Applications of the CRISPR/Cas9 System in Clinical Diagnostic Techniques

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Abstract. The CRISPR/Cas9 system, originating from the adaptive immune mechanisms of bacteria and archaea, has become a revolutionary tool in gene editing and molecular diagnostics due to its high specificity, programmability, and trans-cleavage activity. This systematic review examines the current applications and developmental trends of this system in clinical diagnostics, focusing on its coupling strategies with various isothermal amplification techniques (such as NASBA and EXPAR). It elaborates in detail on...The principles and performance characteristics of multiple diagnostic technologies, including NASBACRISPR Cleavage, CAS-EXPAR, Paired dCas9, and nanopore-based targeted sequencing (nCATS). Research indicates these methods demonstrate high sensitivity, strong specificity, and rapid response advantages in pathogen detection (e.g., Zika virus, Staphylococcus aureus), tumor gene mutation screening, genetic disease diagnosis, and pharmacogenomics analysis. Although challenges remain, including sensitivity dependence on preamplification, PAM sequence limitations, and interference from clinical sample matrices, this technology holds significant potential for advancing molecular diagnostics towards portability, low cost, and point-of-care implementation.

Keywords: CRISPR/Cas9 system; Cas9 protein; gene editing; isothermal amplification; clinical diagnostic technology.

1. Introduction

Precision targeted therapy relies heavily on the efficient identification of specific biomarkers and particular sequence variations. Clinically, molecular diagnostic techniques are employed to analyze biomolecules such as nucleic acids and various proteins within bodily fluids. Over the past decade, this approach has significantly advanced the early detection of diseases and the development of personalized treatments. Its applications span the detection of infectious pathogens (such as HIV, HBV, SARS-CoV-2), early tumor screening and drug guidance, diagnosis of single-gene hereditary disorders (e.g., SCA), and pharmacogenomic analysis (e.g., CYP2C19, MTHFR, ADH1B/ALDH2 polymorphisms) and disease susceptibility assessment.

qPCR has become a core method for routine clinical testing due to its reliability and flexibility, but its reliance on thermal cyclers and specialized operating environments limits its applicability in resource-constrained settings. FISH is applicable for detecting gene rearrangements and amplifications, while NGS, though advancing genomic medicine, faces constraints of high cost and complex data analysis. Therefore, there is an urgent need to develop biosensing technologies that combine high sensitivity, strong specificity and cost-effectiveness to meet the demands of diverse clinical applications. Against this backdrop, the CRISPR/Cas system has pioneered an entirely new technical pathway for molecular diagnostics.

The CRISPR/Cas system is an RNA-guided adaptive immune mechanism found in archaea. This system integrates exogenous DNA into CRISPR gene sequences, which are then transcribed into crRNA to guide Cas proteins in targeting and cleaving specific sequences [1]. Its ability to recognize genes with single-base discrimination overcomes limitations of traditional nucleic acid detection techniques, establishing CRISPR as a pivotal tool and research focus in gene editing and molecular engineering [2]. Moreover, diagnostic technologies based on the CRISPR/Cas9 system have achieved significant advances in molecular diagnostics in recent years. The core functionality of the CRISPR/Cas9 system lies in its programmable, highly specific DNA cleavage capability, enabling it

to recognize target DNA and trigger or terminate detection signals by cleaving specific DNA sequences. With deep researching into the CRISPR system and the development of detection technologies built upon it will further expand its application prospects in disease diagnosis and biosensing.

2. Composition and Core Mechanism of the CRISPR/Cas9 System

2.1. Cas9 Protein

Cas9 comprises approximately 1,000 to 1,600 amino acids. It is a class of endonucleases containing multiple structural and functional domains, and facilitates the maturation of crRNA, the recognition of exogenous target DNA, and the cleavage of nucleic acids. Streptococcus pyogenes Cas9 (SpCas9) is the most extensively studied Cas9 protein to date, possessing both recognition and nuclease lobe. These two lobes are linked via a helical structure containing a proline residue and a disordered linker. The nuclease lobe possesses both HNH and RuvC domains, with the connection between these domains dependent upon a bridging helix. [3]. Prior to gRNA attachment, the Cas9 protein remains in an inactive state, exhibiting only non-specific, loose binding to DNA. Upon encountering gRNA, the disordered PAM-interacting region of apoCas9 undergoes pre-displacement, causing the Hel-III domain to shift towards the HNH. These structural changes manifest that After gRNA is loaded onto a specific delivery system, the critical transition state from molecular recognition to chemical cleavage by Cas9. Cas9 forms a complex with gRNA, and upon binding to target DNA, gRNA pairs with the target DNA to activate Cas9. Cas9 locally cleaves the DNA double strand near the PAM site, allowing the crRNA strand to invade and the target strand to shift, forming an R-loop from the proximal to the distal end of the PAM. The HNH domain rotates and shifts, enabling the non-target strand to precisely locate the RuvC domain [4]. Ultimately, the fully active Cas9 cleaves the DNA double strand at the third base upstream of the PAM. The HNH domain cuts the target strand, while the RuvC domain cleaves the non-target strand, resulting in a blunt-ended break. [5].

2.2. Core Mechanism

The Cas9 protein and gRNA form a targeting complex. The sgRNA (single-guide RNA) contains a spacer sequence of approximately 20 nucleotides, designed to be complementary to the target DNA sequence via the principle of base complementary pairing. SgRNA is a chimeric molecule formed by the binding of mature tracrRNA to crRNA. It guides Cas9 to a 20-nucleotide target sequence at a specific site and provides the domains (such as the stem-loop structure) required for Cas9 protein binding, ensuring the stability of the complex [3]. The sgRNA binds tightly to the Cas9 protein, forming a functional Cas9-sgRNA ribonucleoprotein complex (RNP). The formation of this RNP is a prerequisite for diagnostic specificity.

Cas9 protein relies on dual recognition of the PAM sequence and the target sequence. Cas9 first scans double-stranded DNA for a specific short sequence upstream of the target sequence (typically at the 3' end), termed the PAM. For the most common Streptococcus pyogenes Cas9 (SpCas9), the PAM sequence is 5'-NGG-3' (where N represents any base). PAM recognition constitutes the key mechanism by which Cas9 distinguishes between host endogenous DNA (such as CRISPR arrays) and exogenous invading DNA [6]. Upon locating the PAM, Cas9 unwinds the downstream DNA double strand, permitting the spacer sequence of the sgRNA to undergo base pairing verification with the complementary strand of the target DNA (termed the target strand). The PAM requirement provides the first layer of specificity, while base complementary pairing offers a second layer of high specificity. Only when both conditions are met does Cas9 bind effectively.

Upon successful binding, the two independent nuclease domains of the Cas9 protein are activated: (1) HNH domain: Cuts the target strand that complements the sgRNA. (2) RuvC domain: Cuts the non-target strand that does not pair with the sgRNA. This synergistic cleavage results in a double-strand break (DSB) in the target DNA. The cleavage site typically occurs at the third base upstream

of the PAM, yielding a blunt-ended incision (for SpCas9). Moreover, considering the interaction between Cas9's two distinct domains and single-stranded DNA, the scientific team discovered a Cas9 nuclease variant capable of generating single-strand nicks on the complementary strand of target DNA. Following the action of two cleavage enzyme complexes at adjacent sites on the complementary strands of the target DNA, bilateral nicks are generated. The cell then repairs the breaks via two distinct repair pathways. This method of breaking the DNA strand produces an effect similar to double-strand breaks and effectively reduces off-target rates [7].

2.3. Detection Mechanism

The CRISPR/Cas9 system comprises Cas9 and gRNA. When bacteria encounter phage invasion, portions of exogenous RNA are incorporated into the genome, where spacer sequences undergo adaptation to generate gRNA containing RNA sequences matching the target site. By guiding recognition of the target DNA sequence and binding to the PAM sequence, Cas9 performs specific cleavage of the target DNA. With the support of in vitro diagnostic technology, the obtained DNA sequences are amplified. Finally, detection of certain pathogenic microorganisms is achieved through sequencing analysis or immediate testing, observing color changes or specific gene locus displays.

3. Diagnostic technologies based on the CRISPR/Cas9 system

3.1. NASBA-CRISPR

Nucleic acid sequence-based amplification (NASBA) technology primarily relies on the synergistic action of AMV reverse transcriptase [8], T7 RNA polymerase [8], and ribonuclease H (RNase H) [8]. RNA amplification is achieved at a constant temperature of 42°C using a pair of specific primers [3]. NASBA-CRISPR employs nucleic acid sequence isothermal amplification technology and Cas9-mediated specific cleavage to generate detectable signals that can be monitored by miniature sensors or displayed as color changes on test strips. This system distinguishes between American and African Zika virus lineages at the single-base level [2,9].

NASBA-CRISPR Cleavage integrates nucleic acid sequence-dependent amplification (NASBA), toe-hold switch RNA sensors, and the CRISPR/Cas9 system. In the presence of specific PAM sequences and gRNA targets, double-stranded DNA synthesized by the NASBA reaction is cleaved by Cas9. The truncated RNA products lack sensor H, thereby failing to activate the toe-hold switch [10]. In the absence of a PAM sequence, however, a full-length RNA product containing the sensor H trigger sequence is produced, thereby activating sensor H. Subsequently, the RBS and start codon are released, activating LacZ gene translation and expression of β-galactosidase. This enzyme converts the yellow substrate (Chlorophenol Red-β-D-galactopyranoside) into a purple product (chlorophenol red), yielding a visible color change. The NLSCas9 Nuclease (E365) effectively cleaves double-stranded DNA, making it suitable for in vitro cleavage of target DNA at specific sites.

During reverse transcription, a Trigger (magenta) binds to the sequence-specific primer. If the template contains the specific PAM sequence (blue), Cas9-induced targeted cleavage results in truncated RNA transcribed by T7, preventing Toehold- activation [11]. If the template lacks the specific PAM sequence, the full-length template transcribed with the Trigger triggers Toehold activation, producing a visible color change.

3.2. CAS-EXPAR

The Cas9/sgRNA complex performs site-specific cleavage on ssDNA substrates, generating product fragments. DNA polymerase catalyzes hybridization between X and the EXPAR template, yielding double-stranded extension products. These double-stranded products are subsequently cleaved by NEase to form gaps. One copy of X is released from the template by Vent (external) DNA polymerase, and the dissociated X triggers a new round of amplification. The Cas9 (D10A) Nickase (E366) variant of the Cas9 protein, derived from the Cas9 Nuclease, has one of its cleavage sites

mutated. It generates nicks on single-stranded DNA complementary to the sgRNA target sequence, thereby creating functional single strand breaks.

3.3. Paired dCas9(PC)

Employing a pair of dCas9 proteins, each fused to one of luciferase's two separate domains (NFluc and CFluc), detection is achieved via two gRNAs targeting adjacent sequences. In the presence of target DNA, the two dCas9 proteins, each bearing a luciferase domain, bind to the adjacent target sequences under gRNA guidance bringing the two separate luciferase domains into proximity to form an active luciferase complex. In the presence of ATP and oxygen, this complex catalyzes the oxidation of luciferin to oxidized luciferin, generating a detectable fluorescent signal [12].

3.4. Nano-Pore Cas9-targeted sequencing Sequencing (nCATS)

This is an enrichment strategy that utilizes Cas9 to target and cleave chromosomal DNA for the attachment of nanopore sequencing adapters. nCATS enables the simultaneous assessment of single nucleotide variants, structural variants, and CpG methylation for haplotype resolution. To avoid amplification, the CRISPR system is employed to precisely target and cleave specific regions within the target genome. The resulting fragments are then enriched for nanopore sequencing. Cas9 RNPs, guided by engineered gRNAs, excise regions of interest (ROIs) from genomic DNA. Sequencing adapters are subsequently ligated to the excision margins outside the ROI. Following Cas9 cleavage, the enzyme remains bound to DNA adjacent to the 5' end of the gRNA, directing adapter ligation preferentially to the 3' end of the cleavage site. We introduced flanking nicks in the region of interest (ROI) to achieve coverage on both strands [13]. Nanopore sequencing can detect alterations in DNA. Utilizing nanopore Cas9-targeted sequencing nanopore Cas9-targeted sequencing (nCATS) enables rapid detection of Staphylococcus aureus whilst simultaneously performing Staphylococcal protein A (spa) typing and Staphylococcal cassette chromosome mec (SCCmec) typing [14,15].

4. Conclusion

This paper systematically summarizes the key technical pathways and clinical diagnostic applications of the CRISPR/Cas9 system, emphasizing the pivotal role of its trans-cleavage-based detection mechanism in identifying biomarkers for multiple diseases. The core of its clinical detection lies in the trans-cleavage activity of the Cas9 protein, which is activated after it specifically recognizes target nucleic acids (such as viral RNA/DNA or human gene sequences) under the guidance of a guide RNA (crRNA). This activity enables indiscriminate cleavage of single-stranded reporter nucleic acids (e.g., RNA or DNA) within the reaction system. Pre-labelled fluorescent and quenching groups on the reporter nucleic acid separate upon cleavage, generating a detectable signal (e.g., fluorescence). Based on this mechanism, detection methods requiring no pre-amplification of target nucleic acids (such as the system developed by Nobel laureate Jennifer Doudna's team) have been realized. Through optimization of components like crRNA, they can complete detection in approximately five minutes and quantify viral loads in samples. This technology not only significantly enhances detection sensitivity and specificity but also enables the development of integrated diagnostic systems capable of delivering results directly from samples. However, the current system still faces a series of bottlenecks in practical clinical application, such as dependence on PAM sequences, interference issues in complex biological samples, and the lack of supporting equipment and standardized protocols.

Looking ahead, CRISPR diagnostic technology may achieve breakthroughs in several directions: Firstly, developing PAM-independent Cas variants and novel reporter systems to broaden the range of targets and enhance signal-to-noise ratios; secondly, advancing the deep integration of CRISPR with microfluidics, nanomaterials, artificial intelligence and other technologies to construct intelligent diagnostic platforms capable of multi-target, automated detection; Thirdly, strengthening

its application in detecting non-nucleic acid targets (such as proteins and metabolites) to broaden its utility in chronic diseases, neurodegenerative disorders, and other fields; fourthly, advancing clinical translation research to establish standardized operating procedures and performance evaluation systems, thereby facilitating its genuine implementation in primary healthcare and remote health scenarios. Through continuous technological innovation and interdisciplinary collaboration, the CRISPR/Cas system holds promise to reshape the next-generation clinical diagnostic paradigm, providing more powerful and accessible tool support for precision medicine.

References

[1] Hryhorowicz M, Lipiński D, Zeyland J, Słomski R. CRISPR/Cas9 Immune System as a Tool for Genome Engineering. Arch Immunol Ther Exp (Warsz). 2017;65(3):233-240.

[2] Sun Wenjun, Huang Xingxu, Wang Xinjie. Rapid, Sensitive and Convenient Molecular Detection Based on CRISPR. Chinese Journal of Biotechnology, 2023, 39(1): 60-73.

[3] Jiang F, Doudna JA. CRISPR-Cas9 Structures and Mechanisms. Annu Rev Biophys. 2017; 46:505-529.

[4] moitra A, chakraborty A, dam B. CRISPR-Cas9 system: A potent tool to fight antibiotic resistance in bacteria. The Microbe, 2024, 5: 100184.

[5] Site-specific cleavage by the two nuclease domains of Cas9 RNA-guided nucleases* Site Selection in the Two Nuclease Domains of Cas9 RNA-Guided Nucleases- Journal of Biological Chemistry [EB/OL].

[6] Di Carlo E, Sorrentino C. State of the art CRISPR-based strategies for cancer diagnostics and treatment. Biomark Res. 2024;12(1):156

[7] Zhang C, Lei Z, Li K, et al. Mis-target effect and detection technology in CRISPR/Cas9 system. Biotechnology Bulletin, 2020, 36 (03):78-87.

[8] Hønsvall BK, Robertson LJ. From research lab to standard environmental analysis tool: Will NASBA make the leap. Water Res. 2017; 109:389-397.

[9] Pardee K, Green AA, Takahashi MK, et al. Rapid, Low-Cost Detection of Zika Virus Using Programmable Biomolecular Components. Cell. 2016;165(5):1255-1266.

[10] Qiu Y, Zhang S, Tan A, et al. Detection of TuMV by a toehold switch sensor coupled with NASBA amplification in Pseudostellaria heterophylla. Plant Methods. 2025;21(1):81.

[11] Green AA, Silver PA, Collins JJ, Yin P. Toehold switches: de-novo-designed regulators of gene expression. Cell. 2014;159(4):925-939.

[12] Zhang Y, Qian L, Wei W, et al. Paired Design of dCas9 as a Systematic Platform for the Detection of Featured Nucleic Acid Sequences in Pathogenic Strains. ACS Synth Biol. 2017;6(2):211-216.

[13] Gilpatrick T, Lee I, Graham JE, et al. Targeted nanopore sequencing with Cas9-guided adapter ligation. Nat Biotechnol. 2020;38(4):433-438.

[14] Pan Shufan, Yang Donglei, Wang Pengfei. Rapid Detection and Typing of Staphylococcus aureus Using Nanopore-Based Cas9 Targeted Sequencing. Journal of Shanghai Jiao Tong University (Medical Science Edition), 2025, 45(6): 774-783.

[15] Zhou J, Li Z, Seun Olajide J, Wang G. CRISPR/Cas-based nucleic acid detection strategies: Trends and challenges. Heliyon. 2024;10(4): e26179. Published 2024 Feb 14.