (0) signal, while analog measurements of ensemble reactions must accurately measure the magnitude. Second, a small reaction volume increases the effective concentration of the target molecule, which improves the sensitivity. Third, effective partitioning of the target excludes compounds that could inhibit the reaction (i.e., iron or immunoglobulin in blood samples) [82]. Digital enzymology combined with Cas12- and Cas13-reliant collateral nuclease assays results in highly sensitive and rapid diagnostics that detect 17.5 copies/μL of DNA in 60 min (Cas12), or 3,400 copies/μL of RNA in 5 min (Cas13) [66-68].

2.1.2. Amperometric biosensors

Amperometric sensors measure the current generated by oxidation or reduction reactions on a conductive surface (Fig. 2C) [83,84].
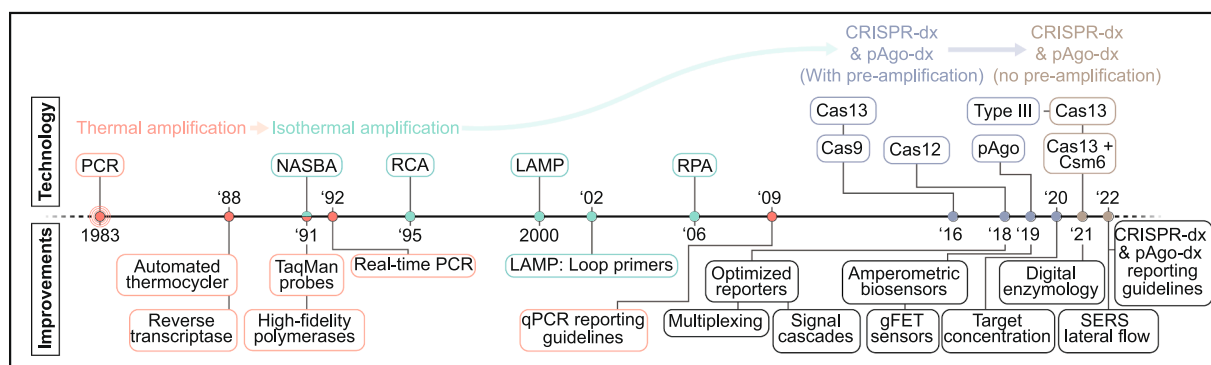
Fig. 1. Evolution of molecular diagnostics. A decade of technological and methodological improvements to PCR resulted in a sensitive and specific amplification method that is the gold-standard of molecular diagnostics. However, guidelines that facilitated reproducible and reliably interpretable qPCR assays were established over a 16-year period. Isothermal amplification methods developed in the 90 s and early 2000 s eliminate the need for thermocyclers and reduce reaction times. While CRISPR-dx and pAgo-dx were initially used as secondary methods to confirm results from isothermal amplification, current research aims to combine CRISPR-dx or pAgo-dx, with novel detection methodologies, signaling cascades and protein circuits that do not rely on pre-amplification.

Fig. 2. Sensitive nucleic acid detection using programmable target recognition and activated collateral nucleases. (A) RNA-guided target recognition by Cas13 (top) or Cas12 (bottom) allosterically activates a collateral nuclease activity, which cleaves a reporter RNA or DNA. Reporter nucleic acids can be labeled with different functional groups that result in a variety of readouts including fluorescent, colorimetric, electrical and Raman scattering. (B) Dilution of the diagnostic reaction into microchambers leads to a digit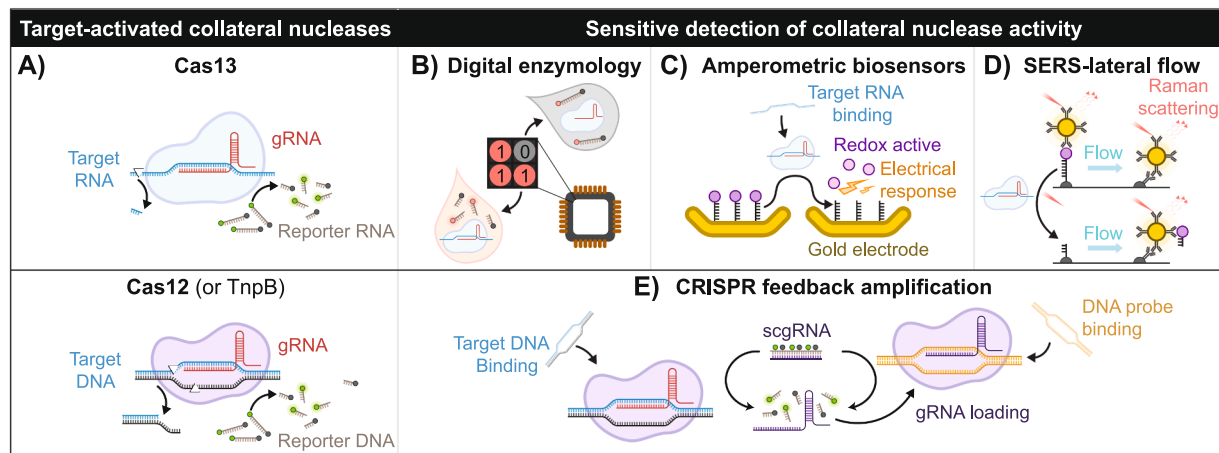al count (0 or 1) of target-containing chambers, enabling the absolute quantification of targets in the original sample and improving sensitivity 10,000-fold over bulk reactions. (C) Amperometric sensors detect the reduction or oxidation of a gold electrode, which results in a change in current. To detect collateral nuclease activity on an amperometric sensor, a redox-active molecule (purple sphere) is tethered to a gold electrode by a nucleic acid tether. Cleavage of the nucleic acid tether changes the current across an electrode. (D) Surface-enhanced Raman scattering (SERS) improves the sensitivity of lateral flow assays > 1,000-fold over visual inspection alone. (E) Feedback amplification circuits of CRISPR enzymes can be designed to mimic the exponential "chain reaction" of DNA amplification that occurs during PCR, leading to a 1,000,000-fold increase in sensitivity.

Amperometric biosensors are attractive because they are inexpensive to manufacture, simple to design, amenable to small volumes (0.1–5 μL), and compatible with complex samples (e.g., human serum). To detect collateral nuclease activity on an amperometric sensor, a redox-active molecule (e.g., methylene blue) is tethered to a gold electrode by a nucleic acid tether (e.g., ssDNA). An intact tether oxidizes the gold electrode (high current). Cas12-mediated detection of a complementary target activates the collateral nuclease activity, which cleaves the ssDNA tether, and liberates the methylene blue from the gold electrode (low current). The first generation of Cas12-based amperometric sensors detected $3x10^7$ copies/μL of target DNA in 60 min [64,65]. This approach has recently been improved by using a stem-loop DNA structure that brings the redox-active molecule closer to the gold electrode. This simple innovation boosts the signal and further increases the sensitivity to $1.8x10^7$ copies/μL [64]. Coincidentally, Cas13 has been recently reported to preferably cleave tRNA anticodon loops, halting protein translation during phage infection [44]. Therefore, the stem-loop structure of an RNA tether may optimally position the redox-active molecule near the gold electrode, while simultaneously mimicking the natural substrate for Cas13 cleavage. Future integration of scalable and disposable amperometric biosensors with electronic handheld devices may enable point-of-care detection of both RNA and DNA.

2.1.3. Surface-enhanced Raman scattering analysis of lateral flow assays

SERS (surface-enhanced Raman scattering) is a "surface-sensitive" spectroscopy technique that has been used to improve the sensitivity of lateral flow assays (Fig. 2D). In specific applications, SERS can detect single molecules [85,86]. While lateral flow assays are a low-cost, paper-based platform that provides a visible result within 5–30 min (e.g., home pregnancy test strips) [87], they are frequently less sensitive than other diagnostics (Fig. 2D) [87]. SERS enhances the sensitivity by coupling a Raman-active reporter molecule (5,5'-dithiobis(2-nitrobenzoic acid); DTNB) to a lateral flow strip that is read by a portable Raman spectrometer [88]. In this assay, the Raman-enhanced reporter is tethered to biotin by a ssDNA. Detection of a DNA target by Cas12, activates the collateral DNase activity, which cleaves the ssDNA tether, and allows the reporter to aggregate at a specific location on the strip. SERS-analyzed lateral flow assays are roughly 1,000–10,000 times more sensitive

than a visual readout, and recent advancements enable SERS analysis in a portable and multiplex format [69,70].

2.1.4. CRISPR feedback amplification circuits

The sensitivity of PCR and isothermal amplification techniques are based on exponential amplification of the nucleic acid target. In these assays, each round of DNA amplification doubles the amount of template for further replication, which leads to an exponential "chain reaction". Similarly, "feedback amplification" circuits of CRISPR enzymes increase sensitivity and transform Cas-based detection into an exponential process [73]. A Cas12-based feedback amplification circuit has been designed for the sensitive detection of DNA without prior polymerase-based amplification (Fig. 2E). A complementary DNA target activates the Cas12-mediated collateral ssDNase activity, which leads to cleavage of the fluorescent DNA reporter in a DNA-crRNA duplex, causing an increase in fluorescent signal (Fig. 2E). Cleavage of the DNA reporter releases the crRNA, which is designed to function as a guide for "empty" Cas12 enzymes. These Cas12 proteins bind to complementary dsDNA, which results in additional collateral cleavage, resulting in the release of more RNA guides and more signal (Fig. 2E). This single enzyme circuit results in 1,000,000-fold improved sensitivity and 5- to 10-fold improved specificity over direct Cas12-based detection [73].

2.2. Target-activated signal amplification

In 2009, Hale *et al.* demonstrated that type III CRISPR systems rely on RNA-guided complexes that bind and cleave RNA targets [89]. This result implied that at least some type III CRISPR systems provide protection by directly cleaving the genome of RNA phages, degrading transcripts from DNA phages, or both. However, these *in vitro* experiments didn't jive with *in vivo* experiments that suggested a role for DNA targeting by these systems [90]. We now know that most type III CRISPR RNA-guided complexes (i.e., Csm or Cmr) cleave the complementary RNA in six nucleotide intervals [91-93], and that RNA binding activates a DNA nuclease in the Cas10 subunit of the complex [94]. In 2017, the versatility and complexity of these systems expanded again when two groups independently reported that the Cas10 subunits of these type III CRISPR complexes are also polymerases that generate cyclic oligonucleotides [95,96]. The cyclic nucleotides generated by the Cas10

polymerase serve as an alarm signal that is recognized by a diverse and growing group of "effectors" that function as RNases [95,96], DNases [97,98], peptidases [99], or a wide array of other downstream effector enzymes [100,101]. The discovery of CRISPR-mediated signaling cascades has inspired several new applications that rely on signaling pathways to improve CRISPR-dx applications [15,76–78].

The type III CRISPR immune signaling pathway has been repurposed for specific and sensitive detection of viral RNA [15,76–78]. RNA-bound Csm complex polymerizes *ribo*-ATP into cyclic oligoadenylate messengers, including cyclic tetra-adenylate (cA4), which bind and activate the *Thermus thermophilus* Csm6 effector nuclease to cleave a fluorescent reporter (Fig. 3**A**). To increase the sensitivity of RNA detection, the backbone subunits (Csm3) of the Csm complex were mutated to prevent Csm3-mediated cleavage of the bound target RNA. This mutation preserves, rather than degrades, the RNA target and locks the polymerase into an activated state. The initial use of these engineered complexes increased sensitivity 3-fold over the wildtype Csm complex [15]. Although the initial iteration of this approach was fast (1–30 min), it wasn't especially sensitive (10^7 copies/μL) [15]. Successive iterations have improved the sensitivity by replacing Csm6 with downstream nucleases (i.e., NucC, Can1 or Can2) that do not degrade the cyclic nucleotide activator [78,102,103], and by pairing effector nucleases (e. g., NucC) with type III complexes that predominantly generate the activating cyclic nucleotide (e.g., cA3) [98,101]. These advancements improved the sensitivity of Csm- or Cmr-based diagnostics to ~ 10^3 copies/μL in a 30-minute assay [76,78]. However, diagnostic parameters are often reported for purified RNA as the starting material, and exclude the time, costs or laboratory equipment typically required for extracting RNA from complex samples. A Histidine-tagged Csm complex from *Thermus thermophilus* was recently repurposed for capturing and concentrating target RNA directly from a patient sample using nickel-derivatized magnetic beads. This approach simultaneously reduces time, cost, and sample handling requirements associated with RNA extraction, and the concentration step increases the sensitivity of this assay [103].

While current Csm- and Cmr-based diagnostics rely on cyclic oligoadenylate-activated effector nucleases and the readout of collateral nuclease activity, nucleases are a common contaminant in human samples, which can result in false-positives. The discovery of new enzymatic activities (i.e., peptidases, pore-forming toxins, etc.) activated by these and other CRISPR immune signaling molecules [99,100] could enable novel detection methodologies that are more robust.

2.2.1. CRISPR-catalyzed ATP Polymerization

Incorporation of a nucleotide during DNA or RNA polymerization reactions coincides with the release of a single proton and a pyrophosphate molecule (Fig. 3**A**). The release of these "byproducts" is the basis for label-free sequencing technologies by Ion Torrent and Pyrosequencing (454 sequencing) [104-106]. Similarly, byproducts of the polymerase have also been repurposed for visual readouts of PCR, LAMP, RPA, and type III CRISPR diagnostics [15,107,108]. Visual methods of detecting polymerization byproducts enable sensitive detection of molecular targets using LAMP (100 copies/μL) [16]. However, since type III CRISPR diagnostics lack the exponential amplification of target nucleic acid that occurs in PCR, LAMP, or RPA, the current sensitivities for visual detection of ATP polymerized by the Cas10 polymerase is relatively low (10^{10} copies/μL). Therefore, more sensitive detection methods must be applied to detect Csm or Cmr-generated polymerization byproducts at clinically relevant sensitivities.

2.2.2. Engineered CRISPR signaling cascades

The first example of an engineered CRISPR-based signaling cascade leveraged the collateral nuclease activity of target-bound Cas13. In this assay, the collateral cleavage activity of Cas13 generates RNA fragments that activate CRISPR effector nucleases, like Csm6 (Fig. 3**B**) [11]. Cas13 cleavage results in a 2′,3′-cyclic phosphate at the 3′ end of the RNA, and certain Cas13 variants preferentially cleave at uridine [11]. Therefore, Zhang and colleagues designed an RNA (i.e., 5′-AAAAUUUUU-3′) that is converted into a Csm6 activator (i.e., 5′-AAAA that ends with a 2′,3′-cyclic phosphate) following Cas13 processing. Activated Csm6 cleaves a fluorescent reporter RNA, resulting in a 3.5-fold increase in sensitivity over Cas13 alone (Fig. 3**B**) [11]. Recently, this approach has been further enhanced by replacing specific hydroxyl groups in the RNA activator with Fluorine. This substitution prevents Csm6-mediated degradation of the activating ligand and improves sensitivity by 100-fold [74,102]. These engineered CRISPR signaling cascades may be further improved by replacing Csm6 with a compatible nuclease that does not have ring-nuclease activity (i.e., NucC, Can1, or Can2). This change would eliminate the costs associated with the synthesis of fluorinated RNAs, and could improve the sensitivity by eliminating the negative impact that the 2′-fluoro modification has on nuclease activation [74,109].

An alternative signaling cascade relies on Cas13 collateral cleavage to snip a chimeric RNA-DNA reporter. The reporter is designed such that snipping of the RNA liberates a binding site for Cas12 in the DNA, and this activates the collateral cleavage activity of Cas12 (Fig. 3**C**) [75]. This results in a 1000-fold improvement over direct Cas13-based detection and the two amplification steps of this circuit result in a limit of detection of 800 copies/μL in 100 min [75]. Intriguingly, Cas13 may cooperate with natural downstream effector proteins to facilitate
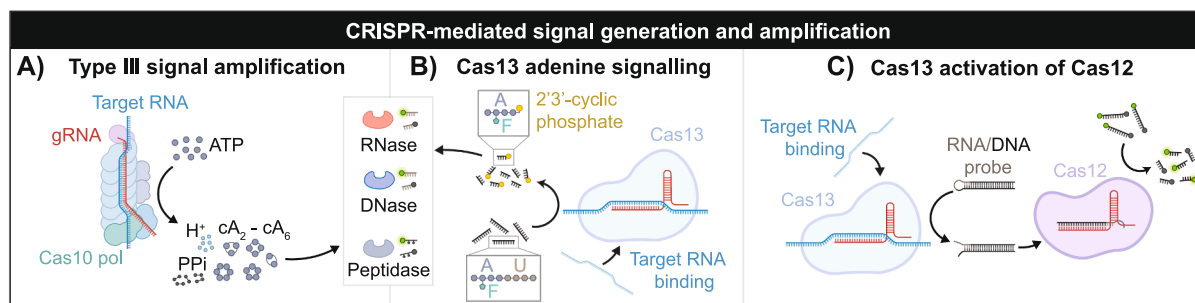

Fig. 3. CRISPR-mediated signaling inspires new CRISPR-dx applications. (**A**) Type III (i.e., Cmr and Csm) CRISPR complexes are used for the direct detection of RNA. Target RNA binding allosterically activates a Cas10 polymerase subunit (Cas10 pol) in Csm or Cmr that produces a mixture of cyclic oligoadenylate molecules (cA2 – cA6). Cyclic oligoadenylate molecules bind and activate CRISPR-associated effectors including RNases, DNases, and peptidases, which can be repurposed to cleave RNA, DNA, peptides, or proenzyme reporters. (**B**) The collateral nuclease activity of Cas13 produces 2′3′-cyclic phosphates at the 3′ ends of cleaved RNAs. Cas13-mediated cleavage of a specially designed reporter RNA thereby produces an artificial linear ligand that activates CRISPR-associated effectors, resulting in > 100-fold increase in sensitivity. (**C**) Cas13-mediated nicking of an inhibiting RNA loop makes an RNA-DNA hybrid duplex accessible for Cas12 RNA-guided DNA binding. One Cas13 thereby activates many Cas12 complexes, which in turn cleave many fluorescent reporter DNAs, increasing the sensitivity 1000-fold over direct Cas13-based detection.

antiviral defense. For example, Cas13 has recently been shown to activate a CRISPR-associated pore-forming toxin (Csx28) that disrupts the integrity of the inner membrane, possibly stimulating an abortive infection. This observation suggests that future Cas13-based RNA target detection applications may be repurposed for the depolarization of a biological membrane that results in electrochemical readouts [110].

2.3. Target-guided nucleases

While most of the attention around Cas9 has focused on applications for RNA-guided genetic engineering, Cas9 has also been used for diagnostics [14,20,52,71,111,112]. In 2016, Collins and colleagues used Cas9 to detect RNA from the Zika virus by first converting the viral RNA to DNA using reverse transcriptase and isothermally amplifying the DNA using NASBA [14]. However, Cas9 is a single turnover nuclease, which limits the sensitivity of detection methods that rely on dsDNA cleavage. A new innovation has transformed Cas9 into a versatile diagnostic that can be multiplexed for the identification of many different RNA targets [17]. In this application, the tracrRNA (*trans*-activating CRISPR RNA), which is required for CRISPR RNA processing [113], has been reprogrammed (reprogrammed tracrRNAs, Rptrs), to be complementary to a sequence on the target RNA of interest. The Rptr thereby "coerces" the target RNA to act as a crRNA for Cas9, which then cleaves a complementary reporter (Fig. 4A) [17]. The multiplexing potential of this approach is a major advantage, however current applications rely on a pre-amplification step to make up for the single-turnover nature of Cas9 cleavage. Further, these Cas9-based diagnostic reactions must be analyzed using electrophoresis. However, coupling this Cas9-based diagnostic to a microarray-based detection methodology could improve this platform to detect millions of different RNA targets in the same reaction [17,114]. Further, single-molecule imaging of fluorescently labeled Cas9 may circumvent the current requirement for a pre-amplification step. Similar applications may pertain to the evolutionary precursors of Cas9 (i.e., TnpB and IscB) that have recently been reported [47,48,115].

Like CRISPR-Cas systems, prokaryotic Argonaute (pAgo) proteins can be repurposed to act as target-guided nucleases that detect DNA. In 2017, the Argonaute protein from *Thermus thermophilus* was shown to "chop" dsDNA in a guide-independent manner and subsequently load cleaved DNA products as gDNAs (guide DNAs). As a diagnostic, short gDNAs are used to program pAgo for cleavage of a DNA target,

generating two target-derived fragments. One of these fragments has a 5' monophosphate, which is required for loading into an unguided (i.e., apo) pAgo protein. This target-derived guide, then directs the pAgo to cleave a complementary fluorescent reporter DNA [18,19]. DNA cleavage by pAgo is a multi-turnover process (Fig. 4B) [116,117], which suggests that the implementation of signaling cascades or sensitive nuclease detection methods could improve pAgo-dx sensitivities. Finally, pAgos are often associated with, or directly fused to diverse enzymatic effectors, some of which have been shown to deplete cellular NAD+ (Nicotinamide adenine dinucleotide) (i.e., Sir2, or TIR) or to depolarize the bacterial inner membrane (i.e., Aga2) [28,34,35]. Therefore, pAgo-dx may eventually be purposed for detection methodologies that do not rely on nuclease activity [34]. In fact, a pAgo-dx that relies on NAD(P)$^+$-ase activity was recently reported [36]. The pAgo-TIR/APAZ heterodimer from *Crenotalea thermophila* forms a tetramer of homodimers upon binding an RNA guide [36]. RNA-guided binding of pAgo-TIR/APAZ to a ssDNA target activates the NAD(P)$^+$-ase activity of the TIR domain to degrade NAD(P)$^+$ into ADPR(P) (adenosine diphosphate ribose) and NAM (nicotinamide). Therefore, ssDNA can be detected with high specificity by pAgo-TIR/APAZ-mediated degradation of ε-NAD$^+$ to the fluorescent molecule ε-ADPR [36]. While the sensitivity of this approach is currently modest (~10^{10} copies/μL) [36], the identification of enzyme variants that are naturally more active may improve sensitivity.

2.4. Target binding

2.4.1. Graphene Field Effect Transistor sensing of DNA

A FET (Field-Effect Transistor) is a transistor that uses an electric field to control the flow of current in a semiconductor and is composed of three electrodes (source, drain, and gate). A voltage applied to the gate alters the conductivity between the drain and source. In graphene FETs (gFETs), the channel between the source and drain electrodes is graphene, which is an excellent electrical conductor with an extraordinarily high surface-to-volume ratio (Fig. 4C). This material is highly sensitive to changes in electric fields caused by any molecule attached to the surface [118]. These properties have been repurposed for the sensitive detection of Cas9 binding to target DNAs in a method that has been termed "CRISPR-CHIP" [71,72]. In this application, a graphene layer is functionalized with catalytically dead Cas9 (dCas9) and sequence-specific binding of dCas9 to a complementary dsDNA target causes
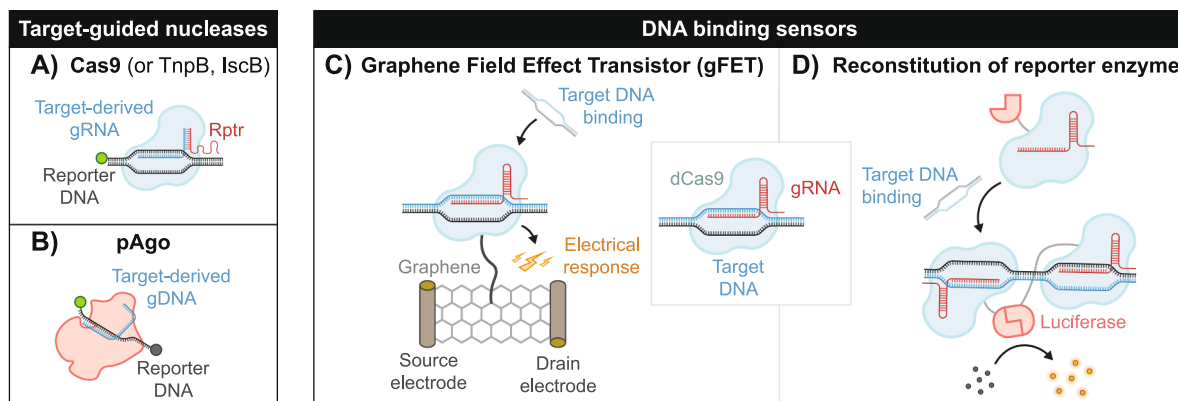

Fig. 4. Target-guided nucleases and sensitive methods to detect target binding. (A) Cas9 can be repurposed to detect RNA in a multiplexed protocol, by using reprogrammed tracrRNAs (Rptrs) that anneal to the target RNA and coerce the adjacent RNA sequence to behave as a typical RNA guide that results in cleavage of a complementary DNA reporter. The presence of many different RNA targets is determined using gel electrophoresis or a Bioanalyzer. Future microarray-based detection methods may enable multiplexed detection of millions of RNAs. **(B)** DNA-guided prokaryotic Argonautes cleave dsDNA targets into fragments that are recognized as new gDNAs (guide DNAs), which direct pAgos to cleave complementary DNA reporters. Unlike target-guided Cas9, target-guided Argonautes are multi-turnover enzymes that cleave many reporter DNAs per target-derived guide. **(C)** RNA-guided Cas9 can be repurposed to detect complementary dsDNA target. Cas9-bound dsDNA induces a large change in the electrical properties of the tethered graphene channel, resulting in rapid and sensitive electrical detection of DNA. **(D)** Two RNA-guided Cas9s, each fused to different halves of a split reporter enzyme can be repurposed to detect dsDNA. Stable association of each Cas9 to the adjacent complementary sites results in the reconstitution of the reporter enzyme (e.g., Luciferase).

large changes in the electric field across the gFET channel (Fig. 4C) [71,72]. These assays are capable of detecting 1000 copies/µL in 15 min [71,72]. Further, extending the assay time to 1 h allows for highly specific detection that discriminates between SNPs [71,72]. gFET-based sensors enable rapid, sensitive, specific, and low-cost detection via an electronic readout [118]. In addition, the multiplexing of gFET sensors is expected to facilitate the testing of up to thousands of targets on a single small device. These properties are expected to enable highly multiplexed, amplification-free detection of DNA-containing infectious agents or human alleles at point-of-care.

2.4.2. Reconstitution of split reporter enzyme

Since Richards' 1958 observation that two proteolytic fragments of bovine pancreatic ribonuclease could interact *in trans* to reconstitute a functional enzyme [119], the reconstitution of "split reporter enzymes" have been used to visualize molecular interactions [120]. Current iterations of this approach rely on tethering two non-functional halves of the reporter to two other proteins that are anticipated to interact [120]. Using this strategy, two halves of a split luciferase can be linked to Cas9s that are programmed to bind complementary target sequences that are adjacent to one another (Fig. 4D) [79]. However, the nucleotide spacing between Cas9s may require optimization since the efficient reconstitution of the split reporter protein is likely to depend on the helical phase of DNA [121,122].

3. Suggested reporting metrics for molecular diagnostics

PCR matured into its current status as a gold-standard diagnostic technique through the formulation of guidelines that standardized how to conduct, analyze and report results from quantitative PCR (qPCR) [123,124]. This initiative for standardization was prompted by a technically flawed study that claimed to detect measles viral RNA in children with autism [125], that could not be reproduced [126]. In the above section of this review, we compare the sensitivities and specificities of CRISPR- and pAgo-dx platforms that have been designed to detect a wide variety of RNA and DNA targets. However, an objective comparison between many of these platforms is not possible due to a lack of standardized reporting guidelines. The concentration of target nucleic acid detected by qPCR is commonly referred to by the cycle threshold (Ct) or quantification cycle (Cq) number, which is the cycle number at which the fluorescence reading for a reaction exceeds the background fluorescence threshold. Conversely, CRISPR-dx and pAgo-dx utilize diverse biochemical activities that are measured via diverse methodologies, resulting in diverse signals including fluorescence, visible wavelengths of light, electrical current, and the inelastic scattering of photons. Therefore, no single experiment-specific metric can be used to compare these emerging diagnostic assays, further highlighting the need to standardize the reporting of the limit of detection, repeatability within an experiment, reproducibility between experiments, clinical sensitivity, and diagnostic specificity (Table 1, Table S1). These minimal diagnostic metrics are informed by previous guidelines established during the maturation of qPCR as the gold-standard diagnostic, and by FDA's EUA (Food and Drug Administration's Emergency Use Authorization) guidelines for molecular diagnostics during the COVID-19 pandemic [124,127].

4. Outlook

It took nine years for PCR to be developed from an error-prone, insensitive, and time-consuming proof-of-concept into a reliable diagnostic [1,2,45,128], and a total of 16 years to establish guidelines that enable reliable interpretation [124]. In the past six years, CRISPR-dx and Ago-dx have undergone rapid development into an emerging class of molecular diagnostics [18,40]. Coupling CRISPR-dx and Ago-dx to pre-amplification methods have improved their sensitivities by six orders of magnitude to detection limits comparable to RT-qPCR, and fast-tracked

Table 1
Minimal diagnostic metrics that facilitate objective performance comparisons between platforms.

Metrics	Description	Suggested units
Limit of Detection*	Minimal target concentration detected with 95% probability (19/20 replicates)	Target concentration *in the sample* (copies/µL, copies/mL or molar concentration)***
Repeatability	Intra-assay variance	S.D. or C.V. within experiment***
Reproducibility	Inter-assay variance	S.D. or C.V. between experiments
Clinical sensitivity	Proportion of true positive samples identified as positive	Percentage (%)
Diagnostic specificity**	Proportion of true negative samples identified as negative	Percentage (%)

* To facilitate comparisons between CRISPR- and pAgo-dx to qPCR, the Limit of Detection should be reported as the minimal concentration of target in the stock or patient sample being tested, as opposed to the concentration of the target after it has been diluted into the diagnostic reaction (which would appear as a more sensitive diagnostic).
** Water is not an adequate negative control for CRISPR- and pAgo-dx because it does not assay for non-specific binding and activation of the biochemical activities of the nucleic acid-guided proteins used. True negative samples should have similar complexities (i.e., nucleic acid content, patient contaminants, etc.) to the positive samples tested.
*** Sample concentrations should not be reported solely in Cq (qPCR, quantification cycle) values. Cq values are a relative measure of target concentration that varies significantly between reagent kits, primer sets, instruments, and laboratories [120]. Therefore, Cq values should be supplemented with a standard curve generated using concentration standards available from the National Institute of Standards and Technology (NIST) and commercial suppliers.
**** S.D. – standard deviation, C.V. – coefficient of variation.

their use as molecular diagnostics under the FDA's Emergency Use Authorization (Fig. 1) [129,130]. However, pre-amplification requires primer design, and optimization, increases the cost, and generally increases reaction times or sample handling. Therefore the next generation of CRISPR- and pAgo diagnostics will rely on the discovery and incorporation of new enzyme activities, development of target concentration methods, sensitive detection methodologies, and protein circuits or signaling cascades that all improve sensitivity, specificity and time-to-result (Figs. 2, 3, and 4) [28,100,131,132]. Several of these innovations have already led to the development of rapid (<60 mins) CRISPR- and pAgo-dx that do not rely on pre-amplification (Fig. 5). Efforts to increase the sensitivity of these methods, and to bypass or obviate the need for RNA extraction, are ongoing (Fig. 5) [22,58,59,67,103]. Machine learning-assisted design of enzymes with improved kinetics, substrate affinity, or selection of more specific and active RNA guides, may facilitate these efforts [133-136]. The continued maturation of these molecular diagnostic tests will assist a transition to a distributed-testing paradigm. The centralized-testing paradigm, which requires shipping samples to a high-complexity lab for RT-qPCR, created a critical bottleneck in SARS-CoV-2 surveillance. Therefore, a distributed-testing model that enables individuals to self-perform and analyze results will prevent similar bottlenecks in the future. An estimated 6.64 billion people, or ~ 84% of the world's population, own a smartphone that includes a high-resolution camera [137]. The SARS-CoV-2 pandemic has bolstered efforts to enhance smartphone-based detection and interpretation of test results, machine learning-assisted interpretation of the results, and cloud-based computing are anticipated to empower point-of-care applications [22,60,138-140]. Collectively, we anticipate that continued innovation, driven by demands for faster diagnostics that can be administered at the point-of-care, will elevate CRISPR-dx and Ago-dx to a new standard in molecular diagnostics.
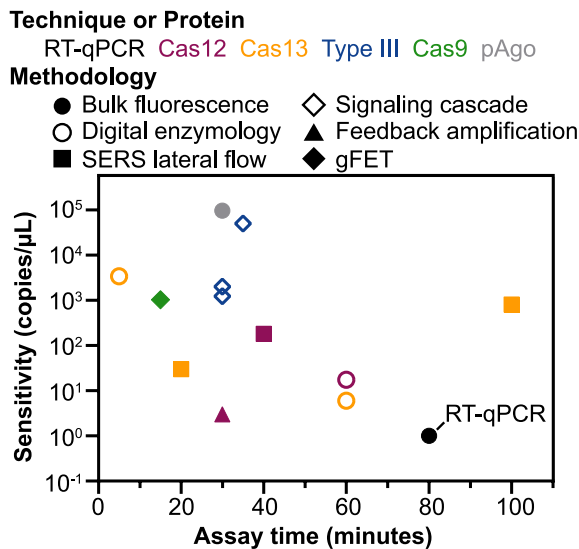
Technique or Protein
RT-qPCR   Cas12   Cas13   Type III   Cas9   pAgo

Methodology
- ● Bulk fluorescence
- ◇ Signaling cascade
- ○ Digital enzymology
- ▲ Feedback amplification
- ■ SERS lateral flow
- ◆ gFET

Fig. 5. The emerging landscape of pre-amplification-independent CRISPR-dx and pAgo-dx. Unique and sensitive Cas12-, Cas13-, Type III CRISPR-, Cas9- and pAgo-based diagnostic methodologies that do not rely on preamplification compared to the CDC's recommended RT-qPCR protocol for SARS-CoV-2 [18,66-69,71,73-76,78,141]. Data and citations included in Table S2.

CRediT authorship contribution statement

Declaration of Competing Interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: B. W. is the founder of SurGene and VIRIS Detection Systems. B.W., A.S.-F., A. Nemudraia, and A. Nemudryi are inventors on patent applications related to CRISPR-Cas systems and applications thereof.

Acknowledgments

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ymeth.2022.06.002.

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