Critical review on where CRISPR meets molecular diagnostics

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Abstract
Simple yet powerful clustered regularly-interspaced short palindromic repeats (CRISPR) technology has led to the advent of numerous developments in life sciences, biotechnology, therapeutics, and molecular diagnostics, enabled by gene editing capability. By exploiting the CRISPR-Cas system's nucleic acid sequence detection abilities, CRISPR-based molecular diagnostics have been developed. Here, we review the development of rapid, sensitive, and inexpensive CRISPR-based molecular diagnostics. We introduce the transition of CRISPR technology to precision molecular diagnostic devices from tube to device. Next, we discuss the various nucleic acid (NA) detection methods by CRISPR. We address the importance of significant sample preparation steps for a future sample-to-answer solution, which is lacking in current CRISPR-based molecular diagnostic technology. Lastly, we discuss the extension of CRISPR-based molecular diagnostics to various critical applications. We envision CRISPR technology holds great promise for widespread use in precision NA detection applications after particular technical challenges are overcome.

1. Introduction

A multitude of applications derived from CRISPR-Cas (clustered regularly-interspaced short palindromic repeats-associated system) systems have been developed since the first demonstration of in vitro CRISPR-Cas9 in 2012 [1] with publications including CRISPR in the title or the abstract having risen 1453% since 2011 [2]. These applications have far ranging effects on human society and health.

The CRISPR-Cas system is a prokaryotic adaptive immune system against viruses present in various archaea and bacteria [3]. CRISPR has advanced our understanding of fundamental biological processes and aided in the discovery of new disease targets [4–7]. By leveraging its ability to identify and cut with single nucleotide precision, many applications have been derived. Some of these CRISPR applications are, genome editing, gene therapy, epigenetic modulation, library generation with large scale CRISPR-Cas9 targeting for genome wide genetic and epigenetic screens [2, 8, 9]. Other CRISPR applications include imaging with CRISPR, which allows targeting and tracking of specific nucleic acid sequences [10] and CRISPR interference (CRISPRi), which allows reversible repression of multiple target genes simultaneously [11]. Over 3000 genes have been identified to be associated with disease-causing mutations [12], leading to an opportunity to correct these mutations through gene therapies. Therapeutic gene therapies are being explored to treat diseases through mechanisms such as disruption [13], deletion [14], and insertion [15] into genes via CRISPR-Cas9. However, the ethical implications of these CRISPR applications which involve the editing of genetic information have recently come to light. Concerns over the propagation of edits through germline editing and the recent case of CRISPR-edited babies have spurred debate into the ethics and the requirement of regulations on the use of CRISPR in gene editing [16–19]. Therefore, the implementation of CRISPR-Cas
systems in molecular diagnostics is likely to be one of CRISPR-derived biotechnology’s first maturation. Unlike CRISPR therapeutics, CRISPR diagnostics do not involve gene-editing. As a result, CRISPR diagnostic platforms will have lower technical, regulatory, and ethical barriers for entry into the market [20].

2. Molecular diagnostics

Molecular diagnostic testing for genetic or pathogenic diseases has importance for improving healthcare outcomes by providing simple, quick, and accurate testing, especially in the developing world [21]. Rapid detection of nucleic acids is necessary for clinical diagnosis of various pathologies [22]. It is essential to identify individuals who require treatment for specific pathologies to prevent undertreatment. It will also reduce the amount of overtreatment leading to resistant microorganisms [21].

Polymerase chain reaction (PCR) is a popular nucleic acid amplification technique used in molecular diagnostics, such as for the detection of infectious disease, due to its reliability and sensitivity [23, 24]. However, the thermocycling requirement reduces its impact in low-resource settings, so low-cost ultrafast photonic PCR chips using LED are developed for both developing and developed countries [25]. Also, isothermal amplification techniques [26] such as loop-mediated isothermal amplification [27] (LAMP), recombinase polymerase amplification [28] (RPA), and nucleic acid sequence-based amplification (NASBA) [29] are adopted as alternatives to PCR to amplify nucleic acid sequences in low-resource settings. Presently, point-of-care nucleic acid amplification tests (NAATs) use PCR and isothermal techniques to amplify such diagnostic tests [30]. These can be revolutionized through the introduction of CRISPR-Cas systems.

The mechanism of CRISPR-Cas systems, which are ‘programmed’ by guide RNA, allows for single base specificity [31] compared to the amplification-based molecular diagnostics techniques such as PCR which use generated primers. Primers can have issues such as primer-dimer hybridization and off-target amplification, leading to non-specific amplification [32]. As a result, NAATs can report false positives, negatives, and lower specificity. Furthermore a trade-off between specificity, sensitivity, and fidelity in PCR limits its accuracy, precision, and applicability, especially in pathologies with low titers of target nucleic acids [33].

This difference in mechanism facilitates CRISPR-Cas systems’ ability to engage in strain identification with single nucleotide specificity [31, 34], a trait that is lacking in PCR techniques. RT-PCR (reverse transcription PCR) tests, which allow the amplification of RNA through reverse transcription of RNA into DNA followed by PCR, are used for detection of RNA-based viruses and suffer from frequent false-negative diagnoses [35]. It is a result of the low sensitivity of amplification-based detection for the Zika virus. Conventional isothermal amplification techniques trade specificity for sensitivity. As a result, incorporating a specific CRISPR-Cas module can improve the specificity in the detection scheme, despite off-target CRISPR-Cas behaviour [36].

The CRISPR-Cas mechanism allows it to be highly programmable allowing for easy adaptation to different targets [1] and higher specificity, while maintaining adequate sensitivity—making this a promising development in molecular diagnostics.

3. Timeline of CRISPR-based molecular diagnostics

Beginning with the discovery of CRISPR locus in 1993 [37], there is an increasing number of discoveries concerning CRISPR-Cas systems since the first demonstration of its use in gene-editing [1]. Recently, there have been exciting developments in CRISPR-Cas based molecular diagnostics.

In (figure 1), the timeline for the development of CRISPR-Cas systems and the subsequent transition from laboratory test tube reactions to diagnostic devices is shown. Earlier diagnostics relied on the CRISPR-Cas9 [34, 38–40] system demonstrated in 2012, while newer methods starting in 2017 make use of the collateral cleavage activity found in orthologues Cas12 [41] and Cas13 [31]. With the exception of CRISPR-Chip [42], all the CRISPR diagnostic methods make use of amplification reactions to detect specific sequences in nucleic acids. This amplification increases the sensitivity but at the cost of increasing the resource requirements. CRISPR-based tests can improve upon the sensitivity and specificity of nucleic acid amplification tests by introducing CRISPR base-pair matching.

4. CRISPR-Cas9 systems

Detection schemes using the CRISPR-Cas9 system require input sgRNA (single-stranded guide RNA) for specified target sequences, which will be used to guide the Cas9 complex to the correct site. Some methods employ dCas9 proteins without endonuclease activity, instead they use the protein solely for sequence identification.
The development of nucleic acid amplification techniques has enabled the adoption of molecular diagnostics for the detection of diseases and pathogens. The recent demonstration of CRISPR's in vitro gene-targeting and editing capabilities has led to the development of new nucleic acid diagnostic tests.\[1, 23, 27–29, 31, 34, 38–52\].

In INSPECTR, synthetic gene networks are used for RNA sensing. The RNA is extracted from a clinical sample before amplification by NASBA. The products of this process are used to rehydrate a paper sensor with the freeze-dried components for the toehold reaction. The toehold switch reaction relies on the interaction between the switch and trigger RNA sequences, resulting in an activated complex that results in the colour change in the paper sensor [53]. This technology has been demonstrated to detect viral Zika RNA [34].

The addition of CRISPR-Cas9 enables strain discrimination in the detection scheme, NASBACC (NASBA-CRISPR cleavage), (figure 2(c)), with single nucleotide accuracy. A trigger sequence is appended to the amplified product through reverse transcription. This presence of a strain-specific protospacer adjacent motif (PAM) will dictate the production of a truncated or full length trigger RNA, determining the toehold activation. The paper disc will undergo a colour change from yellow to purple upon activation [34]. The ability to freeze-dry the reagents allows for more practical applications by reducing the cost and complexity of storage [54].

CAS-EXPAR, (figure 2(d)) combines the rapid amplification and sequence-dependent extension of EXPAR [50] (exponential amplification reaction) and the specific cleavage of CRISPR-Cas9 to develop a diagnostic scheme which can detect nucleic acids and DNA methylation. The readout of this method is
through transient fluorescence intensity measurements and analysis. The authors reported a sensitivity of 0.82 amol with higher specificity compared to traditional EXPAR [40].

The adoption of CRISPR-Cas9 into the EXPAR reaction eliminates the need for exogenous primers to amplify long DNA or RNA, possibly lowering target-independent triggering [40]. The ‘primers’ are generated and accumulated through site-specific cleavage of the target DNA sequence by the CRISPR-Cas9 complex. The method cycles through CRISPR-Cas9 mediated cleavage, nicking endonuclease cleavage, polymerase extension, and strand displacement to generate dsDNA copies. This method was applied to detect the mRNA of foodborne pathogen L. monocytogenes in extracted samples [40].

The PC (paired dCas9) reporter system, (figure 2(a)) takes advantage of the single-nucleotide specificity of the CRISPR-dCas9 to improve upon molecular beacon designs. Previously, these beacons were limited mostly by the constraints on the probe sequence due to secondary structures and energetics as well as the limitations on sensitivity due to incomplete quenching and unstable signal output of common fluorophores. The alteration to the design decouples the probing sequence with the reporter moieties using a pair of dCas9 proteins, which when in close proximity through binding to close proximity segments in the substrate DNA, form luciferase and generates fluorescence from the catalytic activity of its entirety [39].

This method was employed to detect isolated M. tuberculosis (Mtb) genomic DNA. Compared to nonspecific E. coli DNA, the Mtb DNA showed a 52-fold increase in fluorescent signal intensity. After amplifying Mtb genomic DNA samples through 35 rounds of PCR, PC reporters were used to detect a target sequence in the genome. An 11.6-fold gain in luminescence was reported from pre-to-post PCR using the PC reporters, with a sensitivity of 1 copy/500 µl. This method adds increased specificity, which is lacking after isothermal amplification for the detection of pathogenic DNA [36, 39].

MiRNA (microRNA) are small noncoding RNAs ~ 22 nt long responsible for the post-transcriptional regulation of gene regulation [56]. They are of diagnostic interest due to linkage with various diseases and their robustness leading to applications in diagnostics [56, 57]. Currently, they are challenging to detect because of complexity, high cost, and low sensitivity [38].

The RCH (RCA-CRISPR-split-HRP) method, (figure 2(b)), combines RCA (rolling circle amplification), CRISPR-dCas9, and split-HRP (horseradish peroxidase) techniques to detect miRNAs. The detection method, with a sensitivity of 35.4 aM, relies on RCA to amplify the low concentrations of miRNAs in the pretreated sample. RCA uses a small circular oligonucleotide as a template for the polymerase to produce long repeating strands that are copies of the circular sequence in a unidirectional amplification. The highly specific nature of the RCA reaction can reduce the off-target effects of dCas9 on specificity. It uses dCas9-split HRP fusion proteins, to achieve secondary amplification and signal output with single-nucleotide specificity. Upon interaction due to the proximity of the dCas9-split HRP fusion proteins, its activity can be detected through the 3, 3'-, 5, 5'-tetramethylbenzidine (TMB) colour reaction [38].

This method was used to detect miR-195 and miR-16, a biomarker of breast cancer and a control respectively, in serum samples from non-small cell lung cancer (NSCLC) patients. Compared to qRT-PCR, the authors claim improved data significance and homogeneity, however there was no significant result for the miR-195 [38]. The authors showed the specificity of the method through let-7a detection compared with let-7c, let-7f, and let-7g, demonstrating the discrimination of NSCLC patients from healthy patients [38].

5. Orthologous CRISPR-Cas systems

Orthologues of the CRISPR-Cas system such as Cas12a and the Cas13 family have been shown to possess a unique ability to unleash indiscriminate cleavage of ssDNA (single-stranded DNA) and RNA, respectively, upon target recognition [31, 41]. This ability can be exploited for use in diagnostics where recognition of specific nucleic acid sequences can be easily linked with a readout through the indiscriminate cleavage activity.

In (figures 2(e), (f)), a scheme for diagnostics using Cas12a and Cas 13 from a clinical sample to a fluorescent readout is presented.

The first demonstration of an indiscriminate cleavage diagnostic CRISPR-Cas system is the Cas13-based SHERLOCK (specific high-sensitivity enzymatic reporter unlocking). Upon target recognition in an ssRNA strand, indiscriminate cleavage activity will separate a fluorescent molecule from an ssRNA bound quencher resulting in fluorescence [31]. It has also been shown that the reporter can be adapted for lateral flow assay strips by replacing the dye-quencher pair with two proteins/antigens which can be bound by antibodies in the capture lines of the strip [55, 58]. It was reported that < 0.22% off-target fluorescence was observed. Thus, the low level of off-target fluorescence has little impact towards reducing the high specificity of this method.

A scheme using Cas12a for nucleic acid detection is named DETECTR (DNA endonuclease-targeted CRISPR trans reporter). It employs Cas12a to target specific DNA sequences and upon target recognition, performs non-specific ssDNA cleavage which separates fluorescent molecules linked by ssDNA to the
quencher resulting in a fluorescent signal. It achieved attomolar sensitivity for DNA detection of prepared human papillomavirus (HPV) clinical samples, consisting of two genotypes varying by six base pairs [41].

Both schemes rely on sample preparation, consisting of nucleic acid extraction and RNase treatments, and amplification. The amplification technique chosen by the two methods is RPA (recombinase polymerase amplification) or RT-RPA with T7 transcription, which was determined to be the most sensitive compared to other isothermal amplification techniques [31].

Using multiple orthologues of the CRISPR-Cas system in one reaction can permit the multiplexing of various targets. In (figure 2(f)), the SHERLOCKv2 technique allows for quantitative measurement and extends SHERLOCK to four-channels through multiplexing with the following four orthogonal Cas12 and Cas13 enzymes: PsmCas13b, LwaCas13 a, CcaCas13b, and AsCas12a to detect Zika ssRNA, Dengue ssRNA, and two synthetic dsDNA and ssRNA targets [55]. Each of these orthologues have a unique and specific cleavage preference for their indiscriminate nuclease activity; combined with uniquely linked quenched-fluorescent probes allows for each orthologue to be paired to one unique probe, forming a set. Each set forms a single channel for the four-channel detection. These differences consist of variation in the constitution of the nucleotides in the link of the probe for the Cas13 orthologues and variation in the nature of the nucleic acid structure (cDNA vs RNA) for the Cas12 orthologue. A consequence of using different Cas enzymes is the difference in sensitivity of each one; SHERLOCKv2 only showed (≈2 aM) attomolar range sensitivity for PsmCas13b and LwaCas13a. Additionally, it was further shown that the signal sensitivity of Cas13 family enzymes could be improved by 350% with the combination of an auxiliary CRISPR-associated enzyme, Csm6 [55].

The variable sensitivity between channels may pose additional difficulties during translation into practical settings. However, this demonstration of single-reaction multiplexing is an essential step in reducing the cost and increasing the effectiveness and efficiency of CRISPR-based diagnostic techniques.

6. Development CRISPR-based molecular diagnostic devices

The previously mentioned CRISPR-based diagnostic methods showed promising developments, offering improvements to existing NAATs. A table comparing various performance metrics of the ‘test-tube’ methods can be found elsewhere [59]. However, those ‘test-tube’ methods need to be integrated with sample preparation and demonstrate the ability to function as a sample-to-answer device to enable practical and functional point of care diagnostics. A workflow of the required steps for a diagnostic device and its expected time is provided in (figure 3(a)). Each of these steps needs to be integrated to one device for a sample-to-answer device. The difficulty in integrating these onto a single device is a significant barrier in progress for more straightforward and robust tests.

Sample preparation in particular poses significant challenges. Various clinical sample types (figure 3(b)) can be used for a diagnostic test, depending on the detection targets’ properties. Each of the different clinical samples presents particular difficulties in achieving CRISPR-based detection, requiring different sample preparation steps. These steps can be simple or complex depending on the downstream requirements of the assay. Furthermore, the nucleic acids must be extracted from the clinical sample to be amiable to the amplification and detection reactions further downstream (figure 3(b)). One common requirement is the removal of inhibitory compounds found in the clinical sample. Promising work has been undertaken to meet these challenges facing the development of a sample-to-answer CRISPR-Cas based diagnostic device.

The workflow from design to a sensor product for INSPECTR is shown in (figure 3(b.i)). This is a good step towards the practical implementation of CRISPR-Cas based diagnostics as it has simple design process and considerable robustness in transportation due to the freeze-drying of the reagents. A problem arises in the sample preparation required before applying to the paper sensor. This sample preparation step, which includes RNA extraction and requires heating to 95 °C [34], is a limiting factor of the sensor. The simple design process will allow this technology to adapt quickly (5+ days) to new and emerging molecular diagnostic needs. Combined with the low cost of (~$1/test), this technology holds promise for affordable and robust CRISPR-based molecular diagnostics.

Most methods require amplification before applying the specific detection offered through CRISPR-Cas systems. The CRISPR-Chip device, (figure 3(b.iii)) can rapidly (~15 min) detect genomic DNA with a sensitivity of 1.7 fM without the need for nucleic acid amplification [42]. This biosensor leverages the gene-targeting ability of dCas9-sgRNA complexes functionalized to a graphene transistor film to provide a digital electronic quantitative readout. Upon the selective hybridization of the target sequence and the dCas9-sgRNA complex, the creation of a Donnan potential and the change of graphene channel conductivity results in the electrical signal output.

This device was applied to clinical applications by detecting the deletion of two exons related to the Duchenne muscular dystrophy (DMD). Swabs were taken from patients and genomic DNA was extracted.
and purified using commercial kits before incubation on the chip with results in ~15 min [42]. The lack of amplification and the rapid readout are two powerful characteristics of the CRISPR-Chip as these increase the effectiveness and decrease the resource consumption of the device leading to a viable path towards a point-of-care (POC) diagnostic device. At the time of writing, all other CRISPR molecular diagnostic techniques/devices require an amplification procedure. The authors claim that the sensitivity of this device could be further improved with the addition or pre-amplification to the device, however that would increase the resource consumption, time, and complexity of the device.

HUDSON (heating unextracted diagnostic samples to obliterate nucleases), (figure 3(b.3)) is a protocol for sample preparation of clinical samples. It consists of heating increments to inactivate nucleases and lyse viral particles in human serum, urine, and saliva samples. Pairing this protocol with SHERLOCK, the authors report instrument-free detection of dengue from patient samples in 2 h [58]. ‘One-pot’ reactions for SHERLOCKv2, (figure 3(b.ii)) have been demonstrated where the amplification and detection steps all occur in a single tube [55]. This consolidation reduces the number of steps performed by the operator and allows for faster sample-to-result. However even with this improvement, this still lacks the ability to obtain an answer from a sample in a single step. SHERLOCK has been shown to be used for the detection of SNPs (single nucleotide polymorphisms) from the 2015–2016 Zika pandemic demonstrating the single-nucleotide specificity of the technique as well as the short development time of tests. Within a week, the authors claim that multiple SHERLOCK assays were developed which could identify the S139 point mutation associated with fetal microcephaly in patient samples via visual readout [58].

7. Accessing practicality

A key parameter in accessing the usefulness of POC diagnostic devices is given in the ASSURED (Affordable, Sensitive, Specific, User-friendly, Rapid and robust, Equipment-free and Deliverable to end-users) guidelines by the WHO [60]. Referring to these guidelines, critically lacking factors of the reviewed methods are the assay runtime, resource intensity, and simplicity (sample-to-answer in one user step).

In table 1, a comparison of the runtime of the various techniques and devices that have been reviewed in this article is made. The durations are split into three stages which are essential for diagnostic devices: sample preparation, amplification, and readout. Point-of-care diagnostic devices should be able to rapidly return a result to the user for maximal effectiveness [21, 61].

Rapid molecular diagnostics can allow the patients to be treated efficiently, which is vital in pathogenic applications. In resource-poor settings this is useful as it allows patients to not have to wait/will not leave before treatment [61]. This will increase the overall health outcomes, as overtreatment will be reduced resulting in reduced resources consumed, undesired side effects, and antibiotic resistance [62].
Undertreatment would also be addressed, limiting the spread of the pathogens. Broadly, one hour represents a respectable runtime for the assay [21]. From (figure 3(a)), we can see that assay runtime is over an hour from sample to answer, which is not rapid.

Having equipment-free diagnostics is especially crucial in point-of-care diagnostics as it allows the test to be easily accessible to a large portion of the population and allows it to be operated by patients instead of being limited to clinicians [21].

8. Sample preparation

A missing piece in achieving useful CRISPR-based POC molecular diagnostics devices is the integration of sample preparation [63]. From table 1, the various sample preparation times are compared. Sample preparation consists of a large part of these diagnostic methods and devices and is dependent on the type of sample. Sample preparation, generally, includes nucleic acid extraction, inhibition of undesired reactions, and lysis. Nucleic acid extraction in low resource settings remains a challenge, particularly with human immunodeficiency virus (HIV) which has low viral titers [63]. Furthermore, the concentration of cell-free nucleic acids is low, resulting in the requirement of lysis. Lysis procedures may vary depending on the target and the source of the sample [58]. The sample source depends on which bodily fluid is the most clinically relevant and minimally invasive to the patient [64].

The sample preparations of the various methods take up a large portion of the total time required to obtain a readout from a sample, table 1. Furthermore, the sample preparations for all the reviewed literature are conducted separately from the amplification and detection reactions. This separation increases the complexity of the diagnostic tests and reduces its effectiveness by requiring more actions to be performed by the user. Furthermore, having the separate reaction environments and requiring the user to transfer the components would not allow the diagnostic test to pass the waiver requirements to the Clinical Laboratory Improvement Amendments (CLIA) in the USA. To obtain this status the diagnostic test must be ‘simple and accurate as to render the likelihood of erroneous results by the user negligible’ or ‘pose no unreasonable risk of harm to the patient if performed incorrectly’ [65]. It is essential to consider the ability to receive regulatory approval for any diagnostic test. If a diagnostic test can gain CLIA-waived status, it will become more accessible and widely adopted than a non-approved test as they are cleared for home or over-the-counter use [66]. As such, it is desirable to have a diagnostic test which can produce a readout from a patient sample with only a single action required from the user. This would address the user-friendly portion of the ASSURED guidelines. Furthermore, the requirement of sample preparation typically involves the use of heating equipment, extraction columns, and other biological laboratory equipment. As a result, to achieve an equipment-free diagnostic test, work will need to be conducted on either achieving effective sample preparation equipment free or through reducing sensitivity to the input requirements for the assays. Chemical and electrical heating are viable alternatives to achieve the high temperatures required for
heat-based sample preparation. Alternatively, non-heat related sample preparation techniques could be attempted such as mechanical lysis and RNase inhibition kits [67, 68].

Amplification is required to obtain higher sensitivities by producing more copies of the starting material allowing for better detection by CRISPR-Cas systems. This would decouple the detection from non-specific amplification resulting in an increased sensitivity at no cost of specificity due to the single-nucleotide specificity of CRISPR-Cas systems. For example, adding an amplification step would increase the sensitivity of the CRISPR-Chip by increasing the number of copies available to be bound to the functionalized dCas9. However, this would increase the cost, resources, and time to result of the CRISPR-chip, thereby reducing its practical advantages. From table 1, the readout is the largest portion of the time required to run these diagnostic tests. These criteria are intrinsically tied to the CRISPR-Cas system and are more challenging to improve compared to the previous stages. The readout is user-friendly so that the result can be interpreted easily by clinicians and non-clinicians alike. The readout method from the aforementioned methods include indication of results through colour changes, fluorescence, and lateral flow strips. Each of these methods has its own benefits for diagnostics. The use of lateral flow strips caused a longer duration required for a result in the case of SHERLOCK (table 1).

Fluorescence and colour changes intensities need to be obvious and not subjective to ensure the results are accurately read to ensure user friendliness and accuracy. Through a combination of hardware and software and the widespread usage and adoption of smartphones, this technology can be exploited to capture and analyse the output of point of care diagnostic tests [69], such as this Zika diagnostic test [70]. These methods should have controls to ensure that the test has been properly run [71].

9. Applications

The development of pathogen detection CRISPR-based diagnostics has progressed dramatically in the past few years with the development of INSPECTR, SHERLOCK, and DETECTR, leading companies to pursue commercialization of the aforementioned techniques. This technology offers clear advantages compared to current NAATs and will help bring diagnostics to a more extensive usage among the developing and developed world. Through its apparent relationship to disease and enormous immediate potential impact, this will be the first application of CRISPR-based sensing to be realized.

Developments in CRISPR-based sensing will expand the scope and depth of our diagnostics, leading to new and more advanced applications.

Beyond the focus of pathogen diagnostics, there are many sensing areas beyond those demonstrated. Some identified applications for CRISPR diagnostics, (figure 4), are mutation detection, drug screening, antibiotic resistance, prenatal genetic testing, circulating tumour cells, tumour cells, and singe cell analysis. A commonality among these applications is that can benefit from the base-pair matching specificity of CRISPR. CRISPR-based sensing confers the ability to achieve high specificities and sensitivities. This is useful in applications where there is a low concentration of nucleic acids due to the nature of the input or technical challenges.

Presently, detection of mutations requires laboratory benchtop based techniques which are not equipment-free to perform tests such as heteroduplex analysis (HA), single nucleotide polymorphism (SNP) genotyping, and genome sequencing to determine the presence of mutations [72]. The demonstration of SNP detection from the SHERLOCK system provides a glimpse into the introduction of CRISPR-based sensing into point-of-care genetic screening for mutations [31]. This can be combined and integrated with existing testing technology to improve current designs or in new devices which can be equipment-free and portable allowing point-of-care usage. Increasing the availability of mutation detections through point-of-care diagnostic tests could improve early stage detection; resulting in improved health outcomes, lower cost, and increased quality of life.

Prenatal genetic testing is an area which could benefit for the inclusion of CRISPR-based sensing technologies. Increasing the accessibility of these tests can reduce infant mortality through early detection of diseases [73, 74]. Treatment can be combined with diagnostics through CRISPR gene regulation and editing [31], bringing forward highly accessible and straightforward diagnostics and treatment.

Applications where the high specificity and sensitivity of CRISPR-based methods will be useful are with circulating tumour cells (CTCs) which have an incidence of 0–10 CTCs/7.5 ml of whole blood [75]. The low incidence of circulating tumour cells in human samples results in the requirement of extremely sensitive detection. The single-nucleotide specificity of CRISPR-Cas systems confers an advantage in the analysis of the nucleic acid components of captured CTCs. The heterogeneity of cells increases the necessity of single cell analysis with CTCs. Single cell analysis using pooled CRISPR screens can empower transcriptome analysis and genomic screening and elucidate transcriptome signalling, and cellular communication [8, 9, 76, 77] by
improving on existing single cell analysis, from diagnostic applications to research on tumour cells and other biological components containing nucleic acids.

Antibiotic resistance is a growing problem stemming from the over prescription of antibiotics [78]. The ability to rapidly determine the correct treatment plan to reduce overtreatment could be achieved through CRISPR-based diagnostics while also enabling an increase in our knowledge on the resistance [79]. CRISPR-based diagnostics can be used to detect mutations resulting in antibiotic resistance in bacterial strains [80, 81]. This will empower the handling of the rising issue of antibiotic resistance and contribute to a greater understanding of the progression, allowing us to have better control over the situation. Similarly, drug screening for different patients using genetic tests for variability among the population can be accomplished using CRISPR-based sensing. Screening can also look for specific mutations, if known, to quickly allow the determination of the effects of certain drugs on specific individuals. A possible technology that enables this would be pooled CRISPR screens [8, 9, 76].

Cell free DNA is of diagnostic interest as it has been linked to various diseases and has been determined to have diagnostic value indetecting disease states [82–85]. As a result of low concentrations compared to cellular DNA, it requires diagnostic techniques with higher sensitivity. This higher sensitivity can be achieved without sacrificing specificity through the use of CRISPR-based sensing systems with single nucleotide specificity, allowing the detection of point mutations. These applications represent a small portion of CRISPR-based sensing’s potential impact, as anything related to nucleic acids could benefit from the inclusion of CRISPR systems.

10. Conclusion

CRISPR-based molecular diagnostics employ the highly specific nature of CRISPR-Cas systems to combine high sensitivity and specificity in assays. This greatly improves upon existing diagnostic solutions and will become the first maturation of CRISPR-based technology due to its lower barriers. Developments using the Cas13 system have shown good progress towards a practical point-of-care diagnostics after integrating an effective sample preparation unit, critical to forming a functional sample-to-answer molecular diagnostics. This progress will help address the growing necessity for quick, accurate, and resource-limited diagnostic tests as the world experiences increasing pressures on medical systems from antibiotic resistance to viral outbreaks.
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Author contributions

C.R. and L.P.L. mentored A.L. A.L., C.R. and L.P.L. wrote, edited and reviewed the manuscript. All authors contributed to the discussion of the content.

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Competing interests

The authors declare no competing financial interests.

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