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Signal Amplification Strategies and Advances in CRISPR/Cas Molecular Diagnostics

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Abstract

Accurate detection of infectious diseases and serious diseases is of great importance to public health. CRISPR/Cas has become a highly sensitive and specific molecular diagnostic tool with its programmable recognition and trans-cleavage activity. This article reviews its classification and mechanisms, nucleic acid detection principle, signal amplification strategy, one-tube detection and clinical translation. It is pointed out that pre-amplification coupling, amplification free enhancement and system optimization can significantly improve the sensitivity. One-tube strategies such as physical isolation, glycerol separation, light control, and suboptimal PAM can alleviate aerosol contamination and problems with amplification compatibility, but multiplexing throughput, sample interference, and POCT integration still constrain clinical translation. This study provides a reference for the optimization of CRISPR molecular diagnosis and "sample-in, result-out" application.

Keywords

CRISPR/Cas, molecular diagnostics, signal amplification strategies, one-tube assays, nucleic acid testing, Cas12a/Cas13a

1. Introduction

Precise detection of infectious diseases and serious illnesses is crucial for public health. Traditional PCR technology relies on expensive equipment and professional operation, making it difficult to meet the needs for rapid detection in the field. Although isothermal amplification simplifies the equipment, it is easy to produce false positives, and the sensitivity and specificity of antigen detection are insufficient [1]. The CRISPR/Cas system, with its programmable recognition and trans-cleavage activity, has evolved into a highly sensitive and specific molecular diagnostic tool; representative platforms such as SHERLOCK and DETECTR can achieve detection at the amol/L level [1,2,3]. However, the clinical translation of this technology still faces bottlenecks such as aerosol contamination, signal amplification compatibility, limitations in multiplex testing, and sample matrix interference [1,4,5]. This article systematically reviews its classification mechanisms, signal enhancement strategies, one-tube methods, and clinical progress, with the aim of providing guidance for technological optimization and application.

The significance of studying the development of CRISPR/Cas molecular diagnostic technology in its application fields can be explored at both theoretical and practical levels: (1) Theoretical significance: The CRISPR/Cas system has evolved from a microbial immune mechanism into a programmable molecular

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detection tool. While the academic community has accumulated a wealth of research data on effector proteins such as Cas9, Cas12, and Cas13, a systematic summary of key optimization approaches—such as signal enhancement strategies and one-tube testing methods—remains insufficient. Building on the achievements of previous research, this paper deepens our understanding of CRISPR/Cas molecular detection methods. “Signal amplification” and “one-tube detection” serve as the primary analytical frameworks of this study, which to some extent fills the gap in systematic summaries of methodological innovations in this field and strengthens the theoretical foundation for systematic research on CRISPR diagnostic technologies.

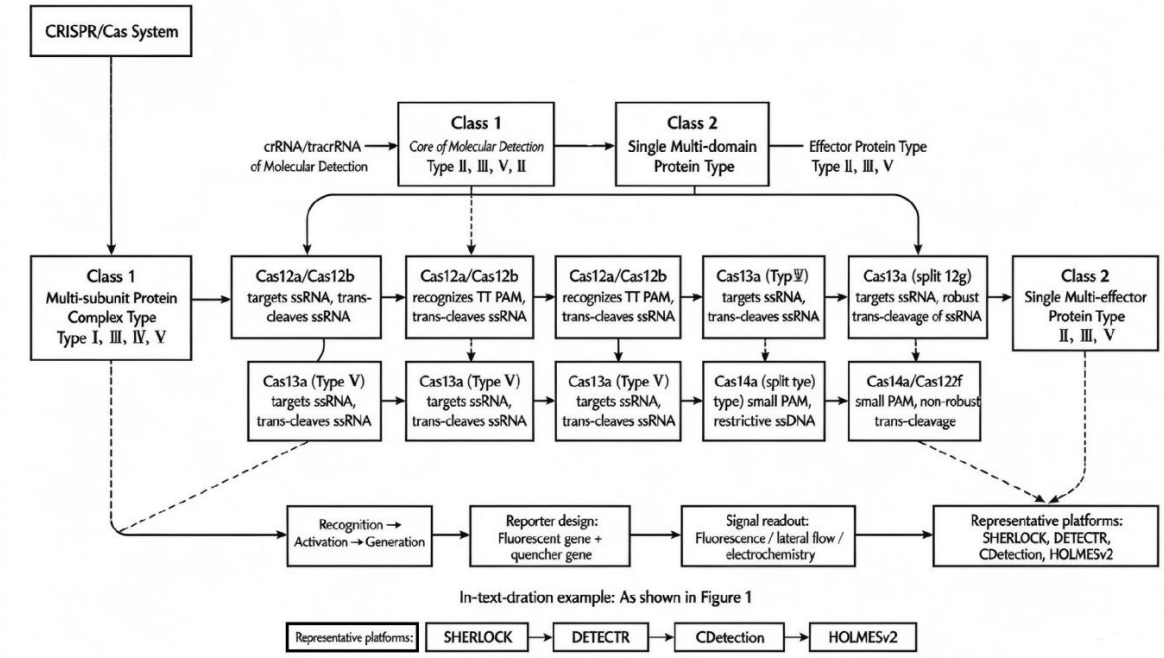
(2) Practical Significance: First, research on CRISPR/Cas molecular diagnostic technology will lay the theoretical foundation and methodological pathways for the development of point-of-care testing (POCT) instruments, promoting the practical application of closed diagnostic systems that operate on a “sample-in, result-out” basis. Second, research on this technology can provide reference methods for rapid pathogen screening, early cancer screening, and basic medical diagnosis. Third, addressing the challenges and strategies involved in the clinical translation of CRISPR-based testing will provide guidance for the formulation of relevant testing standards and the refinement of regulatory measures, thereby facilitating the transition of molecular testing technologies from the laboratory to clinical applications.

2. Classification, Functional Mechanism and Nucleic Acid Detection Principle of CRISPR/Cas System

2.1 Classification of CRISPR/Cas Systems

The definition and differentiation of CRISPR/Cas systems is fundamental to work in this industry. Based on the differences in structural composition of effector proteins, they are divided into two major categories: Class 1, which mainly includes type I, III, IV, and VII, depends on multi-subunit protein complexes to achieve their functions. The second class (Class2) system consist of a single multidomain effector protein and are mainly classified into types II, V, and VI. Due to their ease of use, they have become a core method in molecular detection [6,7]. The specific process is illustrated in Figure 1: by recognizing a specific PAM sequence or targeting a specific nucleic acid (dsDNA or ssRNA), the system triggers trans-cleavage activity (as shown in the middle branch of Figure 1). Subsequently, the system enters the “recognition–activation–cleavage–signal generation” phase, in which the cleavage of a reporter probe bearing a fluorescent group and a quencher group ultimately enables the detection of fluorescent, lateral flow, or electrochemical signals. Based on this principle, representative detection platforms such as SHERLOCK, DETECTR, CDetection, and HOLMESv2 have been developed (as shown at the bottom of Figure 1).

Figure 1: Classification of CRISPR/Cas systems and diagnostic testing workflow



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2.2 Functional Mechanism of the CRISPR/Cas System

From a functional perspective, different Cas proteins exhibit distinct substrate specificities and cleavage activities. The Cas9 protein, mediated by sgRNA, can recognize target dsDNA containing the PAM sequence (5'–NGG–3') and utilize its HNH and RuvC domains to perform double-strand cleavage; further studies have shown that it also possesses trans-cleavage activity. Cas12a (Cpf1) requires only a single crRNA to recognize T-rich PAM sequences (such as TTTV); upon target recognition, it activates non-specific trans-cleavage activity with a catalytic efficiency of 1,250 times per second. Cas12 recognizes both dsDNA and ssDNA, where dsDNA recognition is PAM dependent and ssDNA does not require a PAM site [1,4]. Cas13a specifically targets RNA, activates nonspecific cleavage of surrounding ssRNA upon discovery, and has two independent RNase activities - one for crRNA processing and one for cleaving target genes [2,6]. Cas14a (Cas12f) is one of the smallest known CRISPR effector molecules (400–700 amino acids); it targets ssDNA in a PAM-independent manner and initiates trans-cleavage [8]. Additionally, novel effectors such as CasΦ (Cas12j) have demonstrated excellent trans-cleavage capabilities and can be applied to ultra-sensitive, amplification-free detection of pathogens [9].

2.3 Principles of Nucleic Acid Detection Using the CRISPR/Cas System

In the theoretical field of nucleic acid detection, the scientific community has established a universal paradigm of "recognition–activation–cleavage–signal generation." Researchers design reporter probes with fluorescent groups at one end and quencher groups at the other; once the Cas protein is activated and cleaves the reporter probe, a detectable signal is generated. Representative detection systems include SHERLOCK (based on Cas13a), DETECTR (based on Cas12a), and CDetection and HOLMESv2 (based on Cas12b) [1,2,3]. Studies have also shown that LbuCas13a can directly target DNA and induce trans-cleavage activity without being constrained by PAM or PFS, greatly expanding the scope of detection. CRISPR/Cas3 technology has also been engineered into nucleic acid detection devices that utilize its ATP-dependent endonuclease/helicase functions for target detection [10].

3. Signal Amplification Strategies and One-Tube Assay Methods for CRISPR Nucleic Acid Detection

Although the CRISPR/Cas system possesses single-base resolution, its inherent sensitivity (at the pmol/L to fmol/L level) falls short of the requirements for clinical trace-level target detection. Therefore, researchers have proposed various signal enhancement methods, which can be roughly summarized into three categories: pre-amplification methods of nucleic acids, signal enhancement methods without amplification, and reaction system optimization methods [1,4].

3.1 Signal Amplification Strategies for the CRISPR/Cas System

In studies on nucleic acid pre-amplification strategies, researchers have coupled the trans-cleavage activity of CRISPR with amplification methods such as PCR, LAMP, and RPA to establish a "pre-amplification–CRISPR" synergistic system, thereby increasing sensitivity by 3 to 6 orders of magnitude. Using DETECTR technology, which combines RPA with Cas12a, the detection of high-risk HPV subtypes was achieved with a limit of detection of 10 copies/μL.

HOLMESv2 achieves efficient detection of DNA and RNA dual targets by fusing LAMP and Cas12b technology. SHERLOCK uses Cas13a, RPA and T7RNA polymerase cascade amplification system [1,2,11]. In addition, based on the characteristic of sticky ends of Cas12a cis-cut products, some researchers proposed a dual recognition system combining cis-cut and strand invasion hybridization, and constructed cc-LFA technology platform, which achieved highly specific analysis at the single-base level and was successfully used for the detection and genotyping of nine high-risk HPV types [12]. In another study, photo-activated digital RPA-CRISPR-Cas12a technology (pd-DETECTR) was applied to the determination of BK polyomavirus, which significantly improved the accuracy of the determination [13].

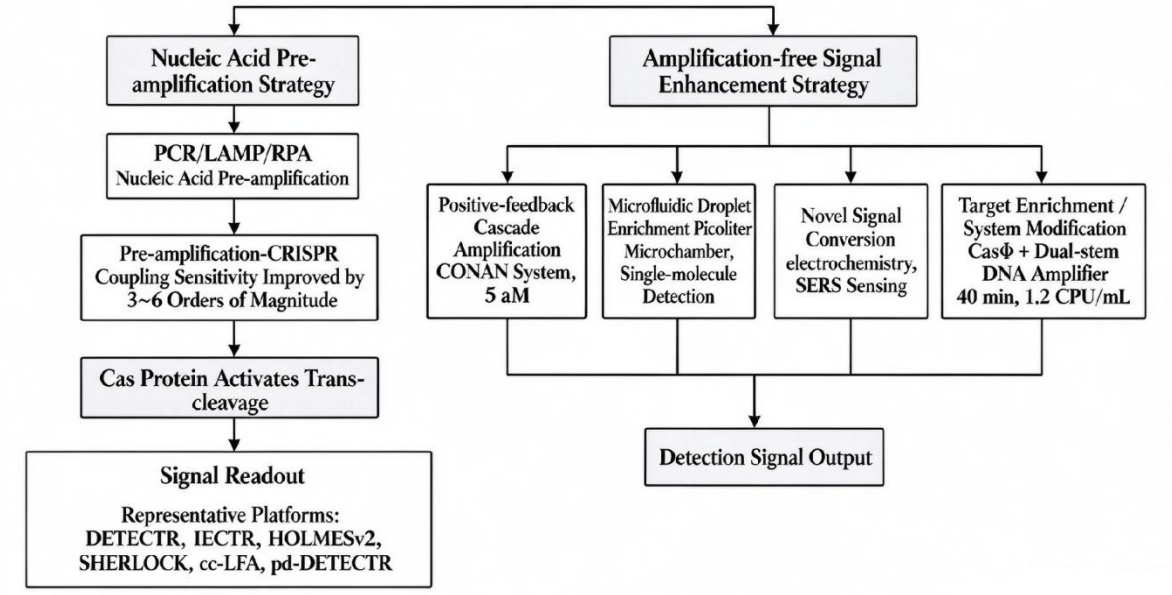
In the field of amplication-free signal enhancement strategies, researchers have proposed various measurement techniques that do not rely on pre-amplification. Firstly, signal exponential growth is achieved through cascade amplification reactions. For example, CONAN system adopts Cas12a positive feedback loop

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to achieve autocatalytic signal amplification, and the detection sensitivity reaches 5 aM[14]. The microfluidic droplet technology is used to confine the reaction system in the picoliter droplets, so as to increase the local target concentration and achieve single molecule detection. Novel signal conversion methods such as electrochemistry and SERS are used. Jiayi Tang et al. systematically reviewed four approaches to enhancing amplification-free CRISPR/Cas-based detection: improving target capture and enrichment, improving CRISPR/Cas system, signal enhancement strategy and combining with advanced biosensors [4]. CasΦ technology has also been reported to be able to improve trans-cleavage efficiency using a double-stem loop DNA amplifier, with detection sensitivity as low as 1.2CFU/mL at 40 min [9].

In summary, as shown in Figure 2, nucleic acid pre-amplification strategies serve as the classic approach, using techniques such as PCR, LAMP, and RPA to enrich targets. Following pre-amplification coupled with CRISPR (resulting in a 3–6-order-of-magnitude increase in sensitivity), the Cas protein is activated to perform trans-cleavage, ultimately enabling signal readout (representative platforms include DETECTR, IECTR, HOLMESv2, SHERLOCK, cc-LFA, and pd-DETECTR). Amplification-free signal enhancement strategies have further given rise to various novel enhancement approaches: such as positive-feedback cascade amplification (the CONAN system, 5 aM), microfluidic droplet enrichment (pico-liter droplets, single-molecule detection), novel signal conversion (electrochemical and SERS sensing), and target enrichment/system optimization (CasΦ + dual-stem-loop DNA amplifier, 40 min, 1.2 CFU/mL). These strategies ultimately achieve high-sensitivity detection signals through various signal conversion and amplification mechanisms, without the need for pre-amplification.

Figure 2: Workflow of signal amplification strategies based on CRISPR/Cas systems



3.2 One-tube Detection Method of CRISPR/Cas System

One-tube technology is the main way to solve the compatibility problem of CRISPR assay and nucleic acid amplification. The traditional two-step method has aerosol hazards caused by cover opening and transfer [5]. To this end, researchers have explored various integration strategies: (1) physical isolation method, the CRISPR system is placed on the tube cover or tube wall in advance, and centrifuged and mixed after amplification is completed [15]; (2) Separation method, which utilizes the viscous property of glycerol or sucrose to separate the two systems at the early stage of amplification. The glycerol enhancement method increased the detection sensitivity by nearly 100 times to 1 aM level [16]. (3) photocontrol method: CRISPR activity was temporarily silenced by modifying the photolytic linker or photocage group, and activated by ultraviolet light after amplification [17,18]; (4) suboptimal PAM regulation to reduce the amplification interference by reducing the cleavage rate of Cas12a through atypical PAM sites. Xu Xiaoling systematically explored the single-tube amplification-CRISPR technology with light control strategy, which could detect as few as 2.5 copies of African swine fever plasmid molecules within 60 minutes [19].

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4. Clinical Translation and Challenges of CRISPR Molecular Diagnostics

4.1 Applications of the CRISPR/Cas System

In the field of rapid viral detection, CRISPR diagnostic technology underwent rapid development during the COVID-19 pandemic. In 2020, Sherlock Biosciences' SARS-CoV-2 test kit received Emergency Use Authorization (EUA) from the FDA, becoming the world's first authorized CRISPR-based diagnostic product [1]. When testing 154 nasopharyngeal swab samples, the SHERLOCK technology achieved 100% specificity and accuracy using fluorescence-based detection. The HUDSON pretreatment technology directly inactivates nucleases in clinical samples through heating, allowing testing without the need for purification [20]. STOPCovid.v2 uses magnetic bead enrichment to shorten the sample processing cycle to 15 minutes [15]. Through Cas13 and microfluidic chips, CARMEN platform can simultaneously analyze 169 types of viruses in a single sample [21]. Wang Xiao et al. developed a Cas13a/Cas12a one-step, double-target assay to simultaneously detect monkeypox virus and its co-infected viruses (HIV and SARS-CoV-2) within 40 minutes. Clinical tests showed that the sensitivity and specificity of DETECTR for SARS-CoV-2 were 95% and 100%[2].

In the field of cancer molecular typing and liquid biopsy, CRISPR/Cas technology has important value in the detection of ctDNA and miRNA. Some studies have used CRISPR/Cas12a to cut RNA-DNA chimeric primers to activate EXPAR and produce G-quadruplexes linked to thioflavin T to enhance fluorescence, with ctDNA sensitivity of 57amol/L[22]. Another study has established a dual signal enhancement system coupling CRISPR/Cas12a with catalytic hairpin ligation, and the sensitivity of miRNA detection can reach the level of subfmol /L [23]. Pan et al. conducted a systematic review of CRISPR applications in early tumor screening and proposed that the integration of CRISPR with microfluidics, electrochemistry, and mobile internet technologies has significantly enhanced the portability and intelligence of detection methods. The cc-LFA system achieved 96.3% sensitivity and 100% specificity in 50 clinical HPV cases; for cases with $Ct \leq 34$, both sensitivity and specificity reached 100% [12].

In the field of non-nucleic acid target analysis, CRISPR methods have been extended to the analysis of proteins, antibodies, and metabolites. By utilizing aptamer technology to convert protein detection into a DNA signal that activates Cas12a, ultra-sensitive analysis of Aβ40/Aβ42 proteins in cerebrospinal fluid from Alzheimer's disease patients has been achieved [24]. UCAD technology converts antibody recognition into DNA barcode information recognizable by CRISPR/Cas12a, achieving sensitivity at the amol/L level [25]. The CaT-SMelor platform integrates allosteric transcription factors with CRISPR/Cas12a to enable nanomolar-level analysis of uric acid-related small molecules [26].

4.2 Difficulties and Challenges

Regarding the difficulties faced in clinical translation, current research identifies three core issues. First, specificity and off-target effects—the CRISPR/Cas12a technology still carries a risk of off-target cleavage in complex samples, which may lead to false-positive results. Second, sample interference and processing—matrix components in clinical specimens, such as proteins, nucleases, and polysaccharides, inhibit Cas12a activity and amplification efficiency; most existing methods rely on complex pretreatment steps [5]. Third, multi-target detection—differences in conditions among orthogonal Cas protein technologies and an insufficient number of signal-tag codes prevent the requirements for high-throughput analysis from being met. Recent studies indicate that reagent reliability, cold-chain dependency, and regulatory coordination are the primary challenges in transitioning CRISPR diagnostics from research prototypes to clinical applications [27]. In the animal pathogen detection industry, Zhang Jinshen et al. believe that both single-system and multi-system CRISPR-Cas multiplex analysis methods have advantages and disadvantages, the former is simple but with limited throughput, and the latter is high-throughput but expensive.

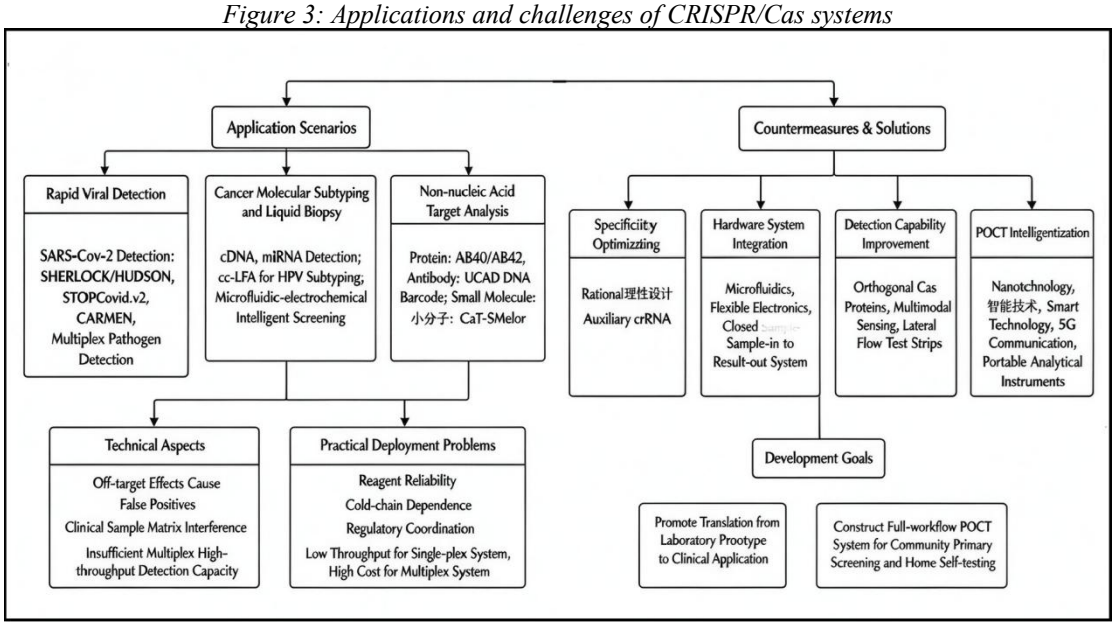
In response to these issues, the academic community has proposed systematic solutions. Regarding specificity optimization, the use of rational protein design and AI-assisted crRNA development reduces off-target effects. In the field of clinical translation, there is a need to develop integrated "sample-in, result-out" closed-system detection platforms utilizing microfluidic chips and flexible electronics [12,28]. In the field of multi-target analysis, high-throughput platforms can be established using orthogonal Cas protein systems and multimodal sensing technologies [21]. Xueqi Wang's systematic review showed that the CRISPR detection

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method could be connected to the lateral flow test strip, and the results could be read directly by the naked eye, which was equal to antigen analysis [29]. In their review, Zhang Yanan et al. noted that molecular POCT diagnostics are evolving from "rapid testing" toward "smart and precise" diagnostics, and will closely integrate nanotechnology, intelligent technologies, and 5G communication technologies [30]. In his doctoral dissertation, Qiu Zhiqiang systematically developed a light-controlled CRISPR single-tube viral analysis platform, a portable dual-color fluorescence analyzer, and a multiplex viral analysis system based on thin-film microreactors, which respectively meet the requirements for high-sensitivity single-tube analysis, portable dual-gene analysis, and sealed automated multiplex testing[31].

Therefore, the current status of CRISPR molecular diagnostic technology is summarized in Figure 3. Regarding the applications and challenges of the CRISPR/Cas system, application scenarios—driven by demand—have further given rise to a variety of testing fields: such as rapid viral detection (SARS-CoV-2 test kits, SHERLOCK/HUDSON, STOPCovid.v2, CARMEN, monkeypox multiplex testing), molecular cancer subtyping and liquid biopsy (ctDNA, miRNA detection, cc-LFA for HPV genotyping, microfluidic-electrochemical smart screening), and non-nucleic acid target analysis (Aβ40/Aβ42 proteins, UCAD DNA-barcoded antibodies, and the small molecule CaT-SMelor). However, in practical implementation, the system still faces dual challenges: technical issues (false positives caused by off-target effects, matrix interference in clinical samples, and insufficient high-throughput capacity for multi-target detection) and practical implementation challenges (reagent reliability, reliance on the cold chain, regulatory coordination, and low throughput in single-system setups versus high costs in multi-system configurations). To address these challenges, countermeasures should be developed along four main lines: specificity optimization (rational protein design, AI-assisted crRNA), hardware system integration (microfluidics, flexible electronics, closed-loop sample-in/result-out systems), enhanced detection capabilities (orthogonal Cas proteins, multimodal sensing, lateral flow test strips), and POCT smartification (nanotechnology, intelligent technology, 5G communication, portable analytical instruments). Based on this series of measures, ultimately advance the achievement of the development goals: advancing the translation of laboratory prototypes into clinical applications and establishing a full-chain POCT system encompassing community-based initial screening and at-home self-testing.

Figure 3: Applications and challenges of CRISPR/Cas systems



5. Conclusion and Outlook

This paper presents a systematic literature review on the application of the CRISPR/Cas system in molecular diagnostics. By organizing and evaluating the research background, technical principles, signal amplification strategies, one-tube detection methods, and the process of clinical translation, it establishes a clear understanding of the research landscape in this field. Based on an analysis of core elements, research in the field of CRISPR molecular diagnostics is driven by the synergistic forces of "the need for prevention and

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control of emerging infectious diseases, advancements in nucleic acid detection technology, and the pressing demand for point-of-care testing at the grassroots level." Internationally, research is characterized by a closed-loop pathway of "discovery of novel Cas effector proteins—development of detection platforms—clinical validation." Domestic research, on the other hand, highlights the key advantages of "optimizing signal amplification strategies and innovating single-tube technologies." Regarding CRISPR detection, both sides agree on the need to balance sensitivity, specificity, and convenience; however, they hold differing views on the path to technological translation—international researchers tend to focus on discovering novel Cas systems (such as CasΦ and Cas14a) and expanding their application scenarios. In China, more attention is paid to the signal amplification and one-tube integrated optimization of existing technology (Cas12a/Cas13a). However, the industry is still facing challenges such as "throughput limitations in multiplex detection", "insufficient compatibility of clinical samples", and "the integration of POCT platform needs to be improved", highlighting the urgency and significance of further exploration.

Therefore, future research can be carried out in the following three directions: first, focus on specific scenarios such as multi-target joint detection, and conduct targeted empirical studies on the problems such as Cas protein activity inhibition in complex samples to find a more efficient integrated method of sample pretreatment and signal enhancement. Second, utilize cutting-edge research methods—such as digital microfluidics, flexible electronic devices, and AI-assisted crRNA design—to bridge the gaps in automation and intelligence in existing technologies, thereby advancing CRISPR detection from the laboratory stage to on-site applications. Third, conduct cross-cultural comparative studies on domestic research in areas such as "universality of single-tube testing" and "non-nucleic acid-targeted expansion." This review can lay a literature foundation and provide conceptual guidance for subsequent explorations, thereby promoting the transformation of CRISPR molecular detection technology from "laboratory prototypes" to full-chain applications ranging from "community-based initial screening" to "home self-testing."

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Conflicts of Interest

The authors declare no conflict of interest.

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