This article is made available via the ACS COVID-19 subset for unrestricted RESEARCH re-use and analyses in any form or by any means with acknowledgement of the original source. These permissions are granted for the duration of the World Health Organization (WHO) declaration of COVID-19 as a global pandemic.

ACS SENSORS

pubs.acs.org/acssensors

Review

Enzyme-Assisted Nucleic Acid Detection for Infectious Disease Diagnostics: Moving toward the Point-of-Care

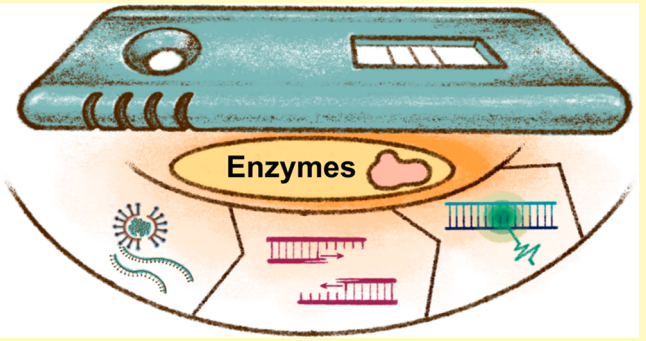
Akkapol Suea-Ngam, Léonard Bezinge, Bogdan Mateescu, Philip D. Howes,* Andrew J. deMello,* and Daniel A. Richards*

Cite This: *ACS Sens.* 2020, 5, 2701−2723

Read Online

ACCESS | Metrics & More | Article Recommendations

ABSTRACT: Driven by complex and interconnected factors, including population growth, climate change, and geopolitics, infectious diseases represent one of the greatest healthcare challenges of the 21st century. Diagnostic technologies are the first line of defense in the fight against infectious disease, providing critical information to inform epidemiological models, track diseases, decide treatment choices, and ultimately prevent epidemics. The diagnosis of infectious disease at the genomic level using nucleic acid disease biomarkers has proven to be the most effective approach to date. Such methods rely heavily on enzymes to specifically amplify or detect nucleic acids in complex samples, and significant effort has been exerted to harness the power of enzymes for in vitro nucleic acid diagnostics. Unfortunately, significant challenges limit the potential of enzyme-assisted nucleic acid diagnostics, particularly when translating diagnostic technologies from the lab toward the point-of-use or point-of-care. Herein, we discuss the current state of the field and highlight cross-disciplinary efforts to solve the challenges associated with the successful deployment of this important class of diagnostics at or near the point-of-care.



KEYWORDS: *enzyme-assisted, diagnostics, field-deployable, infectious disease, isothermal amplification, microfluidic, miniaturization, point-of-care*

Downloaded via 157.7.194.173 on February 3, 2021 at 20:21:17 (UTC).
See https://pubs.acs.org/sharingguidelines for options on how to legitimately share published articles.

INTRODUCTION

The ability to detect disease with a high degree of sensitivity and specificity is an essential part of any healthcare pipeline, and an important tool in biomedical research. In vitro diagnostics (IVDs) inform clinical decisions, and play a primary role in determining therapeutic pathways at both the individual and the population level. This is particularly important for infectious diseases, where IVDs provide key data for epidemiological models and aid in disease tracing, helping to slow or prevent the spread of disease. The global impact of infectious disease is perfectly exemplified by the current SARS-CoV-2 outbreak (COVID-19), which, at the time of writing, has caused over 13.3 million infections and 782,000 deaths globally.[1] Unfortunately, the ultimate cost of the COVID-19 pandemic is yet to be felt, as the resultant economic damage is likely to have a lasting impact on multiple sectors including healthcare and agriculture, which will almost certainly exacerbate morbidity and mortality, particularly in resource limited settings. Although the current outbreak is receiving significant global attention, the more general problem of infectious disease is broadly underestimated. In 2016, the World Health Organization (WHO) found that infectious disease was implicated in three of the top ten causes of death globally.[2] This picture worsens still when looking at populations in low income regions such as Africa, where infectious diseases such as human immunodeficiency virus (HIV), malaria, tuberculosis (TB), and lower-respiratory tract infections (LRTs) accounted for over 35% of total deaths in 2016.[3] Fortunately, some progress is being made in this area. For example, it is widely accepted that increased access to diagnostics played a key role in controlling the HIV epidemic in Uganda in the 1990s, helping to drive a 67% reduction in prevalence between 1991 and 2001.[4] This trend has continued across Africa; between 2000 and 2016 deaths from HIV in the region decreased by 61%.[3] While tackling epidemics is a multifaceted problem, it is clear that the correct use of diagnostic data can have a significant impact by enabling early

Received: July 20, 2020
Accepted: August 25, 2020
Published: August 25, 2020



ACS Publications

© 2020 American Chemical Society

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

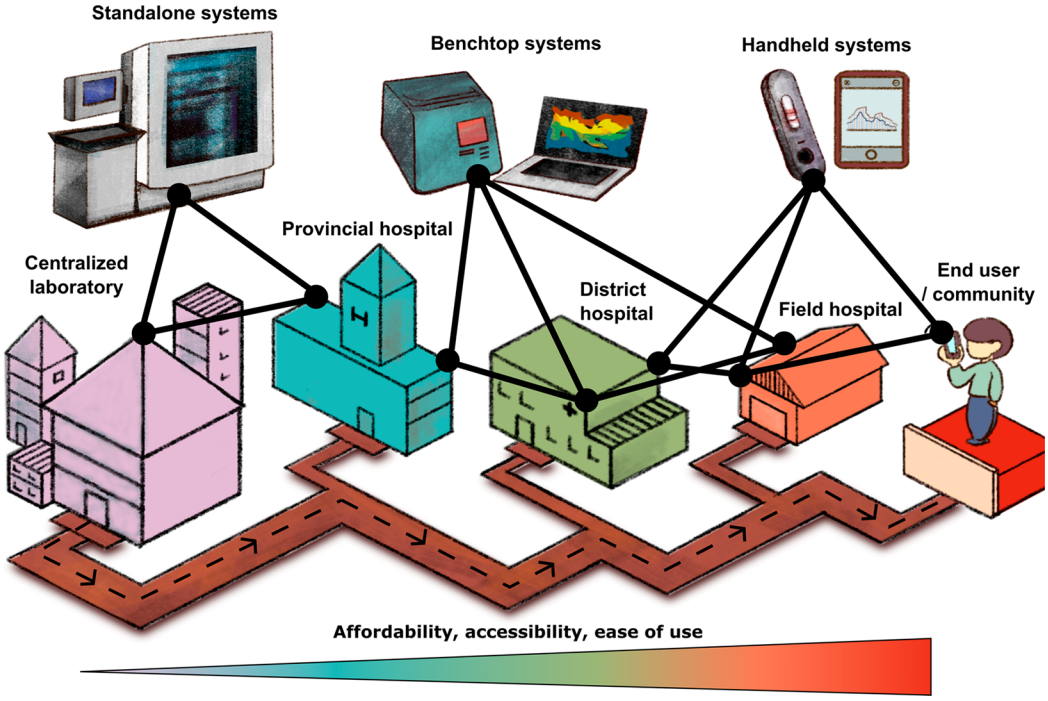


Figure 1. Path toward the point-of-care. From left to right, the schematic represents the pathway from centralized laboratories through to the end user, and the different healthcare setting that exists between those two extremes. Also shown are the different types of in vitro diagnostic systems used within each setting, with lines representing the overlap that can occur between systems and settings.

intervention, thus reducing the overall burden on healthcare systems and the infrastructure that supports them. Additionally, sensing, tracking, and analyzing markers of infectious disease within a research setting can help scientists to develop fundamental disease models, find new druggable targets, and test the efficacy of medicines both in vitro and in vivo. Accordingly, developing new technologies for sensing disease is of paramount importance.

Point-of-Care Infectious Disease Diagnostics. While multiple technologies have proven effective for in vitro infectious disease diagnostics, currently employed systems are largely dependent on complex equipment, centralized laboratories, and highly trained personnel. These requirements introduce several limitations that severely diminish their overall utility. Diagnostics laboratories require substantial infrastructure to operate effectively, and training personnel to use complex equipment is labor-intensive. In addition, samples must be effectively stored at the point of collection and transported to the testing location. These factors ultimately serve to increase the cost per test, and also introduce a lag period between sample collection and test result, limiting the utility for disease tracing and containment. Unfortunately, these problems are intensified within resource-limited communities where infectious diseases are likely to have the greatest impact.

In the context of IVDs, the term point-of-care (PoC) refers to systems that have been developed to address the aforementioned limitations, commonly through miniaturization and simplification. PoC systems are designed as self-contained devices that ideally contain a complete diagnostic platform, from sample collection through to analysis, data generation, and diagnosis, within a single unit. These systems should also be portable (or at least transportable), provide results quickly, and be easy to operate either by the end user or a field-deployed care worker. Diagnostic assays that meet these requirements are typically termed rapid diagnostics tests (RDTs). It is important to consider the transition from centralized laboratories toward the point-of-care as a continuum, rather than a discrete series of steps, and understand that there is significant overlap between the different systems and the situations in which they are effectively deployed. Thus, while highly portable PoC systems are designed for the end user, many systems can also be highly beneficial in more developed settings (Figure 1). It is also important to note that while PoC diagnostics fill an essential, and often neglected, gap in the diagnostic process, centralized laboratories continue to play an important role due to their ability to process samples at high-throughput. This can help to offset some of the negatives associated with centralized testing (high cost, slow response), and can be particularly valuable once a pandemic is established.

The efficacy of PoC testing for infectious disease is well documented, and the development of RDTs has been highlighted as a priority by organizations such as the WHO. In 2003, the WHO Special Programme for Research and Training in Tropical Diseases (WHO/TDR) published a list of criteria for in vitro infectious disease PoC diagnostics. These criteria were labeled ASSURED: Affordable, Sensitive, Specific, User friendly, Rapid and robust, Equipment-free, and Deliverable to end users. In 2019, Peeling and colleagues evaluated the effectiveness of ASSURED and proposed two new criteria, namely, Real-time connectivity and Ease of specimen collection, to create the new REASSURED standards for infectious disease diagnostics.[5] The addition of connectivity and specimen collection bridges apparent gaps in the original criteria, and also reflects the advent of mobile health (mHealth) and improved communications infrastructure.

Nucleic Acid Biomarkers in Infectious Disease. IVDs can be broadly categorized by the type of biomarker that they detect, e.g., antigen, antibody, metabolite, or nucleic acid.

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

Though each of these markers has unique advantages, and it is important to consider this when designing a diagnostic assay, nucleic acids are particularly promising in the context of infectious diseases. Viral and bacterial pathogens contain genomes which encode them. Parts of this genetic information, which is highly specific to the disease, can be exploited as a direct marker of the disease. This genotypic specificity is not always replicated at the phenotypic level, a factor which can lead to cross-reactivity and false diagnoses when sensing phenotypic biomarkers such as proteins. A good example of this is the spike protein of SARS-CoV, which is maintained across both SARS-CoV-1 and SARS-CoV-2.[6] Tests based on this antigen are not readily able to differentiate between the two strains, though genotypic differences can be easily distinguished.[7] The ability to differentiate between closely related diseases at the genetic level is particularly important for bacterial or viral pathogens, which are able to rapidly mutate and form new strains. A further advantage of the extraneous nature of infectious disease nucleic acid biomarkers is that they can be detected in the body immediately after infection. This, in theory, enables earlier diagnosis and can be invaluable in slowing or preventing the spread of the disease. Perhaps the biggest advantage of nucleic acid testing in the context of infectious disease diagnostics is the speed at which the tests can be developed after the emergence of a new disease. Such agility is a result of the relative simplicity of both nucleic acid biomarker identification and disease-specific targeting-ligand generation. Thanks to advances in automated gene sequencing, it is now possible to sequence entire viral or bacterial genomes quickly, often within a matter of days.[8,9] Similarly, generating target-specific ligands (in the form of primers) is simpler for nucleic acid targets due to the well understood process of complementary base pairing. Computational techniques, coupled with high-throughput screening, can generate optimized nucleic acid-specific ligands within hours, whereas antibody ligands for protein-based biomarkers may take months to develop.[10] These factors culminate in a rapid turnaround time between disease identification and the deployment of a working assay; reports of assays being developed in under one week exist in the literature.[11] Unfortunately, life is rarely simple, and nucleic acid biomarkers present several diagnostic challenges. They are frequently present in vanishingly small quantities among a large background of indigenous nucleic acids, can be sensitive to extraction techniques and storage methods, and are readily degraded by common contaminants. Researchers from across the scientific spectrum have dedicated significant resources to developing, adapting, and exploiting technologies to overcome these barriers and develop effective diagnostics for nucleic acid targets.

Role of Enzymes in Nucleic Acid Biosensing. The vast majority of nucleic acid biosensors employ enzymes to amplify, bind, degrade, or otherwise manipulate nucleic acids. This is unsurprising, given that enzymes have been evolved by nature to perform a wide array of complex operations on nucleic acids during the process of transcription and translation. Discovering novel enzymes and elucidating their interactions with nucleic acids has played an important role in the development of nucleic acid biosensors, and has formed the basis for a new generation of diagnostic platforms. While the most common role of enzymes remains the specific amplification of target nucleic acids, they also play important roles throughout the entire diagnostic workflow, from sample preparation and extraction through to signal generation and analysis. Though it is clear at this time that enzymes are essential for effective nucleic acid sensing, our reliance on them presents several practical problems. Enzymes generally require quite specific physical and chemical conditions (e.g., temperature, pH) in order to operate effectively, and can rapidly degrade if not handled correctly. This creates both technological and logistical challenges that must be overcome to ensure practical impact. We will now describe the roles that enzymes play in both traditional and emerging nucleic acid diagnostics, and discuss the advantages and disadvantages of each approach in the context of infectious disease RDTs. We will also highlight how established technologies for exploiting enzymes to detect nucleic acids are gradually being adapted toward field-deployable and/or PoC systems that aim to meet the REASSURED criteria. Emerging technologies that provide entirely new methodologies for sensing nucleic acids, and how these novel approaches could change the way we approach PoC infectious disease diagnostics, will also be discussed.

■ ENZYME-ASSISTED NUCLEIC ACID AMPLIFICATION

Arguably, the most important role of enzymes in nucleic acid biosensors, and particularly disease diagnostics, is the amplification of nucleic acids. Amplification of a target biomarker from the background greatly simplifies the process of detection and enables the use of multiple downstream signal and detection technologies. Fortunately, many enzymes have been discovered or developed to achieve efficient nucleic acid amplification.

Polymerase Chain Reaction. Polymerase chain reaction (PCR), first reported in 1985,[12,13] is still the most popular method for amplifying DNA. PCR employs thermostable polymerase enzymes, most commonly Taq polymerase from the thermophilic bacterium *Thermus aquaticus*. Since its inception, PCR has become one of the most important tools in molecular biology and the primary driver for IVDs that target nucleic acids. PCR mixtures comprise several reagents, including forward and backward DNA primers specific to a target sequence, a polymerase, and a dNTP-rich buffer. Optimal amplification requires cycling through three temperatures, each with a specific function: 95 °C for double-stranded DNA (dsDNA) dissociation, 55−72 °C for primer annealing, and 70−80 °C for polymerization. PCR can also be used to detect RNA by first employing the enzyme reverse transcriptase to transcribe RNA into DNA prior to amplification. Early PCR technologies were hindered by the relatively high error rates in DNA replication caused by Taq polymerase. These issues were remedied by the characterization of alternative thermostable enzymes, such as Pfu polymerase, that possess exonuclease proofreading abilities. These features allow the enzymes to correct errors in nucleotide incorporation during PCR, though the polymerization rate is generally lower than Taq.

Quantitative Polymerase Chain Reaction. Over the last 30 years, there have been multiple advances in PCR technology, particularly through the development of detection reagents and novel systems which allow for real-time monitoring of amplicon generation. These approaches have engendered the widespread implementation of quantitative PCR (qPCR) for DNA, and quantitative reverse transcriptase PCR (qRT-PCR) for RNA. As the names suggest, qPCR and qRT-PCR are capable of delivering a quantitative readout of the degree of

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

Table 1. Overview of Isothermal Amplification Techniques

method	enzyme(s)	operating temperature/°C	reaction time/min	target	amplicons	amplification capacity	number of primers
PCR	Polymerase: Taq, Pfu, Vent, Klenow,	95, 55, 72	45−120	dsDNA	dsDNA	10^7−10^{10}	2
LAMP	Polymerase: Bst	55−65	15−60	dsDNA	concatemer	10^9	4, 6
RCA	Polymerase: Φ29	rt−37	30−60	ssDNA	ssDNA	10^3	1
RPA	Polymerase: Bsu	rt−42	5−20	dsDNA	dsDNA	10^{11}	2
SDA	Polymerase: Φ29, Bst, Exo-Klenow	rt−65	20−120	ssDNA,dsDNA	dsDNA	10^9	1, 2
	Endonuclease: Nb.BbVCI, Nt.BstNBI, HincII···						
HDA	Polymerase: *Taq*, *Bst*, *Exo*-Klenow, *Gsp*, *Gst*	60−65	60−120	dsDNA	dsDNA	10^6	2
	Denaturing: *Helicase*						
NASBA	Polymerase: T7 polymerase	65, 41	90−120	RNA	RNA	10^9	2
	Endonuclease: RNase H						

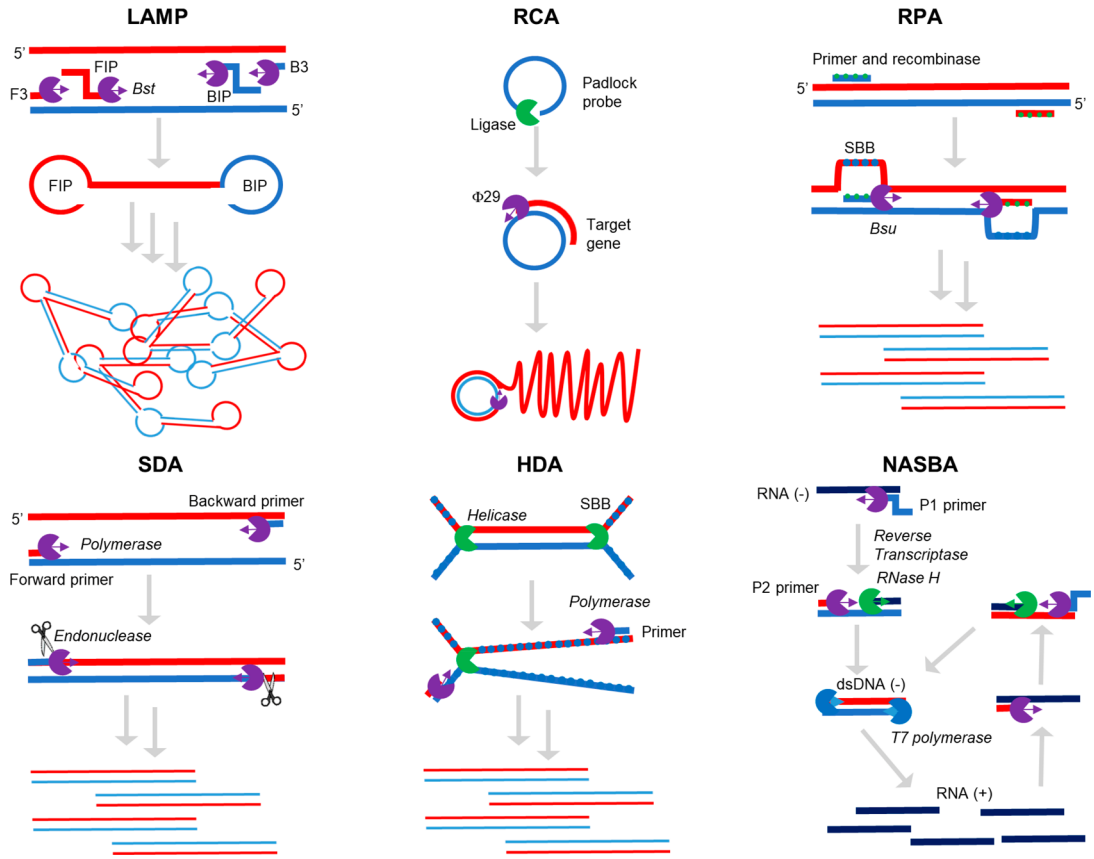


Figure 2. Schematic representations of different isothermal amplification techniques. LAMP: Binding of the inner primers (FIP, BIP) is followed by strand displacement and polymerization by Bst polymerase. Subsequent binding of an outer primer (F3, B3), and polymerization, displaces the newly synthesized strand which forms a self-hybridizing loop structure. This process repeats on the opposite end of the target sequence with the reverse primer, forming short "dumbbell" loop structures that can be amplified into dsDNA concatemers. RCA: Target DNA binds to a padlock probe template, circularized by DNA ligase, followed by Φ29 polymerase which rolls around the circular template to produce an ssDNA amplicon. RPA: A recombinase enzyme inserts forward and reverse primers into a dsDNA target template, resulting in strand displacement. Single-strand binding proteins (SSBs) bind to prevent strand recombination, and Bsu polymerase amplifies the template. SDA: After an initial round of amplification, an endonuclease nicks the newly synthesized strands between the primer and the target, allowing a new polymerase enzyme to bind and amplify the target while simultaneously displacing the old strand. HDA: Initial DNA denaturation is performed by helicase, which is followed by an SSB to prevent recombination. Subsequent binding of the primers is followed by polymerization. NASBA: A P1 forward primer, containing complementary regions for both the target and a T7 promoter region, binds to a target RNA(−) strand. In the initial stage, reverse transcriptase extends the primer to produce a DNA:RNA duplex, and the RNA strand is subsequently degraded by RNase H to produce an ssDNA template. Binding of the P2 reverse primer and extension by reverse transcriptase produces a dsDNA, which can then enter the amplification stage. T7 polymerase binds the dsDNA to create an RNA strand that is antisense to the original target, the P2 reverse primer can bind, and the whole process begins again, resulting in RNA amplicons.

amplification in real-time, which in turn can be used to determine the concentration of the target biomarker from a calibration curve. First developed in the 1990s,[14] qPCR has rapidly grown to become the gold standard for laboratory-

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

based nucleic acid diagnostics. Semiautomated commercial qPCR systems, such as the GeneXpert from Cepheid or Cobas from Roche, are the mainstay of diagnostics laboratories throughout the developed world, and are capable of processing up to 4000 tests per day.[15]

Signal Generation in PCR. Obtaining a signal in PCR is achieved by detecting the amplicon during amplification (qPCR) or post-amplification by addition of a detection probe, most commonly a dye that exhibits an enhanced fluorescence yield upon intercalation into DNA. These methods can be cost-effective and easy to implement into a biosensing system, and thus offer significant utility in the context of diagnostics. Multiple intercalating dyes have been developed for visualization of DNA, including cyanine-based dyes such as DNAzure, Novel Juice, SYBR, and TOTO. These dyes emit at distinct wavelengths, from the blue through to the red, providing a degree of flexibility in regard to signal generation. However, one key drawback of these compounds is that they bind indiscriminately to dsDNA, and thus emit even in the presence of background DNA or off-target amplicons. Sequence-specific probes based on FRET-molecular beacons provide an elegant solution to these issues. Here, a fluorophore is attached to the 5′-end of an oligonucleotide probe specific to the target sequence, and a FRET quencher is attached to the 3′-end to quench the fluorescence of the fluorophore when nearby. After the denaturation and annealing of the probe to its target, the exonuclease activity of the polymerase can degrade the beacon, cleaving the quencher and releasing the fluorophore. The complementarity between the nucleotide linker on the probe and the target sequence provides a high degree of specificity (down to the single nucleotide level) and decreases background signal. The most famous and commonly employed of such probes are the TaqMan series of probes, first reported in 1991,[16] which work with Taq polymerase. Multiple different fluorophores and quencher pairs are commercially available, providing flexibility and facilitating target multiplexing.

Isothermal PCR. Although qPCR is recognized as the gold standard for nucleic amplification, multiple fundamental limitations hinder the application of the technology for RDTs. The largest barrier is the reliance on thermal cycling for amplification. Thermal cycling is a slow, energy-intensive process that requires relatively complex heating elements to provide the necessary temperature control. In addition, the majority of PCR systems are relatively large and require a steady power supply; this limits the portability of the technology and excludes many PoC applications. Significant effort has been put into the development of isothermal techniques to overcome this issue (Table 1, Figure 2). Isothermal amplification techniques can operate optimally at a single temperature, greatly simplifying the heating requirements of the system.

Loop-Mediated Isothermal Amplification. Loop-mediated isothermal amplification (LAMP), first reported in 2000,[17] is one of the most popular isothermal techniques for nucleic acid amplification. LAMP requires four primers: inner primers, termed the forward inner primer and backward inner primer (FIP and BIP), and forward and backward outer primers (F3 and B3). These primers bind to six unique sequences on the target sense and antisense strands. Additional loop forward and loop backward (LF and LB) primers can be introduced to accelerate the reaction. Amplification in LAMP relies on Bst polymerase, which exhibits strand displacement activity, i.e., the ability to unwind double-stranded DNA (Figure 2). This eliminates the need for high-temperature DNA denaturation, but since Bst polymerase optimally operates between 55 and 65 °C, heating is still required for optimal results. Since LAMP yields dsDNA products, signal can be generated using methods commonly employed for PCR and qPCR. Interestingly, the high amplification capacity of LAMP facilitates unique methods for generating signal. For example, during elongation of dsDNA, a significant amount of pyrophosphate is generated, which sequesters magnesium from the buffer to create magnesium pyrophosphate. This process can be exploited through the use of metal indicators that fluoresce or change color upon chelation of Mg^{2+}. For example, Goto et al. used hydroxy napthol blue to detect bacterial DNA from *Pseudomonas aeruginosa*, a common cause of secondary infections in hospitalized patients, in fecal samples, achieving a limit of detection down to 130 CFU per 0.1 g of sample.[18,19] Similar colorimetric methods have been successfully employed for the detection of *Shigella*, *Salmonella*, and *Vibrio cholerae*, though these tests were performed on spiked samples rather than clinical specimens.[20] The colorimetric nature of this approach is clearly advantageous for PoC settings as it precludes the need for additional equipment, and facilitates naked eye qualitative analysis.

The most compelling feature of LAMP is its high amplification capacity. Indeed, a 10^9-fold amplification can be achieved within an hour. Additionally, the use of multiple primers enhances specificity, a feature that is particularly valuable in the context of infectious disease IVDs. Due to these advantages, LAMP has become a common component of many PoC infectious disease IVDs, particularly for paper-based systems.[21,22]

Recombinase Polymerase Amplification. Recombinase polymerase amplification (RPA) relies on the coordinated actions of multiple enzymes to achieve isothermal amplification. First reported in 2006,[23] RCA has since been developed and patented as a proprietary technology of TwistDX. RPA requires only two primers, in addition to three enzymes: A recombinase enzyme, an ssDNA binding protein (SSB) named gp32, and Bsu DNA polymerase. The recombinase and SSB act in a complementary fashion to initiate strand dissociation and insert the primers, negating the need for high temperature dissociation (Figure 2). Bsu polymerase has been shown to work optimally between 37 and 42 °C. RPA is compatible with all signal generation techniques applicable to dsDNA, such as fluorescence staining or FRET-beacons.[24]

Amplification using RPA is exponential in nature, and has demonstrated a 10^9−10^{11}-fold amplification of target DNA, making it an excellent option for the detection of low abundancy nucleic acids. Unfortunately, the rapid amplification can be a significant source of error and background if the primers bind off-target, an issue made worse by the relatively long primer lengths required in RPA (32−35 nt).[24] This is problematic when sensitivity and specificity are paramount, e.g., in infectious disease PoC diagnostics. Additionally, the proprietary nature of RPA poses a problem for PoC diagnostics. Here, a reliance on a single supplier can complicate supply chains and increase costs, particularly when compared to conventional "open source" alternatives. Nevertheless, RPA is a popular choice for PoC infectious disease diagnostics, and has been successfully employed for multiple diseases including influenza,[25] Dengue,[26] and Ebola.[27]

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

Rolling Circle Amplification. Rolling circle amplification (RCA) takes inspiration from the natural rolling circle transcription of circular plasmids and viral genomes to generate an amplicon around a circular DNA template. The first examples of RCA appeared in the 1990s,[28,29] and many iterations have since been reported.[30] In RCA, an ssDNA "padlock-probe" sequence containing a target binding sequence is ligated end-to-end using DNA ligase to generate circular template. Binding of the target DNA to the complementary sequence on the circular template is followed by binding of Φ29, a polymerase isolated from bacteriophage Φ29, which then proceeds to "roll" around the circular template to create a linear ssDNA amplicon (Figure 2). The Φ29 polymerase is capable of debranching double-stranded DNA, precluding the need for thermal DNA denaturation. Furthermore, Φ29 can operate efficiently at temperatures between 35 and 40 °C, and has an innate exonuclease proofreading capacity that minimizes copy errors. As with conventional PCR, fluorescent probes can be used for quantification and assessing disease load. Another approach specific to RCA has been the incorporation of G-quadruplex sequences, i.e., guanidine-rich sequences, into the RCA template to facilitate the generation of DNAzymes during RCA.[31] These structures are capable of sequestering metal complexes such as the Hemin porphyrin, which can subsequently catalyze the oxidation of chromogenic substrates to produce a colorimetric signal. This strategy provides for a more rapid signal by exploiting the catalytic nature of both the RCA reaction and the oxidation of 3,3′,5,5′-tetramethylbenzidine (TMB) by the G-quadruplex-Hemin complex.

Due to the linear nature of the amplification, RCA is relatively slow (approximately a 10^3-fold amplification per hour); this can limit sensitivity in diagnostic tests, particularly when minimizing the total assay time is paramount. This drawback has somewhat decreased the popularity of RCA in PoC applications, although many successful examples exist.[32] Moreover, adaptations to RCA designed to improve the amplification rate have been moderately successful. One notable approach has been to utilize multiple target binding sequences on the linear padlock probe, and thus provide multiple points for primer attachment on a single circular template. This method, termed hyperbranching RCA (HRCA), enables concurrent amplification of multiple ssDNA targets from a single template and thus increases the overall rate of amplification.[33]

Strand Displacement Amplification. Strand displacement amplification (SDA) was first reported in the early 1990s,[34,35] and as such is one of the earliest described isothermal amplification techniques. SDA exploits specific restriction endonucleases to initiate strand dissociation, and so negates the need for thermal dissociation. The technique requires two target-specific primers, a suitable polymerase enzyme and the presence of a nicking exonuclease capable of cleaving the newly synthesized strand between the primer and target sequence. This nicking process allows partial dissociation and unwinding of the newly synthesized strand and the template strand, allowing a new polymerase to bind. As the polymerase moves along the template, the new amplicon displaces the old amplicon, eliminating the need for heat induced dissociation (Figure 2). Early reports of SDA required an initial denaturation step at 95 °C, so could not be considered truly isothermal, though modern developments have incorporated additional "bumper" primers to accomplish initial denaturation and achieve true isothermal amplification.[36] SDA has proven to be a flexible technique, and works well with multiple polymerases including Bst, Φ29, and Exo- Klenow; this flexibility allows SDA to operate at temperatures ranging between 21 and 65 °C. Depending on the polymerase used, target amplification between 10^7- and 10^9-fold in 20−120 min can be achieved using SDA. However, the dependence of SDA on restriction enzymes, which can only cut at specific restriction sites, is a limitation. Indeed, successful SDA requires that the primers are designed to bind upstream of an endogenous nicking site, thus allowing the restriction enzyme to cleave the primer and facilitate strand dissociation. The optimal temperature of operation for the nicking enzyme must also be considered. However, multiple nicking endonucleases for distinct restriction sites have been successfully employed in SDA, including Nb.BbvCI, Nt.BstNBI, HincII-*Eco*RI(Gl11), BsoBI, Nt.AlwI, Nt.BsmAI, and HincII. Unsurprisingly, several examples of SDA being employed in with PoC diagnostics for infectious disease have been reported.[36,37]

Helicase-Dependent Amplification. Helicase-dependent amplification (HDA), first reported in 2004 by scientists at BioHelix,[38] takes inspiration from in vivo cellular DNA replication, and achieves isothermal amplification by employing a helicase enzyme to perform dsDNA denaturation (Figure 2). Initial embodiments employed UvrD helicase isolated from *E. coli.*, though the process was rapidly adapted to work with Tte-UvrD, a thermostable helicase capable of operating at significantly higher temperatures.[39] HDA is flexible with regard to polymerase, and has notably been implemented using Bst, Gsp, and Gst polymerases, among others.[40,41] As with SDA, this theoretically enables efficient operation across a broad range of temperatures. However, helicase-mediated strand separation is most efficient at 60 °C; thus, for isothermal amplification, a polymerase that operates in the range of 60−65 °C is usually chosen.

Despite being an exponential technique, the amplification efficiency of HDA is relatively low, achieving a 10^6-fold amplification after 1 h. This is likely due to the unwinding of DNA by helicase, which is the rate-determining step, and significantly constrains the sensitivity of systems based on HDA.[42] Additionally, early iterations of HDA suffered from low specificity due to the reliance on long primers, which can lead to false positives in diagnostic applications.[42] Despite these limitations, HDA has been successfully incorporated into PoC IVDs for HIV,[43] *Staphylococcus aureus*,[44] and *Herpes simplex* virus,[45] among others.[42]

Nucleic Acid Sequence-Based Amplification. Nucleic acid sequence-based amplification (NASBA) is a partially isothermal technique that mimics retroviral RNA replication, generating ssRNA as the product. NASBA was first introduced in 1991,[46] and has remained popular because it works innately with RNA, rather than DNA, making it ideal for detecting single-stranded viral RNA or endogenous RNA (e.g., mRNA or miRNA). NASBA requires two primers (forward P1 and reverse P2), as well as three enzymes: T7 DNA dependent RNA polymerase, RNase H, and reverse transcriptase. The process works optimally at 41 °C, though an initial primer annealing that requires higher temperatures (55−65 °C) step is necessary. The coordinated action of reverse transcriptase and RNase H produces single-stranded DNA, precluding the need for high-temperature strand dissociation. Target specificity is imparted by the P1 forward primer, which contains a region that is complementary to the target RNA, and another

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

distinct region that is complementary to a T7 promoter region (Figure 2).

NASBA is an efficient amplification process and is able to amplify RNA by 10^9-fold in 90 min. Though NASBA is regarded as a robust isothermal method, it is not without disadvantages. As RNA stability can be greatly impacted by pH, temperature, and the presence of RNase, additional precautions must be taken during sample preparation and reaction assembly. Moreover, the enzymes required for NASBA are also thermolabile and can suffer significant performance degradation if the temperature goes above 42 °C.[47] Despite this, examples of NASBA being effectively utilized within PoC systems for infectious diseases do exist. Gulliksen et al. employed NASBA in a proof-of-concept PoC device for detecting human papillomavirus (HPV),[48] and the RNA can be detected through the use of reverse transcriptase. This technique is also integral to the emerging synthetic gene network/CRISPR diagnostics reported by Collins and co-workers for the detection of Zika virus (see Enzyme-Assisted Signal Generation and Translation-Based Approaches).[49]

■ ENZYME-ASSISTED SIGNAL GENERATION

Enzymes have evolved an incredible variety of mechanisms, and the range of chemical and biochemical processes that they can catalyze is exceptionally broad. These processes can be readily exploited to generate signals in the presence of disease biomarkers. In this section, we will highlight some of the more commonly employed approaches.

CRISPR-Cas Enzymes. Enzymes that catalyze the degradation of nucleic acids (nucleases) are common, and have been employed in nucleic acid amplification for the last several decades (see Enzyme-Assisted Nucleic Acid Amplification). More recently, various nucleases have found use as tools for gene editing, though many groups are now beginning to harness their potential for diagnostic applications. Of the various gene editing tools available, the CRISPR-Cas systems have established themselves as the most useful in terms of diagnostics. Clustered regularly interspaced short palindromic repeats (CRISPR) provides adaptive immunity against invasive genetic elements in bacteria and archaea. CRISPR notably allows for the processing and storage of exogenous invasive genetic sequences in the form of short DNA sequences (protospacers). Once integrated, these pathogen-specific sequences are transcribed and processed into small noncoding RNAs, which can subsequently bind to Cas nucleases and guide them to selectively degrade the corresponding target disease nucleic acids. CRISPR-Cas systems are currently categorized into two classes, class 1 and class 2, with further subcategorization into types and subtypes.[50] Determination of the CRISPR-Cas class is guided primarily by the makeup of the Cas effector protein complexes; Class 1 systems typically comprise multiple proteins that complex to form an active nuclease, whereas Class 2 systems are characterized by a single effector Cas nuclease. The types and subtypes are typically categorized by the presence of a signature Cas protein, or complement of multiple Cas proteins. The capacity to reprogram these nucleases in vitro to target specific nucleic acid sequences, e.g., disease-specific targets, has established Cas enzymes as promising candidates for diagnostics applications. Below, we will highlight the properties of four distinct Cas proteins (Cas9, Cas12, Cas13, and Cas14), and highlight their promise in the context of infectious disease diagnostics.

Cas9. Cas9 (class 2, type II) is an RNA-guided endonuclease that primarily cleaves dsDNA,[51] but specific orthologs of the protein have also been shown to promote the cleavage of ssDNA or ssRNA under certain conditions.[52,53] The specific targeting and function of Cas9 is guided by two RNAs, CRISPR RNA (crRNA) and trans-activating CRISPR RNA (tracrRNA). The crRNA is formed by cleavage of a crRNA precursor (pre-crRNA) directly transcribed from the CRISPR loci, and can base pair with complementary protospacer sequences of invading viral or plasmid target. Importantly, the crDNA contains a spacer sequence which can be modified to target a specific nucleic acid target. The tracrRNA participates in the maturation of pre-crRNA, and associates with crRNA to form an essential base-paired structure within Cas9. Importantly, Jinek et al. demonstrated the possibility of fusing crRNA and tracrRNA to form a single guide RNA (gRNA) without compromising the capacity of the protein to cleave dsDNA.[51] After binding between the crRNA (or gRNA) and the target sequence, the Cas9 enzyme is activated and the target DNA can be cleaved (Figure 3). It

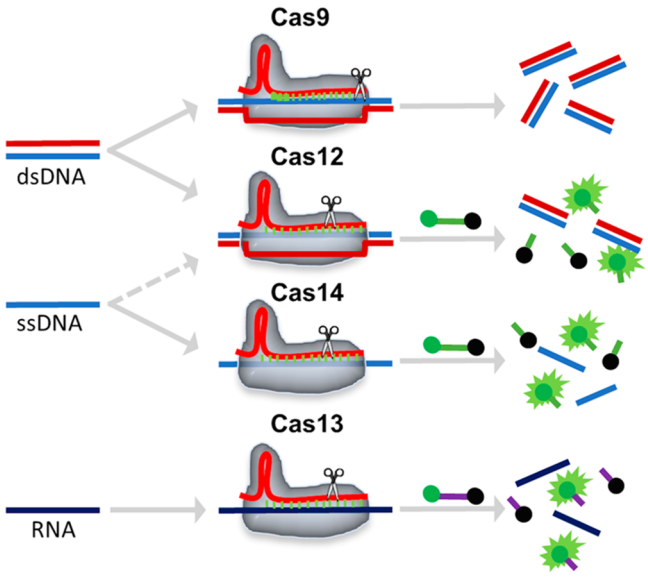


Figure 3. Schematic representations of different Cas enzyme activities. Cas9: Binding of target dsDNA to the gRNA−Cas9 complex is followed by cis-cleavage of the DNA. Cas12/14: Binding of the target ds/ssDNA to the gRNA-Cas12 complex and subsequent cis-cleavage of the DNA is followed by collateral trans-cleavage of ssDNA molecular beacon probes to generate fluorescence. Cas13: Binding of the ssRNA to the gRNA-Cas13 complex and subsequent cis-cleavage of the RNA is followed by collateral trans-cleavage of ssRNA molecular beacon probes to generate fluorescence.

should be noted that the presence of a specific sequence on the target DNA, termed the protospacer adjacent motif (PAM), is required for Cas activation. In the case of Cas9, the PAM is a 5′-NGG-3′ sequence which is present downstream of the target sequence.

Though not as widely employed for diagnostics as Cas12 and Cas13 (see following sections), there are examples of Cas9 being used within infectious disease RDTs.[54] For example, Pardee et al. successfully combined Cas9 with toehold switches (see Translation-Based Approaches) to enable differentiation of the American and African strains of Zika virus.[49] The team designed a gRNA specific to a nucleic acid sequence upstream

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

of a PAM region on American ZIKV, enabling Cas9 to cleave the dsDNA produced during NASBA. The cleaved dsDNA was unable to undergo transcription and translation, and thus expression of the reporter protein (β-gal) was repressed. The African ZIKV did not contain the necessary gRNA/PAM combination, and thus expression was able to continue unimpeded, producing a colorimetric signal to indicate disease load and differentiate the two strains. Huang et al. also employed Cas9 to develop a primer-free DNA biosensor capable of detecting DNA down to low attomolar concentrations.[55] In this elegant strategy, the DNA released after Cas-mediated cleavage serves to initiate an isothermal exponential amplification reaction (EXPAR), producing a fluorescent readout. By employing reverse transcriptase alongside this technique, the group were able to detect RNA from *L. monocytogenes*, the bacteria responsible for listeriosis, in spiked samples. Taking a slightly different approach, Müller et al. designed Cas9-gRNA complexes able to selectively target and cleave genes associated with antibiotic resistance in plasmids isolated from *E. coli* and *K. pneumoniae*.[56] By coupling the Cas9 enzymes' ability to cleave plasmids in particular locations with optical DNA mapping, the group were able to quantify the prevalence of different resistance genes in patient samples. In a similar approach, Crawford and co-workers combined Cas9 with next-generation sequencing to detect antibiotic resistance genes in their Finding Low Abundance Sequences by Hybridization (FLASH) system.[57] Though these systems are not yet being employed at the point-of-care, it is possible that the technology could be moved in that direction in the future.

Cas12/14. The success of Cas9 for both genome editing and biosensing led to researchers devoting significant effort toward finding and exploiting alternative CRISPR-Cas systems. This line of research proved fruitful, and multiple new Cas enzymes were successfully characterized. Cas12a (class 2, type V) is capable of targeting and cleaving dsDNA and ssDNA, and, like Cas9, contains a PAM region (5′-TTTV-3′) which directs the cleavage of the nucleotide after complexation with the gRNA. However, Cas12 enzymes have an additional catalytic activity which facilitates a novel modality for generating signal. After initial cleavage of the dsDNA, termed cis-cleavage, Cas12a goes on to randomly cleave nearby ssDNA via a process known as trans-cleavage, or "collateral" cleavage. This trans-cleavage occurs indiscriminately and requires minimal sequence specificity. Thus, quenched ssDNA probes can be combined with the molecular specificity of the Cas12a-gRNA complex to develop all-in-one systems that generate fluorescence in the presence of target DNA. Chen et al. demonstrated this in their DNA Endonuclease-Targeted CRISPR Trans Reporter (DETECTR) assay. In this assay, the target DNA, amplified using RPA, and Cas12a-gRNA complex are first mixed together, followed by addition of ssDNA probes carrying a fluorescent dye and a quencher. After cis-cleavage of the target DNA, the collateral trans-cleavage was able to release the fluorophore from the quencher to provide a concentration-dependent signal.[58] The team used this technique to positively identify DNA associated with human papillomavirus in clinical samples, and obtained results that were in agreement with PCR. More recently, Broughton et al. combined the DETECTR platform with RT-LAMP to detect RNA associated with SARS-CoV-2.[11] The team combined this with reporter probes that could be captured on a lateral flow test line to develop a paper-based PoC test with a limit-of-detection of 10 copies per microliter in 40 min. Similar assays based around the trans-cleavage mechanism of Cas12 have been reported. One example is the Hour Low-Cost Multipurpose Highly Efficient System (HOLMES),[59] which utilizes PCR for the initial amplification, and its second-generation analogue HOLMESv2 which employs a Cas12 orthologue (Cas12b) and LAMP.[60] These techniques have been shown to detect target DNA down to 10 aM in biofluid samples.

The collateral cleavage activity of Cas12a is shared among other reported type V CRISPR-Cas nucleases.[61] Of particular note are the Cas14 (class 2, type V) enzymes, which are typically much smaller than Cas12 enzymes (~400−700 amino acids vs ~1000 amino acids) and demonstrate exquisite selectivity for ssDNA targets. Doudna and co-workers showed that Cas14a1 was able to identify ssDNA with single-nucleotide specificity independent of a PAM sequence, and also displays efficient collateral cleavage.[62] The team were able to incorporate this enzyme into a redesigned DETECTR assay to detect specific single-nucleotide polymorphisms conferring blue and brown eye phenotypes. To the best of our knowledge, Cas14a has not yet been employed for infectious disease diagnostics, though we assert that its ability to differentiate single nucleotide mismatches presents a powerful diagnostic opportunity.

Cas13. While Cas9 and Cas12/14 enzymes evolved to recognize DNA, Cas 13 (class 2, type VI) enzymes recognize RNA with a high degree of specificity. Similar to their type V counterparts, Cas13 enzymes possess collateral cleavage activity, though most Cas13 orthologues require a protospacer flanking site (PFS) rather than a PAM to initiate cleavage. The combination of these two traits makes them attractive for use in diagnostics. In 2017, the Collins and Zhang groups reported the Cas13a-driven Specific High Sensitivity Enzymatic Reporter Unlocking (SHERLOCK) platform.[63,64] The SHERLOCK system employs either RPA or RT-RPA to produce a DNA template, which is subsequently amplified into RNA amplicons that feed into Cas13a. This triggers collateral cleavage of RNA quencher−fluorophore pairs to produce a concentration dependent fluorescent signal. The system was able to detect specific strains of Zika and Dengue virus at attomolar concentrations, and also differentiate single-nucleotide polymorphisms.

Interestingly, reported Cas13 orthologues have displayed different dinucleotide specificity (e.g., PolyA/GA for PsmCas13b, PolyU/UA/UC for CcaCas13) with regard to their collateral cleavage activity. This has enabled multiplexed assays in which different RNA targets can be coupled with different Cas13 orthologues (or even Cas12/14) and orthogonal fluorescent molecular probes.[65] Akerman et al. leveraged this strategy in their Combinatorial Arrayed Reactions for Multiplexed Evaluation of Nucleic Acids (CARMEN) assay.[66] This assay relies on nanoliter droplets containing the CRISPR reagents, and was notably able to detect up to 4500 targets on a single microarray chip. Though the relatively complexity of this approach reduces its appeal for PoC applications, the low volumes significantly minimize reagent usage, and thus substantially reduce the overall cost per test.

As previously demonstrated for genome engineering, the flexibility offered by CRISPR-Cas nucleases, i.e., their ability to be rapidly reprogrammed to target novel sequences using simple synthetic gRNA, has enabled them to revolutionize the field of nucleic acid biosensors. This flexibility facilitates the rapid development (within weeks) of tests in response to novel

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

infectious disease outbreaks. CRISPR-based systems, coupled with isothermal amplification, frequently achieve sensitivities that parallel PCR, and can easily be adapted to work with PoC friendly systems (e.g., paper-based lateral flow). On 6 May 2020, the FDA granted Emergency Use Authorization (EUA) to the SHERLOCK CRISPR SARS-CoV-2 kit, making it the first CRISPR-based diagnostic to be authorized by the FDA for clinical use.[67] We predict that CRISPR-based diagnostics will play an important role in infectious disease RDTs.

Redox Enzymes. Redox enzymes generate signal through their ability to oxidize or reduce substrates, such as chromogenic small molecules or nanoparticles,[68] to produce a detectable change e.g., in the optical or electrochemical properties of the substrate. Redox enzymes are a popular choice for immunoassays, particularly ELISA, due to their ability to rapidly produce a colored signal that can be easily analyzed using a variety of techniques. Unsurprisingly, these enzymes have also been adapted to aid in signal generation in nucleic acid diagnostics; some of the more commonly used approaches are highlighted below.

Oxidases. Oxidase enzymes catalyze oxidation−reduction reactions, commonly utilizing molecular oxygen as the electron acceptor and hydrogen as the electron donor to reduce oxygen to water or hydrogen peroxide. The reduced species, such as hydrogen peroxide, can subsequently go on to induce a signal through downstream redox processes. The most common oxidase enzyme is glucose oxidase (GOx), which oxidizes glucose into glucono-1,5-lactone, producing hydrogen peroxide in the process. The redox process can be detected directly, commonly via electrochemical methods. For example, Zhang et al. reported a system based on electrochemiluminescence that was able to detect single nucleotide mismatches down to 1 pM. The group developed a sandwich assay using thiolated oligonucleotide capture probes, biotinylated oligonucleotide detection probes, and an avidin−GOx conjugate. The hydrogen peroxide produced by the GOx in the sandwich complex was used oxidize luminol to 3-aminophtalate, producing incident photons as a measurable signal.[69] While the team used this method to determine mutations in genes associated with breast cancer, this technique could be generalized and used for infectious disease monitoring.

Peroxidases. Peroxidase enzymes catalyze the splitting of peroxidases into either water or alcohol in the presence of an electron donor. The most popular and commonly employed peroxidase is horseradish peroxidase, a metalloenzyme that derives its redox capacity from an iron-containing heme group. A variety of chromogenic organic compounds that produce a color change upon oxidation, such as 3,3′,5,5′-tetramethylbenzidine (TMB) or 4-chloro-1-naphthol/3,3′-diaminobenzidine tetrahydrochloride (CN/DAB), have been developed to exploit the redox activity of peroxidases. Though most commonly employed in protein-based sandwich immunoassays, there have been several reports of HRP being used to generate signal for the detection of nucleic acids in a similar fashion. Bodulev et al. utilized HRP-labeled oligonucleotide detection probes to specifically hybridize to hepatitis B viral DNA that was captured onto microtiter plates.[70] By exploiting the HRP-mediated oxidation of 3-(10′-phenothiazinyl)-propionic acid/*N*-morpholinopyridine, the group was able to detect the target DNA down to 3 pM using a chemiluminescent signal. A more recent and promising application of peroxidases is in electrochemical signal generation. Many electroactive species can be utilized for electrochemical detection, often with excellent sensitivity. For example, Zhang et al. utilized HRP-modified DNA probes to oxidize TMB, and measured the resulting electrochemical signal using a gold electrode. The group was able to measure genomic DNA from *E. coli* down to 1 pM when coupled with PCR amplification.[71] Hydroquinone and *o*-phenylamine-based substrates have also been employed in a similar fashion for electrochemical detection.[72,73] A similar approach was taken by Mao and co-workers, who attached biotin-conjugated DNA capture probes to a gold electrode. Upon binding of the target DNA, a conformational change enables binding of streptavidin-HRP to the electrode. Subsequent oxidation of hydroquinone by HRP could be detected electrochemically.[74] The group were able to achieve a limited of detection of 0.1 nM, and could specifically detect a single base mismatch in their target ssDNA.

TRANSLATION-BASED APPROACHES

The vast majority of work in the field of nucleic acid biosensing has focused on exploiting the enzymes involved in transcription (see Enzyme Assisted Nucleic Acid Amplification). However, recent developments have moved one step further and begun to utilize translational catalytic machinery. New technologies that enable precise control over translation have enabled researchers to couple protein expression to the presence of a target analyte, and thus harness the power of synthetic biology for biosensing. By utilizing common reporter proteins, researchers have been able to develop expression-driven assays that can be easily and quickly interpreted.

Translational Riboregulators. The primary method for controlling translation is through riboregulation, i.e., by regulating the catalytic ability of ribosomes to translate mRNA into proteins. Methods for controlling translation first gained traction at the turn of the 21st century, most notably through the use of antisense RNA and RNA silencing strands to down-regulate (silence) gene expression.[75] Seeking ways to expand upon this, several teams have reported methods to enable gene activation as well as deactivation. The majority of these systems rely on preventing ribosomal binding by introducing secondary structure into RNA sequences.[76−78] The conformational change caused by this secondary structure prevents ribosomal binding and effectively halts translation. Introduction of cognate RNA that can hybridize specifically to the altered structure can relieve the steric hindrance, enable ribosomal binding, and in turn initiate translation (Figure 4). For biosensing, these systems are designed so that the target nucleic acid fulfills the role of the cognate RNA responsible for initiating translation. Despite their benefits, early nucleic acid riboregulators were somewhat limited in their application. The degree to which these early switches are able to modulate gene expression is low; gene activation is typically enhanced 55−100-fold, and gene repression is lower still.[79−81] This leads to relatively low signal-to-noise ratios, and hinders both the sensitivity and dynamic range of any resulting biosensor. Fortunately, several technologies that address the issues with traditional riboregulation have been developed, primarily through de novo and in vitro synthetic RNA engineering.

Toehold Switches. Early development of riboregulators highlighted a number of limitations due to a reliance on ribosome binding site (RBS) binding to regulate translation. This reliance introduces significant sequence constraints into the resulting trigger RNAs, which in turn limits the number of targets and results in reduced specificity. To circumvent this

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

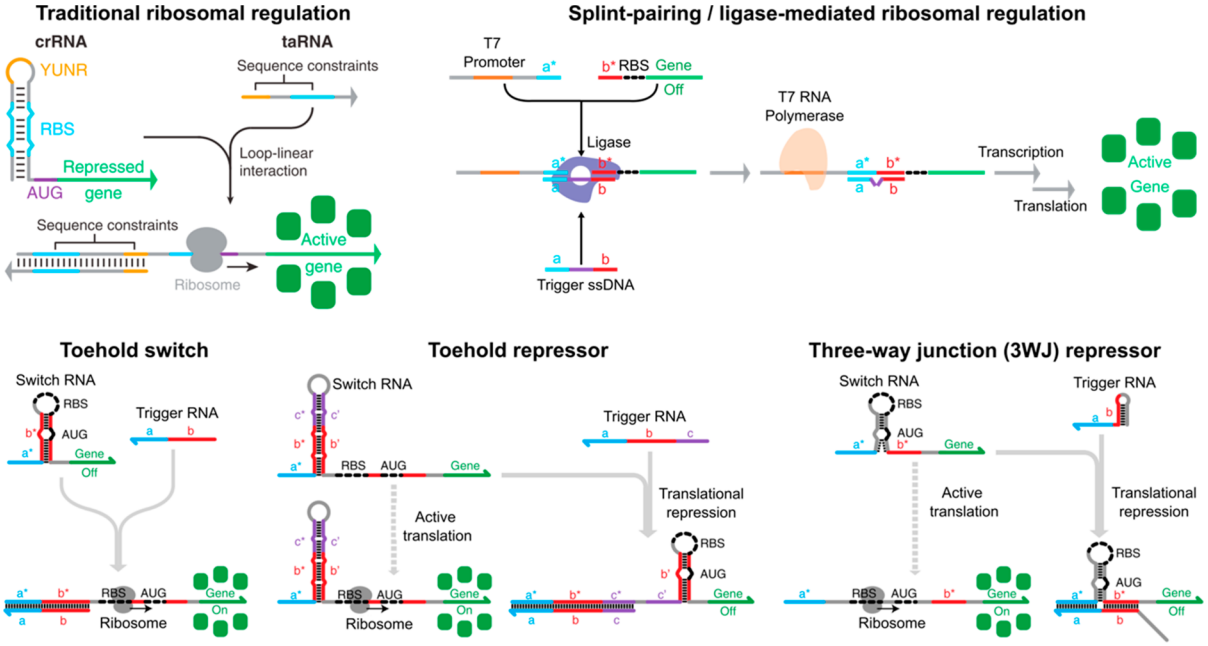


Figure 4. Schematic representations of different methods for regulation translation. Traditional ribosomal regulation: A transducer RNA contains the RBS sequestered in a loop. Binding of a trans-acting RNA (taRNA) results in a conformational change that relieves the sequence constraints and facilitates translation. Toehold switches: A transducer (switch) RNA in which the RBS is sequestered by an upstream "toehold" switch. Binding of a trigger RNA relieves the constraints and facilitates translation. Toehold repressors: A switch RNA in which the RBS is not sequestered is able to actively translate the gene of interest. Upon binding of the trigger RNA, conformational restraint is introduced to the RBS via a strong hairpin structure and translation is repressed. Three-way junction (3WJ) repressors: A switch RNA in which an unstable hairpin structure sequesters the RBS and start codon, but still allows ribosomal binding and thus translation. Binding of the trigger RNA creates a three-way junction and translation is repressed. Splint-pairing: Separate ssDNA sequences containing a T7 promoter and gene of interest are brought into close proximity by a target (trigger) sequence containing complementary ends on both. DNA ligation is performed by a ligase, generating an intact cassette which can transcribe and translate the gene of interest. The figure for traditional ribosomal regulation was reprinted with permission from ref 82. Copyright 2014 Elsevier. The figures for toehold switch, toehold repressor, and 3WJ were reprinted by permission from ref 84. Copyright 2019 Springer Nature.

issue, Green et al. developed de novo designed, in vitro engineered synthetic riboregulators, termed toehold switches, which are able to sequester the RBS by introducing a synthetic upstream sequence.[82] Binding of the trigger RNA to the toehold switch still efficiently relieves RNA secondary structure hindrance on the RBS and thus modulates translation, but is no longer reliant on sequence complementarity with the RBS (Figure 4). Furthermore, due to their reliance on engineered linear−linear rather than linear−loop interactions, toehold switches display more favorable binding kinetics and thermodynamics, leading to more efficient riboregulation and higher levels of expression. The authors reported enhanced expression (up to 400-fold) over negative controls and minimal cross-reactivity between different switches and targets. Since this first publication, multiple improvements on toehold switch technology have been reported, including the development of toehold repressors, three-way junctions (3WJs) (Figure 4), and single-nucleotide-specific programmable riboregulators (SNIPRs).[83,84] These improvements vastly increase the capacity for target multiplexing, and facilitate specificity down to the single nucleotide level. Overall, the use of synthetic RNA sequences in riboregulation has led to lower background, improved dynamic range, and increased specificity in the resulting protein expression. Such benefits drastically improve the utility of riboregulation for a range of diagnostic applications.

The main barrier to the early development of biosensors based on riboregulation was the reliance on cellular machinery. Early investigations were cell-based and exploited the natural machinery of prokaryotic cells to achieve translation.[76,77] However, these systems are not amenable to disease biosensing, since they are typically operated by trigger RNA produced within the cell. Sensing external target nucleic acids would require transport across the cell membrane prior to detection. Fortunately, in vitro transcription/translation (IVTT) systems provide an effective means to circumvent this issue. IVTT systems utilize cellular extracts to perform protein expression outside of the cell, thus presenting opportunities to operate riboregulators using extraneous nucleic acid targets. Cellular extracts are typically isolated from prokaryotic cells, which can be easily and rapidly scaled up to industrial quantities, though "bottom up" systems built from recombinantly expressed proteins are becoming more popular.[85] The flexibility provided by cell-free expression has sparked interest in the use of IVTT for disease biosensing.

Though the technology is relatively novel, infectious disease biosensors based on synthetic RNA riboregulators have already been developed. For example, Pardee and co-workers developed a toehold switch-based biosensor for that was capable of discerning Zika virus RNA sequences from closely related Dengue virus sequences.[49] Using computational methods, the authors scanned the Zika virus genome to determine disease-specific target sequences and filter out sequences with potential cross-reactivity with the human genome or closely related viruses. A library of toehold switches specific to the identified targets was subsequently designed and

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

used to repress the expression of β-galactosidase on a synthetic gene. β-Galactosidase was used as a reporter protein since its activity can be readily assessed using common chromogenic substrates. Using this approach, the team were able to identify 25 (52% of the total) toehold switches capable of modulating an increase in β-galactosidase expression in the presence of isolated Zika virus RNA, with the best switch demonstrating a 34-fold increase over background. Importantly, no cross-reactivity with homologous sequences in the related Dengue virus was observed. In the optimized format, and without further amplification, the test was able to detect Zika RNA at a concentration of 30 nM. Moreover, when coupled with an isothermal amplification technique (NASBA), detection of clinically relevant levels of Zika virus (3 fM) was possible. To facilitate the use of the test at the point-of-care, the team utilized a paper-based format consisting of an IVTT mixture freeze−dried onto paper discs (see Point-of-Care Systems). This is particularly important in the context of Zika virus, where the disease burden is typically largest in resource-limited settings. Importantly, the authors emphasized that the rapid development pipeline of this technology makes it amenable to fast prototyping in the advent of a new infectious disease outbreak. Indeed, their specific toehold switch library was designed within 4 days, with subsequent screening and device manufacture taking a further 2 days.

Splint-Pairing. An alternative method for controlling translation is through the use of ligase-mediated splint-pairing to generate functional expression cassettes (Figure 4). In splint-pairing, a designated target sequence is responsible for mediating the ligation of a gene encoding for a specific reporter protein and a sequence containing a transcriptional promoter region. In the absence of the target the gene cannot be expressed, as it lacks a promoter, but in the presence of the target, a DNA ligase can couple the gene to the promoter and transcription can occur, followed by translation. This process requires a suitable ligase enzyme, e.g., DNA ligase or SplintR ligase, to facilitate efficient construction of the expression cassette.[86] To the best of our knowledge, there have been no reports in the academic literature of splint-pairing being used specifically for infectious disease biosensing, although its ability to detect miRNA has been demonstrated.[87] Splint-pairing forms the basis of Sherlock Biosciences INSPECTR (Internal Splint-Pairing Expression Cassette Translation Reaction) molecular diagnostics platform, and thus we expect that many examples of splint-pairing for infectious disease diagnostics will be forthcoming.[88]

It is clear that emerging developments have begun to solve many of the prohibitive limitations associated with early translational modulation, and have increased the applicability of this approach for PoC diagnostics. The rapid advances in de novo and in vitro nucleic acid engineering, coupled with the advent of accessible IVTT technology, have led to significant increases in the sensitivity, specificity, and practicality of translation-based sensors. Early-stage diagnostic tests based on this technology have demonstrated exquisite specificity, point-of-care utility, and the potential for rapid adaptation to new diagnostic challenges. While we have limited the scope of the current discussion to applications within nucleic acid PoC biosensing, it is important to highlight that these technologies are finding significant use in biosensors for other analytes, such as organic molecules and amino acids.[89−91] We direct the readers to reviews by Silverman and Lee for more comprehensive overviews.[85,92]

POINT-OF-CARE SYSTEMS

System Requirements. We have previously highlighted the vast array of different enzymes that can be employed for nucleic acid amplification, and the different signals that enzymes can generate to detect nucleic acids. It is imperative that any RDT designed to take advantage of enzymes be capable of exploiting these functions (Figure 5A). While these features are essential for any nucleic acid IVD, there are additional contextual requirements for field-deployable or PoC devices, particularly in the context of infectious disease. These requirements broadly parallel the REASSURED criteria, with a few notable additions specific to enzyme-assisted nucleic acid detection.

Biocompatibility. To fully exploit the benefits of enzymes, it is imperative that a system is capable of maintaining an environment that supports their function. This includes important chemical and physical factors such as pH, salinity, cofactor content, temperature, and pressure. Materials, electronics, physical dimensions, and mode of operation can all be tweaked to influence these parameters. The approach taken is often specific to the type of system employed, and the context in which the diagnosis is going to take place. For example, large benchtop systems are typically able to take advantage of simple heating elements and premanufactured reaction cartridges to maintain temperature and pressure. Conversely, microfluidic devices require a more carefully considered approach, with factors such as material, device footprint, and flow rate playing an important role in regulating pressure and temperature.

Signal Transduction Capacity. As previously highlighted, enzymatic mechanisms can be transduced into a variety of signals, including colorimetric, fluorescence, and electrochemical, and diverse schemes for each of these signals have been developed. These detectors vary in complexity and can provide different degrees of sensitivity and dynamic range, depending on the need. The simplest colorimetric systems can even take advantage of the human eye; this is particularly useful for PoC IVDs as it greatly minimizes reliance on external technology. While this approach is purely qualitative, it is still useful for infectious diseases where a positive confirmation of disease holds value. Fortunately, the simple addition of a CMOS sensor, even within a smartphone camera, can be enough to enable quantitative interpretation of systems designed around colorimetric signals. Systems built around fluorescence generally require further engineering, though they commonly offer greater signal-to-noise compared to colorimetric signals. Fluorescence detection has successfully been incorporated into a variety of commercial tests, and is particularly amenable to microfluidic systems where the small volumes enable high localized concentrations of fluorescent substances.[93] Historically, electrochemical signals have not been widely reported for PoC applications, with a few notable exceptions (e.g., glucose monitoring). Nevertheless, recent advances in miniaturization have facilitated the development of micrometer-scale electrical components and renewed interest in electrochemical signals for PoC nucleic acid diagnostics.

Usability. One of the primary REASSURED criteria is "user friendliness", and highlights the importance of designing a system that is both practical for its application and easy to operate. Multiple factors determine how "easy" a device is to operate in a PoC setting, including portability, user interface, robustness, and degree of automation. An ideal system will be

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

able to meet the disease-specific requirements for sensitivity and specificity, while maintaining a portable and robust form factor, simple user interface, and be able to automate the entire analytical process from sample preparation through to signal output. Automation is of particular importance for infectious disease diagnostics, where manual human input can increase operator risk, in addition to introducing errors into the process.

Manufacturability. The ultimate utility of a diagnostic system is dependent on the ability to deliver it to the desired location, at an affordable price and in sufficient quantity to meet demand. System simplicity is clearly favorable, though multiple factors can affect the manufacturability of a device. The ability to source the necessary materials globally, rather than relying on a single material source, is advantageous, particularly in the context of infectious disease epidemics where transport chains can be easily disrupted. Minimizing reliance on cold-chain transport is also beneficial as it can dramatically reduce transport costs. In the context of enzyme-dependent systems, methods for lyophilizing enzymes and their substrates onto paper or other easily transportable materials (to enable transportation at ambient temperatures) are being explored.[94] Due to their low manufacturing costs, and the simplicity of the resources required to manufacture them, paper-based devices are particularly promising candidates when large scale, global manufacturing is paramount. A major challenge here is the robustness of such devices, particularly with respect to their ability to withstand the varied environmental conditions experienced during the supply chain. Although industrial partners are ideally suited to tackling these issues, the reality of these requirements must be considered from the outset.

Adaptability. An ideal diagnostic platform can be quickly and easily retargeted to many different diseases with minimal changes in operation. This is particularly important for infectious disease diagnostics due to the possibility that rapid mutations will lead to novel strains, invalidating existing diagnostics tests within short time periods. If a system can be easily adapted to a new strain, then it can be more rapidly deployed. For more complex systems, there is also the additional burden of building the prerequisite infrastructure and training personnel. Utilizing a single system for multiple diseases can minimize this impact, since a single user can run multiple tests simultaneously. Once again, the current COVID-19 pandemic exemplifies perfectly the benefits of adaptable systems; the most promising systems (Abbott ID Now, Cepheid GeneXpert) utilize existing frameworks, and have been able to quickly adapt to the new challenge.

Connectivity. Once nucleic acid IVDs are moved from centralized laboratories and into the field, it is essential to have mechanisms in place to interpret test data and remotely transmit results to the appropriate authorities. Fortunately, the smartphone boom of the last ten years has provided technologists with powerful and portable solutions for connecting diagnostic systems. Indeed, many modern devices exploit smartphones themselves to connect their data when necessary.[95] The smartphone revolution has also helped to drive investment into global communication infrastructure, even in the developing world, and many countries have significantly improved their communications networks over the past decade.[96] These technological innovations are simplifying the process of developing connected diagnostic devices, and it is anticipated that going forward, most major diagnostic systems will include connectivity options. This concept of mobile health, or mHealth, is particularly key for the decentralization of diagnostics and the transition toward the end user.

Benchtop Systems. While benchtop systems are typically associated with centralized laboratories, many manufacturers have realized the need for smaller, more portable systems. Though these systems still require significant infrastructure, they can be effectively operated from regional or even field hospitals. These types of devices are termed "near point-of-care", and provide an important function in the fight against infectious disease.

Benchtop PCR. Recent outbreaks have highlighted the reliance of nucleic acid IVDs on PCR, and the historical prevalence of PCR-based benchtop IVDs reflects this.[97] The first thermal cycler for PCR was launched in 1987 by PerkinElmer, with the aim of limiting the interaction of the user with the system, thus streamlining the analytical process. Since then, devices have evolved to include higher levels of automation and real-time quantification capabilities (qPCR), though they still require significant input from trained personnel. Unfortunately, these benchtop instruments require power and can be highly sensitive to temperature variations, limiting their utility in resource-limited settings. Furthermore, the large number of temperature cycles fundamentally limits the assay time due to thermal inertia, and multiplexing capabilities remain limited.[98] Recent developments have focused on making PCR instruments more compact, battery powered, and smartphone connected, such as the two3 thermocycler from Biomeme. Similarly, a number of compact qPCR systems have been developed commercially, including Open qPCR from CHAIbio, Franklin from Biomeme, and Mic Real-Time PCR from Labgene Scientific. These systems provide multiple accessibility options including remote operation and data analysis using cloud-based frameworks. More recently, there has been a focus on incorporating additional workflows into existing PCR technologies to enhance diagnostic utility. For example, sample extraction is a common bottleneck in high-throughput diagnostics. To address this issue, the private sector is moving toward modular systems that integrate nucleic acid extraction and preconcentration, aiming to create fully automated sample-to-result platforms. Many of these platforms are highly versatile and can be rapidly adapted to specific diseases and enzymatic amplification reactions, such as the QIAstat-Dx Respiratory 2019-CoV Panel by Qiagen.[99]

Isothermal Approaches. In recent years, isothermal amplification approaches have gained traction. By doing away with complicated temperature programs, the instrumentation associated with isothermal amplification can be greatly simplified, making isothermal methods highly promising as field-deployable IVDs. For example, Crannell and co-workers reported the use of body heat to regulate RPA reactions, an approach with obvious advantages at the point-of-care.[100] Similar PoC-friendly approaches have been combined with various detection methods including fluorescence and electrochemical detection,[101,102] and can be performed using lyophilized reagents (in test tubes) for in-the-field use.[101,103] Benchtop instruments for isothermal amplification, such as the ID Now by Abbott, are now commercially available and commonly employ disposable cartridges containing dried-down reagents.[104] With that said, these test tube-based, semiopen systems require a skilled operator to perform complex reagent manipulations, potentially exposing the assays

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

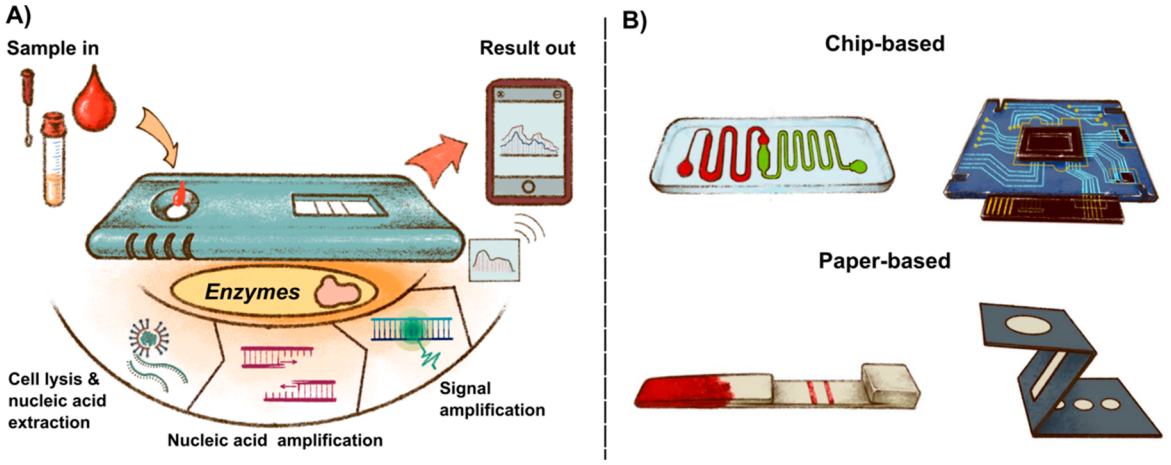


Figure 5. (A) Idealized point-of-care diagnostic utilizing enzyme-assisted nucleic acid biosensing. (B) Different microfluidic devices used in point-of-care diagnostics.

to contamination. Greater automation could help to minimize human interaction during the diagnostic pipeline and unlock the true potential of isothermal technologies.

Chip-Based Microfluidic Systems. Over the past three decades, microfluidic systems have transformed the way in which chemical and biological experiments are performed.[105] By shrinking the length scales associated with an analytical process down to the micrometer scale, both mass and heat transport can be significantly enhanced. This in turn means that fluidic mixing occurs in a rapid and controllable fashion, thermal homogeneity can be maintained across the system, and rapid heat transfer will occur between the device and the contained fluid. In practical terms, microfluidic technologies are ideally suited to perform complex operations on small volumes and have paved the way for portable diagnostic systems (Figure 5B).[106] Their ability to perform a range of functional operations (such as sample preconcentration, filtration, extraction, reaction, separation, and product detection) in an automated and integrated fashion enables the creation of fully integrated platforms able to perform sample-to-answer assays without the need for an expert operator.

On-Chip PCR. Microfluidic-based PCR was perhaps the first application of microfluidics in diagnostics.[107−109] In such systems, thermal cycling may proceed in a batch, continuous flow, or segmented flow fashion, with the extent of the reaction being monitored in real time or via end-point analysis. Flow-based systems are especially advantageous in this regard, since thermal masses are significantly reduced, and thus the time constraints associated with heating and cooling can be as short as a few milliseconds. Multiplexing can be achieved by parallelizing batch microsystems or by sequential addition of sample in flow-based systems.[110] It is important to note that monolithic systems comprising, for example, sample lysis, nucleic acid extraction, sample preconcentration, target amplification, and signal detection can be realized through standard micromachining methods.[109]

Several commercial platforms leverage proprietary microfluidic cartridges to achieve fully automated molecular diagnostic workflows (e.g., GeneXpert from Cepheid or Filmarray from bioMérieux). While such systems provide for fully automated workflows, with no user-controlled operation, their high cost, large size, and reliance on single-use cartridges somewhat limits their utility, restricting them to near point-of-care settings. Nevertheless, there are a number of reports of these systems being used successfully in RDTs for infectious disease. For example, Opollo and co-workers employed the GeneXpert HIV-1 Qual for in-field testing of infant HIV in Kenya and found that it performed well in comparison to the gold standard laboratory test.[111] Moroever, Andrews et al. compared the Filmarray system to traditional laboratory testing for monitoring of upper respiratory tract infections in the UK, and found that the microfluidic system facilitated faster treatment decisions.[112]

On-Chip Isothermal Amplification. The last ten years have seen a renewed effort in harnessing the power of nucleic acid amplification in increasingly miniaturized, hand-held devices.[113,114] Unfortunately, the majority of these are still at a very early stage of development and require significant off-chip manipulations during sample preparation and loading. The integration of nucleic acid extraction and amplification within microfluidic devices remains a significant challenge. Fortunately, isothermal amplification greatly simplifies the hardware required to perform nucleic acid amplification in chip-based systems, leaving more resources dedicated to on-chip sample pretreatment without compromising with respect to footprint or cost. For example, Yeh et al. recently presented the low-cost and self-powered "SIMPLE" platform for digital nucleic acid amplification.[115] Based on RPA, the chip-based system uses a pre-evacuated chamber to slowly draw fluid through a channel network. DNA extraction is achieved in one step using a branched design with a deep main channel. The extracted DNA, mixed with the RPA reagents, is then directed into an array of microwells where the reaction is triggered by the rehydration of prepatterned reagents and the fluorescence signal can be read. Using this approach, the authors were able to successfully detect methicillin-resistant *Staphylococcus aureus* bacteria, achieving a limit of detection of 10 DNA copies per μL. Delamarche and colleagues showcased the use of a silicon chip capable of dispensing enzymes and other RPA reagents in a timed and passive manner, through control of coalescence in capillary-driven flows.[116] Their approach, while still being preliminary from a diagnostic point of view, is simple and modular, and uses standard microfabrication technologies. The simplicity and portability of these approaches demonstrate the potential of microfluidic systems to perform complex biological workflows in a fully integrated manner.

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

When designing a microfluidic device, it is important to consider the range of signals that can be generated by the assay under investigation. As discussed previously, enzyme-assisted nucleic acid sensors can output a broad range of signals, each with their own advantages and disadvantages. A problematic aspect of fluorescence-based approaches remains the need for bulky and often expensive optical components (such as lenses, filters, cameras, and photodiodes), though progress is being made in developing miniaturized optical components for the quantification of fluorescence in small-volume environments, even using mobile phone cameras.[93,117−119] On the other hand, electrochemical detectors are intrinsically more compact than optical systems, with electrical signals readily processable using mobile phones. For example, Hsieh and co-workers reported a compact electrochemical microfluidic platform capable of detecting as little as 16 copies of *S. typhimurium* genomic DNA in a single microfluidic chamber.[120] In this system, LAMP reactions were monitored quantitatively in real-time using an intercalating electroactive dye. However, it should be noted that spiked DNA samples in buffer were used in all experiments, with no consideration of sample pretreatment in real-world application. That said, cell lysis and DNA preconcentration have been achieved within microfluidic platforms,[121] and we expect that fully integrated microfluidic systems with electrochemical detection will play a crucial role in bringing nucleic acid detection to the field. Finally, we would like to highlight that label-free detection methods have also been used to good effect in nucleic acid analysis. For example, Toumazou and colleagues fabricated microfluidic devices with integrated ion-sensitive field effect transistors using conventional CMOS fabrication methods.[122] Such a device is able to monitor ssDNA amplification in real time by taking advantage of the small change in pH that results from the release of hydrogen ions during amplification (the greater the initial concentration of the target, the more rapid the pH change). Although the technique has yet to be used for the detection of disease biomarkers, the method shows significant promise.

Paper-Based Systems. Microfluidic paper-based analytical devices (or μPADs) have emerged as a promising point-of-care diagnostic platform owing to their ultralow cost, biocompatibility, and relative independence from external power sources.[123,124] In recent years, several paper-based immunoassays have been approved by the WHO for malaria and HIV antigen/antibody detection.[125] μPADs for nucleic acid detection are currently less developed due to the increased complexity of the assays, but are the subject of intense research.[126,127] Several challenges remain with paper-based nucleic acid tests, including reduced specificity due to nonspecific adsorption, reproducibility, and storage lifetimes.

Paper-Based PCR. Lateral flow assays are the most recognizable μPAD format. Here, sample flows unidirectionally along a paper strip and the targets and/or laboratories are captured on a line for detection. In the case of a colorimetric label, the result can be read qualitatively by the naked eye, or quantitatively analyzed using digital cameras and image analysis software. The detection of PCR amplicons was among the first use of μPADs for nucleic acid detection.[128] In such applications, primers are typically modified with a hapten or a unique overhang so that they can be captured and labeled with plasmonic gold nanoparticles on the test line. Similarly, line probe assays have also been developed using the same approach and are now commercially available for the rapid detection of genes associated with resistance to first- and second-line antibiotics.[129,130] For example, the GenoType MTBDRplus assay system from Hain Lifescience can detect up to 27 different PCR amplicons on a single strip, using alkaline phosphatase enzymes to amplify the colorimetric signal. Multiplexing is achieved by printing the capture oligonucleotides sequentially along the flow path, with results analyzed as a barcode.

Motivated by the need for equipment-free methods for field testing, μPADs are becoming established tools for the quantification of isothermal amplification reactions. TwistDx is now offering TwistAmp info kits for RPA with a lateral flow colorimetric readout.[131] Amplification is typically performed in a test tube, and the lateral flow strip is subsequently dipped into the reaction mixture to generate a readout without the need for additional equipment. Similar approaches have also been developed using LAMP.[22,132] Due to the nature and large size of the amplification product, other strategies based on size discrimination have also been explored in LAMP-based techniques. For example, Du et al. reported a "signal-off" approach, using the fact that the LAMP product is too large to migrate through cellulose paper, effectively detecting the excess of unbound labels on the test line.[133] The team reported a limit of detection of 20 copies of the Ebola virus in spiked serum within 150 min.

Paper-Based CRISPR-Cas Diagnostics. After their initial discovery, Cas-based nucleic acid detection platforms were swiftly incorporated into μPAD systems. This is unsurprising, as the collateral cleavage activity of CRISPR-Cas13 combines ideally with probes commonly used for lateral flow assays. An early system, termed specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), developed in the Collins and Zheng laboratories at MIT, exploits oligomers with FAM- and biotin-modified ends to enable lateral flow readout.[63] After RPA, anti-FAM gold nanoparticles are added to the sample and flowed through a lateral flow strip modified with a streptavidin test line. The assay can then be read visually using the plasmonic properties of the gold nanoparticles. The power of this approach was showcased by detecting RNA targets for both Zika and Dengue viruses, and clinically relevant limits of detection were achieved in patient samples. Following on from this, the team reported SHERLOCKv2, in which they achieved multiplexing by employing orthogonal CRISPR-Cas systems and fluorophores.[65] Myhrvold et al. subsequently refined the SHERLOCK workflow to incorporate sample pretreatment, comprising reducing agents and two subsequent heating steps to lyse viral particles and inactivate ribonucleases found in bodily fluids.[134] This protocol enables testing for nucleic acids in unprocessed whole blood, saliva, or urine, thereby providing an instrument free sample-to-answer assay. A major advantage of Cas-based diagnostic platforms is that they can be rapidly adapted to new sequences, and thus new diseases. This adaptability was perfectly illustrated in the recent COVID-19 pandemic, with protocols based on RT-LAMP and the SHERLOCK or DETECTR platforms being rapidly developed once the virus had been sequenced.[11,135]

Paper-Based Translational Diagnostics. Perhaps one of the most promising technologies to be combined with μPADs is IVTT (see Translation-Based Approaches), demonstrating great potential in point-of-care nucleic acid IVDs.[49,94] Remarkably, all of the transcription/translation components required for this complex reaction network showed long-term stability when freeze−dried on paper, with activity remaining

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

even after a year of storage at room temperature.[94] When rehydrated with the diluted sample solution, the mixture is incubated on small paper discs for 2 h at 37 °C to yield a fluorescent or colorimetric signal. As proof-of-concept, Collins and colleagues developed a test capable of differentiating the Sudan and Zaire strains of the Ebola virus with good sensitivity.[94] The colorimetric test, based on a LacZ-expressing toehold switch, requires 25−60 min to complete and is quantifiable using a smartphone camera or an in-house developed optical reader (<100 USD). Each test is cheap (<65 cents per sensor) and quick to manufacture, and can be rapidly adapted to a new disease by high-throughput screening (240 reactions in parallel on a single sheet of paper). Still, as a standalone method, the detection limit of approximately 30 nM is insufficient for clinical application. In a follow-up study, the authors showed that synthetic gene networks can be combined with NASBA for increased sensitivity, and when integrated with an additional CRISPR/Cas9-mediated selection downstream of the amplification yielded single-nucleotide discrimination.[49] This approach resulted in a sample-to-answer test in under 3 h with low femtomolar sensitivity for both Zika and Dengue viruses. Nevertheless, the method still required a nucleic acid extraction step performed by a skilled user.

Fully Integrated Paper-Based Systems. A major limitation of the paper-based methods discussed thus far is that the sample preparation and amplification steps are performed "off-paper". This increases the number of liquid-transfer steps, increasing the chances of error. The reliance on such preprocessing is not surprising; the complete integration of a nucleic acid test on paper is a considerable challenge. Controlling all the necessary parameters (flow rate, temperature, concentration) for both extraction and amplification on paper is complicated by factors such as evaporation and the absorptivity of the paper itself. Additionally, cellulose paper has been shown to inhibit enzymatic isothermal amplification reactions,[136] though the exact cause of inhibition remains unclear. Nonetheless, many groups have found creative ways to exploit the peculiar properties of paper to perform complex operations, ranging from nucleic acid extraction to elaborate detection methods.[127] For example, Byrnes et al. showed that paper strips are able to extract DNA from cell lysate by flow separation, effectively removing possible amplification inhibitors for subsequent nucleic acid analysis.[137] The same group also demonstrated an in-flow DNA concentration method using chitosan-coated paper as a solid-phase material for anion exchange chromatography.[138] DNA capture and purification can also be performed with reasonable efficiency on cellulose paper.[139,140]

An interesting example of a highly integrated μPAD was reported by Cooper and co-workers for malaria detection in low resource settings.[141,142] This origami-based μPAD comprises different panels containing the necessary reagents for cell lysis and DNA amplification (via LAMP), which are folded to sequentially perform each step. Multiplexing is achieved by having a 2D-patterned layer to dispense the lysed sample to different reaction zones: up to five, including a positive control for LAMP.[143] The required temperatures can be realized by simply laying the device on a hot plate, or even a cooking stove, with the top and bottom layer covered with a plastic film to limit evaporation. Signal can be generated either directly via fluorescence or by contacting the bottom layer to multiple lateral flow strips containing dried nanoparticle labels. Their method is able to process crude clinical samples, such as whole blood, and if the sample is too viscous lysis can be performed off-device before dilution. This platform was recently deployed in rural Uganda for the diagnosis of malaria. Enzymes and reagents were stored and transported in lyophilized form and, once rehydrated, retained their activity for more than 3 days without refrigeration. Buffers for the LAMP reaction and the lateral flow assay were stored in sealed plastic chambers that opened when pressed with the finger, thus limiting the contact of users with the sample. During field tests, the device worked well, demonstrating 93% specificity and sensitivity when compared to qPCR. A similar origami-based approach was developed by Magro et al. for diagnosing Ebola using reverse-transcriptase RPA.[144] Here, the necessary reagents were freeze−dried onto the paper, and no significant reduction in the signal-to-noise ratio was observed after one month of storage; after three months of storage, a 20% loss in performance was reported. However, it should be noted that these stability tests were performed using high viral loads, and it is unclear how the limit-of-detection will be affected at lower concentrations. Yager and colleagues also presented a prototype of a fully integrated μPAD with no moving/foldable parts for methicillin-resistant *Staphylococcus aureus* detection from nasal swabs.[37] The 3D-printed device comprising several resistive heating blocks with associated electronics and a μPAD. Heat-actuated wax valves were printed on the channel to achieve automated timing of the various steps. Achromopeptidase, an enzyme with bacteriolytic properties, was dried in the fluid introduction tubing, and iSDA and detection reagents (nanoparticle labels and capture probes) were freeze−dried onto the lateral flow strips. The device provided robust results even when stored for more than 6 weeks at room temperature, and confirms the feasibility of fully automated, low-cost nucleic diagnostic tools for in-field testing. With that said, the prototypes did exhibit some reliability issues, in particular, overheating during valve actuation and nonideal flow conditions due to evaporation, thus highlighting the ongoing challenges associated with fully automated paper-based nucleic acid detection.

OUTLOOK

Throughout this review, we have highlighted some of the issues associated with the use of enzymes within PoC tests, while simultaneously acknowledging the unrivaled specificity, versatility, and efficacy that enzyme-assisted nucleic acid IVDs can achieve. Accordingly, we contend that technological developments that enable these assays to join the march out of centralized laboratories toward simple and robust PoC tests are extremely important. Using the recent COVID-19 crisis as a case study, we now illustrate the current state of PoC nucleic acid IVDs, before turning our attention to where we go next.

Where Are We Now? The initial stages of the COVID-19 pandemic highlighted the critical role of IVDs in infectious disease control.[145] A key issue in this respect has been the need for gene-based (i.e., nucleic acid) or antigen-based (i.e., protein) testing as opposed to serology; antibody testing has been ineffective at revealing early stage infections when people were at their most infectious.[146] Unfortunately, the unavailability of widespread and rapid diagnostics led to a severe data deficit in the initial phase of the disease, data that were urgently needed to inform critical time-sensitive interventions (i.e., test, trace, and isolate). Notably, regions that managed to perform extensive testing, e.g., South Korea, Germany, Singapore, Taiwan, and Hong Kong, had the greatest success

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

in containing early outbreaks, highlighting the vital role of epidemiology alongside healthcare.[147] As researchers in the field of IVDs, our community was uniquely positioned to analyze diagnostic efforts and draw conclusions to aid in future outbreaks. Importantly, we observed a deep gulf between the "state-of-the-art" technologies and the "ready now" technologies. For COVID-19, the "ready now" turned out to be the decades-old but reliable PCR, run in centralized laboratories by highly skilled professionals. This restricted the global capacity for testing, and often resulted in significant delays between sampling and result. A more agile technology, capable of providing faster diagnoses to a larger proportion of the population, would have substantially changed the course of the pandemic. As highlighted previously, at a fundamental research level, many technologies to facilitate this already exist; the problems arise primarily from our ability to translate these technologies from the research lab to the end user in a timely fashion. In the spirit of the REASSURED criteria, despite past experience and knowledge of the challenges,[148,149] we failed on the "D"—delivery. Delivery refers to the organizational structures and relationships established with the purpose of coordinating and steering the logistics of selecting, procuring, shipping, storing, distributing and delivering a new health technology to ensure it reaches the end users.[5]

Although the causes of our failure to deliver are myriad, particular problems include the danger of a market-driven approach to technology transfer in pandemic preparedness, the fact that the majority of funding remains in high income nations where research agendas are often disconnected from the needs of countries where outbreaks commonly originate, a general lack of funding and interest in IVD R&D compared to drugs and vaccines, and the inevitable politicization of actions surrounding a crisis. There could also be a case made that relevant higher-level decision makers were assuming that researchers would be able to deliver diagnostic technologies on demand, when in reality there needs to be a solid pipeline from fundamental research right through to implementation. We must find ways to solve these problems and facilitate efficient technology transfer if we are to successfully deliver the promising technologies coming out of the lab.

Where can we improve? Although many of the problems in the IVD development pipeline are beyond the control of technologists, there are distinct areas where we can effect improvements. By focusing on transferability from the outset of a project, rather than solely on novelty, we can ensure that the time and resources we invest in our research have the best chance of achieving real impact further down the line. Clear, concise, and easily accessible guidelines exist to help researchers develop effective IVDs. The REASSURED (see the Introduction) criteria stand out as widely applicable, comprehensive, and easily understandable by any technologist.[5] By considering these criteria from the outset of a project, we can design affordable PoC tests that mitigate common problems such as market forces, supply chain issues, and a reliance on highly trained personnel. In addition to REASSURED, national and international regulatory bodies provide comprehensive guidelines and requirements for IVD device development, designed to aid in the realization of marketable and deliverable products. Although the specifics differ between bodies, the process essentially divides into the following phases: proof-of-concept, assessment of technical feasibility (e.g., sensitivity, specificity, and precision in the relevant matrix), safety and performance evaluation, certification, market launch, and post-market surveillance. As technologists, we spend the majority of our time and energy on the first two steps—translating fundamental discoveries into a working concept. If this process represents the foundation of a new IVD, then everything that comes after represents the concrete realization of a project. The best way to ensure that you build an appropriate foundation is to make yourself fully aware of everything that the foundation must support. We refer readers to the US Food and Drug Administration IVD documentation,[150] the European Commission's directive on IVD devices,[151] and to the International Organization for Standardization (ISO) requirements for quality management in the manufacture of medical devices,[152] including IVDs.

Excellent resources and assistance can be attained by engaging with bodies that exist to facilitate the development and delivery of diagnostics to combat major diseases. One example is the Foundation for Innovative New Diagnostics (FIND), a global nonprofit organization headquartered in Switzerland with regional hubs in India, Kenya, South Africa, and Vietnam.[153] FIND can assist in the entire diagnostic development pipeline, including identifying needs, developing fit-for-purpose tests, generating evidence for regulators and policy-makers, and introducing new products to the markets. Another excellent example is PATH, a nonprofit organization focused on increasing public health equity, which includes work in diagnostics.[154] In summary: *It does not matter how excellent a diagnostic approach is if it is not deliverable at the time and place that it is required. So, as technologists, we must consider all of the enabling steps between that spark of inspiration at the lab bench and the delivery of an impactful diagnostic to the point-of-need.*

Where Are We Going? Widespread availability of cheap and accurate IVDs will play an increasing and integral role in global management of infectious disease. Furthermore, it will also play a key part in moving our general healthcare model from pure "crisis intervention" toward "preventative maintenance", a transition that is vital for improving global health. In this review, we contend that nucleic acid diagnostics will play a key role in achieving these ambitious goals. Going forward then, what are key areas of consideration for the next generations of nucleic acid IVD assays and devices?

New research and discoveries in enzymology and synthetic biology undoubtedly hold immense potential for impact in enzyme-assisted nucleic acid IVDs. Regarding natural enzymes, even considering humanity's expansive investigations and knowledge in enzymology, we have likely only scratched the surface of nature's pool of useful biocatalysts.[155] Thus, further fundamental studies in biology will continue to reveal new and important enzymes for advanced applications. For example, further study of bacteria and viruses, in particular, the mechanisms by which bacteria defend themselves,[156] could give rise to new opportunities in nucleic acid assays; this is precisely what happened after the discovery of CRISPR-Cas enzymes.[157] Another exciting area of exploration is the field of enzyme engineering. We believe that advances in de novo enzyme design, for example, using protein structure modeling, computational enzyme design, and molecular dynamics simulations, will give rise to ever more advanced enzymes for manipulating and detecting of nucleic acids.[158] These techniques are already having a huge impact in other fields of research;[159] therefore, we have good reason to be optimistic

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

about the impact they will have in enzyme-assisted nucleic acid IVDs.

The ultimate goal for point-of-care testing, in the context of infectious disease, is to create a simple sample-in, answer-out platform that requires minimal user input and infrastructure to operate effectively. Despite the multitude of advances described in this review, it is clear that we have not yet achieved this goal—at least not in a format that has been practical enough for widespread adoption during recent pandemics. Considering the complexity, the drive toward making these tests simpler (i.e., smaller, easy to operate) is a challenge, particularly while maintaining affordability. After all, these challenges are what necessitate the current reliance on complex machinery and centralized laboratories. Accordingly, we should ask, how far can we go to bridge the gap between a well-equipped lab and an end user self-test? Can we really envisage people performing nucleic acid IVDs in the same way we use home pregnancy tests now? The technologies discussed in this review suggest that this is indeed a possible future, at least from a technological standpoint. Nevertheless, we have to be smart in identifying whether our new ideas in the lab have any chance of finding real-world application, with a particular focus on practicality and affordability. Furthermore, we must not lose sight of the importance of sample preparation in the testing process; we feel that this crucial area has been relatively overlooked by technologists, and that there is great potential for impact here.

Versatile and Marketable Platform Technologies. The versatility and adaptability of IVDs are vital considerations in their marketability. We suggest that a key opportunity exists in looking for synergies between IVDs for chronic and infectious diseases. Chronic diseases such as cancer, diabetes, and obesity are increasing in wealthier populations, which is establishing an attractive market for companies looking to maximize their profits in the area of IVDs. However, the burden of infectious diseases is predominant in less wealthy nations, and the market for IVDs can be unstable given the transient or inconsistent demand for particular tests. One way to reconcile this conflict is to look for synergies in IVD requirements between chronic and infectious disease, such that IVDs that are developed to serve the chronic market could be rapidly reengineered and deployed against an infectious disease threat. In the context of epidemics, this would yield platform technologies that are "epidemic sensitive" rather than "epidemic specific". However, in academic publications there is pressure to go for specificity, where we write papers tackling a specific disease/problem of interest. We suggest that it would be more useful to allow the versatility of an approach to shine through. The target then must be to make systems where a minimal number of components need to be changed to detect a new target, with minimal reoptimization.

Mobile Health and Connected Diagnostics. Although the power of combining nucleic acid IVDs and mHealth solutions is obvious, there are still many challenges in practical implementation. For example, the policy and regulation governing mHealth concepts, software, and devices provide a headache for regulatory bodies that have evolved to work within the traditional centralized medicine model, though there are advanced global efforts to make vital reforms here.[160] There must be caution with regard to how mHealth solutions are marketed and implemented, taking great care not to impose upon people's privacy, and balancing this against potential stifling of innovation and impact. Another consideration is that the deployment and operation of diagnostics should not become completely reliant on digital connectivity, given that 33% of the global population are not connected to mobile Internet.[161]

Advanced Multiplexing Capabilities. The seemingly simple transition from "does the patient have disease X?" to "which disease does the patient have?" encompasses a huge technological challenge. It requires tests to be multiplexed to cover a range of infectious diseases, which increases the complexity of the molecular biology and the device design, with inevitable ramifications for end-point price. However, the power of this approach is well worth the technological investment required in engineering new solutions, and in this review, we have discussed existing approaches that do possess remarkable multiplexing potential. Beyond multiplexing, conceptually the most powerful gene-based diagnosis of an infectious disease is to perform genome-sequencing and compare the results to extensive libraries of known sequences. Therefore, instead of performing many tests in parallel, one is performing a single test that can output the data necessary to diagnose multiple diseases. However, current methods for sequencing are still technologically demanding, and it is an open question as to how far toward the point-of-care this technology can penetrate. Nevertheless, there are some extremely exciting examples of how sequencing can be used in infectious disease diagnostics,[162,163] and enzyme-assisted amplification and/or detection could play a key role here.

Focus on Antimicrobial Resistance. Finally, but by no means least, we would like to highlight the severe and increasing threat to human health and wellness posed by antimicrobial resistance (AMR). Currently, it is estimated that AMR leads to at least 700,000 global deaths each year, a figure that could rise to 10 million per year by 2050 if no action is taken.[164] While this number represents a worst case scenario, it is clear that we must do something to combat AMR. Part of our battle against the evolution of AMR is being able to quickly discern whether an infection is viral or bacterial in nature, and preferably what the pathogen is.[165] With this knowledge, deciding which treatment is required is far simpler, thus circumventing the current approach of using broad spectrum antibiotics as a first port of call for undiagnosed infections. Future nucleic acid IVDs will likely play a key role here.

CONCLUSIONS

Historically, researchers have been incredibly adept at finding ways to repurpose natural processes to drive technologies that ultimately benefit the human race. This is particularly true in the context of nucleic acid IVDs; after all, it was Mullis's intelligent repurposing of polymerases in the 1980s that yielded the polymerase chain reaction, which forms the basis of many of the nucleic acid amplification techniques discussed in this review. Thanks to advances in synthetic biology, e.g., protein engineering, directed evolution, and DNA/RNA engineering, we are now in a position to not only exploit molecular biology, but actively modify and direct it toward new and exciting challenges. We anticipate that these powers will facilitate the development of promising next-generation nucleic acid IVDs that can rapidly adapt to new threats.

Infectious diseases strike indiscriminately, leaving us all at risk regardless of national boundaries and economic status. Further, with the heaviest burden of infectious disease being felt in resource-limited areas, the world's poorest people are, by no fault of their own, the most susceptible to life-threatening

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

infections. Diagnosis is an absolutely key, but often overlooked, part of disease treatment. The shift toward the point-of-care requires a concerted, multidisciplinary effort; advances in enzyme engineering, molecular biology, chemistry, and engineering all contribute to the development of fully integrated RDTs. While a substantial amount of work remains to realize truly effective PoC nucleic acid diagnostics, each step forward has brought the technology closer to the end user. It stands to reason that further exploration will continue this process, and enable the development of previously unimaginable diagnostic modalities. Moving forward, we must find ways to address the challenges associated with not just the development of diagnostic technologies, but more importantly our ability to successfully deliver them where they are needed most. Current and future research into nucleic acid IVDs, encompassing both curiosity-driven fundamental science and impact-focused engineering, provides an opportunity to positively impact global health. It is our job now to maximize this opportunity.

AUTHOR INFORMATION

Corresponding Authors

Philip D. Howes − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland;* orcid.org/0000-0002-1862-8395; Email: philip.howes@chem.ethz.ch

Andrew J. deMello − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland*; Email: andrew.deMello@chem.ethz.ch

Daniel A. Richards − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland;* orcid.org/0000-0001-8827-9170; Email: daniel.richards@chem.ethz.ch

Authors

Akkapol Suea-Ngam − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland;* orcid.org/0000-0002-2463-4548

Léonard Bezinge − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland;* orcid.org/0000-0002-9733-9697

Bogdan Mateescu − *Institute for Chemical & Bioengineering, Department of Chemistry & Applied Biosciences, ETH Zürich, 8093 Zürich, Switzerland; Brain Research Institute, Medical Faculty of the University of Zürich, 8057 Zürich, Switzerland*

Complete contact information is available at: https://pubs.acs.org/10.1021/acssensors.0c01488

Notes

The authors declare no competing financial interest.

ACKNOWLEDGMENTS

B.M. is supported by grant UG3CA241703 from the NIH Common Fund, through the office of Strategic Coordination/Office of the NIH Director, and a translational fellowship from the RNA and disease Swiss National Center of Competence (NCCR). All authors would like to acknowledge the support of the Swiss Federal Institute of Technology.

ABBREVIATIONS

CRISPR, clustered regularly interspaced short palindromic repeats; dsDNA, double-stranded DNA; FRET, Förster resonance energy transfer; gRNA, guide RNA; HDA, helicase-dependent amplification; IVDs, in vitro diagnostics; IVTT, in vitro transcription/translation; LAMP, loop-mediated isothermal amplification; μPADS, microfluidic paper-based analytical devices; NASBA, nucleic acid sequence-based amplification; nt, nucleotide; PAM, protospacer adjacent motif; PFS, protospacer flanking site; PoC, point-of-care; qPCR, quantitative PCR; RBS, ribosome binding site; RCA, rolling circle amplification; RDT, rapid diagnostic test; RPA, recombinase polymerase amplification; RT-PCR, reverse transcriptase polymerase chain reaction; SDA, strand displacement amplification; ssDNA, single-stranded DNA

VOCABULARY

In vitro diagnostic - Diagnostic device that measures analytes outside of the body.

Mobile health - Healthcare which exploits advancements in mobile technologies (e.g., smartphones) and increased access to communications infrastructure

Multiplexing - Detecting multiple biomarkers simultaneously either through the use of parallelization or orthogonal signaling modalities (e.g., different colored dyes).

Platform technology - Versatile technology which can be easily adapted, built upon, or integrated into existing systems to address multiple diagnostic challenges, including novel or emerging pathogens.

Point-of-care - Location at which a patient typically receives care, e.g., a field hospital or doctor's clinic.

Rapid diagnostic test - Diagnostic test that can deliver a result at the point-of-care and within a practical time scale.

REFERENCES

(1) *World Health Organization Coronavirus Disease (COVID-19) Dashboard*; covid19.who.int (accessed August 20th, 2020).

(2) *Top Ten Causes of Death, Global Health Observatory (GHO) Data*; www.who.int/gho/mortality_burden_disease/causes_death/top_10/en (accessed August 20th, 2020).

(3) World Health Organization List of Prequalified in Vitro Diagnostic Products; www.who.int/diagnostics_laboratory/evaluations/PQ_list/en/ (accessed August 20th, 2020).

(4) Slutkin, G.; Okware, S.; Naamara, W.; Sutherland, D.; Flanagan, D.; Carael, M.; Blas, E.; Delay, P.; Tarantola, D. How Uganda Reversed Its HIV Epidemic. *AIDS Behav.* **2006**, *10*, 351−360.

(5) Land, K. J.; Boeras, D. I.; Chen, X. S.; Ramsay, A. R.; Peeling, R. W. REASSURED Diagnostics to Inform Disease Control Strategies, Strengthen Health Systems and Improve Patient Outcomes. *Nat. Microbiol.* **2019**, *4*, 46−54.

(6) Yuan, M.; Wu, N. C.; Zhu, X.; Lee, C. C. D.; So, R. T. Y.; Lv, H.; Mok, C. K. P.; Wilson, I. A. A Highly Conserved Cryptic Epitope in the Receptor Binding Domains of SARS-CoV-2 and SARS-CoV. *Science* **2020**, *368*, 630−633.

(7) Gussow, A. B.; Auslander, N.; Faure, G.; Wolf, Y. I.; Zhang, F.; Koonin, E. V. Genomic Determinants of Pathogenicity in SARS-CoV-2 and Other Human Coronaviruses. *Proc. Natl. Acad. Sci. U. S. A.* **2020**, *117*, 15193.

(8) Zhu, N.; Zhang, D.; Wang, W.; Li, X.; Yang, B.; Song, J.; Zhao, X.; Huang, B.; Shi, W.; Lu, R.; et al. A Novel Coronavirus from Patients with Pneumonia in China, 2019. *N. Engl. J. Med.* **2020**, *382*, 727−733.

(9) *Institut Pasteur Sequences the Whole Genome of the Coronavirus, 2019-NCoV*. www.pasteur.fr/en/press-area/press-documents/institut-

pasteur-sequences-whole-genome-coronavirus-2019-ncov (accessed August 20th, 2020).
(10) Kelley, B. Developing Therapeutic Monoclonal Antibodies at Pandemic Pace. *Nat. Biotechnol.* **2020**, *38*, 540−545.
(11) Broughton, J. P.; Deng, X.; Yu, G.; Fasching, C. L.; Servellita, V.; Singh, J.; Miao, X.; Streithorst, J. A.; Granados, A.; Sotomayor-gonzalez, A. CRISPR − Cas12-Based Detection of SARS-CoV-2. *Nat. Biotechnol.* **2020**, *38*, 870.
(12) Saiki, R. K.; Scharf, S.; Faloona, F.; Mullis, K. B.; Horn, G. T.; Erlich, H. A.; Arnheim, N. Enzymatic Amplification of β-Globin Genomic Sequences and Restriction Site Analysis for Diagnosis of Sickle Cell Anemia. *Science* **1985**, *230*, 1350−1354.
(13) Mullis, K.; Faloona, F.; Scharf, S.; Saiki, R.; Horn, G.; Erlich, H. Specific Enzymatic Amplification of DNA in Vitro: The Polymerase Chain Reaction. *Cold Spring Harbor Symp. Quant. Biol.* **1986**, *51*, 263−273.
(14) Higuchi, R.; Fockler, C.; Dollinger, G.; Watson, R. Kinetic PCR Analysis: Real-Time Monitoring of DNA Amplification Reactions. *Nat. Biotechnol.* **1993**, *11*, 1026−1030.
(15) *Cobas(R) 6000 Analyzer Series*; diagnostics.roche.com/global/en/products/systems/cobas_-6000-analyzer-series.html (accessed August 20th, 2020).
(16) Holland, P. M.; Abramson, R. D.; Watson, R.; Gelfand, D. H. Detection of Specific Polymerase Chain Reaction Product by Utilizing the 5′ → 3′ Exonuclease Activity of Thermus Aquaticus DNA Polymerase. *Proc. Natl. Acad. Sci. U. S. A.* **1991**, *88*, 7276−7280.
(17) Notomi, T.; Okayama, H.; Masubuchi, H.; Yonekawa, T.; Watanabe, K.; Amino, N.; Hase, T. Loop-Mediated Isothermal Amplification of DNA. *Nucleic Acids Res.* **2000**, *28*, e63.
(18) Goto, M.; Honda, E.; Ogura, A.; Nomoto, A.; Hanaki, K. I. Colorimetric Detection of Loop-Mediated Isothermal Amplification Reaction by Using Hydroxy Naphthol Blue. *BioTechniques* **2009**, *46*, 167−172.
(19) Goto, M.; Shimada, K.; Sato, A.; Takahashi, E.; Fukasawa, T.; Takahashi, T.; Ohka, S.; Taniguchi, T.; Honda, E.; Nomoto, A.; et al. Rapid Detection of Pseudomonas Aeruginosa in Mouse Feces by Colorimetric Loop-Mediated Isothermal Amplification. *J. Microbiol. Methods* **2010**, *81*, 247−252.
(20) Soli, K. W.; Kas, M.; Maure, T.; Umezaki, M.; Morita, A.; Siba, P. M.; Greenhill, A. R.; Horwood, P. F. Evaluation of Colorimetric Detection Methods for Shigella, Salmonella, and Vibrio Cholerae by Loop-Mediated Isothermal Amplification. *Diagn. Microbiol. Infect. Dis.* **2013**, *77*, 321−323.
(21) Connelly, J. T.; Rolland, J. P.; Whitesides, G. M. Paper Machine" for Molecular Diagnostics. *Anal. Chem.* **2015**, *87*, 7595−7601.
(22) Zhang, H.; Xu, Y.; Fohlerova, Z.; Chang, H.; Iliescu, C.; Neuzil, P. LAMP-on-a-Chip: Revising Microfluidic Platforms for Loop-Mediated DNA Amplification. *TrAC, Trends Anal. Chem.* **2019**, *113*, 44−53.
(23) Piepenburg, O.; Williams, C. H.; Stemple, D. L.; Armes, N. A. DNA Detection Using Recombination Proteins. *PLoS Biol.* **2006**, *4*, 1115−1121.
(24) Li, J.; Macdonald, J.; Von Stetten, F. Review: A Comprehensive Summary of a Decade Development of the Recombinase Polymerase Amplification. *Analyst* **2019**, *144*, 31−67.
(25) Yehia, N.; Arafa, A. S.; Abd El Wahed, A.; El-Sanousi, A. A.; Weidmann, M.; Shalaby, M. A. Development of Reverse Transcription Recombinase Polymerase Amplification Assay for Avian Influenza H5N1 HA Gene Detection. *J. Virol. Methods* **2015**, *223*, 45−49.
(26) Abd El Wahed, A.; Patel, P.; Faye, O.; Thaloengsok, S.; Heidenreich, D.; Matangkasombut, P.; Manopwisedjaroen, K.; Sakuntabhai, A.; Sall, A. A.; Hufert, F. T.; et al. Recombinase Polymerase Amplification Assay for Rapid Diagnostics of Dengue Infection. *PLoS One* **2015**, *10*, 1−17.
(27) Faye, O.; Faye, O.; Soropogui, B.; Patel, P.; El Wahed, A. A.; Loucoubar, C.; Fall, G.; Kiory, D.; Magassouba, N.; Keita, S. Development and Deployment of a Rapid Recombinase Polymerase Amplification Ebola Virus Detection Assay in Guinea in 2015. *Eurosurveillance* **2015**, *20*, 1 DOI: 10.2807/1560-7917.ES.2015.20.44.30053.
(28) Fire, A.; Xu, S. Q. Rolling Replication of Short DNA Circles. *Proc. Natl. Acad. Sci. U. S. A.* **1995**, *92*, 4641−4645.
(29) Kool, E. T. Rolling Circle Synthesis of Oligonucleotides and Amplification of Select Randomized Circular Oligonucleotides. US Patent 5,714,320 A, 1998.
(30) Mohsen, M. G.; Kool, E. T. The Discovery of Rolling Circle Amplification and Rolling Circle Transcription. *Acc. Chem. Res.* **2016**, *49*, 2540−2550.
(31) Zhuang, J.; Lai, W.; Chen, G.; Tang, D. A Rolling Circle Amplification-Based DNA Machine for MiRNA Screening Coupling Catalytic Hairpin Assembly with DNAzyme Formation. *Chem. Commun.* **2014**, *50*, 2935−2938.
(32) Nayak, S.; Blumenfeld, N. R.; Laksanasopin, T.; Sia, S. K. Point-of-Care Diagnostics: Recent Developments in a Connected Age. *Anal. Chem.* **2017**, *89*, 102−123.
(33) Lizardi, P. M.; Huang, X.; Zhu, Z.; Bray-Ward, P.; Thomas, D. C.; Ward, D. C. Mutation Detection and Single-Molecule Counting Using Isothermal Rolling-Circle Amplification. *Nat. Genet.* **1998**, *19*, 225−232.
(34) Walker, G. T.; Little, M. C.; Nadeau, J. G.; Shank, D. D. Isothermal in Vitro Amplification of DNA by a Restriction Enzyme/DNA Polymerase System. *Proc. Natl. Acad. Sci. U. S. A.* **1992**, *89*, 392−396.
(35) Walker, G. T.; Fraiser, M. S.; Schram, J. L.; Little, M. C.; Nadeau, J. G.; Malinowski, D. P. Strand Displacement Amplification - an Isothermal, in Vitro DNA Amplification Technique. *Nucleic Acids Res.* **1992**, *20*, 1691−1696.
(36) Toley, B. J.; Covelli, I.; Belousov, Y.; Ramachandran, S.; Kline, E.; Scarr, N.; Vermeulen, N.; Mahoney, W.; Lutz, B. R.; Yager, P. Isothermal Strand Displacement Amplification (ISDA): A Rapid and Sensitive Method of Nucleic Acid Amplification for Point-of-Care Diagnosis. *Analyst* **2015**, *140*, 7540−7549.
(37) Lafleur, L. K.; Bishop, J. D.; Heiniger, E. K.; Gallagher, R. P.; Wheeler, M. D.; Kauffman, P.; Zhang, X.; Kline, E. C.; Buser, J. R.; Kumar, S.; et al. A Rapid, Instrument-Free, Sample-to-Result Nucleic Acid Amplification Test. *Lab Chip* **2016**, *16*, 3777−3787.
(38) Vincent, M.; Xu, Y.; Kong, H. Helicase-Dependent Isothermal DNA Amplification. *EMBO Rep.* **2004**, *5*, 795−800.
(39) An, L.; Tang, W.; Ranalli, T. A.; Kim, H. J.; Wytiaz, J.; Kong, H. Characterization of a Thermostable UvrD Helicase and Its Participation in Helicase-Dependent Amplification. *J. Biol. Chem.* **2005**, *280*, 28952−28958.
(40) Yang, Z.; McLendon, C.; Hutter, D.; Bradley, K. M.; Hoshika, S.; Frye, C. B.; Benner, S. A. Helicase-Dependent Isothermal Amplification of DNA and RNA by Using Self-Avoiding Molecular Recognition Systems. *ChemBioChem* **2015**, *16*, 1365−1370.
(41) Tong, Y.; Tang, W.; Kim, H. J.; Pan, X.; Ranalli, T. A.; Kong, H. Development of Isothermal TaqMan Assays for Detection of Biothreat Organisms. *BioTechniques* **2008**, *45*, 543−557.
(42) Barreda-Garcia, S.; Miranda-Castro, R.; de-los-Santos-Alvarez, N.; Miranda-Ordieres, A. J.; Lobo-Castanon, M. J. Helicase-Dependent Isothermal Amplification: A Novel Tool in the Development of Molecular-Based Analytical Systems for Rapid Pathogen Detection. *Anal. Bioanal. Chem.* **2018**, *410*, 679−693.
(43) Jordan, J. A.; Ibe, C. O.; Moore, M. S.; Host, C.; Simon, G. L. Evaluation of a Manual DNA Extraction Protocol and an Isothermal Amplification Assay for Detecting HIV-1 DNA from Dried Blood Spots for Use in Resource-Limited Settings. *J. Clin. Virol.* **2012**, *54*, 11−14.
(44) Frech, G. C.; Munns, D.; Jenison, R. D.; Hicke, B. J. Direct Detection of Nasal Staphylococcus Aureus Carriage via Helicase-Dependent Isothermal Amplification and Chip Hybridization. *BMC Res. Notes* **2012**, *5*, 430.
(45) Miller, N. S.; Yen-Lieberman, B.; Poulter, M. D.; Tang, Y. W.; Granato, P. A. Comparative Clinical Evaluation of the IsoAmp® HSV Assay with ELVIS® HSV Culture/ID/Typing Test System for the

Detection of Herpes Simplex Virus in Genital and Oral Lesions. *J. Clin. Virol.* **2012**, *54*, 355−358.
(46) Compton, J. Nucleic Acid Sequence-Based Amplification. *Nature* **1991**, *350*, 91−92.
(47) Fakruddin, M.; Mazumdar, R.; Chowdhury, A.; Mannan, K. Nucleic Acid Sequence Based Amplification (NASBA)-Prospects and Applications. *Int. J. Life Sci. Pharma Res.* **2012**, *2*, 106.
(48) Gulliksen, A.; Keegan, H.; Martin, C.; O'Leary, J.; Solli, L. A.; Falang, I. M.; Grnn, P.; Karlgrd, A.; Mielnik, M. M.; Johansen, I. R. Towards a Sample-in, Answer-out Point-of-Care Platform for Nucleic Acid Extraction and Amplification: Using an HPV E6/E7MRNA Model System. *J. Oncol.* **2012**, *2012*, 1.
(49) Pardee, K.; Green, A. A.; Takahashi, M. K.; Braff, D.; Lambert, G.; Lee, J. W.; Ferrante, T.; Ma, D.; Donghia, N.; Fan, M.; et al. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. *Cell* **2016**, *165*, 1255−1266.
(50) Makarova, K. S.; Wolf, Y. I.; Iranzo, J.; Shmakov, S. A.; Alkhnbashi, O. S.; Brouns, S. J. J.; Charpentier, E.; Cheng, D.; Haft, D. H.; Horvath, P.; et al. Evolutionary Classification of CRISPR−Cas Systems: A Burst of Class 2 and Derived Variants. *Nat. Rev. Microbiol.* **2020**, *18*, 67−83.
(51) Jinek, M.; Chylinski, K.; Fonfara, I.; Hauer, M.; Doudna, J. A.; Charpentier, E. A Programmable Dual-RNA-Guided DNA Endonuclease in Adaptive Bacterial Immunity. *Science* **2012**, *337*, 816−821.
(52) Ma, E.; Harrington, L. B.; O'Connell, M. R.; Zhou, K.; Doudna, J. A. Single-Stranded DNA Cleavage by Divergent CRISPR-Cas9 Enzymes. *Mol. Cell* **2015**, *60*, 398−407.
(53) Strutt, S. C.; Torrez, R. M.; Kaya, E.; Negrete, O. A.; Doudna, J. A. RNA-Dependent RNA Targeting by CRISPR-Cas9. *eLife* **2018**, *7*, e32724.
(54) Xiang, X.; Qian, K.; Zhang, Z.; Lin, F.; Xie, Y.; Liu, Y.; Yang, Z. CRISPR-Cas Systems Based Molecular Diagnostic Tool for Infectious Diseases and Emerging 2019 Novel Coronavirus (COVID-19) Pneumonia. *J. Drug Targeting* **2020**, 1.
(55) Huang, M.; Zhou, X.; Wang, H.; Xing, D. Clustered Regularly Interspaced Short Palindromic Repeats/Cas9 Triggered Isothermal Amplification for Site-Specific Nucleic Acid Detection. *Anal. Chem.* **2018**, *90*, 2193−2200.
(56) Müller, V.; Rajer, F.; Frykholm, K.; Nyberg, L. K.; Quaderi, S.; Fritzsche, J.; Kristiansson, E.; Ambjörnsson, T.; Sandegren, L.; Westerlund, F. Direct Identification of Antibiotic Resistance Genes on Single Plasmid Molecules Using CRISPR/Cas9 in Combination with Optical DNA Mapping. *Sci. Rep.* **2016**, *6*, 1−11.
(57) Quan, J.; Langelier, C.; Kuchta, A.; Batson, J.; Teyssier, N.; Lyden, A.; Caldera, S.; McGeever, A.; Dimitrov, B.; King, R.; et al. FLASH: A next-Generation CRISPR Diagnostic for Multiplexed Detection of Antimicrobial Resistance Sequences. *Nucleic Acids Res.* **2019**, *47*, e83.
(58) Chen, J. S.; Ma, E.; Harrington, L. B.; Da Costa, M.; Tian, X.; Palefsky, J. M.; Doudna, J. A. CRISPR-Cas12a Target Binding Unleashes Indiscriminate Single-Stranded DNase Activity. *Science* **2018**, *360*, 436−439.
(59) Li, S. Y.; Cheng, Q. X.; Li, X. Y.; Zhang, Z. L.; Gao, S.; Cao, R. B.; Zhao, G. P.; Wang, J.; Wang, J. M. CRISPR-Cas12a-Assisted Nucleic Acid Detection. *Cell Discovery* **2018**, *4*, 20.
(60) Li, L.; Li, S.; Wu, N.; Wu, J.; Wang, G.; Zhao, G.; Wang, J. HOLMESv2: A CRISPR-Cas12b-Assisted Platform for Nucleic Acid Detection and DNA Methylation Quantitation. *ACS Synth. Biol.* **2019**, *8*, 2228−2237.
(61) Yan, W. X.; Hunnewell, P.; Alfonse, L. E.; Carte, J. M.; Keston-Smith, E.; Sothiselvam, S.; Garrity, A. J.; Chong, S.; Makarova, K. S.; Koonin, E. V.; et al. Functionally Diverse Type V CRISPR-Cas Systems. *Science* **2019**, *363*, 88−91.
(62) Harrington, L. B.; Burstein, D.; Chen, J. S.; Paez-Espino, D.; Ma, E.; Witte, I. P.; Cofsky, J. C.; Kyrpides, N. C.; Banfield, J. F.; Doudna, J. A. Programmed DNA Destruction by Miniature CRISPR-Cas14 Enzymes. *Science* **2018**, *362*, 839−842.
(63) Gootenberg, J. S.; Abudayyeh, O. O.; Lee, J. W.; Essletzbichler, P.; Dy, A. J.; Joung, J.; Verdine, V.; Donghia, N.; Daringer, N. M.; Freije, C. A.; et al. Nucleic Acid Detection with CRISPR-Cas13a/C2c2. *Science* **2017**, *356*, 438−442.
(64) Kellner, M. J.; Koob, J. G.; Gootenberg, J. S.; Abudayyeh, O. O.; Zhang, F. SHERLOCK: Nucleic Acid Detection with CRISPR Nucleases. *Nat. Protoc.* **2019**, *14*, 2986−3012.
(65) Gootenberg, J. S.; Abudayyeh, O. O.; Kellner, M. J.; Joung, J.; Collins, J. J.; Zhang, F. Multiplexed and Portable Nucleic Acid Detection Platform with Cas13, Cas12a and Csm6. *Science* **2018**, *360*, 439−444.
(66) Ackerman, C. M.; Myhrvold, C.; Thakku, S. G.; Freije, C. A.; Metsky, H. C.; Yang, D. K.; Ye, S. H.; Boehm, C. K.; Kosoko-Thoroddsen, T. S. F.; Kehe, J.; et al. Massively Multiplexed Nucleic Acid Detection with Cas13. *Nature* **2020**, *582*, 277−282.
(67) *Sherlock CRISPR SARS-CoV-2 Kit*; www.fda.gov/media/137747/download (accessed August 20th, 2020).
(68) Bezinge, L.; Suea-Ngam, A.; deMello, A. J.; Shih, C. J. Nanomaterials for Molecular Signal Amplification in Electrochemical Nucleic Acid Biosensing: Recent Advances and Future Prospects for Point-of-Care Diagnostics. *Mol. Syst. Des. Eng.* **2020**, *5*, 49−66.
(69) Zhang, L.; Li, D.; Meng, W.; Huang, Q.; Su, Y.; Wang, L.; Song, S.; Fan, C. Sequence-Specific DNA Detection by Using Biocatalyzed Electrochemiluminescence and Non-Fouling Surfaces. *Biosens. Bioelectron.* **2009**, *25*, 368−372.
(70) Bodulev, O. L.; Gribas, A. V.; Sakharov, I. Y. Microplate Chemiluminescent Assay for HBV DNA Detection Using 3-(10′-Phenothiazinyl)Propionic Acid/N-Morpholinopyridine Pair as Enhancer of HRP-Catalyzed Chemiluminescence. *Anal. Biochem.* **2018**, *543*, 33−36.
(71) Zhang, J.; Lao, R.; Song, S.; Yan, Z.; Fan, C. Design of an Oligonucleotide-Incorporated Nonfouling Surface and Its Application in Electrochemical DNA Sensors for Highly Sensitive and Sequence-Specific Detection of Target DNA. *Anal. Chem.* **2008**, *80*, 9029−9033.
(72) Azek, F.; Grossiord, C.; Joannes, M.; Limoges, B.; Brossier, P. Hybridization Assay at a Disposable Electrochemical Biosensor for the Attomole Detection of Amplified Human Cytomegalovirus DNA. *Anal. Biochem.* **2000**, *284*, 107−113.
(73) Wu, S. H.; Huang, X. B.; Tang, Y.; Ma, L. M.; Liu, Y.; Sun, J. J. Temperature Controllable Electrochemical Sensors Based on Horseradish Peroxidase as Electrocatalyst at Heated Au Disk Electrode and Its Preliminary Application for H2O2 Detection. *Anal. Chim. Acta* **2020**, *1096*, 44−52.
(74) Mao, X.; Jiang, J.; Xu, X.; Chu, X.; Luo, Y.; Shen, G.; Yu, R. Enzymatic Amplification Detection of DNA Based on "Molecular Beacon" Biosensors. *Biosens. Bioelectron.* **2008**, *23*, 1555−1561.
(75) Opalinska, J. B.; Gewirtz, A. M. Nucleic-Acid Therapeutics: Basic Principles and Recent Applications. *Nat. Rev. Drug Discovery* **2002**, *1*, 503−514.
(76) Isaacs, F. J.; Dwyer, D. J.; Ding, C.; Pervouchine, D. D.; Cantor, C. R.; Collins, J. J. Engineered Riboregulators Enable Post-Transcriptional Control of Gene Expression. *Nat. Biotechnol.* **2004**, *22*, 841−847.
(77) Callura, J. M.; Dwyer, D. J.; Isaacs, F. J.; Cantor, C. R.; Collins, J. J. Tracking, Tuning, and Terminating Microbial Physiology Using Synthetic Riboregulators. *Proc. Natl. Acad. Sci. U. S. A.* **2010**, *107*, 15898−15903.
(78) Rodrigo, G.; Landrain, T. E.; Jaramillo, A. De Novo Automated Design of Small RNA Circuits for Engineering Synthetic Riboregulation in Living Cells. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 15271−15276.
(79) Liu, C. C.; Qi, L.; Lucks, J. B.; Segall-Shapiro, T. H.; Wang, D.; Mutalik, V. K.; Arkin, A. P. An Adaptor from Translational to Transcriptional Control Enables Predictable Assembly of Complex Regulation. *Nat. Methods* **2012**, *9*, 1088−1094.
(80) Callura, J. M.; Cantor, C. R.; Collins, J. J. Genetic Switchboard for Synthetic Biology Applications. *Proc. Natl. Acad. Sci. U. S. A.* **2012**, *109*, 5850−5855.
(81) Mutalik, V. K.; Qi, L.; Guimaraes, J. C.; Lucks, J. B.; Arkin, A. P. Rationally Designed Families of Orthogonal RNA Regulators of Translation. *Nat. Chem. Biol.* **2012**, *8*, 447−454.

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

(82) Green, A. A.; Silver, P. A.; Collins, J. J.; Yin, P. Toehold Switches: De-Novo-Designed Regulators of Gene Expression. *Cell* **2014**, *159*, 925−939.
(83) Hong, F.; Ma, D.; Wu, K.; Mina, L. A.; Luiten, R. C.; Liu, Y.; Yan, H.; Green, A. A. Precise and Programmable Detection of Mutations Using Ultraspecific Riboregulators. *Cell* **2020**, *180*, 1018−1032.
(84) Kim, J.; Zhou, Y.; Carlson, P. D.; Teichmann, M.; Chaudhary, S.; Simmel, F. C.; Silver, P. A.; Collins, J. J.; Lucks, J. B.; Yin, P.; et al. De Novo-Designed Translation-Repressing Riboregulators for Multi-Input Cellular Logic. *Nat. Chem. Biol.* **2019**, *15*, 1173−1182.
(85) Silverman, A. D.; Karim, A. S.; Jewett, M. C. Cell-Free Gene Expression: An Expanded Repertoire of Applications. *Nat. Rev. Genet.* **2020**, *21*, 151−170.
(86) Jin, J.; Vaud, S.; Zhelkovsky, A. M.; Posfai, J.; McReynolds, L. A. Sensitive and Specific MiRNA Detection Method Using SplintR Ligase. *Nucleic Acids Res.* **2016**, *44*, e116.
(87) Ying, Z. M.; Tu, B.; Liu, L.; Tang, H.; Tang, L. J.; Jiang, J. H. Spinach-Based Fluorescent Light-up Biosensors for Multiplexed and Label-Free Detection of MicroRNAs. *Chem. Commun.* **2018**, *54*, 3010−3013.
(88) *INSPECTR: Direct-to-Consumer Molecular Diagnostic*; wyss.harvard.edu/technology/inspectr-a-direct-to-consumer-molecular-diagnostic (accessed August 20th, 2020).
(89) Thavarajah, W.; Silverman, A. D.; Verosloff, M. S.; Kelley-Loughnane, N.; Jewett, M. C.; Lucks, J. B. Point-of-Use Detection of Environmental Fluoride via a Cell-Free Riboswitch-Based Biosensor. *ACS Synth. Biol.* **2020**, *9*, 10−18.
(90) Liu, X.; Silverman, A. D.; Alam, K. K.; Iverson, E.; Lucks, J. B.; Jewett, M. C.; Raman, S. Design of a Transcriptional Biosensor for the Portable, on-Demand Detection of Cyanuric Acid. *ACS Synth. Biol.* **2020**, *9*, 84−94.
(91) Jang, Y. J.; Lee, K. H.; Yoo, T. H.; Kim, D. M. Complementary Cell-Free Translational Assay for Quantification of Amino Acids. *Anal. Chem.* **2017**, *89*, 9638−9642.
(92) Lee, K. H.; Kim, D. M. In Vitro Use of Cellular Synthetic Machinery for Biosensing Applications. *Front. Pharmacol.* **2019**, *10*, No. 1166, DOI: 10.3389/fphar.2019.01166.
(93) Ryu, G.; Huang, J.; Hofmann, O.; Walshe, C. A.; Sze, J. Y. Y.; McClean, G. D.; Mosley, A.; Rattle, S. J.; deMello, J. C.; deMello, A. J.; et al. Highly Sensitive Fluorescence Detection System for Microfluidic Lab-on-a-Chip. *Lab Chip* **2011**, *11*, 1664−1670.
(94) Pardee, K.; Green, A. A.; Ferrante, T.; Cameron, D. E.; Daleykeyser, A.; Yin, P.; Collins, J. J. Paper-Based Synthetic Gene Networks. *Cell* **2014**, *159*, 940−954.
(95) Wood, C. S.; Thomas, M. R.; Budd, J.; Mashamba-Thompson, T. P.; Herbst, K.; Pillay, D.; Peeling, R. W.; Johnson, A. M.; McKendry, R. A.; Stevens, M. M. Taking Connected Mobile-Health Diagnostics of Infectious Diseases to the Field. *Nature* **2019**, *566*, 467−474.
(96) *International Telecommunication Union*; www.itu.int/en/ITU-D/Statistics/Pages/stat (accessed August 20th, 2020).
(97) Loeffelholz, M. J.; Tang, Y. W. Laboratory Diagnosis of Emerging Human Coronavirus Infections−the State of the Art. *Emerging Microbes Infect.* **2020**, *9*, 747−756.
(98) Yang, S.; Rothman, R. E. PCR-Based Diagnostics for Infectious Diseases: Uses, Limitations, and Future Applications in Acute-Care Settings. *Lancet Infect. Dis.* **2004**, *4*, 337−348.
(99) *Qiagen*; www.qiagen.com/ (accessed August 20th, 2020).
(100) Crannell, Z. A.; Rohrman, B.; Richards-Kortum, R. Equipment-Free Incubation of Recombinase Polymerase Amplification Reactions Using Body Heat. *PLoS One* **2014**, *9*, No. e112146.
(101) Hopkins, H.; González, I. J.; Polley, S. D.; Angutoko, P.; Ategeka, J.; Asiimwe, C.; Agaba, B.; Kyabayinze, D. J.; Sutherland, C. J.; Perkins, M. D.; et al. Highly Sensitive Detection of Malaria Parasitemia in a Malaria-Endemic Setting: Performance of a New Loop-Mediated Isothermal Amplification Kit in a Remote Clinic in Uganda. *J. Infect. Dis.* **2013**, *208*, 645−652.
(102) Martin, A.; Grant, K. B.; Stressmann, F.; Ghigo, J. M.; Marchal, D.; Limoges, B. Ultimate Single-Copy DNA Detection Using Real-Time Electrochemical LAMP. *ACS Sensors* **2016**, *1*, 904−912.
(103) Lucchi, N. W.; Gaye, M.; Diallo, M. A.; Goldman, I. F.; Ljolje, D.; Deme, A. B.; Badiane, A.; Ndiaye, Y. D.; Barnwell, J. W.; Udhayakumar, V.; et al. Evaluation of the Illumigene Malaria LAMP: A Robust Molecular Diagnostic Tool for Malaria Parasites. *Sci. Rep.* **2016**, *6*, 1−8.
(104) *Alere*; www.alere.com/en/home/product-details/id-now.html (accessed August 20th, 2020).
(105) Chiu, D. T.; deMello, A. J.; Di Carlo, D.; Doyle, P. S.; Hansen, C.; Maceiczyk, R. M.; Wootton, R. C. R. Small but Perfectly Formed? Successes, Challenges, and Opportunities for Microfluidics in the Chemical and Biological Sciences. *Chem.* **2017**, *2*, 201−223.
(106) Yager, P.; Edwards, T.; Fu, E.; Helton, K.; Nelson, K.; Tam, M. R.; Weigl, B. H. Microfluidic Diagnostic Technologies for Global Public Health. *Nature* **2006**, *442*, 412−418.
(107) Kopp, M. U.; De Mello, A. J.; Manz, A. Chemical Amplification Continuous-Flow PCR on a Chip. *Science* **1998**, *280*, 1046−1048.
(108) De Mello, A. J. DNA Amplification: Does "Small" Really Mean "Efficient"? *Lab Chip* **2001**, *1*, 24N.
(109) Woolley, A. T.; Hadley, D.; Landre, P.; deMello, A. J.; Mathies, R. A.; Northrup, M. A. Functional Integration of PCR Amplification and Capillary Electrophoresis in a Microfabricated DNA Analysis Device. *Anal. Chem.* **1996**, *68*, 4081−4086.
(110) Schaerli, Y.; Wootton, R. C.; Robinson, T.; Stein, V.; Dunsby, C.; Neil, M. A. A.; French, P. M. W.; deMello, A. J.; Abell, C.; Hollfelder, F. Continuous-Flow Polymerase Chain Reaction of Single-Copy DNA in Microfluidic Microdroplets. *Anal. Chem.* **2009**, *81*, 302−306.
(111) Opollo, V. S.; Nikuze, A.; Ben-Farhat, J.; Anyango, E.; Humwa, F.; Oyaro, B.; Wanjala, S.; Omwoyo, W.; Majiwa, M.; Akelo, V.; et al. Field Evaluation of near Point of Care Cepheid GeneXpert HIV-1 Qual for Early Infant Diagnosis. *PLoS One* **2018**, *13*, No. e0209778.
(112) Andrews, D.; Chetty, Y.; Cooper, B. S.; Virk, M.; Glass, S. K.; Letters, A.; Kelly, P. A.; Sudhanva, M.; Jeyaratnam, D. Multiplex PCR Point of Care Testing versus Routine, Laboratory-Based Testing in the Treatment of Adults with Respiratory Tract Infections: A Quasi-Randomised Study Assessing Impact on Length of Stay and Antimicrobial Use. *BMC Infect. Dis.* **2017**, *17*, No. 671, DOI: 10.1186/s12879-017-2784-z.
(113) Ahrberg, C. D.; Ilic, B. R.; Manz, A.; Neužil, P. Handheld Real-Time PCR Device. *Lab Chip* **2016**, *16*, 586−592.
(114) Li, T. J.; Chang, C. M.; Chang, P. Y.; Chuang, Y. C.; Huang, C. C.; Su, W. C.; Shieh, D. B. Handheld Energy-Efficient Magneto-Optical Real-Time Quantitative PCR Device for Target DNA Enrichment and Quantification. *NPG Asia Mater.* **2016**, *8*, e277.
(115) Yeh, E. C.; Fu, C. C.; Hu, L.; Thakur, R.; Feng, J.; Lee, L. P. Self-Powered Integrated Microfluidic Point-of-Care Low-Cost Enabling (SIMPLE) Chip. *Sci. Adv.* **2017**, *3*, No. e1501645.
(116) Gökçe, O.; Castonguay, S.; Temiz, Y.; Gervais, T.; Delamarche, E. Self-Coalescing Flows in Microfluidics for Pulse-Shaped Delivery of Reagents. *Nature* **2019**, *574*, 228−232.
(117) McLeod, E.; Nguyen, C.; Huang, P.; Luo, W.; Veli, M.; Ozcan, A. Tunable Vapor-Condensed Nanolenses. *ACS Nano* **2014**, *8*, 7340−7349.
(118) Bueno, D.; Muñoz, R.; Marty, J. L. Fluorescence Analyzer Based on Smartphone Camera and Wireless for Detection of Ochratoxin A. *Sens. Actuators, B* **2016**, *232*, 462−468.
(119) Vietz, C.; Schütte, M. L.; Wei, Q.; Richter, L.; Lalkens, B.; Ozcan, A.; Tinnefeld, P.; Acuna, G. P. Benchmarking Smartphone Fluorescence-Based Microscopy with DNA Origami Nanobeads: Reducing the Gap toward Single-Molecule Sensitivity. *ACS Omega* **2019**, *4*, 637−642.
(120) Hsieh, K.; Patterson, A. S.; Ferguson, B. S.; Plaxco, K. W.; Soh, H. T. Rapid, Sensitive, and Quantitative Detection of Pathogenic

DNA at the Point of Care through Microfluidic Electrochemical Quantitative Loop-Mediated Isothermal Amplification. *Angew. Chem., Int. Ed.* **2012**, *51*, 4896−4900.
(121) Marshall, L. A.; Wu, L. L.; Babikian, S.; Bachman, M.; Santiago, J. G. Integrated Printed Circuit Board Device for Cell Lysis and Nucleic Acid Extraction. *Anal. Chem.* **2012**, *84*, 9640−9645.
(122) Toumazou, C.; Shepherd, L. M.; Reed, S. C.; Chen, G. I.; Patel, A.; Garner, D. M.; Wang, C. J. A.; Ou, C. P.; Amin-Desai, K.; Athanasiou, P.; et al. Simultaneous DNA Amplification and Detection Using a PH-Sensing Semiconductor System. *Nat. Methods* **2013**, *10*, 641−646.
(123) Martinez, A. W.; Phillips, S. T.; Whitesides, G. M.; Carrilho, E. Diagnostics for the Developing World: Microfluidic Paper-Based Analytical Devices. *Anal. Chem.* **2010**, *82*, 3−10.
(124) Yamada, K.; Shibata, H.; Suzuki, K.; Citterio, D. Toward Practical Application of Paper-Based Microfluidics for Medical Diagnostics: State-of-the-Art and Challenges. *Lab Chip* **2017**, *17*, 1206−1249.
(125) *World Health Organization List of Prequalified in Vitro Diagnostic Products, 2020*; www.who.int/diagnostics_laboratory/evaluations/PQ_list/en/ (accessed August 20th, 2020).
(126) Choi, J. R.; Yong, K. W.; Tang, R.; Gong, Y.; Wen, T.; Li, F.; Pingguan-Murphy, B.; Bai, D.; Xu, F. Advances and Challenges of Fully Integrated Paper-Based Point-of-Care Nucleic Acid Testing. *TrAC, Trends Anal. Chem.* **2017**, *93*, 37−50.
(127) Magro, L.; Escadafal, C.; Garneret, P.; Jacquelin, B.; Kwasiborski, A.; Manuguerra, J. C.; Monti, F.; Sakuntabhai, A.; Vanhomwegen, J.; Lafaye, P.; et al. Paper Microfluidics for Nucleic Acid Amplification Testing (NAAT) of Infectious Diseases. *Lab Chip* **2017**, *17*, 2347−2371.
(128) Aveyard, J.; Mehrabi, M.; Cossins, A.; Braven, H.; Wilson, R. One Step Visual Detection of PCR Products with Gold Nanoparticles and a Nucleic Acid Lateral Flow (NALF) Device. *Chem. Commun.* **2007**, No. 41, 4251−4253.
(129) Nathavitharana, R. R.; Cudahy, P. G. T.; Schumacher, S. G.; Steingart, K. R.; Pai, M.; Denkinger, C. M. Accuracy of Line Probe Assays for the Diagnosis of Pulmonary and Multidrug-Resistant Tuberculosis: A Systematic Review and Meta-Analysis. *Eur. Respir. J.* **2017**, *49*, 1601075.
(130) *Line Probe Assays for Drug-Resistant Tuberculosis Detection. Interpretation and Reporting Guide for Laboratory Staff and Clinicians*; www.stoptb.org/wg/gli/assets/documents/LPA_test_web_ready.pdf (accessed August 20th, 2020).
(131) *TwistDx, TwistAmp Nfo*; www.twistdx.co.uk/en/products/product/twistamp-nfo (accessed August 20th, 2020).
(132) Chander, Y.; Koelbl, J.; Puckett, J.; Moser, M. J.; Klingele, A. J.; Liles, M. R.; Carrias, A.; Mead, D. A.; Schoenfeld, T. W. A Novel Thermostable Polymerase for RNA and DNA Loop-Mediated Isothermal Amplification (LAMP). *Front. Microbiol.* **2014**, *5*, 395.
(133) Du, Y.; Pothukuchy, A.; Gollihar, J. D.; Nourani, A.; Li, B.; Ellington, A. D. Coupling Sensitive Nucleic Acid Amplification with Commercial Pregnancy Test Strips. *Angew. Chem., Int. Ed.* **2017**, *56*, 992−996.
(134) Myhrvold, C.; Freije, C. A.; Gootenberg, J. S.; Abudayyeh, O. O.; Metsky, H. C.; Durbin, A. F.; Kellner, M. J.; Tan, A. L.; Paul, L. M.; Parham, L. A.; et al. Field-Deployable Viral Diagnostics Using CRISPR-Cas13. *Science* **2018**, *360*, 444−448.
(135) *Point-of-Care Testing for COVID-19 Using SHERLOCK Diagnostics, 2020, v.20200505*; www.stopcovid.science/docs/STOPCovid%20Whitepaper.pdf (accessed August 20th, 2020).
(136) Linnes, J. C.; Rodriguez, N. M.; Liu, L.; Klapperich, C. M. Polyethersulfone Improves Isothermal Nucleic Acid Amplification Compared to Current Paper-Based Diagnostics. *Biomed. Microdevices* **2016**, *18*, 1−12.
(137) Byrnes, S. A.; Bishop, J. D.; Yager, P. Enabling Lateral Transport of Genomic DNA through Porous Membranes for Point-of-Care Applications. *Anal. Methods* **2017**, *9*, 3450−3463.
(138) Byrnes, S. A.; Bishop, J. D.; Lafleur, L.; Buser, J. R.; Lutz, B.; Yager, P. One-Step Purification and Concentration of DNA in Porous Membranes for Point-of-Care Applications. *Lab Chip* **2015**, *15*, 2647−2659.
(139) Su, X.; Comeau, A. M. Cellulose as a Matrix for Nucleic Acid Purification. *Anal. Biochem.* **1999**, *267*, 415−418.
(140) Zou, Y.; Mason, M. G.; Wang, Y.; Wee, E.; Turni, C.; Blackall, P. J.; Trau, M.; Botella, J. R. Nucleic Acid Purification from Plants, Animals and Microbes in under 30 Seconds. *PLoS Biol.* **2017**, *15*, No. e2003916.
(141) Xu, G.; Nolder, D.; Reboud, J.; Oguike, M. C.; van Schalkwyk, D. A.; Sutherland, C. J.; Cooper, J. M. Paper-Origami-Based Multiplexed Malaria Diagnostics from Whole Blood. *Angew. Chem., Int. Ed.* **2016**, *55*, 15250−15253.
(142) Reboud, J.; Xu, G.; Garrett, A.; Adriko, M.; Yang, Z.; Tukahebwa, E. M.; Rowell, C.; Cooper, J. M. Paper-Based Microfluidics for DNA Diagnostics of Malaria in Low Resource Underserved Rural Communities. *Proc. Natl. Acad. Sci. U. S. A.* **2019**, *116*, 4834−4842.
(143) Yang, Z.; Xu, G.; Reboud, J.; Ali, S. A.; Kaur, G.; McGiven, J.; Boby, N.; Gupta, P. K.; Chaudhuri, P.; Cooper, J. M. Rapid Veterinary Diagnosis of Bovine Reproductive Infectious Diseases from Semen Using Paper-Origami DNA Microfluidics. *ACS Sensors* **2018**, *3*, 403−409.
(144) Magro, L.; Jacquelin, B.; Escadafal, C.; Garneret, P.; Kwasiborski, A.; Manuguerra, J. C.; Monti, F.; Sakuntabhai, A.; Vanhomwegen, J.; Lafaye, P.; et al. Paper-Based RNA Detection and Multiplexed Analysis for Ebola Virus Diagnostics. *Sci. Rep.* **2017**, *7*, 1−9.
(145) Udugama, B.; Kadhiresan, P.; Kozlowski, H. N.; Malekjahani, A.; Osborne, M.; Li, V. Y. C.; Chen, H.; Mubareka, S.; Gubbay, J. B.; Chan, W. C. W. Diagnosing COVID-19: The Disease and Tools for Detection. *ACS Nano* **2020**, *14*, 3822−3835.
(146) Zhou, F.; Yu, T.; Du, R.; Fan, G.; Liu, Y.; Liu, Z.; Xiang, J.; Wang, Y.; Song, B.; Gu, X.; et al. Clinical Course and Risk Factors for Mortality of Adult Inpatients with COVID-19 in Wuhan, China: A Retrospective Cohort Study. *Lancet* **2020**, *395*, 1054−1062.
(147) Sheridan, C. Fast, Portable Tests Come Online to Curb Coronavirus Pandemic. *Nat. Biotechnol.* **2020**, *38*, 515−518.
(148) Soper, G. A. The Lessons of the Pandemic. *Science* **1919**, *49*, 501−506.
(149) *Wellcome Trust. Advancing Epidemics R&D to Keep up with a Changing World: Progress, Challenges and Opportunities*; wellcome.ac.uk/sites/default/files/advancing-epidemics-rd-2019.pdf (acessed August 20th, 2020).
(150) *Overview of IVD Regulation*; www.fda.gov/medical-devices/ivd-regulatory-assistance/overview-ivd-regulation (accessed August 20th, 2020).
(151) *Regulation (EU) 2017/746 of the European Parliament and of the Council of 5 April 2017 on in Vitro Diagnostic Medical Devices, 2017*; eur-lex.europa.eu/eli/reg/2017/746/oj (accessed August 20th, 2020).
(152) *ISO 13485: Medical Devices - Quality Management Systems - Requirements for Regulatory Purposes, 2016*; www.iso.org/standard/59752.html (accessed August 20th, 2020).
(153) *Foundation for Innovative New Diagnostics (FIND)*; www.finddx.org (accessed August 20th, 2020).
(154) *PATH*; www.path.org/diagnostics/(accessed August 20th, 2020).
(155) Sheldon, R. A.; Pereira, P. C. Biocatalysis Engineering: The Big Picture. *Chem. Soc. Rev.* **2017**, *46*, 2678−2691.
(156) Seed, K. D. Battling Phages: How Bacteria Defend against Viral Attack. *PLoS Pathog.* **2015**, *11*, No. e1004847.
(157) Lander, E. S. The Heroes of CRISPR. *Cell* **2016**, *164*, 18−28.
(158) Sheldon, R. A.; Brady, D. The Limits to Biocatalysis: Pushing the Envelope. *Chem. Commun.* **2018**, *54*, 6088−6104.
(159) Chen, K.; Arnold, F. H. Engineering New Catalytic Activities in Enzymes. *Nat. Catal.* **2020**, *3*, 203−213.
(160) Russell, S. M.; De La Rica, R. Policy Considerations for Mobile Biosensors. *ACS Sensors* **2018**, *3*, 1059−1068.

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723

(161) Global Systems for Mobile Communications Association. *The Mobile Economy, 2019*; www.gsma.com/mobileeconomy (GMSA, 2019) (accessed August 20th, 2020.
(162) Schlaberg, R.; Chiu, C. Y.; Miller, S.; Procop, G. W.; Weinstock, G. Validation of Metagenomic Next-Generation Sequencing Tests for Universal Pathogen Detection. *Arch. Pathol. Lab. Med.* **2017**, *141*, 776−786.
(163) Deng, X.; Achari, A.; Federman, S.; Yu, G.; Somasekar, S.; Bártolo, I.; Yagi, S.; Mbala-Kingebeni, P.; Kapetshi, J.; Ahuka-Mundeke, S.; et al. Metagenomic Sequencing with Spiked Primer Enrichment for Viral Diagnostics and Genomic Surveillance. *Nat. Microbiol.* **2020**, *5*, 443−454.
(164) World Health Organization. *No Time to Wait: Securing the Future from Drug-Resistant Infections*; www.who.int/antimicrobial-resistance/interagency-coordination-group/final-report/en/ (accessed August 20th, 2020).
(165) Leonard, H.; Colodner, R.; Halachmi, S.; Segal, E. Recent Advances in the Race to Design a Rapid Diagnostic Test for Antimicrobial Resistance. *ACS Sensors* **2018**, *3*, 2202−2217.

https://dx.doi.org/10.1021/acssensors.0c01488
ACS Sens. 2020, 5, 2701−2723