CRISPR IN NEXT-GENERATION MOLECULAR DIAGNOSTICS

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ABSTRACT

Nucleic acid testing, a key component of molecular diagnostics, is essential for understanding physiological processes and improving human and livestock health. This involves various techniques such as EIAs, PCR, RT-PCR, qPCR, FISH, and NGS, which, despite their high sensitivity and specificity, require sophisticated labs, costly equipment, significant time, and skilled personnel, limiting their use for quick point-of-care (POC) diagnostics. Emerging POC platforms like LAMP and RPA integrated with electrochemical optical biosensors address some of these limitations but often fall short in sensitivity. The CRISPR-Cas systems, especially the simpler Class 2 systems with single crRNA-binding proteins, offer a promising next-generation diagnostic approach. Technologies like NASBACC, DETECTER, and SHERLOCK utilize CRISPR-Cas systems to achieve highly sensitive, specific, and cost-effective diagnostics with single-nucleotide specificity, vital for detecting mutations linked to antibiotic or antiviral resistance.

KEYWORDS: Molecular diagnostics, Nucleic acid testing, Disease prognosis, Point-of-care testing, CRISPR-Cas systems, *Mycobacterium tuberculosis*, SHERLOCK, DETECTR.

ABBREVIATIONS: PCR- Polymerase Chain Reaction; RT-PCR- Real-Time Polymerase Chain Reaction; qPCR- Quantitative PCR; FISH - Fluorescence In Situ Hybridization; NGS- Next-Generation Sequencing; CRISPR - Clustered Regularly Interspaced Short Palindromic Repeats; crRNA - CRISPR RNA; NASBACC- Nucleic Acid Sequence-Based Amplification-CRISPR Cleavage; PAM- Protospacer Adjacent Motif; DETECTER- DNA Endonuclease-Targeted CRISPR Trans Reporter; SHERLOCK - Specific High-sensitivity Enzymatic Reporter unlocking.

INTRODUCTION

Molecular diagnostics, in particular, nucleic acid testing, is fundamental understanding of pathogen detection, disease prognosis and diagnosis. Nucleic acid detection relies on the identification of nucleic acid sequence, thereby providing superior sensitivity and specificity. Techniques such as Enzyme immunoassays (EIAs), Polymerase Chain Reaction (PCR) its derivatives, Real Time Polymerase Chain Reaction (RT-PCR), quantitative PCR (qPCR), Fluorescence in Situ Hybridization (FISH), and Next-generation Sequencing (NGS) are effective, but require specialized laboratories, expensive equipment, significant time, and skilled personnel these limitations make them less suited for quick point of care (POC) diagnostics. Platforms such as electrochemical optical biosensors used Loop-mediated isothermal amplification (LAMP)

recombinase polymerase amplification (RPA) by incorporating nucleic acid complementarity into POC diagnostic. But, these attempts still suffer sequence amplification step or compromised detection sensitivity in comparison to qPCR. It is crucial to develop highly sensitive, highly specific, and cost-effective techniques; such next-generation diagnostics should also have single-nucleotide specificity, which is integral to the detection of mutations conferring resistance against antibiotics or antiviral drugs. CRISPR– Cas system are defined by the nature of the ribonucleoprotein effector complexes encompass a single crRNA (Crispr RNA)-binding protein (cas), primarily been applied for diagnostics, these systems are simpler to reconstitute, enzymes with collateral activity, which serves as the backbone of many CRISPR-based diagnostic assays. Cas9-based method is first approach which referred to as NASBACC (for nucleic acid sequence-based amplification - CRISPR cleavage) combines nucleic acid sequence-based amplification for the isothermal pre-amplification of targets, Cas9 cleavage for PAM (protospacer adjacent motif)-dependent target detection and a toehold sensor for the readout. In brief, If a PAM sequence is present in the RNA fragment, Cas9-mediated cleavage leads to a truncated RNA without the trigger sequence. In the absence of the PAM sequence, the trigger containing full-length RNA activates the toehold switch, as indicated by a colour change. By sensing strain-specific PAM sites, the method allows for viral-lineage discrimination, as shown for the detection of the Zika virus (ZIKV) in plasma at concentrations in the low femtomolar range. Similarly, DETECTER (DNA endonuclease-targeted CRISPR trans reporter) and specific high-sensitivity enzymatic reporter unlocking (SHERLOCK), and allows multiplexed, portable, and ultrasensitive identification of RNA or DNA from clinically applicable samples.

2. COMPARISON OF CRISPR - DIAGNOSTIC ASSAYS TO TRADITIONAL PCR ASSAYS

Modern molecular biology techniques, which have improved their sensitivity, specificity and versatility, and helped rapidly, develop personalized medical diagnosis for both clinical laboratories and point-of-care tests (POCT). However, conventional molecular diagnostic techniques, including PCR and ELISA, require professional laboratories, specific testing equipment, and rigorously trained operators, involving a relatively long detection time. RT-PCR tests that detect viral mRNA have low sensitivity of about 63%–78%, which means that false negatives are common and false negatives are usually a result of low viral load from patients in the early or late stages of infection. In that case the virus still contagious and dangerous a more sensitive test is urgently needed to identify whether a patient is infected. So CRISPR/Cas molecular diagnostic systems can be applied for both nucleic acid and non-nucleic acid molecular diagnoses for inherent properties. CRISPR/Cas-based non-nucleic acid molecular diagnostic tools convert a non-nucleic acid into a nucleic acid signal identifiable by a Cas, with inherent properties of high sensitivity and specificity, fast operation, and affordability, which can be employed to test complex samples, series of molecular diagnostic tools based on Cas proteins included Cas12, Cas13 and Cas14 nucleic acid and non-nucleic acid molecules. Example, cas13 based diagnosis system such as SHERLOCK is also convenient because it is rapid. The first steps completed in 30–45 min. The second detection step is set up in less than 15 min, and provides fluorescence results in1 hr, purification steps are included improved by the fact that the assay can be performed at room temperature (25°C) (Gao et. al.,2021). CRISPR-Cas-based diagnostic tools, such as DETECTR and SHERLOCK, offer advantages in sensitivity and user-friendliness (Table 1) with sensitivity at attomolar level, nearly 99%

specificity with limit of detection (LOD) 10 copies/ul up to 3.13CFC/ml (incase viral detection). Additionally, provide specificity and the ability to distinguish between closely related genetic sequences, which are crucial for accurate diagnosis.

CRISPR system	**Cas9**	**Cas12**	**Cas13**
PAM	Yes	Yes	No
Target	ds DNA	ds/ss DNA	ds/ss DNA
Sensitivity	Approx. 95%	Approx. 97%	Attomolar (aM)
Specificity	Approx. 98%	Approx. 99%	Near 100%
Limit of detection	10 copies/ul	10copies/ul	3.13CFU/ml; 1aM
Application	SARS- COV2	HPV, MT	DENV,ZIKV
Diagnosis	DETECTR	LFA, SHERLOCK	SHERLOCK
Reference	Myhrvoid et al.,2018	Ibrahim et al.,2022	Srivastava et al.,2020

Table: 1: Different CRISPR platforms with different parameters. PAM (protospacer adjacent motif recognized by CRISPR RNA); aM – attomolar (concentration); HPV – Human papilloma virus; MT- *Mycobacterium tuberculosis*; SARS-COV2 (Severe Acute Respiratory Syndrome Coronavirus 2); DENV (Dengue virus); ZIKU (zika virus; DETECTR (DNA endonuclease-targeted CRISPR trans reporter); SHERLOCK (Specific high sensitivity enzymatic reporter unlocking), LFA – Lateral flow assay.

3. VERSATILITY IN TARGETING DIVERSE PATHOGENS

Diagnostic methods depend primarily on identifying a certain sequence associated with mutation sequences or viral and bacterial sequences derived from the infectious agent and then cleaving it in order to produce a readable signal. Most are able to utilize fluorescent probes which are highly sensitive and specificity arises from the binding to the target by pairing between DNA-RNA or RNA-RNA. The tests can proceed at a rapid, it is not necessary to culture isolates or extract genome. Several researchers have developed methods based on the CRISPR-Cas12a and Cas13a families, dubbed DETECTR and SHERLOCK, respectively; with in the field of viral infections. DETECTR uses the Cas12a enzyme to directly bind to DNA targets in a three-stage process: a guide RNA first directs the Cas12a enzyme to a double-stranded sequence of DNA within a specified viral genome (Chen JS *et al.,* 2018). Once bound to its viral genetic target, a single-stranded DNA molecule bound to a quencher molecule and a reporter fluorophore are cleaved indiscriminately by the Cas12a enzyme, this collateral cleavage is detected as a fluorescent signal released from the fluorophore and quencher. SHERLOCK protocol can be optimized for diagnosis of human immunodeficiency virus (HIV), flavi viruses such as Zika, Dengue, West Nile, and yellow fever viruses, N and E gene variants specific to SARS-CoV-2. CRISPR/Cas9 system is an extensively used method Detection and characterization of diseases caused by bacterial such as E. coli, mycobacteria, salmonella. A single guide RNA (sgRNA) directs the endonuclease Cas9 to DNA sequence which has been targeted, and initiates site-specific manipulation (Quan J et al.,2019) as a result of the ability to design CRISPR-guided nucleases in this system easily and relatively quickly dubbed FLASH (Finding Low Abundance Sequences by Hybridization), uses Cas9 enzyme recombination along with multiplex guide RNAs for precise identification of a pathogen by eliminating background sequences, and the Cas9 system cleaves target sequences into fragments. CRISPR/Cas-based diagnostic

methods are suitable for complex samples, and they have been mainly utilized in nucleic acid and non-nucleic acid targets. Example, nucleic acid targets include DNA, RNA, SNP, miRNA (Li, L *et.al.,*2022), non-nucleic acid targets such as metal ions (Pb2+), extracellular vesicles (EVs,), small molecules (ATP, uric acid), enzymatic activity (PNK,NAD). Several strategies have been found to detect the signalling for each type of non-nucleic acid targets, example, DNAzyme-based CRISPR/Cas non-nucleic acid molecular diagnostic system can be used to detect metal ions such as lead ion, OP pesticides Enzyme-linked immunosorbent-based CRISPR/Cas non-nucleic acid molecular diagnostic systems have been developed. A biosensor CRISPR/Cas13a signal amplification-linked immunosorbent assay termed was developed to enhance ELISA sensitivity applying CRISPR/Cas13a (Zhou, J *et al.*,2023).

4. CONCLUSION

The successful application of the CRISPR-Cas9 system to gene editing has quickly become the most promising tool in medicine, microbiology, and other fields. In 2017, CRISPR-Cas systems significantly, revolutionized molecular diagnostics, enabling the accurate and rapid detection of diseases, particularly in point-of-care (POC) settings. CRISPR has high specificity toward nucleic acids of interest, and the detection mechanism is simple and straightforward CRISPR- Cas9, Cas12a, Cas13a, and Cas14 are powerful bio recognition components used in many bio sensing systems because of their enhancing accuracy, specificity and selectivity for targeted nucleic acids. Cas9 protein has a cis-cleavage activity, recognizing the PAM sequence that binds specifically to its guide RNA, resulting in efficient cleavage. Cas12, Cas13, and Cas14 also have a trans cleavage activity to cleave non-selective collateral nucleic acids. The characteristics of Cas12a and Cas13a to amplify target recognition opened the gates for novel nucleic acid detection systems such as SHERLOCK, DETECTR, and HOLMES. Furthermore, combined with the biosensor platform, it holds immense potential as a sensitive, specific, and efficient detection technology for viruses, bacteria, small molecules, proteins, and nucleic acids associated with various diseases. Additionally, integrating CRISPR-Cas diagnostic systems with NGS technologies could further enhance their capabilities and broaden their applications. Researchers have applied engineering principles to both the enzymes and the biosensors to lower the limit of detection. Developing rapidly by the contributions from multiple disciplines as researchers are racing to find more solutions, toolkit designs with fewer steps and simpler materials have emerged while maintaining the detection specificity, integration of CRISPR into diagnostic platforms, including electrochemical biosensors, fluorescence-based assays, and lateral fowl assays (LFAs), has enabled rapid and highly sensitive testing procedures. Innovations in paper-based diagnostics and microfluidic devices are further improving the affordability, integrating CRISPR with artificial intelligence (AI) and machine learning (ML) algorithms are enhancing diagnostic accuracy, speed, and scalability.

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