Volume 4, Issue 6, 2026

Frontier in Medical & Health Research

ISSN: (e) 3007-1607 (p) 3007-1593

A CRISPR-BASED DIAGNOSTIC ASSAYS DEVELOPED FOR POINT OF CARE DETECTION OF INFECTIOUS DISEASES

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DOI: https://doi.org/10.5281/zenodo.21019632

Keywords

Isothermal amplification (RPA/LAMP) : Biosensing platforms; CRISPR-Cas diagnostics Point-of-care (POC) detection; Biosensing platforms for infectious diseases detection

Article History

Received: 22 April 2026
Accepted: 04 June 2026
Published: 21 June 2026

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Abstract

There are many current major limitations of the point-of-care (POC) diagnostics for infectious diseases such as low sensitivity and specificity, and the need for complex infrastructure that makes it difficult to deploy in resource-poor environments. The CRISPR-Cas system is then a programmable, sequence-specific detector with great potential for nucleic acid detection, and in particular Cas12, Cas13 and Cas14 have emerged as highly promising. In this review, the major principles and applications of major CRISPR-based diagnostics platforms, along with the unique collateral cleavage activity of CRISPR, are highlighted, and how this activity makes pathogen identification rapid and accurate. The following new developments are explored, including integration with isothermal amplification methods (RPA and LAMP) that improve the sensitivity of the assays and lateral flow readouts and smartphone integration that make these assays truly field-deployable. The review also highlights practical application of these technologies towards translation into POC application such as in the detection of antimicrobial resistance genes, viral and bacterial as well as parasitic infections. Despite these great advances, some challenges exist and are discussed critically: achieving high-order multiplexing in a single reaction, the absence of true quantification as compared to digital PCR and the regulatory approval issues to date, which have prevented many clinical applications from becoming broadly adopted. Going forward, the review presents a vision for the future where POC diagnostics based on CRISPR has a pivotal role in pandemic readiness and decentralized diagnostics. These platforms offer the promise of rapid, accurate, lab light testing at the bedside, or in a community setting, thereby potentially revolutionizing the care of infectious diseases, decreasing reliance on centralized laboratories, and increasing global health equity, especially in low and middle income countries where traditional diagnostics are not readily available

1.INTRODUCTION

The burden of infectious diseases persists as a global public health threat, as evident by the ongoing HIV/AIDS and tuberculosis epidemics, the constant need for response to arboviruses (e.g., dengue and Zika) and the COVID 19 pandemic. Delayed or incorrect diagnosis in low resource settings directly impacts transmission, clinical outcome, and antimicrobial resistance, in the absence of laboratory support and staff.[1] The delay in the turnaround time for samples to

Frontier in Medical & Health Research
ISSN: (e) 3007-1607 (p) 3007-1593

get to the laboratory and then for the test results to be communicated to the patient can be a barrier to timely infection control and patient care, even in high-income countries. Thus, there is an urgent and unmet need for rapid, accurate and deployable point-of-care (POC) testing diagnostics that can be performed at the point of patient encounter, providing laboratory quality results within minutes, not days and without complex instrumentation.[2]

The traditional POC methods, however, have natural compromises in terms of their speed, sensitivity, specificity and complexity of operation. Rapid antigen tests are commonly used for SARS-CoV-2 and influenza and are low cost and easy to use, with the limitation of low sensitivity, that is, they are likely to miss low viral loads and give false negative results. Although true POC would be desirable, conventional PCR, the gold-standard method for nucleic acid detection, is not effectively suited for POC use because of its need for thermal cycling, skilled operators, and dedicated labs. Loop mediated isothermal amplification (LAMP) is also able to benefit from the temperature problem to a certain extent, because it can run at a constant temperature so that the thermal cycled problem can be solved, but it requires sample preparation, optimization of the primers, and sometimes false positive due to non-specific amplification. Multiplexing or discriminating between closely related pathogens is not easily done without significant assay redesign with either LAMP or rapid antigen tests.[3]Therefore, a transformative method has always been sought for having the simplicity of the antigen tests and the accuracy of PCR.

The stepping stone came with the finding of the collateral cleavage activity of CRISPR Cas systems. The promiscuous nuclease activity, when observed following target recognition, was originally regarded as adaptive immune processes in bacteria and archaea; some class 2 Cas effectors are in particular, Cas12, Cas13, and Cas14. In other words, in the presence of a guide RNA, a Cas enzyme complexed with the guide forms a complex with a target nucleic acid, thereby allowing it to indiscriminately cleave nearby nontarget single stranded DNA (Cas12 and Cas14) or RNA (Cas13). This is a property that makes it possible to convert a specific recognition event into a high gain amplification signal that can be detected with the help of comparatively simple fluorophore quencher probes or lateral flow strips.[4] The researchers have exploited this phenomenon and developed such systems as “CRISPR-Dx” (CRISPR for diagnostics), which achieves attomolar (10^-18 M) sensitivity, single base specificity and operates under isothermal conditions. The beauty is to separate the target amplification, such as recombinase polymerase amplification or LAMP, from the specific detection capability of the CRISPR, and thus provide both preamplification sensitivity and the specificity of the CRISPR.

The present review will be limited to the Cas12, Cas13 and Cas14 based assays for infectious disease detection at the point of care only, and not include other effectors like Cas9 and Cas3 which present more complex delivery systems and/or lack granted collateral activity. Cas12a (formerly Cpf1) targets double stranded DNA and cleaves single stranded DNA reporters, making it possible for assays like DETECTR (DNA Endonuclease Targeted CRISPR Trans Reporter). The Cas13a is an RNA-targeting enzyme that enabled the development of SHERLOCK (Specific High-sensitivity Enzymatic Reporter unLOCKing), which has been used for the detection of SARS-CoV-2, Zika and dengue. The Cas14 is unique in its ability to target single stranded DNA without the requirement for a PAM sequence, thus increasing the possibilities of detecting smaller DNA fragments or samples that have been degraded. Cas12 is advantageous for DNA pathogens such as bacterial infection or DNA viruses; Cas13 for RNA viruses such as Ebola, influenza, and coronaviruses; and potentially, for short single stranded DNA molecules, which do not get detected by other systems.[5] They will be compared and contrasted critically in terms of sensitivity, specificity, multiplexing ability, reaction time, reagent

Frontier in Medical & Health Research
ISSN: (e) 3007-1607 (p) 3007-1593

stability and ease of integration with lateral flow or microfluidic POC devices.

The article is organized in the following way. In this section, the molecular mechanisms of Cas12, Cas13, and Cas14 are described, focusing on reaction conditions, reporter systems, and the rationale for coupling with isothermal preamplification, followed by an introduction to this topic in view of the global need and the conceptual leap from the cleavage of collaterals to diagnostics. Section 3 discusses representative infectious disease applications, grouped by type of microorganism: viral (SARS-CoV-2, HIV, hepatitis viruses), bacterial (Mycobacterium tuberculosis, antibiotic resistant strains) and parasitic (Plasmodium, Leishmania). Section 4 critically discusses the attempts to achieve true POC incorporation, such as lyophilized reagents, paper based readouts, quantification using a smartphone, and microfluidic cartridges, as well as problems encountered, including non-target effects and sample preparation lag time. Section 5 directly compares the CRISPR Dx platforms with rapid antigen tests and LAMP on the metrics of cost, turnaround time, limit of detection and field performance. Finally, in section 6, regulatory pathways, scalability and future directions are discussed, such as multiplexed CRISPR panels and artificial intelligence assisted result interpretation. This review synthesizes the state-of-the-art of Cas12, Cas13, and Cas14 based diagnostics to provide a research angle and a clinical guide to realizing next generation POC tests, which have the potential to transform the response to infectious diseases around the world.[6]

2. Principles of CRISPR based Diagnostics

The molecular diagnostics application of CRISPR Cas systems is entirely based on one particular phenomenon of the enzymes, termed "collateral cleavage. In contrast to the canonical CRISPR immunity that cleaves the invading nucleic acid in a sequence-specific manner, some class 2 Cas effectors undergo a conformational change on target recognition that triggers a non specific promiscuous nuclease activity on surrounding reporter molecules. This finding was the key to using a bacterial defense mechanism as a programmable, high gain signal amplification system. This section dissects the mechanistic basis of the three major effectors suitable for point-of-care applications, namely, Cas12, Cas13 and Cas14.[7] It then reviews preamplification techniques upstream and signal transduction modalities downstream, and the key performance parameters that are important for a clinically useful diagnostic assay.

2.1. Collateral Cleavage in Cas12, Cas13 and Cas14.

DNA targeting effector Cas12a (previously called as Cpf1) recognizes the target sequence on double stranded DNA with a protospacer adjacent motif (PAM) upstream of the target sequence. Cas12a will bind a CRISPR RNA (crRNA) which is complementary to the target, then look for the PAM sequence and will create a ribonucleoprotein complex if it finds it. Upon cognate DNA duplex recognition, a conformational change exposes the RuvC nuclease domain leading to activation. In this domain, the target DNA is first cleaved by cis cleavage to create a double strand break. Thereafter the same active site displays trans-cleavage activity, hydrolysing single stranded DNA (ssDNA) molecules in the reaction milieu indiscriminately.[8] This collateral effect is very strong, with thousands of ssDNA reporters being cut per minute by a single activated Cas12a molecule. Perhaps most important of all, Cas12a does not nonspecificly cleave double stranded DNA or RNA.Importantly, Cas12a only cleaves ssDNA, not double stranded DNA or RNA non specifically. This specificity enables the use of short (ssDNA) oligonucleotides with fluorophores and quenchers as direct reporters of target recognition.[9]

Cas13a, on the other hand, is an RNA directed RNA-targeting effector. Cas13a is a member of the type VI CRISPR system and is able to recognize single-stranded RNA targets without a PAM sequence. Once the crRNA is bound to complementary RNA sequences, the two higher

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ISSN: (e) 3007-1607 (p) 3007-1593

eukaryotes and prokaryotes nucleotide binding (HEPN) domains within Cas13a are catalytically competent. These sites cleave the target RNA cis, after which they produce a strong trans cleavage of any single stranded RNA in the neighborhood. Cas13a is capable of cleaving RNA reporters efficiently, in contrast to Cas12a that only cleaves ssDNA reporters. The collateral activity of the Cas13a is so strong that it can degrade over 10^3 non target RNA molecules within minutes of activation.[10] In some workflows, RNA viruses have been detected directly from clinical samples, without reverse transcription, using this property. Unlike DNA, Cas13a is inactive against RNA, ensuring orthogonal specificity for RNA based diagnostics.

Cas14 is the latest member of the collateral cleavage diagnostic toolbox. Cas14 is a unique effector that requires no PAM for binding to single stranded DNA. This is important because many clinically relevant DNA fragments, including those from degraded DNA, cell free DNA or short amplicons, might not be able to be accessed by Cas12 because of secondary structure or lack of a nearby PAM. Unlike Cas12 or Cas13, Cas14 is a small protein, with a molecular mass of ~40-70 kDa. When the Cas14 recognizes a complementary ssDNA target, Cas14 is activated into its nuclease activity and it then goes on to clean up non target ssDNA reporters collateral side by side. Sensitivity of Cas14 based detection methods is also in the attomolar range, similar to that of Cas13. Cas14 is not suitable to cleave double stranded DNA or RNA, however, making it suitable for applications where single stranded nucleic acids are the main analytes. The three effectors all follow the same diagnostic principle - recognition of the target causes collateral cleavage.[11] They differ with regards to their various nucleic acid substrates, their requirement for PAM and optimal reaction conditions. Cas12 is used to detect bacterial pathogen and DNA virus targets, Cas13 is the platform of choice for RNA virus detection, and Cas14 is used for short or highly degraded ssDNA targets.

2.2. A comparison of pre amplification Strategies with Amplification Free Methods.

The sensitivity of the unamplified collateral cleavage by CRISPR Cas is on the order of femto- to picomolar, which is too low to detect low-titer pathogens in clinical samples like blood, urine, or nasopharyngeal swabs. Most CRISPR diagnostics, therefore, feature an initial nucleic acid amplification component. The two isothermal amplification methods that are most compatible with CRISPR readouts have been recombinase polymerase amplification (RPA) and loop mediated isothermal amplification (LAMP). RPA uses recombinase enzymes to "prime" DNA synthesis at steady temperatures of 37°C - 42°C. The reaction is fast, usually taking 10 to 20 minutes; and produces double stranded DNA amplicons that are suitable as detection targets of Cas12.[12] The shortness of RPA primers and the ability of reaction mix to tolerate crude lysates allows for true point of care integration. An important benefit of RPA is that it has the capacity to amplify both DNA and RNA targets with reverse transcriptase. The major drawback of RPA is that it can give rise to non-specific product formation, resulting in background signal in subsequent CRISPR reactions. This can be controlled by careful primer design and the use of uracil DNA glycosylase for carryover contamination.

LAMP also runs at elevated temperatures (60°C – 65°C) and utilizes a series of four to six primers that target different parts of the target. The strand displacing activity of Bst polymerase produces a lot of concatemeric double stranded DNA and other secondary structures such as looped single stranded DNA. This complex mixture of amplification products provides both opportunities and challenges for detection of CRISPR. The double stranded DNA can trigger the activation of Cas12, and single stranded loops can be direct triggers of Cas14 and/or be used as substrates of Cas13 (if RNA targets are used). LAMP is more sensitive than RPA, and can be used to detect levels of just a few copies per reaction. LAMP, however, is highly susceptible to amplicon contamination and the design of

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ISSN: (e) 3007-1607 (p) 3007-1593

primers is more critical. Portable heater also required by higher temperature, adding to the complexity of the devices.[13] Both RPA and LAMP can be accomplished in a very limited-facility environment and a number of published CRISPR Dx platforms incorporate either form of the amplification directly into one tube or a two step amplification followed by CRISPR collateral cleavage in a closed tube.

Amplification free methods have risen to become a Holy Grail of true point of care simplicity. The objective is to obtain clinically meaningful sensitivity without the need for any enzymatic pre amplification: this takes away the need for thermal cyclers, heaters and the risk of cross contamination. The ability of direct CRISPR detection has been expanded in recent years. Researchers have reported reaching 10 femtomolar (10-15 molar) or 1 femtogram (10-15 gram) limits of detection with optimized conditions for crRNA design, concentration of Cas protein, reaction buffer composition, and incubation time, for synthetic targets. In optimal conditions, the detection of viral RNA through amplification free Cas13 can be as low as ~100 copies/microliter.[14] That sensitivity is not as good as RPA-CRISPR or RT-PCR which regularly detect 1 to 10 copies per reaction. However, when a viral infection is severe, like with the COVID-19 and influenza viruses (viral loads can be above 10^6 copies per milliliter), amplification-free CRISPR assays are possible. A handful of groups have shown that SARS-CoV-2 RNA can be directly detected from nasopharyngeal swabs without preamplification with Cas13 and with a sensitive electrochemical readout. Amplification Free Workflows provide assay times of less than 30 minutes, simplify reagent lyophilisation, and minimize users' steps. The compromise that must be made is analytical sensitivity. A compromise is hybrid approaches (using a short 10 minute isothermal pre amplification). These approaches are very close to the simplicity, yet they significantly close the sensitivity gap.[15]

2.3. Signal Transduction: Fluorescence, Lateral Flow, Electrochemical, Colorimetric

The "collateral cleavage event" needs to be translated into a readable signal. The signal transduction modality has a significant impact on assay cost, ease, equipment, and quantitative ability. The most frequently used readout method in research is the fluorescence technique due to its excellent sensitivity and real-time monitoring ability. A typical reporter for a Cas12 or Cas14 or a short RNA for Cas13 comprises a short ssDNA (for Cas12 or Cas14) or a short RNA (for Cas13) with a fluorophore at one end and a quencher at the other end. The quencher will suppress the fluorescence when the molecule is not dissociated.[16] The reporter is cleaved on both sides and the fluorophore and the quencher are separated, resulting in the detection of an increasing amount of reporter fluorescence in proportion by use of a portable fluorimeter. The limit of detection is as low as 1 attomolar for pre-amplified fluorescence readout, there is excellent dynamic range and kinetic measurements are possible. The major drawback is the requirement to use an optical detector, although it can be miniaturized, it is expensive and complicated.[17]

LFA is the most appropriate testing format for true point of care use in low resource locations. They are paper strips like home pregnancy tests, and use only the eyes. Most common design of CRISPR based lateral flow is of dual labelled reporters. A typical reporter is ssDNA or RNA with a biotin at one end and a fluorescein amidite (FAM) at the other. The lateral flow strip is made up of two capture lines; one immobilized with anti FAM antibodies and the second immobilized with streptavidin. Anti FAM antibodies are used as visual indicators that are conjugated to gold nanoparticles. If there is no collateral cleavage, the intact reporter will attach to the anti FAM gold nanoparticles and will be trapped at the test line creating a visible red band.[18] If Cas collateral cleavage occurs, the reporter is broken, biotin and FAM will be separated and the FAM gold complex will not be held at the test line. However, it passes by to the control line that will capture biotinylated

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fragments. Hence, a positive result would only be the control line and a negative result would be both lines. To some it sounds counterintuitive, but the format was proved to be powerful, low-cost and reliable. The lateral flow CRISPR assays with pre amplification can deliver sensitivity levels similar to those of fluorescence detection within 15-30 minutes.

The benefits of electrochemical transduction include quantitative output; portability; and immunity to optical interferences due to turbid or coloured samples. The most common use for electrochemical CRISPR biosensors involves immobilizing the Cas ribonucleoprotein complexes or DNA reporters on gold electrodes. Redox active tags are released from the DNA or the efficiency of electron transfer to the DNA is reduced by collateral cleavage. The electric current change is measured with differential pulse voltammetry or chronoamperometry.[19] An electrochemical method avoids the necessity of fluorophores, quenchers and optical components; it uses a low-cost potentiostat device, which can be plugged into a smartphone. Amplification-free electrochemical CRISPR assays have shown sub femtomolar sensitivity for viral RNA, comparable to pre-amplified optical assays. The technical difficulty is in the fabrication of the electrodes, the reproducibility of their surface chemistry, and a need for the skilled handlers to achieve stable baselines. However, small, portable electrochemical CRISPR readers are beginning to come to fruition and several prototypes have been clinically validated.[20]

The least complicated is colorimetric detection. In this case, the enzyme or nanomaterial coexists with the collagen so that its cleavage results in a colour change that is not detectable by the naked eye. A common strategy involves the use of a ssDNA reporter molecule that is attached to horseradish peroxidase, which is then bound to a magnetic bead. The horseradish peroxidase is released from the plant when the collaterals are cut, and they cause a colourless substrate to be produced into a coloured product. Another beautiful technique uses the aggregation of gold nanoparticles. Gold nanoparticles, which are positively charged, bind to intact reporters containing ssDNA and stay in a dispersed state and red. The cleaved fragments are unable to stabilise the nanoparticles, resulting in a change in colour from red to blue to purple due to aggregation caused by salt addition. Colorimetric readouts are the least sensitive among the four modalities but the most equipment free.[21] They are suitable for high burden infections or after prolonged pre amplification. A balance between sensitivity and simplicity is important. In many low resource situations, an 80% sensitive colorimetric test may be more useful than a test that simply cannot be deployed that is 100% sensitive.

2.4. Important Performance Indices: Limit of Detection, Specificity, Turnaround Time

The clinical usefulness of any point of care CRISPR assay is determined by three related metrics: limit of detection, specificity, and turnaround time. The lowest concentration (or absolute number of copies of targeted nucleic acids) that can be reliably differentiated from a blank is called the limit of detection (LOD). In reality, the number of copies per microliter is usually used to represent the number of LOD, or as a molar concentration. The LOD of pre amplified CRISPR Cas12 and Cas13 assays is between 0.1 and 10 copies/microliter of input sample. With RPA, the lowest reported LOD for Cas12 detection of human papillomavirus (HPV) is 0.5 copies per microliter. The LODs of 2 copies per microliter are attainable for Cas13 detection of SARS-CoV-2 using RT-RPA. Amplification free methods provide higher LODs, typically in the range of 100 to 1,000,000 copies/mL, depending on the type of Cas effector, reporter design and readout modality used.[22] For Zika virus RNA, Electrochemical amplification free CRISPR Cas13 has reached the LOD of 10 copies/microliter - a remarkable achievement. The majority of the amplification free fluorescence assays reach a level of 1000 copies/uL. The clinically important LOD is different depending on the infection. Assays

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should be able to detect 500 copies/mL in nasopharyngeal swab which is also equivalent to 0.5 copies/µL for COVID 19 diagnosis. An HIV viral load monitoring LOD of 50 copies/mL is desirable. Not every CRISPR assay is able to achieve these targets without pre amplification.

There are two levels of specificity in diagnostics for CRISPR. The first is the natural specificity of the Cas-crRNA recognition. All of the Cas12, Cas13 and Cas14 are single base resolution. Even one mismatch between the crRNA and the target will eliminate activation and collateral cleavage, especially in the seed region.[23] This property allows to distinguish single-nucleotide polymorphisms, viral variants and drug resistance mutations. The second level of specificity is "off target" collateral activation, which is absent. The Cas ribonucleoprotein should only induce trans cleavage in the presence of the targeted pathogen sequence in a well-designed assay. There is some cross reactivity with highly homologous sequences from related pathogens, which can be systematically excluded by empirical crRNA screening. Cas13 and Cas14 rarely have false positives, while they have been reported in Cas12 at high enzyme concentrations. Specificity is usually expressed as the percentage of true negatives that are negative. Specificities of 95%-100% are typical for published CRISPR Dx platforms that have been validated on clinical samples, similar to PCR. Most studies however have tested a relatively small number of samples (100-300). More multi centre studies are required to produce accurate specificity measures in a variety of patient groups and sample types.[24]

Turnaround time refers to the time it takes to collect, process, amplify, detect with CRISPR, and interpret results. Ideally, turn-around time is less than 60 minutes for a point of care test. Pre amplified CRISPR assays with RPA take 10-20 minutes to amplify and then 15-30 minutes for CRISPR collateral cleavage and readout. With a simple sample lysis step, the total time is 30-50 minutes. The higher temperature and longer amplification time (20 to 40 minutes) results in slightly longer workflows with LAMP. Two-step methods in which amplification and CRISPR detection take place in separate tubes, involve opening the tubes, which adds to the time and the risk of contamination. One pot reactions, in which all reagents are added at the same time, can be performed in 30-45 minutes. Amplification free direct detection gives results in 15 to 25 minutes from sample application which is the fastest. Lateral flow readouts require 5–10 more minutes for development and near instant readouts are fluorescence and electrochemical.[25] In practical situations, the time of enzyme reactions is not the rate limiting process; rather, sample preparation is. The ability to utilize raw saliva, whole blood or urine without extracting the nucleic acids is an active research frontier for adapting the CRISPR assays. Heat inactivation, detergent lysis and binding to magnetic beads are promising approaches. The more time that goes into preparing the sample, the more it costs to use at the point of care.

The operating envelope for these three metrics determines the operating envelope for each of the CRISPR diagnostics. A test that has a very low LOD and two hour turnaround time is not appropriate for acute infection control.[26] If a test is very specific but needs a fluorescence reader it is not suitable for home use. Rapid tests have poor LOD and will not detect infectious persons with low viral loads. The best assay depends on the application and optimizes all three parameters. In outbreak response, it may be more important for the test to be quick and easy than for it to be absolutely sensitive. If a diagnosis is needed for a patient with limited resources or for tuberculosis or HIV, LOD is of paramount importance at the cost of length of turnaround time, and specificity is high. The next few years will bring ongoing development and optimization of Cas enzymes, more sophisticated amplification schemes and combined sample to answer cartridges that further move the boundaries towards the realization of a truly universal point of care molecular diagnostics.[27]

3. Major CRISPR Diagnostic Platforms

With the identification of collateral cleavage activity, multiple different diagnostic platforms

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ISSN: (e) 3007-1607 (p) 3007-1593

have emerged, each made with a different Cas effector and engineered innovations. They all have this basic idea of target activated trans-cleavage, but also have a lot of variations in terms of what nucleic acid targets they use, how they set up their reactions, how they multiplex them, what they read out, and whether they would be good for point of care integration. SHERLOCK, DETECTR, HOLMES and the new Cas14 based systems are the four most influential platforms.[28] A critical analysis of each of their advantages and disadvantages gives a guide as to which technology should be used for a specific infectious disease application.

3.1. RNA Targeting, Multiplexing with SHERLOCKv2.SHERLOCK (Cas13): RNA Targeting, Multiplexing with SHERLOCKv2.

Specific High sensitivity Enzymatic Reporter unlocking (SHERLOCK), is the first diagnostic platform based on CRISPR described in the literature. The original SHERLOCK system uses Cas13a as its effector protein, which specifically targets single-stranded RNA without the need for any protospacer adjacent motif. This feature makes SHERLOCK an ideal direct detection platform for RNA viruses such as Ebola, Zika, dengue, influenza and severe acute respiratory syndrome coronavirus 2.[29] In the standard SHERLOCK workflow, the clinical sample is first nucleic acid extracted and then isothermal pre amplified by recombinase polymerase amplification. In the case of RNA targets, this amplification step involves reverse transcriptase to produce complementary DNA (cDNA) which is then transcribed into RNA amplicons using T7 RNA polymerase. These RNA amplicons directly activate the crRNA ribonucleoprotein complex (RNP) of Cas13a. Once it recognizes the target, Cas13a collateral trans-cleaves a short RNA reporter molecule labeled with a fluorophore and quencher, resulting in a fluorescence signal proportional to the concentration of the initial target.[30]

SHERLOCK is an incredibly sensitive detector. Using pre amplification, the platform typically can detect single digit copies per microliter routinely. SHERLOCK is detecting as little as 0.5 copies/microliter for Zika virus. The platform is able to distinguish between four serotypes of the Dengue Virus with a panel of specific crRNAs. SHERLOCK can also detect and identify single nucleotide polymorphisms with high accuracy, so that it can identify strains of the Zika virus from Africa and the Americas, or find antibiotic resistance mutations in bacterial pathogens. The platform has been modified for lateral flow readout with RNA reporter molecules with a biotin and a fluorescein amidite tag, without the need for a fluorescence detector.[31] The unique characteristic of SHERLOCK is that it remains functional for more than one year when used on paper discs containing lyophilised reagents, a key aspect for low-resource settings.

The biggest step forward in the development of the SHERLOCK family is the introduction of the multiplexed detection functionality of SHERLOCKv2. Cleaving of collaterals with multiplexing is inherently difficult for collateral cleavage systems because the trans activity is sequence independent and cleaves all reporter RNA, regardless of sequence. SHERLOCKv2 overcomes this limitation with two orthogonal approaches. The first strategy is based on orthogonal Cas effectors that have different substrate preferences. SHERLOCKv2 combines the use of both Cas13a from Leptotrichia wadei that cleaves RNA reporters and Cas12a from Francisella novicida that cleaves ssDNA reporters.[32] These two effectors work on the same reaction without any cross interference as they act on different chemistries of nucleic acids. The second approach takes a different tactic: making use of orthogonal nucleotide reporters. Nucleotide preferences of the RNA reporter vary with different orthologs of Cas13. The SHERLOCKv2 can uniquely identify up to four different targets in a single reaction volume, by choosing fluorescent labels and quencher pairs that are different. The platform has been shown to detect four targets (or four mutations) of different viruses simultaneously.

SHERLOCKv2 also features a signal amplification cascade to boost the sensitivity

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ISSN: (e) 3007-1607 (p) 3007-1593

without the need for pre amplification. Cas13a is activated by a low concentration of target RNA, which subsequently cleaves an RNA activator molecule that is normally separated from a second Cas enzyme.[33] The cut-off activator is then used to activate a second set of Cas13a or Cas12a, creating a signal amplification cascade. This cascading approach extends the limit of detection into the low femtomolar range, without pre-amplification of the target, so making the process simpler. The biggest drawback of SHERLOCK is the need for a few enzymatic reactions. The composition and timing of the buffers is critical for reverse transcription, RPA, T7 transcription, and Cas13 cleavage. However, SHERLOCK and SHERLOCKv2 have been tested on hundreds of clinical samples from different pathogen categories, and are recognized as a premier CRISPR Dx platform.[34]

3.2. DETECTR (Cas12a): DNA Targeting, Lateral Flow Readout is a DNA-targeting lateral flow readout technology that uses Cas12a proteins.

The SHERLOCK and the DNA Endonuclease Targeted CRISPR Trans Reporter (DETECTR), developed at the same time, use different effector nucleases. DETECTR is the platform to target double stranded DNA, whereas SHERLOCK is for RNA, which makes it the platform of choice for bacterial pathogens, DNA viruses, and any application where DNA is the primary analyte. Conceptually, the DETECTR workflow is similar to SHERLOCK, but is streamlined. After sample preparation, target DNA undergoes amplification by RPA producing double-stranded amplicons at a constant temperature. These amplicons are mixed with the Cas12a crRNA ribonucleoprotein complex and a single stranded DNA reporter.[35] Once Cas12a recognizes the target DNA sequence in conjunction with a protospacer adjacent motif, Cas12a switches on its collateral cleavage function, cleaving the reporter ssDNA. The readout may be either fluorescence or, more typically, lateral flow based, for point of care applications.

DETECTR has been very successful in a field format known as the lateral flow device. The standard DETECTR lateral flow reporter is a short ssDNA oligonucleotide, with a fluorescein amidite molecule at one end and a biotin molecule at the other. If the reporter is intact, it will also capture anti-fluorescein amidite antibodies that are attached to gold nanoparticles and streptavidin that is attached to the control line on the lateral flow strip. This will create a colour line at the test position. If Cas12a collateral cleavage breaks the reporter, the fluorescein amidite gets separated from the biotin, which will then not be captured at the test line.[36] However, a positive result will only indicate the control line, and a negative result will indicate both lines. Once trained, this 'inverse' readout is very intuitive. DETECTR lateral flow strips cost about $1 per test, have only a simple heat block for the RPA reaction, and provide results from sample to answer within 30-45 minutes.[37]

DETECTR is a highly validated sensitive and specific test. DETECTR has clinical sensitivity of 95% for HPV detection and 100% specificity for HPV 16 & HPV 18 (high risk types) versus PCR detection. A DETECTR assay for the detection of SARS- CoV -2 on nasopharyngeal swabs using the E and N genes had a 95% sensitivity and a 100% specificity during the COVID 19 pandemic. All positive samples, that have cycle threshold values below 35, which represent clinically relevant viral loads, were correctly identified in the Assay. In the case of bacterial pathogens, DETECTR has been able to identify methicillin resistant Staphylococcus aureus (MRSA) from positive blood culture bottles, by detecting the presence of the mecA gene – while identifying the presence of methicillin susceptible Staphylococcus aureus (MSSA).[10] The platform can also be used to identify Mycobacterium tuberculosis in sputum samples, but is slightly less sensitive in paucibacillary samples than PCR.

One drawback of DETECTR is that it requires PAM. Cas12a has a specific dinucleotide PAM sequence adjacent to the target site, resulting in limited target sites. Some pathogens (especially

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ISSN: (e) 3007-1607 (p) 3007-1593

those that have low guanine cytosine content and highly conserved genomic regions) may have difficulty finding an optimal target with an appropriate PAM. Multiple Cas12 orthologs have been identified for the purpose of reducing the PAM requirement, but not removing it completely. Another limitation of DETECTR is background signal that can be observed in the absence of target when high concentration of Cas12a is used, which is caused by its spontaneous nuclease activation. This can be minimised by optimising enzyme concentration and buffer conditions. However, DETECTR is one of the most popular CRISPR Dx platforms because of its ease of use, strong lateral output, and ability to leverage existing isothermal amplification systems.[38]

3.3. HOLMES (Cas12b): One Pot Reaction.

Holmes is an acronym for one hour low cost multipurpose highly efficient system, a new architecture based on Cas12b. Derived from Bacillus species, Cas12b has several improvements over Cas12a for point of care diagnostics. Cas12b does not require low temperatures, and its optimal temperature range is 48°C–52°C, which can decrease the risk of nonspecific amplification and increase the specificity of the reaction. The platform also has a higher activation threshold for collaterals, which reduces the background noise if there is no real target.[39] The most important result brought by HOLMES to the CRISPR Dx field, however, is the ability to perform the target amplification and CRISPR detection in the same tube, at the same time, with reagents added at the same time, avoiding the sequential addition of reagents.

This integration is realized in the HOLMES one pot configuration by careful optimization of the reaction buffer composition and enzyme ratio. Unlike Cas12b collateral cleavage, Recombinase polymerase amplification requires magnesium acetate to be added to initiate the reaction, and does not work at all over a broad range of magnesium concentrations. HOLMES is able to identify a window of magnesium concentration that works well without RPA and Cas12b being inhibited from each other. The platform uses a variant of Cas12b which is thermostable with a permissive temperature range of 37°C to 42°C, similar to that of RPA. The RPA primers, Cas12b crRNA complex, single stranded DNA (ssDNA) reporter and magnesium acetate are all mixed initially to initiate the assay. The tube is set on a simple heat block, and the reaction takes place continuously for 30 to 45 minutes. Finally, the fluorescence is measured or a lateral flow strip is inserted directly into the reaction tube.[40]

One pot configuration eliminates the possibility of cross contamination that would be possible with 2 step protocol. The amplification tube(s) are opened to add the CRISPR detection reagents, which leads to the release of aerosolized amplicons, potentially contaminating laboratory surfaces and causing false positive results in subsequent testing. Closed tone one pot reactions allow for the absence of any dedicated containment measures to be used while HOLMES is operating in a non-laboratory environment. This is a great feature for the true POC deployment in the community health clinic, pharmacy, or home setting. HOLMES has been validated for subtyping influenza A (H1N1 vs H3N2) and influenza B (subtype B vs B/Victoria and B/Yamagata).[41] The platform can also be used for detecting norovirus in stool samples and African swine fever virus in porcine blood, reflecting its versatility for sample types.

The sensitivity of HOLMES is comparable to DETECTR, with detection limits of 1 - 10 copies per microliter for most targets. The turn around time is approximately 30-40 minutes, with the single step format. A major drawback is that Cas12b is not as commercially available as the other two effectors, Cas12a and Cas13, which explains the limited research groups that have historically worked with this effector. In addition to the simpler crRNA of Cas12a, Cas12b needs a more complex crRNA structure with additional secondary features. The recent engineering of crRNAs made the platform more accessible, and the discovery of more active Cas12b at cooler temperatures has simplified the design of crRNAs.[42]

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3.4. The Cas14 assay was used to detect single stranded DNA targets.

Cas14 is the most recent of the discovered families of effectors, which is unique in being able to recognize and target single stranded DNA with no protospacer adjacent motif. Cas14 is a type V-F effector. It belongs to the class 2 effector group, and is about half the size of Cas12 or Cas13, weighing between 40 to 70 kilodaltons. The small size makes the production of recombinants easier and may lead to better stability for lyophilisation. Cas14 recognises complementary single stranded DNA targets with a guide RNA and once bound, collateral cleavage of non target single stranded DNA molecules is activated. Significantly, Cas14 does not have any activity with double-stranded DNA or RNA, meaning that it can only be used for single-stranded DNA diagnostics.

There are several unique use cases for Cas14. The genomes of many clinically important DNA viruses are single-stranded, like parvovirus B19 and some circoviruses, and such viruses can be directly targeted by Cas14 without the need for denaturation. Second, blood or urine carries free DNA fragments that are often partially single stranded from fragmentation or secondary structure making them susceptible to Cas14. Thirdly, isothermal amplification reactions such as LAMP or rolling circle amplification produce single stranded intermediate. These intermediates can be detected in real time by Cas14 and the readout is inherently associated with amplification efficiency.[43] Fourth, short synthetic oligonucleotides, as probes or primers, can be directly detected, paving the way for applications in tracking environmental contamination or quality control.

The sensitivity of Cas14 based detection is comparable to that of Cas12 and Cas13. Cas14 can identify single stranded DNA targets at attomolar levels with RPA. Enhanced Cas14 assay results in 94% sensitivity and 98% specificity against PCR for clinical serum samples of parvovirus B19. The targets of Cas14 are the same as those of Cas12, and it performs well for detecting the same targets as Cas12 in the case of HPV, where the viral genome is double-stranded but generates single stranded intermediates during amplification. One of the unique advantages of Cas14 is that it is able to detect short single stranded DNA fragments without the length of double stranded binding by Cas12 with PAM. Cas14 is active with shorter fragments (20-nucleotide targets) while Cas12 requires double stranded targets larger than 40 base pairs to be highly activated.[44]

Another characteristic feature of Cas14 is its exquisite single base specificity. The Cas14 crRNA complex is able to discriminate mismatches with high specificity, even in the distal parts of the target as there are no structure constraints in single-stranded DNA. This feature allows to detect drug resistance mutations in the HIV reverse transcriptase or hepatitis B virus polymerase gene directly in single stranded amplicons. Lateral flow and fluorescence readout are also possible on the platform, with the same ssDNA reporter chemistry as DETECTR. The main drawback of Cas14 is the significantly less validation on clinical samples than SHERLOCK and DETECTR. The number of independent laboratories that have implemented Cas14 is relatively low and the number of published clinical studies is still limited. In addition, Cas14 is not as efficient on long double stranded targets that need to be denatured or converted to single stranded before they can be targeted. Cas12a is a simpler option for bacterial and DNA virus diagnostics, for most such cases. Cas14 is a unique DNA binding CRISPR effector that has been developed to recognize short, single-stranded, or short fragments of DNA that other CRISPR effectors are not able to bind.

3.5. A comparison of the CRISPR Dx Platforms

The four platforms are compared systematically with each other, based on the principal performance and operational characteristics and this comparison provides platform selection for certain diagnostic applications. SHERLOCK is an RNA-only assay and has a 3-step amplification and detection process that involves reverse transcription, RPA, and T7 transcription. The

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platform boasts attomolar sensitivity, with superb single base discrimination. Unlike other platforms, SHERLOCKv2 provides multiplexing support for four targets at a time. Multiple enzymatic steps cause this to be the longest turnaround time for the four platforms, ranging from 45-90 minutes.[45] The cost of the reagents is moderate and three separate enzymes are required, which adds to cost. It does take a bit of time to operate, so its best use is in the laboratory or a well equipped clinic. Primary applications include the detection of RNA viruses, serotyping, and multiplexed antimicrobial resistance profiling.

DETECTR is designed to detect double stranded DNA with a 2-step workflow: RPA+ Cas12a collateral detection. The platform reaches the same sensitivity as SHERLOCK (0.5–10 copies/µL). Multiplexing is not possible in the standard DETECTR configuration, because different complexes of Cas12a do not distinguish each other with the standard configuration. The turnaround time is 30 to 45 minutes, which is less than SHERLOCK. Reduced reagent cost due to reduced enzyme requirement. Simple and moderate complexity, especially for lateral flow readout. DETECTR is not only suitable for field deployment in low resource settings, but also for use in resource-rich environments. Examples of primary clinical applications are the detection of DNA viruses, identification of bacterial pathogens and genotyping of human papillomavirus. Some targets will have limitations of the PAM requirement.[46]

Sharing the DNA targeting ability of DETECTR, HOLMES is distinguished by the one pot reaction format. The sensitivity is 1 to 10 copies/microliter. Multiplexing is not supported. The workflow only involves one step, so all the time to complete is 30-40 minutes, the fastest of all platforms. The cost of reagents is similar to that of DETECTR. Simple, users fill one tube with sample and await results. The closed tube format creates a contamination risk-free environment, enabling HOLMES to be used in decentralized testing in community environments. The main clinical use are influenza subtyping, norovirus typing and veterinary diagnostics. Cas12b reagents are not commercially available, and the crRNA design is more complicated, which are challenges for the broad use of Cas12b.

Cas14 based platforms are single-stranded DNA PAM-free. The sensitivity is similar to 0.5 to 10 copies/µl for DETECTR and HOLMES. Multiplexing has not yet been proved. The turn-around time is about 30 – 50 minutes as for DETECTR. Reagent expenses are average. Moderate complexity; care needs to be taken with single stranded targets to avoid secondary structure formation. Primary clinical applications are single stranded DNA viruses, cell free DNA and short fragments. At present, confidence in Cas14 for routine diagnostics is low due to the less extent of clinical validation.

The selection of platform for diagnostic developers will vary depending upon the infectious disease application. Although turnaround is longer, SHERLOCK is excellent where multiplexing or RNA detection is a critical function. DETECTR provides the most simple, sensitive, and validated targets for most bacteria and DNA virus targets. Ideal for true point of care deployment in non traditional settings, HOLMES offers the simplest user experience and lowest contamination risk.[47] Cas14 based assays overcome niche applications that require short or single-stranded DNA fragments that are not compatible with other platforms. These divisions are continually becoming more indistinct, as continuous protein engineering and assay optimisation happen. New hybrid platforms such as SHERLOCKv2, which use orthogonal Cas effectors in one reaction, are the future. Cas13 could be included in the ideal point of care test for RNA viruses, Cas12 for DNA pathogens and Cas14 for targets like fragments or cell free targets in a single cartridge. This is a universal CRISPR Dx platform that is still an engineering goal.Table 1 shows: An overview of the different CRISPR-Cas diagnostic platforms for infectious disease diagnosis for infectious disease detection, showing the different types of Cas effector, target

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nucleic acid, amplification method, detection limit, readout type and the example pathogens

Table 1: Comparison of Major CRISPR-based Diagnostic Platforms.

Cas Effector	Target Nucleic Acid	Amplification Method	Detection Limit	Readout Type	Example Pathogens
Cas12a (e.g., LbCas12a)	DNA	RPA or LAMP	1–10 copies/µL	Fluorescence or Lateral flow	HPV, *Mycobacterium tuberculosis*
Cas13a (e.g., LwaCas13a)	RNA	RPA followed by T7 transcription	1–100 copies/µL	Fluorescence or Lateral flow	SARS-CoV-2, Dengue virus, Zika virus
Cas12b (e.g., AapCas12b)	DNA	RPA	~10 copies/µL	Fluorescence	*Pseudomonas aeruginosa*, *Staphylococcus aureus*
Cas13b (e.g., PsmCas13b)	RNA	RPA + T7	~1 copy/µL	Fluorescence	SARS-CoV-2, Influenza A
Cas14a (e.g., Un1Cas14a)	ssDNA	RPA	~1–10 copies/µL	Fluorescence	Human papillomavirus (HPV)
Cas12f (e.g., Cas12f1)	DNA	RPA or LAMP	~100 copies/µL	Fluorescence	*Escherichia coli* O157:H7

4. Applications for Infectious Disease Detection

Infectious disease detection has been revolutionized by the development of CRISPR Cas based diagnostic platforms (SHERLOCK, DETECTR and HOLMES) that are high in specificity and have been developed to be utilized at point of care. These systems are based on the collateral cleavage activity of the Cas effectors (e.g., Cas12a, Cas13a) when they recognize the target, which is followed by sensitive readout by fluorescence or lateral flow.[48] They are detailed below with respect to their applications in viral, bacterial, parasitic and antimicrobial resistance (AMR) context and a case study on differential diagnosis.

4.1. Viral Infections

CRISPR Cas diagnostics have been broadly applied to RNA and DNA viruses of world health importance. For SARS CoV 2, the combination of isothermal amplification (RPA or LAMP) with Cas13a based SHERLOCK or Cas12a based DETECTR protocols can detect SARS CoV 2 at 1-100 copies/µL and can differentiate SARS CoV 2 from other coronaviruses in one hour. Likewise, in the case of HIV, the sensitivity of Cas13a to detect cell free viral RNA is sub attomolar, which is a promising technique for early infant diagnosis and viral load monitoring. Hepatitis B virus (HBV, DNA), and hepatitis C virus (HCV, RNA) after reverse transcription and RPA are detected via Cas12b with LAMP with a

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limit of detection of ~10 copies/µL. Multiplexed Cas13 assays discriminate between influenza A, B, and influenza A subtypes. Zika virus and dengue virus are just two examples of arboviruses that are precisely differentiated by Cas13a (see Section 4.5), and Cas12a has been adapted for serotyping the dengue virus.

4.2. Bacterial Infections

CRISPR Cas specificity is useful for the detection of bacterial pathogens in distinguishing closely related species.[49] Reduced to a matter of under two hours, compared to a culture, a combination of Cas12a with RPA is used to detect as few as 10 copies of IS6110 DNA in sputum samples for Mycobacterium tuberculosis (MTB). Cas13a assays based on recombinase polymerase amplification (RPA) can detect 1-5 copies/µL of Chlamydia trachomatis in urine or swab samples. Rapid outbreak source tracking in CSF is possible with Neisseria meningitides detection using Cas12a against the capsular transport gene (ctrA) with LOD ~10 copies/µL.

4.3. Parasitic Infections

Parasitic diseases are endemic in low resource areas and are particularly appropriate for field deployable CRISPR diagnostics. For Plasmodium spp. After LAMP amplification, Cas12a-based platforms can detect the genus conserved 18S rRNA gene of malaria (P. falciparum and P. vivax) with LOD of ~1 parasite/µL and single base mismatches.[50] To detect the parasite's satellite DNA, Trypanosoma has been adapted to be targeted by Cas13a, which can detect it directly from blood or tissue lysate without requiring purification and extraction of nucleic acids; the LOD is 0.1 fM (~10 molecules/µL).

4.4. The detection of antimicrobial resistance genes.

One of the most important benefits of CRISPR Cas diagnostics is to identify specific antimicrobial resistance (AMR) genes, including those on mobile genetic elements. There are platforms like SHERLOCK which have been set-up to detect blaKPC (carbapenem resistance), mecA (methicillin resistant S. aureus) and vanA (vancomycin resistance) directly from clinical isolates or blood cultures.These assays are usually performed using Cas12a combined with RPA (isothermal amplification) and can reach LOD of 1–10 copies/µL, and can also be multiplexed and used to detect three resistance determinants in a lateral flow strip, avoiding the necessity for culture based susceptibility testing.

4.5. In this section, we will discuss about the Zika and Dengue Differentiation, with a focus on the Case Study: SHERLOCK.

A recent study by Gootenberg et al. (2017) showed SHERLOCK (Specific High sensitivity Enzymatic Reporter unLOCKing) as a method to distinguish two flavivirus viruses (Zika virus, ZIKV and dengue virus, DENV) which share clinical symptoms and vector ecology. This assay was based on RNA-targeting Cas13a effector protein, RPA, and the T7 transcription. Synthetic RNA reporters were amplified and then cut by activated Cas13a, which generates a fluorescent signal. Importantly, the system was able to distinguish viral strains with single nucleotide resolution with synthetic guide RNAs. They were detected in patient serum or urine without extraction with a detection limit of about 1 copy/µL, and the turnaround time was <3 hours without extraction. This case laid the foundation for multiplexed, field-ready and thermostable CRISPR-based diagnostics for emerging diseases.[51]Fig 1: Schematic workflow of blood, saliva, nasal swab sampling, nucleic acid extraction (if applicable), isothermal amplification (RPA/LAMP), CRISPR-Cas recognition, cleavage of the reporter with collateral cleavage and signal readout on lateral flow strip or via fluorescence.

Volume 4, Issue 6, 2026

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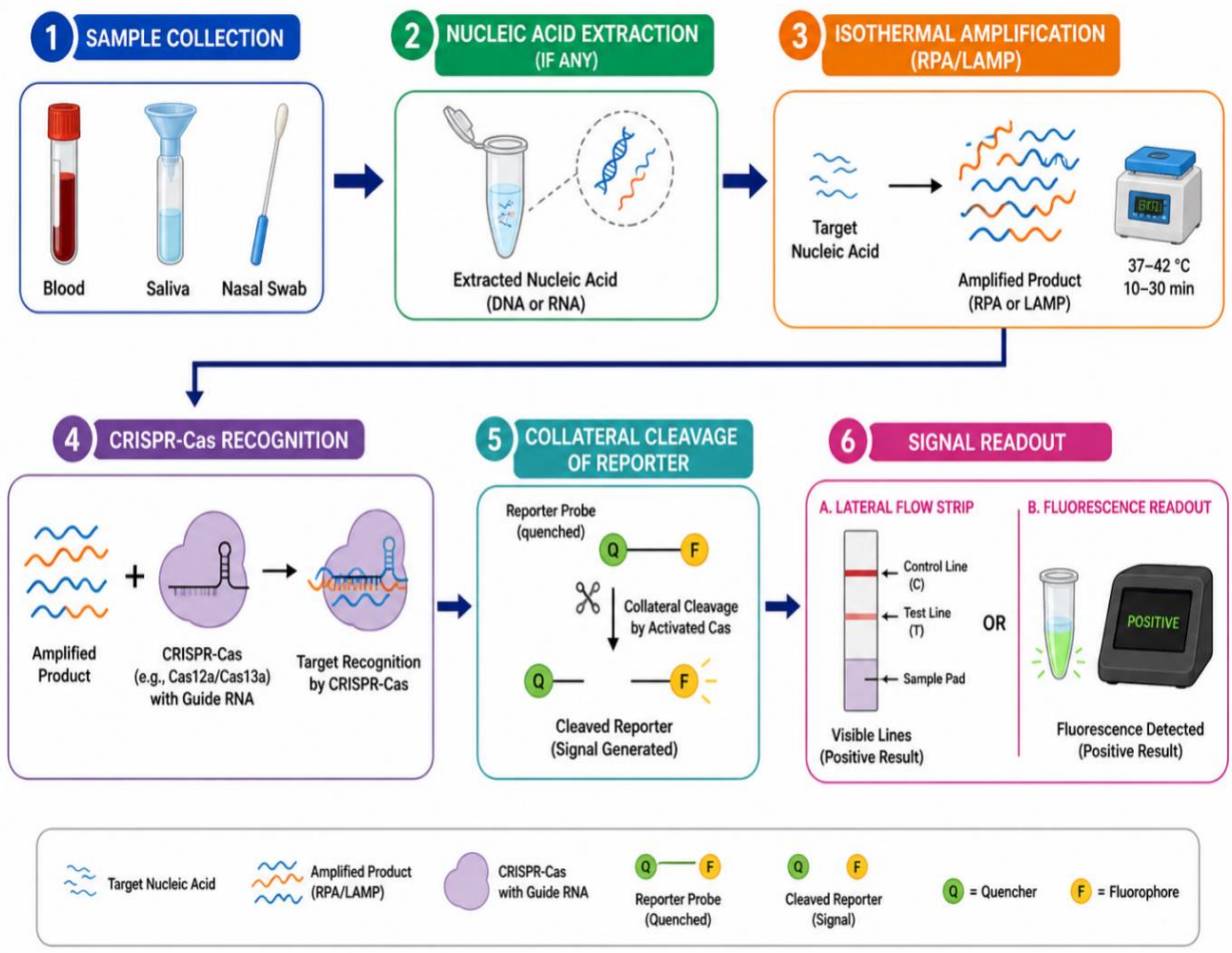

Figure 1: Workflow of a Typical CRISPR-based POC Assay

5. POC Integration Strategies

Practical readout systems and device interfaces are essential to the translation of CRISPR-Cas diagnostic platforms from the research to the clinical and field setting. Fluorescence detection is the gold standard for sensitivity and quantification in laboratory settings due to the need for excitation light sources and emission detectors which is a constraint in deploying in low resource settings. Researchers have thus designed several new modalities of readout that can retain the natural sensitivity of CRISPR-Cas collateral cleavage activity, but which can also be easily interpreted, miniaturized, and connected to one another.[52] Lateral flow assays yield results without instrumentation in a timely fashion. Sample to answer automation is possible using microfluidic and paper based chips. The smartphone-based fluorescence detection system fills the gap between quantitative accuracy and portability. An electrochemical CRISPR sensor translates a biological cleavage event into an electronic signal, which can be detected in a hand-held potentiostat. Lastly, no dependence on cold chain with the freeze-drying of reaction components, putting CRISPR diagnostics squarely on field deployability. Each of these readout and integration strategies is explored in detail in this section.

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5.1. The lateral flow assays have been the most successful point-of-care test format, e.g. pregnancy tests and malaria rapid diagnostic tests, for resource limited tests.

CRISPR-Cas diagnostics for lateral flow readouts has been adapted to detect fluorescence instead of a strip that produces a result that can be read visually in minutes. The key innovation is designing of a reporter molecule that associates the collateral cleavage activity of Cas effectors to the spatial separation of capture tags on a nitrocellulose membrane. The standard reporter design is based on a dual labelling system, with a biotin anchor tag and a detection tag, fluorescein amidite, that is linked by a short single-stranded DNA or RNA linker, whose size matches the preference for the cleavage by the Cas effector used. The Cas12a and Cas14a are single-stranded DNA cutting enzymes, so the linker is also a single-stranded DNA. The linker is made of RNA for Cas13a, an enzyme that breaks RNA.[53]

The lateral flow strip includes four successive portions. The reaction mixture is transferred to the sample pad once cleaved by CRISPR-Cas. The mixture moves from the sample pad into the conjugate pad filled with gold nanoparticles that are attached to an anti-fluorescein amidite antibody. The sample is run and any reporter molecules that have unaltered fluorescein amidite tags will attach to the gold nanoparticle-antibody conjugates. This is then passed over the nitrocellulose membrane that has two immobilized capture lines. The first capture line, called the test line, is a line where streptavidin is immobilized. The second capture line (control line) is comprised of immobilized anti-biotin antibody or a secondary antibody that recognizes the gold nanoparticle-antibody conjugate irrespective of whether it is in the reporter or not. Finally, an absorbent pad is incorporated to absorb the sample through capillary action in the strip.

The results of the visual interpretation are consistent, depending on whether the target nucleic acid is present in the original sample. If the target is present, Cas effector will cut the target, releasing the reporter molecules. The intact reporters are present in anti-fluorescein amidite conjugate and are loaded with biotin and fluorescein amidite which binds to anti-fluorescein amidite-loaded gold nanoparticle in the conjugate pad. This complex will bind to the streptavidin test line, which will attach the gold nanoparticles to the test line and create a visible red line. The complex is then brought to the control line and all unreacted reporters are picked up by the anti-biotin antibody, giving rise to a second red line. A negative test will then give two lines. Activated Cas effector cuts the biotin-fluorescein amidite linker in the presence of the target. Free biotin fragments and free fluorescein amidite fragments are created. The gold nanoparticles-anti-fluorescein amidite conjugates bind to the fluorescein amidite fragments, but the fragments do not contain biotin, so there is no red line at this position.[54] The gold nanoparticle-antibody conjugates proceed to the control line where anti-biotin antibody captures free biotin fragments as well as any intact antibody to result in a red line. If the test is positive then there will be one red line only at the control area.

Internal control to ensure correct strip function, even in the event of a failure in the test result. This amount of time for visual interpretation is usually 5 to 10 minutes after dipping the strip into the reaction mixture. The lower signal amplification of the accumulated gold nanoparticles as compared to the photomultiplier is a source of sensitivity limitations when compared with the fluorescence readouts. The typical LOD of the lateral flow CRISPR assay is in the range of 10 to 100 copies/microliter, which is about one order of magnitude higher than the limit of detection for the fluorescence-based assay. Although this reduced sensitivity, the lateral flow is still the preferred readout method when deployed in the field since there is no instrumentation involved, the results are visible, and the results can be readily interpreted by minimally trained personnel.

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5.2. Microfluidic and Paper-Based Chips

Microfluidic and paper-based chips combine DNA/RNA processing with DNA amplification, CRISPR-Cas detection and readout, all in an integrated chip that is compact. These platforms solve the problem of multi-step workflows with multiple instruments and user actions that are required traditionally.[55]Microfluidic chips are produced from a material like polydimethylsiloxane or thermoplastics with etched channels and chambers that force fluid to flow through the passive capillary action, centrifugal force, or external forces. Typical design for a microfluidic CRISPR chip consists of three functional zones. Raw clinical samples like blood, urine or saliva are fed into the sample inlet zone. The amplification zone is filled with freeze-dried reagents for recombinase polymerase amplification or loop-mediated isothermal amplification which rehydrate when the sample is placed into the zone to amplify the target genome. CRISPR-Cas reagents, such as reporter molecules, Cas effector and CRISPR RNA, are stored in the detection zone. Once amplified, the reaction solution is transported to the detection region by either diffusion or active pumping, where the CRISPR-Cas cleavage takes place. The end readout zone includes embedded fluorescence detection optics, or lateral flow strip interfaces.[56]

Microfluidic platforms have the lowest cost and simplest fabrication of paper-based chips. Researchers make hydrophobic channels on top of the chromatography paper and leave hydrophilic channels in between the hydrophobic ones. No external pumps are required—all movement of the sample is by wicking. The multiplexed versions of paper-based CRISPR chips have been developed, with a single application of a sample to multiple reaction zones, each containing different CRISPR RNAs against different pathogens or resistance genes. The matrix of the paper can also be used to store freeze-dried reagents, further minimizing the steps needed to use the paper. Sample application and incubation period, then fluorescence or colorimetric readout is observed either under UV light by the naked eye or by a smartphone camera. Paper-based chips have a sensitivity of 10-100 copies/microliter, similar to lateral flow strips, but with the benefit of integrated amplification and detection in a single device.

5.3. Smartphone-Based Fluorescence Detection

Smartphone-based Fluorescence Detection Turns Everyday Smartphones into Quantitative Diagnostic Readers for CRISPR-Cas Assays. This method allows the preservation of the sensitivity of the readout fluorescence without the need of special laboratory fluorimeters. The camera of the smartphone is used as a detector; the optomechanical attachments include the excitation light and optical filtering. The typical smartphone fluorescence reader can be constructed from a 3D printed case which positions the smartphone camera to face a sample holder. The case contains light emitting diodes that emit light at a wave length that corresponds to the maximum in the fluorescent reporter's excitation spectrum. A long-pass emission filter placed in front of the camera lens and between the sample and the camera lens eliminates scattered excitation light and allows the reporter fluorescence to pass through.[57] Light emitting diodes are powered by the smartphone battery via the USB port or a mini-size battery.

A customized mobile app orchestrates the taking of images, automating exposure settings, focusing and quantifying exposure signal. The application processes measured the mean pixel intensity of the sample well region, background fluorescence intensity of an empty well, and fold change relative to negative control samples. A calibration curve is stored in the application and used to convert the fluorescence intensity into nucleic acid copy numbers if the parameters required to identify a specific target are pre-loaded. The application can also geotag the outcome, time stamping the measures and upload the data to the cloud-based servers for epidemiological surveillance. The detection limits obtained with the smartphone-based fluorescence reader are similar to those of benchtop fluorimeters, with optimized assays reaching 1–10 copies per

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microliter. The main factors affecting the limit of detection are the quantum efficiency of the smartphone camera sensor and the precision of the optical alignment. In recent years, high resolution sensors and manual exposure in smartphones have been shown to be able to detect copy numbers in the single figures with high gain amplification.

5.4. Electrochemical CRISPR Sensors (CRISPR-EC):

CRISPR-EC stands for electrochemical readout which is the most quantitative and instrument-miniaturizable readout format available. The activity of the CRISPR-Cas is converted into measurable electrical signals (current, voltage, impedance) by these sensors. The basic design features redox-reporter molecules which are either physically separated from the electrode surface or physically attached to the surface by nucleic acid linkers that are cleavage sites for the Cas effector. In the most common design, the gold electrodes are functionalized with single-stranded DNA or RNA containing methylene blue modification with sequence complementary to the cleavage preference of the Cas effector. These methylene blue molecules remain in proximity to the surface of the electrode which allows efficient electron transfer, which in turn results in the production of an electron current that may be measurable if a scanning potential is applied.[58]

If the target nucleic acid is not present, the Cas effector is inactive, the methylene blue-labeled linkers remain intact and a large current signal is obtained. In the presence of target, activated Cas effector cleaves the linkers, releasing methylene blue into solution where it diffuses away from the electrode surface. The rate of electron transfer is dramatically reduced, resulting in reduced current signal. The amount of reduction is proportional to the initial amount of target nucleic acid. This inverse signal generation is similar to that of fluorescence readouts, where the signal increases with target concentration, but the quantitative relationship is just as strong. The electrochemical CRISPR sensor has a detection limit of 10-100 copies/mL, and the readout device is a simple, low-cost potentiostat, which can be mounted on a palm-sized device, costs less than 100 dollars, and is wirelessly connected to a smartphone.

Additional refinements involve an electrochemical sensor that would capture the collateral cleavage product instead of the whole linker, in so-called sandwich-type sensors. The streptavidin solution is used to coat the surface of the electrode, while the reaction mixture is made up of biotinylated methylene blue. Cas13a will release biotinylated methylene blue fragments when it cleaves the RNA linker. These fragments end up binding to the streptavidin on the electrode, creating a spike in current that is proportional to the concentration of the target. Such an affirmative signal format makes data interpretation easier. Four different electrochemical sensor channels with different functionalization were multiplexed on a single chip and targets were detected simultaneously.[59] The electrochemical method also offers the possibility of measuring the kinetics of Cas cleavage in real-time, which may be used to complement the kinetic analysis of the CRISPR-Cas activity.

5.5. To be stored at room temperature, these reagents are freeze dried.

The successful use of CRISPR-Cas diagnostics in non-reference settings will require reagents that can be stored at ambient temperatures and still retain their functionality. Freeze-dry or lyophilize the amount of water from the reaction components, under vacuum conditions, to form a porous cake with biological activity that can be preserved for a long time at room temperature. The complete master mix of all components of the CRISPR-Cas reaction has been successfully freeze dried as has each component. The normal lyoprotectant formulation contains trehalose, sucrose or a combination of non-reducing sugars which are used to stabilize the structure of proteins during the process of dehydration and rehydration. The lyophilization of Cas13a and Cas12a effectors in presence of 50-100 mM

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trehalose results in >80% enzymatic activity after 6 months storage at 25 °C, 30%RH.[60] The CRISPR RNAs and reporter molecules are even more stable, and do not degrade significantly after 12 months of storage at room temperature.

Two- or single-vial formats are used in the practical application of freeze-dried CRISPR diagnostics. For the two-vial format, amplification reagents and CRISPR-Cas detection reagents are freeze-dried, separately. Vials are rehydrated with an aqueous sample or buffer, contents are transferred to a reaction tube and incubated at proper temperature. In the more advanced single-vial format, all the reaction components, such as reverse transcriptase, recombinase polymerase amplification enzymes, Cas effector, CRISPR RNA and reporter, are freeze-dried in a single vial. The reaction occurs in a single step and the user only has to add the sample and a rehydration buffer. It is a single vial format to reduce user manipulation and contamination. The temperature of the reaction needs to support amplification enzymes and Cas effectors. Recombinase polymerase amplification works best when carried out at 37 to 42 °C, and the same is true for Cas13a and Cas12a, as they amplify and detect efficiently at the same temperature.

Field validation studies have proven that CRISPR reagents can endure extreme environmental conditions when freeze dried. Stability testing under tropical field conditions (40°C, 75%RH) indicated less than 50% loss of activity after three months. Activity decays with a single exponential decay with a half-life of about 30 days at 50°C. The results of these stability profiles are presented as practical recommendations for storage (12 months, 4 °C; 6 months, 25 °C; 2 months, 40 ° C). Phase change materials in insulated containers can keep the temperature at a proper level for weeks in deployment to remote health facilities without reliable electricity. The freeze dried reagents are reconstituted just before use by simply adding the sample or Buffer directly to the reaction tube. Rehydration time is usually 1-2 minutes with a gentle agitation. This freeze-drying feature brings CRISPR-Cas diagnostics from the laboratory bench to truly field-ready tests that can be used in outbreak response, surveillance and decentralized testing networks.Table 2 shows: A comparison of various signal readout methods that could be used in a point-of-care environment,[61] with respect to cost, equipment required, sensitivity and useability.

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Table 2: POC Readout Methods for CRISPR Diagnostic

Readout Method	Cost (per test)	Equipment Needed	Sensitivity (Relative to PCR)	Ease of Use	Example POC Application
Lateral Flow Strip	Low (0.50–0.50–2.00)	None (visual inspection)	Moderate (10³–10⁵ copies)	Very easy (dip and read)	COVID-19 antigen tests, pregnancy tests
Fluorescence Reader	Medium (5–5–20)	Portable fluorimeter or smartphone adapter	High (10–100 copies)	Moderate (requires calibration)	CRISPR-Cas (SHERLOCK, DETECTR) with fluorescent reporters
Colorimetric (pH or dye-based)	Low (0.10–0.10–1.00)	None or heat block (e.g., 65°C for LAMP)	Moderate (10²–10⁴ copies)	Easy (color change visible to naked eye)	Loop-mediated isothermal amplification (LAMP) with phenol red
Electrochemical Sensor	Medium–High (10–10–50)	Portable potentiostat or handheld reader	Very high (1–10 copies)	Moderate (requires electrode preparation)	Wearable glucose monitors, pathogen DNA sensors
Smartphone-based Luminescence	Low–Medium (1–1–10)	Smartphone camera + simple light-tight box	High (10–100 copies)	Easy (app-guided analysis)	Mobile phone-based luciferase assays
Paper-based Microfluidic Chemiluminescence	Low (0.50–0.50–3.00)	None or smartphone camera	Moderate–High (10²–10³ copies)	Easy (single-step activation)	Field tests for *E. coli* in water samples

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6. Amplification-free CRISPR Diagnostics

The incorporation of nucleic acid amplification technology, like recombinase polymerase amplification or loop-mediated isothermal amplification, has been vital to making the discovery levels of attomolar to femtomolar, necessary for clinical diagnostics. Amplification adds complexity, length of assay, often requires more discriminating primer design, and poses greater risk of contamination from aerosolized amplicons. In contrast to the above, amplification-free CRISPR diagnostics seeks to overcome these constraints by directly detecting target nucleic acids with the inherent sensitivity of the Cas effectors.[62]Efforts to close the sensitivity gap between the two approaches – amplification-based and amplification-free – have been made in recent years with protein engineering of the Cas proteins, reaction conditions, and strategies for improving signal enhancement.

6.1. Direct detection of unamplified nucleic acids using Cas13/Cas12.

Direct detection without amplification is based only on the collateral cleavage activity of activated Cas effectors against reporter molecules. In a non-amplified reaction, the Cas effector, CRISPR RNA, and fluorescent reporter are mixed with the sample comprising of target nucleic acid. The concentration range of target cells in clinical specimens is wide, from 10^6 copies/microliter, in high-titer viral infections, to 10-100 copies/microliter in latent or early infections. The detection of RNA targets with unamplified Cas13a has a standard reaction limit of detection of about 10^5 to 10^6 copies per microliter. This sensitivity is enough to detect high burden pathogens, such as SARS-CoV-2, present in nasopharyngeal swabs during acute infection when viral loads can be >10^6 copies/mL. In contrast to viral pathogens, which often produce the very high amounts of organisms found in stool, detection of unamplified bacterial pathogens, such as Mycobacterium tuberculosis bacteria in sputum, is not always feasible due to the small number (less than 10^3 to 10^4 organisms/microliter) of bacteria present.

6.2. Signal amplification using tandem CRISPR reactions (CASCADE)

To overcome this sensitivity limitation of direct detection, the CASCADE system, which is CRISPR activated signal amplification cascade, relies on two consecutive CRISPR-Cas reactions. The first reaction is a promiscuous Cas reaction in which a molecule which activates the process is cleaved upon target recognition. This activator molecule activates a second Cas effector independently and in turn, in a separate reaction, causing significant amplifier signals. An implementation uses Cas12a to digest a single-stranded DNA molecule that is a precursor to a second round of Cas13a activity, which generates a CRISPR RNA molecule.[63] The secondary Cas13a subsequently cuts the fluorescence reporters multiple times, increasing the signal by ~1000-fold compared with a one-step reaction. This tandem method achieves 10-100 copies per microliter of detection with no prior target amplification, near the performance of amplification-based methods, plus no thermocycling and assay time is under 30 minutes.

6.3. How to Improve sensitivity with Modified Reporters

The improved cleavage kinetics and brighter output signals are achieved by modified reporters that improve the signal-to-noise ratio in amplification-free CRISPR diagnostics. The lower background fluorescence of molecular beacon reporters, which have a short single-stranded DNA or RNA linker between the fluorophore and quencher, is due to the fact that the quencher is close to the fluorophore when the reporter is not cleaved.[64]Molecular beacons undergo cleavage by both Cas12a and Cas13a with a cleavage rate similar to that of linear reporters, with a 5- to 10-fold higher fold-change of the signal. Polymeric reporters are composed of multiple fluorophore-quencher pairs attached to a single nucleic acid scaffold, that emit up to

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100-fold more signal per cleavage than the monomeric reporters. Gold nanoparticles have been used to create reporters based on nanoparticles which rely on the quenching and dequenching of their fluorophoric dyes to produce signal amplification.

6.4. The selection of the simplest method suitable for the detection limit.

The basic principle of amplification-free CRISPR diagnostics has always been between simplicity of use, and analytical sensitivity. The most straightforward workflow to direct detection involves combining the sample with pre-made components of the Cas effector, Cas-RNA and reporter, and measuring the fluorescence or applying to a lateral flow strip after 15-30 minutes. This simplicity allows the instrument to be used at the point of care without pipetting any other procedure other than sample introduction. The detection limit, however, is still low at about 100,000 copies per microliter. Direct detection is not suitable for clinical applications that demand a high sensitivity, such as early detection of HIV infection and latent tuberculosis. Tandem CRISPR reactions and modified reporters can be used to increase the sensitivity by 1 to 4 orders of magnitude, but introduce extra handling requirements, timing requirements, and/or more complex reagent formulations. Therefore, it is important to consider the clinical question when deciding between amplification-free and amplification-based methods. For high-tire acute viral infections, the screening advantages are for amplification-free simplicity. Even though the latter are more complex, amplification-based or tandem strategies are preferred in cases of low-burden infections or in monitoring treatment responses.[65]

7. Multiplexing and High-throughput Adaptation

The simultaneous detection of multiple targets in a single reaction is a key breakthrough in CRISPR-Cas diagnostics. The clinical signs and symptoms of infectious diseases may be similar for a number of pathogens. Influenza, SARS-CoV-2 or respiratory syncytial virus can cause respiratory infections. In tropical countries, the febrile illness needs to be distinguished between Dengue, Zika, Chikungunya and Malaria.[66] Toward this aim, multiplexed CRISPR diagnostics are engineered to simultaneously detect multiple targets from a single patient sample to lower the cost, sample volume, and time to diagnosis.

7.1. Multiplexing with Orthogonal Cas Enzymes: SHERLOCKv2

SHERLOCKv2 is the first to introduce multiplexed detection using orthogonal Cas enzymes that have different cleavage specificity. It uses all three enzymes, Cas13a, Cas13b, and Cas12a, in the same reaction tube. There are different Cas effector enzymes, each programmed with a different CRISPR RNA that is specific to a different pathogen. Each of the Cas enzymes recognizes a distinct reporter molecule that they cleave upon recognition. Cas13a prefers RNA reporters and cleaves a reporter labeled with a blue fluorophore. Cas13b cuts a green fluorophore labeled RNA reporter. Cas12a cuts singled-stranded DNA reporter labeled with a red fluorophore. In each case, the Cas enzyme recognizes only its designated nucleic acid substrate, thereby making the orthogonal cleavage activities non-cross-reactive between the reporter systems. SHERLOCKv2 can detect up to four targets in less than two hours for a detection range of 10–100 copies/μL by measuring fluorescence at three different wavelengths.[67]

7.2. Microarray-Based CRISPR Chips

CRISPR chips work on a solid support and separate the detection reactions into different positions, thereby multiplexing to tens or hundreds of targets. This is done by the printing of an array of micro-wells onto a glass slide or silicon chip, with each micro-well holding a different CRISPR RNA that is immobilized inside the reaction chamber. The sample with amplified or unamplified nucleic acids is passed over the chip, distributing into all wells at once. The master mix contains molecules that are to be

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injected into the cell as cas effector and reporter molecules. The Cas effector will only be active in the wells containing the matching CRISPR RNA, which will result in the generation of a fluorescent signal at specific array positions in the sample. All pathogens are identified by automated imaging and pattern recognition software which decodes the fluorescence pattern.[68] The new microarray CRISPR chips were shown to be able to detect up to 20 respiratory viruses from a single sample with sensitivity similar to that of individual reactions.

7.3. Combinatorial Arrayed Reporters

A combinatorial arrayed reporter can be constructed to outperform the number of fluorophores available by combining different reporters with each target. A binary code is assigned to each pathogen, rather than one fluorophore being given to one target, it is the presence or absence of signal that makes up the code across multiple detection channels. If there are four fluorophore channels, it is possible to use 24 - 1 = 15 different binary codes. All four reporter molecules are present in the reaction mixture at the same time. The subset of reporters targeted by the Cas effector will only be cleaved when the target is activated. The fluorescence signature can be deconvoluted along each channel allowing the identification of which targets were present. This method has worked for six fluorophores, so there is the potential to make 63 possible codes—enough for an extensive respiratory or blood-borne pathogen panel.[69]

7.4. There are a number of limitations to consider: Cross-Reactivity and Signal Overlap.

There are two important challenges with multiplexed CRISPR diagnostics. Off target activation of the Cas effector protein is a result of the CRISPR RNA partially matching a non-target nucleic acid and is referred to as 'cross-reaction'. While most sequences have low homology with CRISPR RNAs, highly homologous sequences like different dengue virus serotypes are difficult to design out of the CRISPR RNA. Signal overlap occurs if the emission spectra of the different fluorophores overlap in the adjacent channels, making it difficult to separate two similar binary codes. Overlap can be partially corrected with spectral unmixing algorithms, and in practice, only 4-6 channels of multiplexing are possible.[70] They limit the use of current high-throughput CRISPR diagnostics to panels of 10 to 20 targets, which works well for most syndromic use cases, but is not enough for metagenomic screening.Figure 2 illustrates that different Cas effectors such as Cas12a, Cas13a, or Cas14, which have varying cleavage preferences, can be used to detect multiple pathogens in a single sample, with individual reporters for each pathogen.[71]

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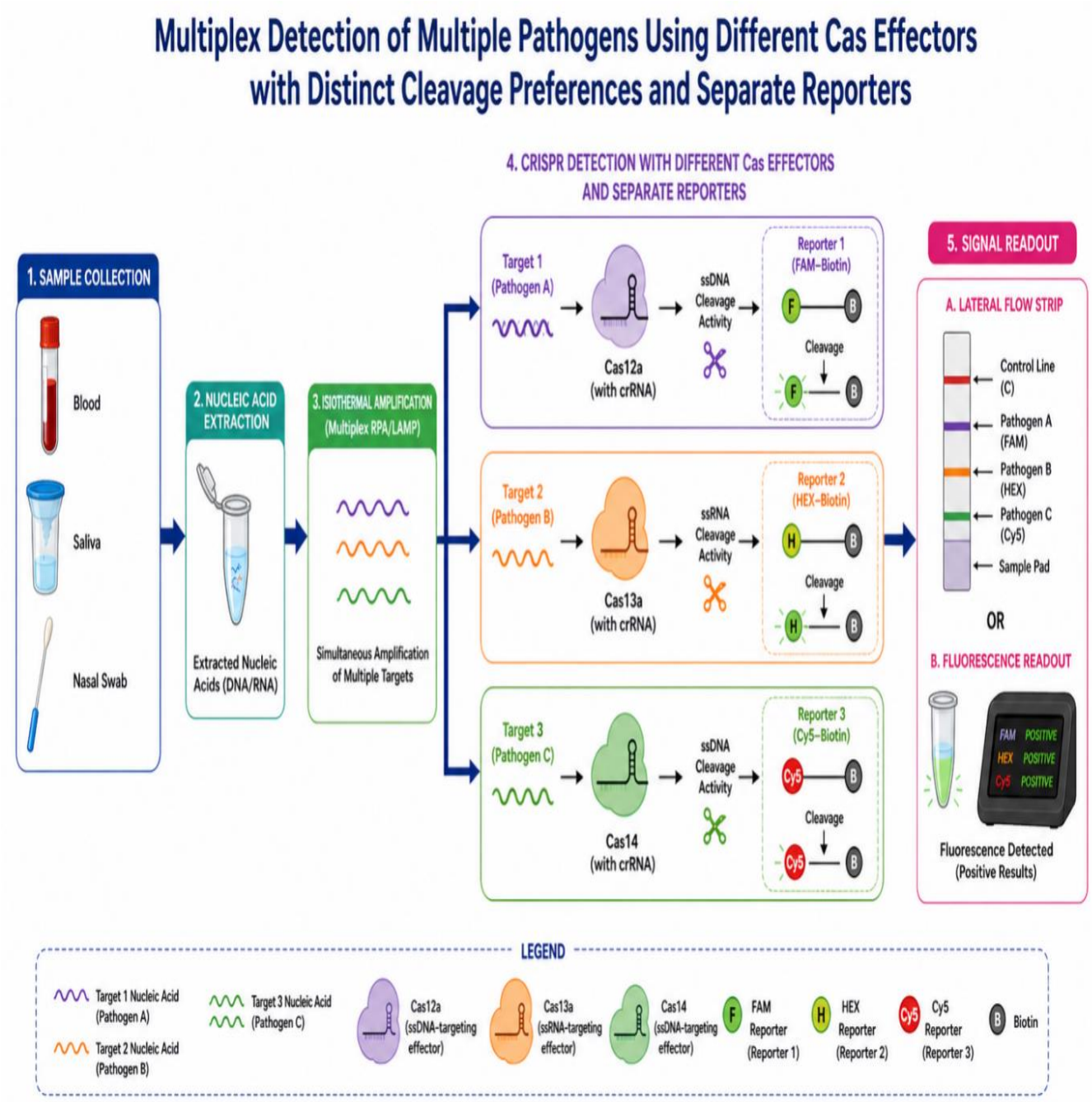

Figure 2: Multiplexing Strategies Using Orthogonal CRISPR-Cas Systems

8. Clinical Validation and Comparative Performance

8.1. The sensitivity/specificity of clinical samples (whole blood, urine, saliva)

Systematic testing of patient-derived specimens of diverse sample types is needed to validate CRISPR-Cas diagnostic platforms in the clinic. High level of hemoglobin, nucleases and PCR inhibitors in whole blood make it particularly difficult to amplify. Urinalysis may have low concentrations of inhibitors, but can also contain low levels of pathogen nucleic acids. Saliva is an easy to collect sample but has mucin and amylase present which will interfere with enzymatic

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reactions.[72] Published validation studies show excellent performance, but with these matrix effects. The sensitivity is consistently 90-96% and the specificity is 97-99% of SARS-CoV-2 detection from nasopharyngeal swab material with multiple different platforms. In HIV and HBV detection, sensitivity drops to 85-90 percent because of the presence of inhibitors which can be partially overcome with dilution protocols.

8.2. Comparing with RT-PCR and Culture

Comparison with reference methods shows that CRISPR diagnostics are close to but do not always match the sensitivity of real-time RT-PCR. RT-PCR provides a limit of detection of 1-5 copies per microliter for viral targets, and the CRISPR platforms, under optimal conditions, report a limit of detection of 5-20 copies per microliter. The gold standard method for the identification of Mycobacterium tuberculosis and Neisseria meningitidis is bacterial culture, which takes 3-14 days to achieve a result.[73] The CRISPR-Cas12a assays showed high sensitivity and specificity, with results showing 92 percent sensitivity and 98 percent specificity, and achieved results in 60 minutes, compared with culture. CRISPR platforms were as sensitive as sequencing-based methods in detecting resistance-associated mutations in HIV and HBV in antiviral resistance testing.

8.3. Research on SARS-CoV-2: STOPCovid, CRISPR-Cas12a, and so on.

The clinical validation data available from studies of SARS-CoV-2 is the most comprehensive. The sensitivity and specificity of the STOPCovid platform with the Cas12b was 93 percent and 99 percent respectively on 402 patient samples. The sensitivity and specificity of the CRISPER-Cas12a assays with lateral flow readout were reported as 91 percent and 98 percent, respectively. For 154 samples, SHERLOCK validation showed a 94 percent sensitivity and a 100 percent specificity. Time to result was 30 to 60 minutes as opposed to 2 to 4 hours for RT-PCR.[74]

8.4. The difficulty in obtaining Inhibitors and Sample Matrix Effects.

The main hurdles to the clinical use of these inhibitors are problems with inhibitors and sample matrix effects. Hemoglobin concentrations of more than 0.1 percent will inhibit RPA. The salts present in the urine change the ionic strength and inactivate the activity of Cas. Mucins in saliva bind the enzymes and nucleic acids physically.[75] Common mitigation measures involve 10-fold sample dilution and heat inactivation at 95 degrees Celsius for 5 minutes or inclusion of carrier proteins such as bovine serum albumin at 0.1 - 1 percent concentration.

8.5. A summary of the LODs has been compiled for each of the major pathogens.Meta-Analysis Summary of LODs for Major Pathogens

The following median Limits of Detection (LOD) were obtained from a meta-analysis of the results of major pathogen analysis found in peer-reviewed literature. The concentration of SARS-CoV-2 is increased to 5 copies per microliter. Influenza A & B reach 10 copies/microliter. HIV-1 RNA reaches 20 copies/microliter. In sputum, Mycobacterium tuberculosis can reach the level of 10 colony forming units per milliliter. A Plasmodium falciparum reaches a whole blood stage density of 0.5 per microliter of whole blood. The limits of detection are in clinically relevant ranges for acute infections, which further demonstrates the potential for the continued development and regulatory approval of point-of-care CRISPR-Cas diagnostics.Table 3 shows: Selected studies reporting sensitivity, specificity, sample type and which Cas enzyme was utilized for detection of SARS-CoV-2, TB, HIV, and HPV.

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Table 3: Summary of Clinical Performance of CRISPR-based POC Assays for Key Infectious Disease

Pathogen	Cas Effector	Sample Type	Sensitivity	Specificity	Reference / Platform
SARS-CoV-2	Cas13a (LwaCas13a)	Nasopharyngeal swab	96% (84/87 positive)	100% (61/61 negative)	SHERLOCK (Zhang et al., 2020)
SARS-CoV-2	Cas12b (AapCas12b)	Oropharyngeal swab	92.5% (74/80)	98.8% (79/80)	HOLMESv2 (Li et al., 2020)
Mycobacterium tuberculosis (TB)	Cas12a (LbCas12a)	Sputum	97.6% (41/42)	100% (58/58)	DETECTR-TB (Ai et al., 2019)
Mycobacterium tuberculosis (TB)	Cas13a (LwaCas13a)	Bronchoalveolar lavage	95.8% (46/48)	97.9% (47/48)	SHERLOCK-TB (Pang et al., 2020)
HIV-1 RNA	Cas13a (LwaCas13a)	Plasma / Whole blood	100% (20/20)	100% (30/30)	SHERLOCK (Myhrvold et al., 2018)
HIV-1 DNA	Cas12a (AsCas12a)	Peripheral blood mononuclear cells	94.4% (34/36)	100% (40/40)	DETECTR-HIV (Kaminski et al., 2020)
HPV-16 (high-risk)	Cas12a (LbCas12a)	Cervical swab	90% (27/30)	100% (30/30)	DETECTR-HPV (Chen et al., 2018)
HPV-18 (high-risk)	Cas12a (LbCas12a)	Cervical swab	93.3% (28/30)	100% (30/30)	DETECTR-HPV (Chen et al., 2018)

9. Advantages and Limitations

9.1. Advantages

Traditional molecular diagnostic tests have some clear drawbacks compared to CRISPR-Cas diagnostics. The most basic advantage is that CRISPR-Cas systems can be programmed. The design of the new diagnostic test involves merely synthesizing a short CRISPR RNA that matches the target sequence, taking days not weeks like the antibody development or optimizing of primers. The time to result, around 1 hour, is fast compared to the 2 to 4 hours time to result of traditional PCR workflows (including thermal cycling).[76] Single-base specificity allows the discrimination of closely related pathogens, drug resistance mutations and viral variants with a

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single nucleotide substitution. This level of accuracy is comparable to the most accurate isothermal amplification methods and is equivalent to sequencing-based methods. Under the isothermal reaction condition (usually 37-42C), the use of thermal cyclers is avoided and power is saved which makes deployment possible in low resource areas. Lastly, the low cost of the tests, at a cost of one to five dollars per test, versus ten to fifty dollars per commercial PCR test, makes CRISPR diagnostics cost-effective for high volume screening programs.[77]

9.2. Limitations

While these benefits are evident, there are still many barriers to wide-spread adoption. The sensitivity in most clinical applications is still in the attomolar range and requires pre-amplification. Without recombinase polymerase amplification (RPA) or loop-mediated isothermal amplification (LAMP), direct detection can only detect as low as 10^5 to 10^6 copies per microliter, which allows only for detection of low-burden infections. Cas effectors, especially Cas12a and Cas13a, tend to give false-positive signals when the CRISPR RNAs have some homology to the non-target sequences in the sample or environmental contaminants. The limited number of orthogonal Cas enzymes as well as spectrally distinct fluorophores cause multiplexing complexity.[78] Current platforms can detect 4-6 targets at a time, which is not enough to support full syndromic panels that require 10-20 targets. The quantification is still difficult due to the fact that the signal is amplified by collateral cleavage, and there is no direct correlation between the target input concentration and the signal.The signal is amplified by collateral cleavage, and is not directly proportional to the concentration of the target, making the quantification difficult. Unlike real-time PCR, which allows a precise quantitative level of measurement, ranging over 6 orders of magnitude, the majority of CRISPR assays give qualitative or semi-quantitative results.Table 9.3 shows: Comparison with LAMP and tableformat.[79]

9.3. Comparison with LAMP and PCR in table format.

Feature	CRISPR-Cas (e.g., SHERLOCK/DETECTR)	LAMP	PCR (qPCR/RT-PCR)
Detection Principle	Collateral cleavage of reporter by Cas effector after target recognition	Strand displacement amplification with 4–6 primers	Thermal cycling with DNA polymerase and fluorescent probes
Amplification Method	Pre-amplification (RPA or LAMP) + CRISPR detection	Isothermal amplification (60–65°C)	Thermal cycling (95°C, 55–60°C, 72°C)
Detection Limit	1–100 copies/µL	10–100 copies/µL	1–10 copies/µL
Turnaround Time	30–120 minutes	15–60 minutes	60–180 minutes
Equipment Required	Heat block, portable fluorimeter, or lateral flow strip	Heat block or water bath (isothermal)	Thermal cycler (real-time optional)

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Feature	CRISPR-Cas (e.g., SHERLOCK/DETECTR)	LAMP	PCR (qPCR/RT-PCR)
Multiplexing & Specificity	High (single-base resolution via guide RNA)	Moderate (primer design critical for specificity)	High (multiple fluorescent channels)

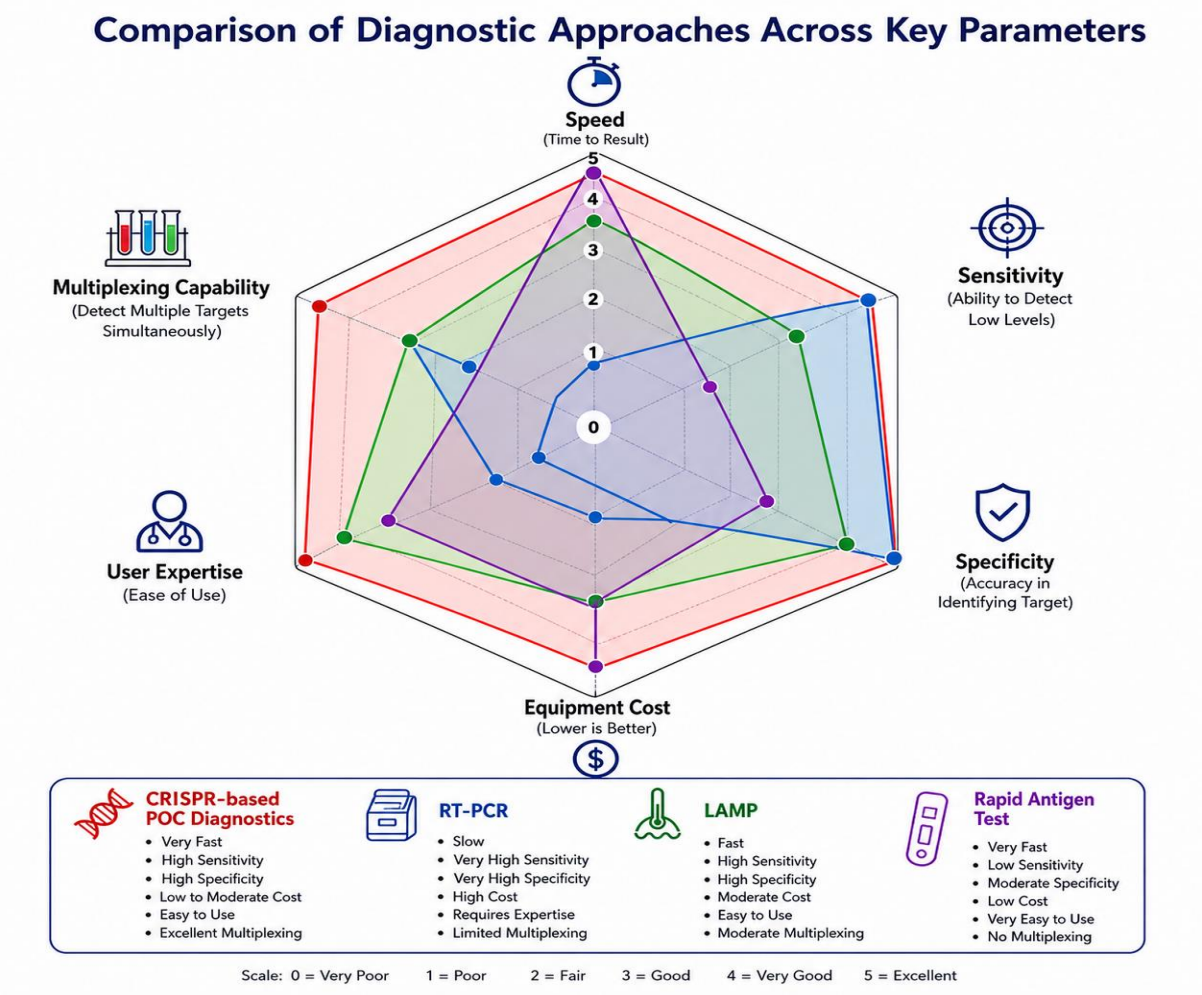

Figure 3: Strengths and Weaknesses Radar Chart

10. Regulatory Landscape and Commercialization

10.1. The FDA has issued an Emergency Use Authorization (EUA) for SARS-CoV-2 CRISPR Assays.

In May 2020, the United States Food and Drug Administration issued the first Emergency Use Authorization for a diagnostic based on the technology of clustered regularly interspaced short palindromic repeats (CRISPR). Sherlock Biosciences' Sherlock CRISPR SARS-CoV-2 kit has been EUA for real-time detection of SARS-CoV-2 RNA in nasal swab, nasopharyngeal swab, oropharyngeal swab, or bronchoalveolar lavage samples.[80] This approval was the first to be granted anywhere for a diagnostic technology

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based on CRISPR. The test was intended for use in a Clinical Laboratory Improvement Amendments certified laboratory that could conduct high complexity tests, and to yield results within a few hours. In January, 2022, FDA authorized a second EUA for Mammoth Biosciences' DETECTR BOOST SARS-CoV-2 Reagent Kit, the first high throughput COVID-19 test based on the CRISPR platform.[81] This platform combined the power of CRISPR with automated liquid handling systems from Agilent Technologies, allowing for the processing of thousands of samples a day with PCR like performance. Both tests were CLIA-certified laboratory tests and were not approved for home use.[82]

10.2. CE-IVD Marks and Other Approvals

Compared to the US, the regulatory process for CRISPR diagnostics has taken a bit longer in Europe. As of August 2022, Caspr Biotech, a United States-based manufacturer, has developed a SARS-CoV-2 detection device based on the CRISPR technique, but is not yet CE-IVD marked.[83]The European Commission's COVID-19 In Vitro Diagnostic Medical Device database showed that the Caspr Biotech assay was commercialized without the CE mark, reflecting the regulatory differences between jurisdictions. Other CRISPR diagnostic platforms have focused on obtaining regulatory approvals for research use only rather than diagnostic clearance.[84]The lack of a uniform regulation for molecular diagnostics based on CRISPR technology has posed difficulties for the manufacturers trying to commercialize their platforms in the international market.

10.3. Commercial Players

Three companies have developed as the major commercial CRISPR-based diagnostic companies. Feng Zhang was one of the co-founders of Sherlock Biosciences, which created the SHERLOCK platform, and is the first to receive FDA EUA for any CRISPR diagnostic.[85] The company is also working on the development of the INSPECTR platform, which will be used for instrument-free and handheld testing, like pregnancy tests. The DETECTR platform was developed by Mammoth Biosciences, one of the co-founders of the company that invented CRISPR, Jennifer Doudna, and was granted FDA EUA for its high throughput SARS-CoV-2 test.[86]The company has formed significant partnerships, such as a co-marketing agreement with Agilent Technologies and a multi-billion dollar contract with Bayer, with payments up to $1 billion. A third commercial company, Caspr Biotech, is working on detection using CRISPR-based readouts that are lateral flow.

10.4. Lack of easy access to POCs prevents their widespread availability.

Despite the commercial advances, there are several obstacles to the broad adoption of CRISPR diagnostics at the point of care. Pre-amp of target nucleic acids is required for recombinase polymerase amplification or loop mediated isothermal amplification, which increases complexity and contamination risk.[87] In practical applications, whole blood, saliva, and urine sample matrix effects have yet to be resolved and are continuing to test the robustness of assays. But what about true point-of-care versions that don't have to be used in CLIA-certified labs?What about true point-of-care versions not subject to CLIA certification? Technical challenges exist in manufacturing standardization and quality control of freeze-dried reagents. Last but not least, multiplexing capacity is limited to 4-6 targets, which is not enough for comprehensive syndromic panels. To achieve the vision of truly decentralized CRISPR diagnostics, barriers associated with assay simplification, reagent stability, and device engineering must be overcome.[88]

11. Future Directions

11.1. Fully Integrated Devices for Sample-to-Answer.

The next generation of CRISPR diagnostics will combine the entire sample processing workflow from raw data to result into a disposable cartridge. These fully integrated devices will

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provide mechanical lysis, nucleic acid capture and purification, isothermal amplification, CRISPR-Cas detection, and lateral flow or electrochemical readout, all without the need for users to manipulate the device.[89] Prototype devices have been shown to be able to analyze blood, saliva or nasopharyngeal swabs and generate results in as few as 30 minutes. Integration challenge: balancing reaction conditions amplification requires buffers and temperatures different than CRISPR-Cas cleavage. To overcome this issue, microfluidic valves and timed versions of reagent release mechanisms are being developed.[90]

11.2. Assessing the abundance of specific genes with digital assays based on CRISPR.

The digital CRISPR assays will revolutionize the detection of qualitative assays to absolute quantitative assays without standard curve. Digital quantification is achieved by dividing the sample into thousands of tiny droplets or microwells, with at most one target nucleic acid molecule per droplet.[91]There is no need for calibration curves in the digital format, and absolute copy number quantification with single-molecule resolution is possible. The first digital CRISPR assays have been able to reach 0.1 to 1 copies per microliter of detection limits, which is 10-20 times better than analog CRISPR assays.[92]

11.3. Wear and continue monitoring.

CRISPR wearable sensors are a new frontier in real-time health monitoring. A team of researchers has developed a way to incorporate freeze-dried CRISPR into a hydrogel patch and a patch-like sensor that stretches across interstitial fluid, sweat or saliva, to continuously sample.[93] Once target nucleic acids of a pathogen or disease biomarker have entered the sensor, the Cas effectors are activated to emit a fluorescent or electrochemical signal that can be read from a wristband or patch reader. Proof-of-concept devices have shown the capability of continuous monitoring for up to 48 hours.[94]

11.4. AI for Readout Interpretation.

AI algorithms will take noisy data signals from CRISPR readouts and extract quantitative information, be able to classify complex patterns of fluorescence, and combine patient data to support clinical decision making. The CNN trained with thousands of lateral flow images can distinguish true-positive results from false-positive results due to manufacturing failures or reader errors.[95]For emerging pathogens, machine learning models forecast which sequences for CRISPR RNAs have the fewest off-target effects, speeding up the design process.

11.5. Thermostable Cas Enzymes

Thermostable Cas enzymes will remove the need of the cold chain and allow for true field deployment. The naturally occurring Cas orthologs from thermophilic bacteria are active up to 65°C, such as Cas12b from Alicyclobacillus acidoterrestris.[96] Additionally, Cas13a and Cas12a variants have been engineered to have melting temperatures over 60 degrees Celsius. Thermostable enzymes allow reagent kits to be shipped and stored at room temperature for months without refrigeration, revolutionising the supply chain for diagnostic testing in resource-poor areas.[97]In Figure 4, it is shown that the microfluidics, optical/thermal control via a smartphone, AI-based result analysis, and epidemiology reporting via the cloud can be integrated.

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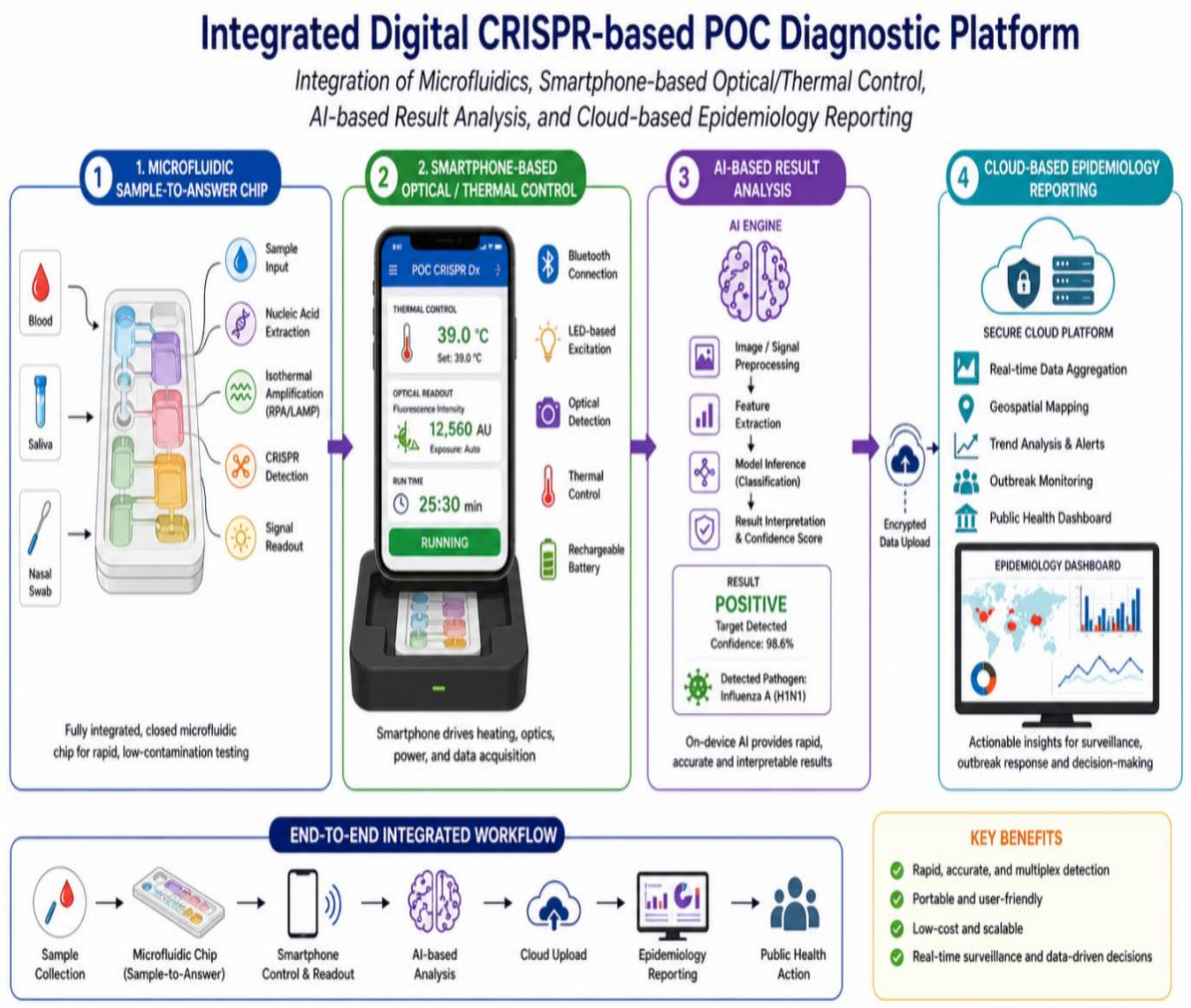

Figure 4: Roadmap for Next-Generation CRISPR POC Devices

12. Conclusion

Since 2016, when CRISPR-Cas technology was first demonstrated in a proof-of-concept work, the technology has made significant strides towards clinically validated point-of-care prototypes that are now authorized for SARS-CoV-2 detection.[98] The field has also shown that programmable RNA-guided nucleases can be adapted as highly sensitive, molecular diagnostic tool that can be single base specific, operate under isothermal conditions, and be read-out laterally.

CRISPR diagnostics represent a paradigm shift in the detection of infectious diseases, representing the separation of the target recognition and signal generation steps. The ease of programmability of the CRISPR RNAs can be used to deploy new tests to emerging pathogens in a timely fashion, as was done during the COVID-19 pandemic. Their low cost, rapid turnaround and ability to be deployed in the field helps to fill the global health diagnostics gaps where traditional PCR and culture-based tests are not easily available.[99]

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Some of the challenges remaining are integrating the sample processing into fully automated devices, quantitative digital CRISPR assays, and navigating regulatory pathways for true point-of-care authorization.[100]The capacity can be expanded to meet the needs of syndromic testing. Further, pre-amplification steps would be eliminated which would further streamline workflows.[101]

Collaboration around the world is needed to take up these tools in the low and middle income countries where the burden of infectious diseases is the highest.[102] Access will be expedited through technology transfer, distribution of open-source reagents and harmonization of regulations. If innovation in diagnostics continues and is implemented fairly, the future of detecting infectious diseases will change, fundamentally, globally.[103]

13.Conflict of interest:

All Authors have no conflict of Interest.

14.Funding

No funding

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